Regulation of ethylene biosynthesis and multiple ethylene signal transduction pathways in a model plant *Arabidopsis*

by

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This is to certify that I have examined the above PhD thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

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Table of contents

Title Page i
Authorization Page ii
Signature Page iii
Acknowledgements iv
Table of Contents v
List of Figures ix
List of Tables xiv
Abstract xv

Chapter 1. Protease purification from wild-type Arabidopsis plant and study of the protease biological function 1

1.1 Introduction 1

1.1.1 Ethylene is a phytohormone 1

1.1.2 Ethylene biosynthesis 4

1.1.3 Protease 10

1.1.4 Fast protein liquid chromatography (FPLC) 12

1.1.5 Proteomics 13

1.1.6 NanoLC-ESI-Q-TOF MS/MS (LC-MS/MS) 14

1.1.7 Fluorescence resonance energy transfer (FRET) 19

1.1.8 Arabidopsis thaliana is a recommended plant in this study 20

1.1.9 Objectives of this study 20

1.2 Materials and Methods 21

1.2.1 Plant materials and growth conditions 21

1.2.2 Other materials and medium 21

1.2.3 Chemicals and reagents 22

1.2.4 Transformation of E.coli with CaCl$_2$ methods 23

1.2.5 Transformation of Agrobacteria (GV3101) with CaCl$_2$ method 23

1.2.6 Transformation of Arabidopsis by infiltration (Floral dip) 24

1.2.7 Expression and purification of recombinant protein in E.coli 26
1.2.8 Preparation of *Arabidopsis* crude extract for protease purification 27
1.2.9 Preparation of plant RNA and synthesis of cDNA 27
1.2.10. Purification of ACC synthase-processing protease from *Arabidopsis* by FPLC 28
1.2.11 Assay for proteolytic activity 29
1.2.12 SDS-PAGE gel separation and protein in-gel digestion 29
1.2.13 NanoLC-ESI-Q-TOF MS/MS analysis 30
1.2.14 β-Estradiol induce protein overexpression in transgenic plant 31
1.2.15 Tissue preparation and protein extraction under fully denature condition 31
1.2.16 Confocal microscopy observation of AtACS2 protein localization and FRET detection 32
1.3 Result 33
1.3.1 Purification of substrate LeACS2-GST fusion protein in *E.coli* 33
1.3.2 Protease activity validation in wild-type *Arabidopsis* plant by using in vitro proteolysis assay 35
1.3.3 Purification of the protease from wild-type *Arabidopsis* plant 38
1.3.4 Purified protease identification with Q-TOF MS/MS 47
1.3.5 Protease homozygous single and double mutants screening 52
1.3.6 Protease activity validation in proteases mutants 57
1.3.7 Protease over-expression in *E.coli* and the activity validation 60
1.3.8 Fusion protein CFP-AtACS2-YFP expression in transgenic plant 65
1.3.9 Different treatment of *CFP-AtACS2-YFP::WT* transgenic plant to find out a condition for transgenic AtACS2 can be processed in vivo 70
1.4 Discussion 77
1.4.1 LeACS2-GST fusion protein overexpresssion in *E.coli* 77
1.4.2 In vitro validation and purification of the protease which can process of ACS2 C-terminus 77
1.4.3 Protease activity validation in T-DNA insersion loss of function muant lines 79
1.4.4 Transgenic protein CFP-AtACS2-YFP overexpression in wild-type plant for detecting in vivo processed by proteases

Chapter 2. Function proteomic study of ethylene up-regulated phosphorylation protein in \textit{ein3-1/eil1-1} double mutant

2.1 Introduction

2.1.1 Over view of ethylene signaling pathway

2.1.2 Ethylene signal perception

2.1.3 Ethylene signal transduction

2.1.4 Protein phosphorylation form in ethylene signal transduction

2.1.5 Quantitative phosphoproteomics study in plant signaling

2.1.6 Water uptake and transport in plant tissues

2.1.7 Plant aquaporin proteins (AQPs)

2.1.8 Post-translational modifications (PTMs) of aquaporins

2.1.9 Objectives of this study

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

2.2.2 Preparation of genomic DNA for amplification of PIP2;1, EIN3 and EIL1 genes from \textit{Arabidopsis} plant

2.2.3 Overexpression of the wild-type and mutant PIP2;1 proteins

2.2.4 Microsomal protein extraction and PIP2;1 phosphorylation peptide quantitation analysis of \textit{ein3-1/eil1-1}

2.2.5 PIP2;1 antigen preparation by purification of recombinant protein from \textit{E.coli} and used for production of polyclonal antibody

2.2.6 In vivo phosphorylated protein quantitation with western blot analysis

2.2.7 Peptides dimethyl labeling and analyzed by LC-MS/MS

2.2.8 Water loss measurement

2.2.9 Protoplast generation

2.2.10 Protoplast swelling and shrinking assays

2.2.11 Confocal laser microscopy
2.3 Result

2.3.1 Overexpression of PIP2;1 protein in *E.coli* and production of PIP2;1 polyclonal antibody

2.3.2 Further validation of ethylene up-regulated phosphopeptide of endogenous PIP2;1/2/3 by quantitative phosphoproteomics analysis

2.3.3 Validation of ethylene up-regulated phosphopeptide of endogenous PIP2;1/2/3 by western blot analysis

2.3.4 Wild-type and mutant PIP2;1 gene overexpression in transgenic lines

2.3.5 Ethylene up-regulated phosphorylation of PIP2;1 isoform protein in transgenic lines

2.3.6 Phosphorylated Ser-280 and Ser-283 of PIP2;1 isoform up-regulated patterns under time courses ethylene treatment

2.3.7 Phosphorylated Ser-280 and Ser-283 of PIP2;1 isoform up-regulated patterns under dose ACC treatment

2.3.8 Ethylene up-regulated Ser-280 and Ser-283 phosphorylation of PIP2;1 isoform increase the cellular water transport activity independent of EIN3/EIL1

2.3.9 Ethylene up-regulated Ser-280 and Ser-283 phosphorylation sites of PIP2;1 isoform increase water loss

2.3.10 Ser-280 and Ser-283 phosphorylation regulation in ethylene mutants under ethylene treatment

2.4 Discussion

2.4.1 Over view of function study of ethylene up-regulated PIP2;1 phosphorylation independent of EIN3/EIL1

2.4.2 Quantitative phosphoproteomics study of ein3-1/eil1-1 double mutant

2.4.3 AtPIP2;1 isoform phosphorylation regulation in ethylene signaling pathway

2.4.4 Ethylene up-regulate phosphorylation of AtPIP2;1 isoform increase water transport activity

Reference
List of Figures

Figure 1.1.1 The triple response of *Arabidopsis* wild-type seedlings 3
Figure 1.1.2 Ethylene biosynthesis pathway in higher plants 9
Figure 1.1.3 Basic components of a mass spectrometer 16
Figure 1.1.4 Operation system of Q-TOF mass spectrometer in MS (upper) and MS/MS mode (lower) modes 17
Figure 1.1.5 Pattern of peptide fragmentation 18
Figure 1.3.1 Fusion protein LeACS2-GST purification result from *E.coli* 34
Figure 1.3.2 Alignment analysis of Tomato LeACS2 and *Arabidopsis* AtACS2 protein sequences 36
Figure 1.3.3 SDS-PAGE profile of proteolysis assay result to validate the protease in wild-type *Arabidopsis* plant 37
Figure 1.3.4 Phenotype of 2-week-old wild-type *Arabidopsis* plant 40
Figure 1.3.5 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC DEAE column purification 41
Figure 1.3.6 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC gel filtration column purification 42
Figure 1.3.7 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC MonoQ column purification 43
Figure 1.3.8 SDS-PAGE profile of proteolysis assay result to compare protease activity after the FPLC steps purification 44
Figure 1.3.9 Western-blot analysis of proteolysis assay result with anti-ACS2 antibody to compare protease activity after the FPLC steps purification 45
Figure 1.3.10 Quantification of protease specific activity after the FPLC steps purification 46
Figure 1.3.11 SDS-PAGE profile of protease after steps purification 48
Figure 1.3.12 MS/MS spectrum of peptides of purified proteases identified by QTOF-MS 50
Figure 1.3.13 PCR validation of proteases loss of function lines 53
Figure 1.3.14 RT-PCR validation of *at1g76140* and *at2g47390* loss of function lines 54
Figure 1.3.15 PCR to validate F1 generation plants of \textit{at1g76140} and \textit{at2g47390} mutants

Figure 1.3.16 PCR to validate F2 generation homozygous \textit{at1g76140/at2g47390} double mutant

Figure 1.3.17 In-vitro proteolysis assay to validate protease activity in the proteases loss of function lines

Figure 1.3.18 Quantification analysis of substrate LeACS2-GST protein after time course in vitro proteolysis assay

Figure 1.3.19 Validation of RNA samples by electrophoresis

Figure 1.3.20 Validation of pET30a-AT1G76140 (A) and pET30a-AT2G47390 (B) constructs by PCR and enzyme digestion

Figure 1.3.21 Validation of \textit{E.coli} over-expression proteases and purification by SDS-PAGE

Figure 1.3.22 SDS-PAGE profile of proteolysis assay to validate the \textit{E.coli} expressed protease activity

Figure 1.3.23 Structure prediction model of AtACS2 protein

Figure 1.3.24 PCR validation of \textit{CFP-AtACS2-YFP::WT} transgenic plants

Figure 1.3.25 Western blot analysis of the fusion protein CFP-AtACS2-YFP inducible expression

Figure 1.3.26 AtACS2 localization observation and CFP-YFP FRET validation with confocal microscopy

Figure 1.3.27 Western blot analysis of AtACS2 in-vivo process in developmental stages

Figure 1.3.28 Western blot analysis of AtACS2 in-vivo process after wounding treatment

Figure 1.3.29 Western blot analysis of AtACS2 in-vivo process after cold treatment

Figure 1.3.30 Western blot analysis of AtACS2 in-vivo process after NaCl infiltration treatment

Figure 1.3.31 Western blot analysis of AtACS2 in-vivo process after UV treatment

Figure 1.3.32 Western blot analysis of AtACS2 in-vivo process after air or ethylene treatment
Figure 1.4.1 Hypothetic model of ACC synthesis 2 processed by protease and promote ethylene production in plant.

Figure 1.4.2 Western blot analysis of CFP-AtACS2-YFP processed during protein extraction.

Figure 2.1.1 Representation of the ethylene signal transduction pathway.

Figure 2.1.2 The mode of action for ethylene in the regulation of phosphorylated ERF110 isoform during *Arabidopsis* bolting.

Figure 2.1.3 Proposed model of ethylene dual-and-opposing effect on plasma membrane intrinsic protein 2;1.

Figure 2.1.4 An overview of methods used for phosphoproteins and phosphopeptides enrichment.

Figure 2.1.5 Co- and post-translational modification profile of *Arabidopsis* PIP2;1.

Figure 2.1.6 PCR and enzyme digestion to validate pET30a-PIP2;1 construct for PIP2;1 overexpression in *E.coli*.

Figure 2.1.7 Sequencing result of 6xHis-PIP2;1-6xHis fusion gene ORF in pET30a-PIP2;1 construct.

Figure 2.1.8 Overexpression of 6xHis-PIP2;1-6xHis fusion protein in *E.coli*.

Figure 2.1.9 6xHis-PIP2;1-6xHis fusion protein validation after purification and re-nature.

Figure 2.1.10 MS/MS spectrum of peptides identified by QTOF-MS to validate PIP2;1 protein purified from *E.coli*.

Figure 2.1.11 Western blot to validate the polyclonal antibody of PIP2;1.

Figure 2.1.12 Genotype validation of ein3-1/eil1-1 double mutant line.

Figure 2.1.13 Western blot to validate Ser-280 and Ser-283 phosphorylation in *pip2;1, pip2;2* and *pip2;1/2* double mutants under ethylene treatment.

Figure 2.1.14 PCR validation of *pip2;1, pip2;2* and *pip2;1/2* double mutant lines.

Figure 2.1.15 RT-PCR validation *pip2;1/2* double mutant line.

Figure 2.1.16 SILIA-based quantitative phosphoproteomic to validate PIP2;1/2/3 phosphorylation in ethylene-treated *ein3-1/eil1-1* double mutant.
Figure 2.3.12 Quantitative western-blot analysis of Ser-280 and Ser-283 phosphorylation of endogenous PIP2;1/2 in wild-type and ein3-1/eil1-1 double mutant lines

Figure 2.3.13 WT/Mutant PIP2;1 overexpression construct map

Figure 2.3.14 Validation of pHUB10-UTR-8xHis-YFP-PIP2;1 (WT, S280A, S283A, S280/3A) constructs by PCR and enzyme digestion

Figure 2.3.15 Sequencing result of WT and mutant PIP2;1 genes on constructs

Figure 2.3.16 PCR validation of His8-YFP-PIP2;1 (WT and mutant PIP2;1) fusion gene in transgenic plants

Figure 2.3.17 Western blot validation of WTPIP2;1 and mutant PIP2;1 transgenic plants

Figure 2.3.18 Phenotype of background plants and PIP2;1 transgenic plants

Figure 2.3.19 Quantitative western-blot analysis of Ser-280 and Ser-283 phosphorylation of transgenic PIP2;1 isoform in wild-type, ein3-1/eil1-1 and pip2;1/2 backgrounds respectively

Figure 2.3.20 Phosphopeptide quantitation and PTM analysis of transgenic PIP2;1 protein in ethylene-treated His8-YFP-PIP2;1::ein3-1/eil1-1 transgenic plant

Figure 2.3.21 Phosphorylation of Ser-280 and Ser-283 of His8-YFP-PIP2;1 regulated by ethylene treatment of different periods of time

Figure 2.3.22 Phosphorylation of Ser-280 and Ser-283 of His8-YFP-PIP2;1 regulated by different concentration of ACC

Figure 2.3.23 Western-blot validation of Ser-280 and Ser-283 phosphorylation in protoplast of His8-YFP-PIP2;1::pip2;1/2 and His8-YFP-PIP2;1::ein3-1/eil1-1 transgenic lines

Figure 2.3.24 PIP2;1 phosphorylation increase protoplast swelling/shrinking rate in transgenic lines

Figure 2.3.25 Protoplast swelling and shrinking assay of ein3-1/eil1-1, WT and pip2;1/2 background lines

Figure 2.3.26 Water loss increase with Ser-280 and Ser-283 phosphorylation level enhanced by ethylene treatment
Figure 2.3.27 Subcellular localization of PIP2;1 protein in epidermal cells of 5 different ACC-treated transgenic plants 159
Figure 2.3.28 Water loss increase in *Arabidopsis* wild type and *ein3-1/eil1-1* double mutant by ethylene treatment 160
Figure 2.3.29 Leaves morphology of plants before and after water loss 161
Figure 2.3.30 Ser-280 and Ser-283 phosphorylation regulation under ethylene treatment in wild type and ethylene mutants 163
Figure 2.3.31 Ser-280 and Ser-283 phosphorylation regulation under ACC treatment in wild-type and ethylene mutants 164
Figure 2.4.1 Bioinformatics prediction motif of Ser-280 and Ser-283 phosphosites on PIP2;1 C-terminus 167
Figure 2.4.2 Western-blot analysis of Ser-280 and Ser-283 phosphorylation in kinase mutant lines after ethylene treatment 170
Figure 2.4.3 The model of ethylene regulation of a Ser-280- and Ser-283-phosphorylated PIP2;1 isoform during water transport in *Arabidopsis* 171
List of Tables

Table 1.3.1 Proteases identified by LC-MS/MS ................................................. 49
Table 2.3.1 Ethylene-regulated phosphopeptides in ein3/eil1 double mutant .......... 127
Table 2.3.2 Peptides of transgenic PIP2;1 protein identified by LC-MS/MS ........... 145
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Abstract

Ethylene is one of important plant hormones, and it plays important role in plant physiological regulation during plant life cycle. Arabidopsis can produce endogenous ethylene through ethylene biosynthesis pathway. ACC synthase (ACS) is one of the key enzymes in ethylene biosynthesis pathway in Arabidopsis plant. Proteases which can cleave ACS2 C-terminal were found in Arabidopsis in this study, and they were purified by FPLC method. Seven proteases were identified by LC-MS/MS, and three protease mutants were validated have lower protease activity than wild-type plant. One in vitro expressed protease still can process ACS2 C-terminus in proteolysis assay. Arabidopsis plant also has response to ethylene by five receptors, then ethylene signaling transduction through ethylene signaling pathway. It is believed that protein phosphorylation plays a key role in ethylene signaling, which is partially mediated by some uncharacterized pathway(s) independent to a known linear signaling pathway from ethylene receptors to transcription factors EIN3 and EIL1. To address how ethylene alters the cellular protein phosphorylation profile through these pathways, a differential and $^{15}$N stable isotope labeling in Arabidopsis (SILIA or SIML)-based quantitative phosphoproteomic analysis was performed on 12 h ethylene-treated Arabidopsis ethylene-insensitive double loss-of-function mutant, ein3-1/eil11-1. Two and four phosphopeptides were identified up- and down-regulated by ethylene, respectively. Amongst the ethylene-regulated phosphoproteins, aquaporin protein PIP2;1 is of highly biological interest since the ethylene regulates the water permeability of plasma membrane through posttranslational regulation. Both LC-MS/MS and western-blot analysis showed PIP2;1 protein phosphorylation up-regulated by ethylene independent of EIN3/EIL1. Further study using transgenic plants confirms that ethylene regulates the water channels opening via a phosphorylation pathway independent to EIN3 and EIL1, which resulted in alteration of water flux rates cross plasma membrane. Taken together, our study suggests that the water channel activity is regulated by ethylene-mediated protein phosphorylation, which is largely independent to EIN3 and EIL1.
Chapter 1.

Protease purification from wild-type *Arabidopsis* plant and study of the protease biological function

1.1 Introduction

1.1.1 Ethylene is a phytohormone

Plant hormones are small organic molecules and function in small amount in almost every aspect of growth and developmental stages. Ethylene as one of important plant hormone exists in the gaseous state under normal physiological condition. It plays important role in plant physiological regulation during plant life cycle. Ethylene can regulate many aspects of plant growth, development, stress response and senescence (Abeles et al., 1992). These physiological processes include seed germination, cell elongation, leaf and flower senescence, fruit ripening, nodulation, as well as responses to some biotic and abiotic environmental stimulation, such as wounding, gravitropic responses (Bingwen Lu, 2002; Bingwen LU, 2001) and pathogen responses (Johnson, 1998).

Biologists study ethylene for more than one hundred years. The Russian plant physiologist Neljubov (1879-1926) observed that contaminating illuminating gas (ethylene) in his laboratory caused the etiolated pea seedlings grew different to outside seedlings. The morphology of ethylene treatment pea seedlings contains agravitropic (horizontal) growth, thickening of stem and inhibition of the stem, so call “triple response” (Figure 1.1.1). That he discovered ethylene is a biologically active gas. Ethylene was maybe the first to be determined as a naturally occurring growth regulator in plant tissues since that original observation that ethylene can influence plant development directly (Neljubov, 1901). In 1910, Cousins found that fungus-infected oranges can produce a gas which caused the ripening of
banana in the mixed commercial shipments (Cousins, 1910). Gane used chemical approach to analyze the gases which release by some ripening apples was ethylene in 1934 (Gane, 1934). The investigation defined that ethylene function as an endogenous signal molecule in plants. Until 1950s, ethylene gas could be identified and quantified by using of syringe pump with advent of gas chromatography, and it was called as a plant hormone, then all doubts about its physiological significance began to be dispelled and physiological and biochemical studies on ethylene became more and more widespread. The physiological function of ethylene was found at beginning is promote fruit ripening, but more and more studies have demonstrated that ethylene production plays an important role in regulating every aspect of plant growth and developmental stage, ranging from seed germination to senescence during the whole life cycle.
Figure 1.1.1 The triple response of *Arabidopsis* wild-type seedlings. Seeds were germinated and grown for 3 days in the dark in air (left) or 10 ppm ethylene (right).
1.1.2 Ethylene biosynthesis

In 1980s, Yang and Hoffman have established the ethylene biosynthesis pathway in higher plants, so called Yang cycle (Figure 1.1.2). The Yang cycle begin from methionine, it is firstly converted to S-adenosylmethionine (AdoMet) by AdoMet synthase, and a major rate-limiting step in the biosynthesis of ethylene in higher plant is the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), catalyzed by ACC synthase (Li et al., 1992; Mattoo and Suttle, 1991; Ravanel et al., 1998; Yang and Hoffman, 1984); the last step is ACC converted to ethylene by ACC oxidase (ACO). As methionine participates in a range of important functions in the cycle, including methylation reactions, protein synthesis and ethylene biosynthesis, so it’s important of regulation of its production. The biosynthesis of ethylene is tightly regulated, and it can be induced by various stimulation, such as wounding, pathogen attack, some stresses, mechanical factors and so on (Mattoo and Suttle, 1991).

1.1.2.1 ACC synthase

ACC synthase (ACS) is a pyridoxal-5’-methylthioadenosine (MTA) requiring enzyme (Adams and Yang, 1979), the key enzyme which responsible for the ethylene biosynthesis by converting AdoMet to ACC. A known inhibitor (AVG) can strongly inhibited the conversion of methionine to ACC in pyridoxal phosphate-mediated enzyme reactions (Rando, 1974), but it can’t block the conversion of methionine to SAM or the conversion of ACC to ethylene. So that AVG inhibits the conversion of SAM to ACC is mediated by a pyridoxal enzyme (Adams and Yang, 1979). The enzymatic conversion from SAM to ACC by ACS was first studied in plant tissue’s extracts. ACC synthase was first identified in homogenates of ripening tomatoes and shown to require pyridoxal phosphate as cofactor, and the enzyme was soluble and was strongly inhibited by AVG. (Boller et al., 1979; Yu et al., 1979). They also demonstrated that low concentrations of pyridoxal phosphate activated ACC synthase was strongly inhibited by another well-known ACC synthase inhibitor of pyridoxal enzymes (AOA).
Based on screening tests of ACC can greatly stimulated ethylene production in a number of plant tissues, Lürssen et al proposed that ACC is a biological precursor of ethylene (Lurssen et al., 1979). ACC synthase activity was shown to limit ethylene biosynthesis in many conditions and can be stimulated by some factors which promote ethylene formation, such as auxin and some stress conditions (Yang and Hoffman, 1984). As it became clear that ACC synthase was relative to regulation of plant developmental processed and responses to stress, then more and more plant biology researchers study the enzyme purification and characterization. But the problem proved to be formidable as the low abundance and unstable of ACC synthase protein in plant. ACC synthase from wounding treated the pericarp of ripening tomato fruits was first purified and had a molecular mass of 50 kDa in SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), but HPLC gel filtration result indicated that ACC synthase in homogenates of wounded pericarp tissue had a molecular mass of 55 to 57 kDa (Acaster and Kende, 1983; Bleecker et al., 1986; Yang, 1980).

In some experiment, tomato ACC synthase was detected by using the monoclonal antibodies in immunoblot, and showed a minor polypeptide of 56 kDa (Bleecker et al., 1986; Bleecker et al., 1988b). It was suggested that ACC synthase undergoes some proteolytic modifications in tissue homogenates because of immunoprecipitation of in vitro translation products was a 56 kDa protein as well (Edelman and Kende, 1990). Based on analysis of amino acid sequence data, Van Der Straeten et al supposed that approximately 85 amino acid residues are proteolytically cleaved from the carboxyl terminus of the enzyme (Vanderstraeten et al., 1990). Proteolysis of ACS was also demonstrated in apple fruit that immunoaffinity purification of radio-labelled ACS showed a molecular weight of 48 kDa by SDS-PAGE, but compared to 52 kDa by gel filtration (Yip et al., 1991). Other demonstrations that ACS purified from wounded winter squash mesocarp tissue can produce a 50 kDa protein, but antibodies raised against the purified protein identified a 58 kDa protein, and immunoprecipitation of in vitro translation products produced the higher molecular weight form of 58 kDa protein (Nakajima and Imaseki, 1986; Nakajima et al., 1988). Therefore, these studies suggest that ACS is proteolytically modified during extraction and purification.
Further study demonstrated that C-terminal of tomato ACS2 protein can be cleaved by a 64 kDa putative metalloprotease from the *Arabidopsis* peptidase M41 family (Li et al., 2005c), and the multiple cleavage sites (at Lys438, Glu447, Lys448, Asn456, Ser460, Ser462, Lys463 and Leu474) have been identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis (Li et al., 2005a). C-terminus truncation of a tomato ACC synthase induced by wounding can affect both enzyme catalysis and the structure of the native protein; and optimal deletion of ACS C-terminus can increase the specific activity of the truncated protein (Li and Mattoo, 1994). However, it is yet to be shown if such a proteolytic phenomenon occurs in vivo and what is its functional significance.

A large number of putative cDNA clones of ACC synthase from a zucchini cDNA expression library were isolated by screening with antibodies and a clone which encoding ACC synthase was identified by expression of enzyme activity in *E. coli* and yeast (Sato and Theologis, 1989). Based on the nucleotide sequence of cDNA clones, the primary structure of ACC synthase shows that the molecular weight of the enzyme from different species is quite similar, between 53 kDa from the tomato (Rottmann et al., 1991) and 58 kDa from the carnation (Park et al., 1992). The quaternary structure of ACS has been a hot point of debating biochemical research field. Some reports indicated that ACS was a dimer (Sato and Theologis, 1989; Satoh et al., 1993; Tsai et al., 1988; White et al., 1994; Yip et al., 1991), but in other cases the data was most consistent with a monomeric form of the enzyme (Acaster and Kende, 1983; Bleecker et al., 1986; Li and Mattoo, 1994; Satoh et al., 1993; Yip et al., 1990). High-resolution X-ray structures of various aminotransferases have showed that the enzymes are dimeric and that both subunits contribute necessary residues to the common active sites (Malashkevich et al., 1995; Mcphalen et al., 1992; Okamoto et al., 1994). Among the various *Arabidopsis* ACS isoforms, the dimeric interactions phenomenon was observed by expression of two different ACS proteins in a single *E.coli* strain (Tsuchisaka and Theologis, 2004). Bimolecular fluorescent complementation (BiFC) study by using transgenic *Arabidopsis* plants confirmed that ACS homodimerization and heterodimerization really exist in plant (Tsuchisaka et al., 2009). It revealed that a combinatorial interaction in the different ACS isoforms could regulate the relative ratio of the different hetero- and homodimers, and
increased the potential biochemical diversity of the ACS protein family.

It’s report that the ACS is encoded by a multigene family in tomato plant and in many other plants (Nakatsuka et al., 1998; Rottmann et al., 1991). The ACS family genes’ transcription level can be induced in plant different environmental stresses and developmental stage have already been validated in recent molecular biology studies (GE et al., 2000). In Arabidopsis, according to the completion of the Arabidopsis genome sequence revealed that ACS genes are putatively encoded by twelve genes (ACS1-12), and they located on five chromosomes (Yamagami et al., 2003). The polypeptides of the ACS gene family have quite similar molecular mass, ranging from about 50.9 to 61.2 kDa. Except for ACS7, ACS10 and ACS12 proteins, other ACS isozymes have the Serine (S) residue in the hypevariable C-terminus, which is a phosphorylation site (Tatsuki and Mori, 2001).

1.1.2.2 Post-translational regulation of ACC synthesis protein

The possibility of post-translational regulation of ACC synthesis protein activity was supported by pharmacological demonstrations (Chappell et al., 1984; Felix et al., 1991; Spanu et al., 1994). Several studies have provided convincing evidence by using phosphatase inhibitors and ordinary kinase that ACS protein stability is regulated by protein phosphorylation. Cells suspension of cultured tomato, addition of fungal elicitors induces a rapid increase in ACS activity, and the serine/threonine (Ser/Thr) protein kinase inhibitors K252a and staurosporine blocked the elicitor-induced increase in ACS activity (Felix et al., 1991; Grosskopf et al., 1990; Spanu et al., 1994). Moreover, consistent with this result that treated with the protein phosphatase inhibitor calyculin A not only can stimulates ACS activity of the tomato cell culture without elicitors but also significantly enhances the effect of elicitor treatment (Felix et al., 1994; Hansen et al., 2009; Spanu et al., 1994). The phosphorylation event appears to be a primary effect of stimulated ACS activity, because the kinetics are very fast, occurring usually within minutes. These results suggested that ACS protein phosphorylation maybe regulate its activity and/or turnover. In later study, that tomato
LeACS2 protein was found to be phosphorylated by a CDPK kinase from wounding tomato fruits crude extract (Tatsuki and Mori, 2001) and a conserved Ser-460 at the C-terminus of the protein was detected as the phosphorylation target site. It is known that ACS proteins have low abundance and is unstable in vivo, so it is conceivable that ACS proteins maybe enhance its activity through increasing its stability by phosphorylation.

Encoding polypeptides of the ACS genes showed high protein similarity within the catalytic domains, but relatively divergent C-terminal domains containing the absence or presence of putative phosphorylation sites. There are three types of ACS proteins on the basis of their C-terminal sequences. Type-1 ACS proteins have extended C-terminal domains and can be phosphorylated by mitogen-activating protein kinase (MAPK) and maybe also phosphorylated by calcium-dependent protein kinase (CDPKs). Type-2 ACS proteins have a shorter C-terminus containing a single potential CDPK phosphorylation site. Type-3 ACS proteins have a minimal C-terminus extension and without phosphorylation sites on it. Type-1 ACS proteins can be phosphorylated and plays an important role in ACS turnover. The C-terminus sequences of some ACS proteins significantly affect the protein stability and activity, and this regulation involves phosphorylation. The variable region of the C-terminus is responsible for phosphorylation (Tatsuki and Mori, 2001) and other post-transcription regulations of ACS proteins. C-terminus phosphorylation can affect the enzyme activity (Tatsuki and Mori, 2001). The *Arabidopsis* ACS2 and ACS6 proteins can be phosphorylated by MAPK6, which leading to accumulation of ACS protein for regulating its abundance and stability (Liu and Zhang, 2004; Xu et al., 2008), then in turn to regulate the ethylene production in *Arabidopsis* plant.
Figure 1.1.2 Ethylene biosynthesis pathway in higher plants. AdoMet is converted to ACC by ACC synthase and then converted to ethylene by ACC oxidase.
1.1.3 Protease

1.1.3.1 Over View of Protease

Proteases (also called peptidases or proteinases) are any enzymes which catalyze the hydrolysis of peptide bonds which link amino acids together in a polypeptide chain. Proteases are distributed widely in nature, they can be found in bacteria, animals, plants, archaea and viruses. Proteases have evolved multiple times, and different classes of protease sometimes can have the same reaction in completely different catalytic mechanisms. In the last decade, there have substantial advances in our understanding of cellular processes involving proteolysis, from the proteolytic processing of nascent polypeptide chains to degradation of proteins to amino acids (Beynon and Bond, 1986; Bond and Beynon, 1987; Schwartz, 1986). Moreover, it has become evident that proteases function is a highly controlled in cells, complex process that takes place actually in all parts of the cells. However, that little is known about the physiological substrates of many cellular proteases, what initiates and regulates proteolytic processes, or which proteases are involved in specific functions. They are so important to control protein process in cells under different environment conditions. Cellular proteases range in size from approximately 20 to 800 kDa, and many of proteases are larger than most cellular enzymes. Proteases can change substrates’ structure, localization or activity, and so on by processing substrate proteins in cells.

1.1.3.2 Classification of Protease

Based on catalytic residue, proteases throughout all living systems are currently classified into six broad groups. These contain serine protease, cysteine proteases, threonine proteases, aspartate proteases, glutamic acid proteases and metalloproteases. In the Arabidopsis thaliana genome, many genes with sequence similarities to known proteases were identified in MEROPS database; it includes 745 genes encoding protease, account for about 3% of the proteome (Rawlings et al., 2010).
Peptidases are classified by ‘clans’ (based on structure, mechanism and catalytic residue order) and ‘families’ (based on sequence similarity) in the MEROPS database. A clan includes all the modern-day peptidases that have arisen from a single evolutionary origin of peptidases and represents one or more families. A family is a set of homologous proteolytic enzymes. Families are grouped by their catalytic type, the first character represent the type: A, aspartic; C, cysteine; G, glutamic; M, metallo; N, asparagine; P, mixed; S, serine; T, threonine; U, unknown. The aspartic peptidases contain two aspartic residues at their active centers that are involved in catalysis; and it is thought that general acid-base catalysis, rather than the formation of covalent enzyme-substrate intermediates. Cysteine peptidases contain an essential cysteine residue that is involved in a covalent intermediate with substrates (Polgar and Halasz, 1982). Cysteine peptidases are the group of plant proteases that have been most thoroughly studied. It takes part in many biological events in plant. They include mobilization of proteins during germination (Dominguez and Cejudo, 1999; Schlereth et al., 2000; Sutow et al., 1999), wounding (Ueda et al., 2000), senescence (Drake et al., 1996; Guerrero et al., 1998; Ueda et al., 2000), and so on. The best-known cysteine-proteinases are caspase-like protein, vacuolar-processing enzymes, papain-like peptides and cathepsin-type proteases. Serine peptidases are characterized by the presence of a uniquely reactive serine side chain at the active center, and the reaction involves the covalent binding of substrates to this serine residue. Metallopeptidases contain metal ions (usually zinc) in the active site. The metal ions are an essential part of their structures, and the nucleophilic attack on a peptide bond is mediated by H_2O, polarize the peptide bond to be cleaved prior to nucleophilic attack (Bond and Beynon, 1985; Rawlings and Barrett, 1995).

Plant genomes encode hundreds of protease, including largely of unknown function. Most of proteases known functions are involved in plant developmental regulation. Therefore, study of proteases biological function become more and more important.
1.1.4 Fast protein liquid chromatography (FPLC)

The rapid growth in the field of biotechnology has led to an increase in the demand of high efficient, large-scale protein purification processes. Fast protein liquid chromatography (FPLC) is a form of medium pressure chromatography technique that is often used to analyze or purify mixtures of proteins. The system consists of one or two high-precision pumps, a control unit, a column, a fraction collector and a detector system. FPLC was developed in Sweden by Pharmacia in 1982 and was originally called fast performance liquid chromatography to contrast it with HPLC or high-performance liquid chromatography.

Purification of the target protein is completed by using a combination of chromatographic methods. The purpose of purifying proteins by using FPLC approach is to get enough purity protein to fit its further study with a biologically active state enzyme. FPLC columns selection is important in the purification process. There are four major types of column chromatography include affinity chromatography, ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) (Goke and Keim, 1992). Most purification approaches maybe require the use of more than one of these types of chromatographic procedures to get the necessary purity protein for downstream experiments. Affinity chromatography depends on the specific and reversible binding of a protein to a matrix-bound ligand. The ligand can bind directly to the interesting protein or a tag that is covalently attached to the protein. The protein of interest can be eluted from the column by competing molecules displace the protein and bind to the ligand (Arakawa et al., 2004). IEX separates proteins based on their net surface charge, through electrostatic interactions that occur between proteins and a charged stationary phase (Duncan et al., 1987). HIC separates proteins depended on their hydrophobicity, and is usually used as an intermediate step in a purification scheme. SEC separates proteins relied on their hydrodynamic radium, a property determined by both the size and shape of the molecule. Proteins can be separated by the speed at which they pass through an inert stationary phase (Corbett and Roche, 1984). Superdex is usually the best choice for typical SEC procedures, because it is compatible with most solvents and offers the highest resolution of all the available stationary phases.
1.1.5 Proteomics

The availability of whole genome sequence (Devine and Wolfe, 1995) or expressed sequence tag (EST) (Adams et al., 1992) libraries allows the entire potential protein complement of organisms to be defined. More and more biologists begin to study the large-scale genes function by using a very powerful proteomic technology. Proteomics is a much newer discipline and more dynamic than genomics in biology study. The term “proteomics” was firstly introduced by Marc Wilkins in 1990s (Domon and Aebersold, 2006). It aims at globally characterizing all components in a proteome simultaneously, and analyzing protein functions, modifications and protein-protein interactions.

Proteomics analysis is based on four technological platforms: (a) a fast and simple procedure for purifying small amounts of proteins from complex mixtures; (b) a fast and sensitive approach for generating limited, but sufficient, structural information from the interesting proteins; (c) access to extended protein or DNA sequence database; (d) computer algorithms able to translate and link the DNA sequence language with various types of protein structural information (Gevaert and Vandekerckhove, 2000). With the demands of proteome analysis approach, high-throughput and high-resolution peptide/protein-based separation technologies are undergoing strong development. Proteomic samples are simultaneously complex, fragile, and usually scarce. They include largely dynamic range of relative abundances, and often contain large quantities of salts, contaminants and surfactants. To solve this big problem, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of protein separation technology has been used for more than twenty years, and it is still plays an important role in analysis of proteome samples, for example cell lines, tissues or biological fluids (Tang et al., 2008a). The critical issues that can limit the utilization of 2D-PAGE are its detection limits, dynamic range and ability to separate proteome-wide protein mixtures.

Proteome analysis is becoming a robust approach in the functional characterization of plants. Because of the availability of larger-scale nucleotide sequence information and based on the progress achieved in rapid and sensitive protein identification with mass spectrometry,
proteomics study open up new perspectives to analyze the complex functions of model plants and crop species at different levels. Proteomic analysis has been used to study protein patterns in plant organs and tissues, such as the developmental processes occurring during seed germination in *Arabidopsis* plant (Canovas et al., 2004; Gallardo et al., 2001, 2002). According to different protein patterns in developmental stages in some case, it can supply some evidence to differential wild type and mutant plants. And the subcellular proteome analysis also performed in plant plasma membrane, mitochondrial, chloroplast, nuclear and cell wall. This data reveals that organelle proteomics is presently can identify most protein containing a specialized subcellular compartment in plants (Canovas et al., 2004).

The original objective of proteomics study was the large-scale identification of all protein species in a cell or tissue. With the development of post-translational modifications (PTMs) study and revolution of mass spectrometry instrument, the applications of proteomics are currently complicated by the need to analyse post-translational modifications of the various functional aspects of proteins, such as phosphorylation, acylation, glycosylation, nitration and ubiquitination (Mann and Jensen, 2003; Seo and Lee, 2004).

**1.1.6 NanoLC-ESI-Q-TOF MS/MS (LC-MS/MS)**

Liquid chromatography-mass spectrometry (LC-MS) is a powerful technique that combines the physical capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). Recently, LC-MS/MS technology has been introduced in proteomics study for identification of large-scale peptides of proteins or complex samples where peptide masses may overlap even with high-resolution mass spectrometer. A mass spectrometer can calculate the accurate mass of a single molecule by measuring the mass-to-charge ratio (m/z) of its ion. The resulting ions flight directly to the mass analyzer where they are separated based on m/z and the detected by a detector, generating a spectrum that provides molecular mass and sequence information (McLuckey and Stephenson, 1999). The detected ions are converted in to electrical signals and transmitted to a computer for further analysis. The proper molecule which fits the m/z ratio can be recognized in a pre-setup database in the
computer. There are four basic components in all mass spectrometers: (a) sample inlet (sample injection); (b) ionization source; (c) mass analyzer; (d) ion detector (Figure 1.1.3).

Additional mass analysis procedure(s) are needed to fully characterize the sample molecules by breaking the molecules into small fragments (tandem mass analysis: MS/MS to MSn). Tandem mass analysis of peptides can profile its sequence. Quadrupole-time-of-flight (QTOF) is a typical tandem mass analysis technology, the target ion is firstly selected by the first analyzer, then dissociated in the collision cell and the fragments which can be analyzed in the second mass analyzer (Figure 1.1.4). Peptide can be broken at three different bonds: NH-CH, CO-NH and CH-CO. The fragment ions be named based on the broken site (Figures 1.1.5). Each fragmentation ion can generate a peak at the spectrum of the sample and can be identified by matching to databases, which have peptide sequences and PTM sites (Biemann, 1992).

Q-TOF mass spectrometers are high mass accuracy and high resolution instruments for peptide sequencing, by connecting with a separation system-liquid chromatography (LC) to form a powerful peptides analysis tool (Nano-LC-ESI-Q-TOF MS/MS). Cis-cinnamic acid regulation genes in Arabidopsis plant were identified by a functional proteomic approach with Nano-LC-ESI-Q-TOF MS/MS instrument (Guo et al., 2011). 224 phosphopeptides were identified in both air-grown and ethylene-treated Arabidopsis ethylene-insensitive 2 (ein2) mutant by using two-dimensional separations coupled with a hybrid quadrupole time-of-flight mass spectrometer (Li et al., 2009). Recently, it has been used in quantitative phosphorproteomic analysis of Arabidopsis plant (Li et al., 2012; Yang et al., 2013; Zhu et al., 2013).
Figure 1.1.3 Basic components of a mass spectrometer.
Figure 1.1.4 Operation system of Q-TOF mass spectrometer in MS (upper) and MS/MS mode (lower) modes.
Figure 1.1.5 Pattern of peptide fragmentation. There are six possible fragment ions for each amino acid residue that labelled in the diagram, with the a-, b- and c- ions having the charge retained on the N-terminal fragment; and the x-, y- and z- ions having the charge retained on the C-terminal fragment.
1.1.7 Fluorescence resonance energy transfer (FRET)

FRET (fluorescence resonance energy transfer) is a physical process in which energy is transferred from an excited donor molecule to an acceptor molecule by long-range dipole-dipole interaction. The phenomenon of FRET was firstly observed by Jean Perrin in 1918 and was theoretically described by Theodor Forster in 1984 (Didenko, 2001; Gordon et al., 1998; Matyus, 1992; Szollosi et al., 2002). FRET requires the following condition: 1) the donor be fluorescent and has long lifetime, 2) the transfer not involve the actual resorption of light by the acceptor, 3) emission spectrum fluorescence from the donor molecule overlaps the excitation spectrum of the acceptor molecule, and 4) the distance between the donor and acceptor molecules within 10 nm (Gordon et al., 1998). Therefore, FRET can be used as a molecular ruler to determine relative molecular distance between two interacting proteins (He et al., 2003; Mori et al., 2004). Based on the spectral properties of CFP and YFP, interactions between two proteins in living cells have been successfully revealed by using FRET, which can be detected by confocal microscopy (Karpova et al., 2003). According to the FRET energy-transfer efficiency is highly distance-dependent, the conformational behavior of peptide linker (flexible glycine- and serine-containing peptide linkers) between CFP and YFP protein is important for FRET energy transfer (Evers et al., 2006). Receptor-mediated activation of heterotrimeric GTP-binding proteins (G-proteins) was observed in living Dictyostelium discoideum cells by monitoring FRET between α- and β- subunits fused to CFP and YFP proteins (Janetopoulos et al., 2001). Yang et al. demonstrated that in vivo interaction of the Arabidopsis basic leucine zipper transcription factor AtbZIP24 as a homodimer was shown by using FRET with CFP and YFP as fused FRET pairs (Yang et al., 2009). B. anthracis proteases which specific for processing D-amino acid were identified (Kaman et al., 2011), trypsin and enteropeptidase activity were detected in complex biological mixtures by using FRET technology (Zauner et al., 2011). Peptide substrates containing luminescent quantum dot (QD) bioconjugates were used to quantify the proteases caspase-1, thrombin, collagenase and chymotrypsin activity with FRET (Medintz et al., 2006). Therefore, FRET approach is a robust way for studying interaction of protease and substrate in vivo.
1.1.8 *Arabidopsis thaliana* is a recommended plant in this study

*Arabidopsis thaliana* is dicot and belongs to mustard family. It is a small flowering plant which is widely used as a model organism in plant biology research. And it offers some important advantages for basic research in genetics, molecular biology and function proteomics study. Such as, it has short life cycle (about 6 weeks from germination to mature seed); prolific seed production and easy cultivation in green house; small size gnome (only 125 magabases); a large number of mutant lines and genomic resources; efficient transformation methods for making transgenic plants by using Agrobacterium tumefaciens. Therefore, *Arabidopsis thaliana* as a model organism were used in this study.

1.1.9 Objectives of this study

The objectives of my Ph.D. thesis in this study are to find out and isolate *Arabidopsis* plant protease which can cleave ACS2 protein C-terminal and study its activity in protease mutant lines and observe protease mutants phenotype. Try to use fret technology to validate ACS2 protein can be processed in vivo.
1.2 Materials and Methods

1.2.1 Plant materials and growth conditions

Seeds of wild-type Arabidopsis thaliana (WT, Col-0), SALK_105351C, SALK_077069C, SALK_054778 and SALK_090550C were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA).

If grown into soil, Arabidopsis seeds were soaked into water for 2-3 days in 4°C, then sowed it into soil. Put plants into inside growth chambers at 22 ± 2 °C under 24-h light condition with a light intensity of 80-100 μmol photons m⁻²s⁻¹. If Arabidopsis were grown on agar plates or in jar with MS culture medium without bacteria and fungus, seeds were surface-sterilized with 70% ethanol for 2 min, followed by 10-15 min in a solution containing 30% (v/v) commercial Clorox bleach and 0.1% Triton X-100, sterilized seeds were stored at 4 °C for 4 d under dark condition before planting. Suspended seeds in sterile 0.1% agar and plant it on MS agar plates (Murashige and Skoog, 1962) or in MS agar jars.

For protease purification experiment, cut leaves of 2-week-old Arabidopsis seedlings growth in soil by using scissors and collected tissues with liquid nitrogen after wounding 5 hours. Plant tissues were ground into a fine powder and kept at -80°C until later use.

1.2.2 Other materials and medium

Bacterial strain BL21(DE3)pLysS: E.coli BL21 star™ (DE3) pLysS cell is used for expression of recombinant protein.

Vector pET30a for cloning protease and was used for transformed into E.coli BL21.

E.coli strain DH10B is used in the cloning experiment. Its genotype: F mcrAΔ(mrr–hsdRMS–mcrBC)φ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139Δ(ara leu)7697 galU galK rpsL endA1 nupG.
Agrobacterial strain \textit{GV3101} is used in plant transformation. 100 \(\mu\)g/mL rifampicin and 50 \(\mu\)g/mL gentamycin were used.

\textbf{LB}: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0.

\textbf{2xYT}: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.4.

\textbf{YEP}: 10 g/L peptone, 5 g/L NaCl, 10 g/L yeast extract, pH 7.0.

\textbf{SOC}: 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM MgCl\textsubscript{2}, 20 mM glucose, pH 7.0.

\textbf{Infiltration medium}: 0.5\(\times\) MS salts, 1\(\times\) B5 vitamins, 50 g/L sucrose, 0.02\% (v/v) Silwet L-77 (freshly prepared), pH 5.7.

\textbf{Selection medium}: 1\(\times\) MS salts, 1\(\times\) Vmix (10 mg/L thiamine HCl; 1 mg/L pyridoxine; 1 mg/L nicotinic acid; 100 mg/L myo-inositol), 1\% sucrose, adjust pH to 5.7 with 2 M KOH, 0.8\% agar. Add antibiotics (50 \(\mu\)g/mL kanamycin was used for screening of pER10 recombinant constructs transformed plants and 25 \(\mu\)g/mL hygromycin was used for screening of pHUB10 recombinant constructs transformed plants).

\subsection*{1.2.3 Chemicals and reagents}

Bleach was purchased from KAO corp (Hong Kong). Methanol, ethanol and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Loughborough, UK). C18 Zip-tips were purchased form Merck Millipore. Acrylamide, bis-acrylamide and dithiotreitol (DTT) were purchased from BioRad (Hercules, CA). The 180-\(\mu\)m \(\times\) 20 mm Symmetry C18 trap column and 75-\(\mu\)m \(\times\) 250 mm BEH130 C18 analytical column was purchased from Waters Corp (Milford, MA). TPCK-treated trypsin was purchased from Amersham Bioscience. Glutathione-agarose beads, Murashige and Skoog basal salt mixture (MS), sucrose, \(\beta\)-estradiol, Diethyl pyrocarbonate (DEPC) and other chemicals were purchase from Sigma. Amersham Hybond PVDF membrane was purchased from GE Healthcare.
1.2.4 Transformation of *E.coli* with CaCl$_2$ methods

1.2.4.1 Preparation of competent cells

Inoculate *E.coli* strain on a LB agar plate and incubate overnight 37°C. Start 2-3 ml LB medium from freshly prepared colonies on LB agar plate, culture overnight in 37°C shaker. Dilute 100 times overnight bacterial culture medium into big volume of LB medium. Usually grown in 37°C shaker for 2-3 h in 100ml of LB medium. When OD$_{600}$ was about 0.6-0.8, put the bacterial medium on ice for chilling 10min, centrifuge at 3,000 rpm at 4°C for 15 min, get rid of supernatant and re-suspend bacterial pellet in 32 mL of 0.1 M CaCl$_2$, cooled on ice for 20 min. Centrifuge at 3,000 rpm at 4°C for 15 min again, and re-suspend bacteria pellet in about 2 mL 0.1 M CaCl$_2$, 10% glycerol store as 100 μL aliquots (chilling with liquid nitrogen for 3-5 min) in -80°C refrigerator.

1.2.4.2 Standard transformation protocol

Add about 1-10 ng of plasmid DNA into 100 μL of melting competent cells, mixed well and left on ice for 30 min. Heat shocked at 42°C for 90 sec, cooled again on ice immediately for about 3-5 min, then add 200 μL SOC medium and recovery growth in 37°C shaker for 30-60 min. Plate out on selective agar plate for screening transformational colonies which can grow on antibiotic medium plate.

1.2.5 Transformation of Agrobacteria (*GV3101*) with CaCl$_2$ method

1.2.5.1 Preparation of competent cells

Inoculate *GV3101* strain on a YEP agar plate with antibiotics (100 μg/mL rifampicin and 50 μg/mL gentamycin), and incubate 2 days at 30°C. Start 2-3 mL YEP medium with antibiotics (100 μg/mL Rifampicin and 50 μg/mL Gentamycin) from freshly prepared colonies on YEP
agar plate, culture one day in 30℃ shaker. Dilute 100 times of agrobacteria culture medium into big volume of YEP medium. Usually grown in 30℃ shaker for 8-10 h in 100 mL of YEP medium. When OD_{600} was about 0.6-0.8, put the agrobacteria medium on ice for chilling 10 min, centrifuge at 3,500 rpm at 4℃ for 15 min, get rid of supernatant and re-suspend agrobacteria pellet in 32 mL of 0.1 M CaCl₂, cooled on ice for 20 min. Centrifuge at 3,500 rpm at 4℃ for 15 min again, and re-suspend agrobacteria pellet in about 2 mL 0.1 M CaCl₂, 10% glycerol store as 100 µL aliquots (chilling with liquid nitrogen for 3-5 min) in -80℃ refrigerator.

1.2.5.2 Agrobacteria transformation protocol

Add about 0.1-1 µg of plasmid DNA into 100 µL of competent cells before melting, left on ice for 30 min. Chilling with liquid nitrogen for 1-5 min, and put it into 37℃ water bath for 5 min immediately, then add 500 µL YEP medium and recovery growth in 30℃ shaker for 3-5 hours. Spread out of agrobacteria on selective agar plate for screening transformational colonies which can grow on antibiotic medium plate after 48 hours.

1.2.6 Transformation of *Arabidopsis* by infiltration (Floral dip)

Plant *Arabidopsis* and growth in the following way: poking three holes at the bottom of white plastic cups and mound soil mixture into it. Put cups in a tray and add some water to let soil wetting. Sow seeds in plastic cups and grow plants in growth chambers at 22 ± 2 ℃ under 24-h light condition with a light intensity of 80-100 µmol photons m⁻²s⁻¹. Thin the plants to three per cup about one week after germination. About 3 weeks growth, the primary inflorescence stem is 1-5 cm tall and the secondary inflorescence stems are appearing at the rosette. Cutting off it to help produce more secondary inflorescence stems (about 6 to 8 days after clipping).

In the meantime, inoculate agrobacteria contains the binary construct into 5 mL YEP medium containing antibiotics (for pER10 series constructs, 100 µg/mL Rifampicin, 50 µg/mL
Gentamycin and 50 μg/mL Spectinomycin; for pHUB10 series constructs, 100 μg/mL Rifampicin, 50 μg/mL Gentamycin and 50 μg/mL Kanamycin) and culture in 30°C shaker for one day. Dilute 100 times of agrobacteria culture medium into big volume (usually 400 mL) of YEP medium containing antibiotics and continue to culture in 30°C shaker for 10-12 h. When OD$_{600}$ was about 0.8-1.0, centrifuge it at 4,000 rpm for 20 min at 10°C; get rid of supernatant and re-suspend agrobacterium pellet with the same volume of infiltration medium. Place re-suspended culture in a 400 mL beaker inside a vacuum desiccator. Inver cups containing plants to dip the flower into the solution. Draw a vacuum of 400 mm Hg and maintain it to let plants stay under vacuum for 15 min. Release the vacuum quickly and put plants on paper towels, place them horizontally in a tray and cover the tray with black plastic wrap to maintain humidity, then place the trays in green house. Uncover the black plastic wrap and set them upright. Put the cylindric transparent plastic wrap around the transformed plants. Let plants to grow under the normal conditions as before. About one month later, harvest mature seeds from each cups plants to make sure get independent transgenic plants from different cups of plants.

Prepare selection agar plates (diameter: 10 cm) with selection medium. Add 50 μg/mL Kanamycin (for pER10 series constructs) or 25 μg/mL Hygromycin (for pHUB10 series constructs) into sterilized 0.8% MS agar-base medium in the sterile hood and let the plates dry well before planting. Usually half an hour with the lids open is sufficient. Sterilize about 100 μL of seeds for each plate according to the protocol. Re-suspend seeds with 1 mL sterile 0.1% agar and transfer it to selection agar plate by using 1 mL pipette, distribute seeds on the whole plate by adding 3ml sterile 0.1% agar and shaking the plant gently. Open the lid of plate in the hood to let the liquid evaporate until no visible water. Seal plates with Parafilm and place to growth chamber. After 7-10 days, transgenic plants will be visible as growth normal. Transfer seedlings into soil and place under normal growth conditions. Further validation of transgenic plants by PCR or western blot analysis after growth for 3 weeks, and then collect mature seeds for later experiments.
1.2.7 Expression and purification of recombinant protein in *E.coli*

Full-length CDS of two proteases (At1g76140 and At2g47390) were used for cloning to pET30a vector and express in *E.coli*. The CDS of two genes were amplified by PCR with the following primers respectively:

At1g76140-*Sal*-F: 5’-ACGCGTCGACAAATGCTCACAGCGTTTGCAAGT-3’ and At1g76140-*Not*-R: 5’-ATTTGCGGCCGCTCCGATGAAGCATTCAC-3’;

At2g47390-*Nde*-F: 5’-GGGCATATGATGCGCTTTCATAAAGCTTG-3’ and At2g47390-*Sac*-R: 5’-GGGGAGCTCGAGAGAAGTGATCTCCTGAGCTTGGA-3’.

For cloning At1g76140 protease, PCR product and pET30a vector were digested with *Sal* and *Not*, and digested CDS was cloned to pET30a vector. For cloning At2g47390 protease, PCR product and pET30a vector were digested with *Nde* and *Sac*, and digested CDS was cloned to pET30a vector. Recombinant plasmids were transformed into *E.coli* strain BL21, and single clone which screened out from kanamycin LB agar plate was inoculated into 10 ml 2× YT medium for overnight incubation in 37°C shaker. The bacterial culture was 1:100 diluted into fresh 2× YT medium and test protein expression by using different temperature incubation. When protein can express, then the bacterial culture was 1:100 diluted into 800 mL 2× YT medium and continue to incubation in 37°C shaker till to OD₆₀₀ reach 0.6. The recombinant proteases were induced expression by IPTG. Cells were harvested by 5,000 rpm for 15 min. Protein was extracted by sonication in lysis buffer pH 8.0 containing 50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole for Ni-NTA beads purification according to its protocol.

The recombination construct pET30a-LeACS2-GST (construct done by Dr.LI Jianfeng) was transformed into *E.coli* BL21 star™ (DE3) pLysS cells for LeACS2-GST overexpress in it. Inoculate freshly transformed single colony into 4 mL 2× YT medium containing 25 μg/mL Kanamycin antibiotic, and culture overnight in 37°C shaker. Dilute 100 times of overnight *E.coli* culture medium into big volume (400ml) of 2×YT medium, and continue to culture in 37°C shaker for 2-3 h until OD₆₀₀ to 0.4-0.6. IPTG was then added to 0.5 mM final concentration, cells were cultured in 30°C shaker for 5 h. Cell cultures were harvested by
centrifugation at 5,000 rpm for 15 min, get rid of supernatant and responded pellet with 16 mL ice-cold GST binding buffer (pH 7.3) containing 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 137 mM NaCl and 2.7 mM KCl. Cells were disrupted by 30 min sonication on ice for 5 sec for optimal times with 10 sec cooling intervals. The cell lysate was centrifuged at 10,000 ×g for 10 min, and the resulting supernatant was mixed with 500 µL glutathione-agarose beads. This affinity beads was pre-equilibrated with GST binding buffer. Binding was performed at 4°C for 1 h under gentle shaking. The glutathione-agarose beads were centrifuged at 400 ×g for 5 min, get rid of supernatant and wash bead with 2 mL GST binding buffer for five times. Finally, the fusion protein Le-ACS2 was eluted with 1 mL elution buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione. Protein concentration of the eluate was measured by DC assay. Protein was stored at -80°C for later proteolytic assay experiment.

1.2.8 Preparation of *Arabidopsis* crude extract for protease purification

Fifty gram *Arabidopsis* wounding tissue powder from -80°C refrigerator were dissolved into 250 mL phosphate-buffered saline buffer pH 8.0 containing 20 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$ and 1 mM MgSO$_4$. The suspensions were incubated at 4°C for 30 min with gently shaking and the extracts were then centrifuged at 12,000 ×g for 10 min at 4°C to get rid of some debris. The supernatants were filtered through two layers of cheesecloth and followed by filtration through a 0.45-µm filter. The extract was used for protease purification with FPLC method.

1.2.9 Preparation of plant RNA and synthesis of cDNA

*Arabidopsis* leaf tissues were harvested by using liquid nitrogen and grinded into powder. Put 0.1 g tissue powder into 1.5 mL Eppendorf tube and add 1 mL TRIzol reagent (Invitrogen) immediately, mix well by vortex. Add 0.2 mL chloroform into tube and shake tube vigorously for 15-30 seconds by hand, incubate at room temperature for 5 minutes. Centrifuge at 11,000 rpm for 10 min at 4°C. Carefully remove the upper aqueous phase which contains the total RNA, and place this into a new tube. Add equal volume of isopropanol and 1/10 volume of
pH 5.3 DEPC NaAC in the tube, then precipitate RNA at -20°C at least 2 hours. Centrifuge at 8,800 rpm for 10 min at 4°C. Wash the pellet with 1 mL 75% DEPC ethanol, centrifuge at 8,800 rpm for 2 min, dry the pellet at room temperature (do not dry completely) and re-suspend the RNA pellet in 50 μL DEPC water. Get rid of DNA by adding 2 μL DNase I (NEB) and incubate at 37°C for 1 h. cDNA synthesis by using SuperScrit®III RT First Strand Synthesis Kit (Invitrogen) according to its protocol.

### 1.2.10 Purification of ACC synthase-processing protease from *Arabidopsis* by FPLC

Place the beaker with the *Arabidopsis* extract on ice water mixture, add -20°C pre-cold ethanol into it slowly (mix well by rotating stir bar at the same time) to 50% final concentration. The protein precipitates were centrifuged at 16,000 ×g for 10 min at 4°C and the pellets were re-suspended in 20 mL extraction buffer containing 20 mM Tris-HCl and 10 mM MgSO₄, pH 8.4. The protein extracts were centrifuged again at 16,000 ×g for 10 min at 4°C and the resulting supernatant was fractionated by FPLC (Pharmacia Biotech AB, Uppsala, Sweden) with a Protein-Pak DEAE 8HR column (5 mm×50mm; Waters, Milford, MA, USA) that was pre-equilibrated with 20 mM Tris-HCl, pH 8.4. Then proteins were eluted from the DEAE column with a linear gradient of 0-0.8 M NaCl in 50 mM MES buffer, pH 6.2. The eluted protein from each fraction was assayed to detect ACC synthase-processing protease activity, and the fractions containing high activities were pooled, desalted, and concentrated to 2 mL by using a Centriprep 30 (Amicon). The concentrated protein extracts were consequently fractionated by FPLC with a Superdex 75 (GE Healthcare) gel filtration column (1 × 30 cm) which was pre-equilibrated with buffer containing 50 mM sodium phosphate and 150 mM NaCl, pH 7.0, and each fraction was used for protease activity assay. Those fractions with high protease activities were again pooled and concentrated to 2 mL using a Centriprep 30. The concentrated sample was loaded into a Mono Q column (5 × 50 mm; GE Healthcare) that was pre-equilibrated with buffer containing 20 mM Tris-HCl, pH 8.4, and eluted with a linear gradient of 0-0.5 M NaCl in 50 mM HEPES buffer, pH 7.4. Protein samples of each
fraction was again assayed for protease activity detection and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purification procedures were performed at 4°C. A flow rate of 0.5 mL/min was applied for FPLC chromatography and the fraction size was 2 mL.

1.2.11 Assay for proteolytic activity

The recombinant proteins LeACS2 which expressed and purified from *E.coli* was use as substrates in proteolytic assay. Refer to the method of Janzik et al. (Janzik et al., 2000), we used a modified buffer containing 20 mM Tris-HCl, 100 mM NaCl, 5 μM ZnCl₂, 2 mM CaCl₂, pH 8.0, as the assay reaction buffer. The in vitro assay was performed at 37°C. The proteolytic products were analyzed by SDS-PAGE gel or western-blot analysis and the degradation rate was calculated by digitization and densitometric measurement of the SDS-PAGE gel with the software Photoshop 7.0.

1.2.12 SDS-PAGE gel separation and protein in-gel digestion

The proteins eluted from each column of FPLC purification were separated on 10% SDS-PAGE mini gel followed by an in-gel trypsin digestion for resolving proteins. The gel was stained with Coomassie Blue and cut into small pieces (1 x 1 mm) of each lane. Gel bands were destained in solution (50% ACN, 25 mM NH₄HCO₃) and dehydrated in 100% ACN. To reduce the proteins by adding freshly prepared 10 mM dithiotreitol (DTT) solution in 25 mM NH₄HCO₃ and place in 56°C water bath for 1 h. The solution was replaced with 55 mM iodoacetamide in 25 mM NH₄HCO₃ to alkylated the thiol groups for 30 min at room temperature with shaking under dark condition. After wash with 25 mM NH₄HCO₃ and dehydration with ACN, the gel pieces were swollen in a trypsin digestion buffer containing 25 mM NH₄HCO₃, 30 μg/mL of TPCK-treated trypsin, buffer covered gel pieces and incubated at 37°C for 16-18 h. Peptides of digested proteins were extracted twice by using a solution containing 50% ACN, 1% TFA, and dry completely by freeze dryer before stored at -80°C.
1.2.13 NanoLC-ESI-Q-TOF MS/MS analysis

To analyze peptides information from in-gel digested proteins, LC-MS/MS was performed with a nanoflow LC (nano Acquity™, Waters) coupled to an ESI-hybrid quadrupole time-of-flight (Q-TOF) Premier tandem mass spectrometer (Waters). A 180-μm × 20 mm Symmetry C18 trap column and 75-μm × 250 mm BEH130 C18 analytical column were used to trap and separate peptides. The MassLynx program (version 4.1, Waters) was used for data acquisition and instrument control. The mobile phases were buffer A (0.1% HCOOH in water) and buffer B (0.1% HCOOH in ACN). LC gradient elution condition was initially 1% to 5% buffer B (5 min), 42% buffer B (90 min), 80% buffer B (95 min), 99% buffer B (105-108 min), and then initial concentration 1% buffer B (109-120 min), with a flow rate of 0.2 μL/min.

The mass spectrometer was operated in positive ion mode with following setting: capillary voltage is 2.4 kV, sample cone voltage is 35 V, source temperature is 80°C, and collision cell gas flow rate is 0.5 ml/min. Data-dependent analysis setting as following: 1 s MS m/z 250-2000 and max 3 s MS/MS m/z 50-2000 (continuum mode), 30 s dynamic exclusion. Three most abundant with +2, +3 and +4 charged ions were selected in each MS/MS scan with the intensity more than 40 counts/s. Raw data were processed with MassLynx 3.5 ProteinLynx (smooth 3/2 Savitzky Golay and center 4 channels/80% centroil) to produce .pkl file, and the resulting MS/MS dataset was searched against Tair7 database (TAIR9_pep_20090619, from www.arabidopsis.org and specific for Arabidopsis). And the settings in the workflow template as follows: 100 ppm mass tolerance for MS precursor ions and 0.1 Da mass tolerance for MS/MS fragment ions; trypsin digestion with up to two missed cleavage sites were allowed; Carbamidomethylation (C) was specified as fixed modification, oxidation (M) and phosphorylation (SYT) were set as variable modifications. Internal calibration mass errors were generally less than 50 ppm in the m/z range 50-2000 with [Glu]-Fibrinopeptide B (m/z of locked mass = 785.8426 for the doubly charged ion).
1.2.14 β-Estradiol induce protein overexpression in transgenic plant

Transgenic *Arabidopsis* plants of pER10-CFP-AtACS2-YFP construct (construct done by Dr. SHI Lin) transformation growth in MS agar jars or soil, and spray 10 μM β-estradiol in 0.1% DMSO solution on leaves of one to three weeks old plants for 36 h to let the transgenic protein express in plants. β-estradiol treatment time can be change according to protein expression level or experimental requirement. The plants which treated with β-estradiol will be used for later experiments.

1.2.15 Tissue preparation and protein extraction under fully denature condition

To study ACC synthesis process in the developmental stages, pER10-CFP-AtACS2-YFP construct transformed transgenic plants growth in MS agar base jars. Spray 10 μM β-estradiol on leaves of 2, 3, 4, 5, 6, 7-week-old transgenic plants for 36 h induction respectively, and harvest tissue by liquid nitrogen. For cold treatment experiment, three weeks old transgenic plants were sprayed with 10 μM β-estradiol for 36 h, and placed at -20°C for 0.5, 1, 2, 4 h respectively, and then placed in room temperature for 0.5 h, untreated plants as control. Tissues were harvested by liquid nitrogen. For wounding treatment experiment, 3-week-old transgenic plants were sprayed with 10 μM β-estradiol for 36 h, then cut leaves into small piece (about 2 mm width) and wounding for 0, 0.5, 1, 2, 4, 12 h respectively, then collected tissue by using liquid nitrogen. In NaCl infiltration treatment experiment, 3-week-old transgenic plants in jars were sprayed with 10 μM β-estradiol for 36 h, then added 100 ml solution with 0, 150, 300, 450, 600 mM NaCl to different jars and infiltration for 15 min, then poured out solution and harvested tissue by liquid nitrogen. In UV treatment experiment, 3-week-old transgenic plants in jars were sprayed with 10 μM β-estradiol for 36 h, treated with UV-A and UV-B at the same time for 0, 2, 4, 8, 12 h respectively, and harvested tissue by liquid nitrogen. For ethylene treatment experiment, 3-week-old transgenic plants in jars were sprayed with 10 μM β-estradiol for 36 h, placed plants in boxes and injected air or
ethylene (5 ppm) respectively, treated for 2, 4, 8, 12 h and harvested tissue by liquid nitrogen. Tissues were stored at -80°C for later use.

The frozen *Arabidopsis* plant tissue (0.6 g) was ground to fine powder with -80°C pre-cold mortar and pestle. The tissue powder was extracted with 5 volume (g/mL) of extraction buffer (Guo and Li, 2011) (150 mM Tris-HCl, pH 7.6, 8 M urea, 0.5% SDS, 1.2% Triton X-100, 5 mM ascorbic acid, 5 mM DTT, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 1% glycerol 2-phosphate, 1 mM PMSF, 0.5% phosphatase inhibitor cocktail 2, 1× protease inhibitor (complete EDTA free; Roche) and 2% polyvinylpolypyrrolidone). The extract was centrifuged at 110,000 ×g for 2 h at 12°C to get rid of cell debris at the bottom of centrifuge tube. The total protein in supernatant was precipitated with 3 volumes of -20°C pre-cooled acetone:methanol (12:1) for at least 2 hours. Collected the protein pellet by centrifugation at 11,000 ×g for 20 min and re-suspended it in protein re-suspension buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 1% SDS, 5 mM DTT, 10 mM EDTA). The concentration of total protein was measured by DC assay method and the proteins were used for western-blot analysis.

### 1.2.16 Confocal microscopy observation for AtACS2 protein localization and FRET detection

Detection of CFP, YFP fusion protein and FRET fluorescence were performed using a Zeiss LSM 710 confocal microscopy. Imaging of the root cells was achieved with a Plan-Apochromat ×40/1.3 oil immersion objective. CFP was excited with 405-nm laser line and emission wavelength from 440-480 nm; YFP was excited with the 488-nm argon laser line, and emitted light was collected through a dichroic mirror on detectors from 520 to 550 nm. A transmitted light detector was used for collecting bright-field images. For FRET fluorescence, 405-nm excitation and 520 to 550 nm emission wavelength were used in the experiment.
1.3 Result

1.3.1 Expression and purification of substrate LeACS2-GST fusion protein in *E.coli*

Protein overexpression in *E.coli* is a common biotechnology to get enough protein for in vitro assay experiment. In previous study, substrate LeACS2-GST fusion protein can overexpression in *E.coli* and can be purified by using glutathione-agarose beads to get pure proteins (Li et al., 2005c). In this study, recombinant construct pET30a-LeACS2-GST was transformed into BL21 strain, and fusion gene LeACS2-GST also can overexpress in *E.coli*. The molecular weight of the fusion protein is 80 kDa (55 kDa full-length LeACS2 protein C-terminus fusion with 25 kDa GST tag protein). The overexpression protein is a soluble fusion protein, and it can be extracted by using native extraction buffer. As the soluble fusion protein contains GST tag protein on C-terminus, and it was purified by using glutathione-agarose beads. The purification result shows that eluted protein from glutathione-agarose beads is very pure, most of *E.coli* intrinsic proteins without GST tag have been washed away from beads (Figure 1.3.1). Figure 1.3.1 also shows that the fusion protein can be processed by itself slowly even without protease existence, small amount of full-length fusion protein was divided to about 55 kDa proteins and 25 kDa proteins.
Figure 1.3.1 Fusion protein LeACS2-GST purification result from *E.coli*.

M: Low molecular weight protein marker.

1-6: Fractions eluted from glutathione-agarose beads.

F: Protein of flow through from glutathione-agarose beads.

W: Protein in buffer after washing beads.
1.3.2 Protease activity validation in wild-type *Arabidopsis* plant by using in vitro proteolysis assay

According to the alignment analysis result of tomato LeACS2 and *Arabidopsis* AtACS2 proteins sequence (Figure 1.3.2), they have high similarity and some cleavage sites which have been validated in previous study on C-terminal homology region of two proteins (Li et al., 2005a), we predict that the protease in *Arabidopsis* plant can cleave C-terminus of both AtACS2 and LeACS2 proteins. Therefore, the purified fusion protein LeACS2-GST was used as the substrate in proteolysis assay to validate protease activity from *Arabidopsis* plant for cleaving C-terminus of ACS2 protein. Based on the proteolysis assay system, C-terminus of tomato ACS2 (LeACS2) can be cleaved in vitro by tomato plant’s protease in previous study (Kuddus et al., 2005). So we used the similar in vitro assay method to find protease from *Arabidopsis* plant which can cleave C-terminal of *Arabidopsis* ACS2 protein. In the experiment, 5 μg LeACS2-GST fusion protein which purified from in E. coli as substrate, and add 3 μg crude extract of wounding *Arabidopsis* wild type plant into it and mix well in tube. Without adding *Arabidopsis* crude extract assay as control. After 3 h digest at 37°C, the result shows that most of substrate was processed by protease from wild-type *Arabidopsis* plant (Figure 1.3.3). About 25 kDa GST tag protein was separated from C-terminal of about 55 kDa LeACS2 protein in vitro. This data indicate that the protease maybe exist in wild-type *Arabidopsis* plant which can cleave C-terminal of ACS2 protein.
Figure 1.3.2 Alignment analysis of Tomato LeACS2 and Arabidopsis AtACS2 protein sequences. Arrow indicate cleavage sites of tomato LeACS2 protein C-terminus.
Figure 1.3.3 SDS-PAGE profile of proteolysis assay result to validate the protease in wild-type *Arabidopsis* plant.

M: Low molecular weight protein marker.

1: Only 5 μg LeACS2-GST substrate in assay.

2: Only 3μg *Arabidopsis* total protein in assay.

3: 5 μg LeACS2-GST substrate plus 3 μg *Arabidopsis* total protein in assay.
1.3.3 Purification of the protease from wild-type *Arabidopsis* plant

Based on the proteolysis assay result that the protease in *Arabidopsis* wild-type plant can cleave C-terminal of ACS2 protein and a 64 kDa putative metalloprotease which purified by FPCL from tomato belongs to a family peptidase of *Arabidopsis* in previous study (Li et al., 2005c), and FPLC method was used to purify the protease in *Arabidopsis* plant. The purification procedure to isolate the putative ACS2-proteolyzing protease from *Arabidopsis* plant extract has been described in ‘Materials and Methods’. Cutting leaves of 2-week-old wild-type *Arabidopsis* plant (Figure 1.3.4), and wounding 5 h before collecting tissue by liquid nitrogen. 200 g wild-type *Arabidopsis* plant tissue powder was used for protease purification. Briefly, 2.34 g protein of crude extract was obtained from tissue powder, and after 50% ethanol precipitation, about 126 mg protein was obtained which can dissolved in DEAE column loading buffer and used for FPLC purification. Proteins which bind to DEAE column were eluted off by using a linear NaCl gradient from 0 to 0.8 M in 60 min. This eluate was collected in 15 fractions and fractions 2-13 were used for an in vitro protease assay to validate protease activity (Figure 1.3.5). The result shows protease in fractions 4-9 has the activity to cleave C-terminal of LeACS2 protein by SDS-PAGE analysis of proteolytic assay result. In order to get rid of other proteins, three fractions (fraction 6-8) were then pooled with Centriprep 30 and applied to the next chromatography (gel filtration column) purification. 22 fractions eluted from gel filtration column were collected and fractions 5-17 were used for in vitro proteolytic assay experiment to validate protease activity (Figure 1.3.6). Figure 1.3.6 shows that protease in fractions 8-13 has relatively higher activity than other fractions by SDS-PAGE analysis of proteolytic assay result. These six fractions were pooled and used for the last purification step involving MonoQ chromatography. Proteins which can bind to MonoQ column were eluted off by using a linear NaCl gradient from 0 to 0.5 M in 64 min. This eluate was collected in 16 fractions and fractions 6-16 were used for an in vitro protease assay to validate protease activity by SDS-PAGE analysis of proteolytic assay result (Figure 1.3.7). The in vitro proteolytic assay result shows that protease in fraction 9-14 has protease activity to process C-terminal of LeACS2 protein by SDS-PAGE analysis. The protein
concentration of these fractions was detected by using DC assay, and about 40 μg putative protease was obtained in these six fractions.

In order to calculate the protease specific activity variation after FPLC purification, 5 μg substrate was used and plus protein after 50% ethanol precipitation of crude extract, eluate from each column respectively in the in vitro proteolytic assay experiment. Based on the proteolytic assay experiment to validate protease activity after DEAE column, gel filtration column and MonoQ column purification, optimal protease and assay time were chosen to differentiate the protease specific activity by using SDS-PAGE and western-blots analysis. Figure 1.3.8 shows that protease specific activity increase after FPLC purification. 0.1 μg protease eluted from MonoQ column can process LeACS2-GST more quickly than from eluted from gel filtration column in 20 min in vitro assay experiment. Proteases in crude extract after 50% ethanol precipitation or eluted from DEAE column have lower activity than eluted from gel filtration column. Western-blot analysis result with anti-AtACS2 antibody also shows the similar result of SDS-PAGE that the protease specific activity has strongly increased after FPLC purification (Figure 1.3.9). The relative degradation rate of the substrate LeACS2-GST on SDS-PAGE profile (Figure 1.3.8) was determined by digitization of pixel intensity with Photoshop 7.0 software. The result shows protease activity in crude extract after 50% ethanol precipitation is only 3.47 ± 0.47 (•10⁻⁴ μg substrate/min/μg protease), after DEAE column purification the protease specific activity increase to 105.47 ± 23.01 (•10⁻⁴ μg substrate/min/μg protease). After gel filtration and MonoQ column purification, the protease specific activity increase to 1271.70 ± 104.38 and 2829.98 ± 77.16 (•10⁻⁴ μg substrate/min/μg protease) respectively (Figure 1.3.10). Therefore, protease purified by FPLC can increase its activity greatly, and then was used for LC-MS/MS analysis to identify peptides which belong to protease.
Figure 1.3.4 Phenotype of 2-week-old wild-type *Arabidopsis* plant. Scale bar = 1 cm
Figure 1.3.5 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC DEAE column purification.

M: Low molecular weight protein marker.

2-13: Fractions eluted from DEAE column, proteolytic activities in these fractions were assayed by incubating 10 μl aliquots of each fraction with 3 μg LeACS2-GST at 37°C for 3 h.
Figure 1.3.6 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC gel filtration column purification.

M: Low molecular weight protein marker.

5-17: Fractions eluted from gel filtration column, proteolytic activities in these fractions were assayed by incubating 10 µl aliquots of each fraction with 3 µg LeACS2-GST at 37°C for 3 h.
Figure 1.3.7 SDS-PAGE profile of proteolysis assay result to validate protease activity after the FPLC MonoQ column purification.

M: Low molecular weight protein marker.

6-16: Fractions eluted from MonoQ column, proteolytic activities in these fractions were assayed by incubating 10 μl aliquots of each fraction with 3 μg LeACS2-GST at 37°C for 3 h.
Figure 1.3.8 SDS-PAGE profile of proteolysis assay result to compare protease activity after the FPLC steps purification.

M: Low molecular weight protein marker.

a: 5 μg LeACS2-GST at 37°C for 120 min

b: 5 μg LeACS2-GST plus 5 μg wild-type Arabidopsis crude extract at 37°C for 120 min.

c: 5 μg LeACS2-GST plus 0.2 μg DEAE column elution at 37°C for 90 min.

d: 5 μg LeACS2-GST plus 0.1 μg gel filtration column elution at 37°C for 20 min.

e: 5 μg LeACS2-GST plus 0.1 μg MonoQ column elution at 37°C for 20 min.
Figure 1.3.9 Western-blot analysis of proteolysis assay result with anti-ACS2 antibody to compare protease activity after the FPLC steps purification.

M: Low molecular weight protein marker.

a: 0.5 μg LeACS2-GST at 37°C for 120 min.
b: 0.5 μg LeACS2-GST plus 0.5 μg wild-type Arabidopsis crude extract at 37°C for 120 min.
c: 0.5 μg LeACS2-GST plus 0.02 μg DEAE column elution at 37°C for 90 min.
d: 0.5 μg LeACS2-GST plus 0.01 μg gel filtration column elution at 37°C for 20 min.
e: 0.5 μg LeACS2-GST plus 0.01 μg MonoQ column elution at 37°C for 20 min.
Figure 1.3.10 Quantification of protease specific activity after the FPLC steps purification.

Step1: 50% ethanol precipitation of wild-type Arabidopsis crude extract with SDS-PAGE analysis.

Step2: DEAE column purification result of SDS-PAGE analysis.

Step3: Gel filtration column purification result of SDS-PAGE analysis.

Step4: MonoQ column purification result of SDS-PAGE analysis.

The relative degradation rate of the substrate LeACS2-GST was determined by digitization of pixel intensity with Photoshop 7.0 software, three times repeat of in vitro assay experiment, error bar = ± SE.
1.3.4 Purified protease identification with Q-TOF MS/MS

Based on in-gel trypsin digestion method can obtain peptides, protease eluted from MonoQ column was used for Q-TOF MS/MS analysis. Protein in *Arabidopsis* crude extract after 50% ethanol precipitation and eluate of each column with FPLC purification was profiled by using 10% SDS-PAGE (Figure 1.3.11). 2 μg protein of before and after steps purification was used for SDS-PAGE and profiled by using coomasie blue stain and silver stain methods. Protein on silver stain SDS-PAGE gel shows eluate from MonoQ column still contains many proteins, protease is not very pure after FPLC purification. SDS-PAGE gel lanes of fraction 11 and 12 eluted from MonoQ column were cut into three parts and in-gel digest proteins with trypsin, the peptides were analyzed by Q-TOF MS/MS. A total of seven proteases were identified after FPLC purification (Table 1.3.1), and some other protein also identified by Q-TOF MS/MS. Proteases ID and description as follows: AT1G76140 (Prolyl oligopeptidase family protein, functions in: serine-type peptidase activity and serine-type endopeptidase activity), AT2G47390 (Prolyl oligopeptidase family protein, functions in: serine-type peptidase activity and serine-type endopeptidase activity, AT3G14067 (Subtilase family protein), AT3G19400 (Putative cysteine proteinase), AT1G47128 (RD21 responsive to dehydration 21 cysteine type peptidase), AT5G45120 (Aspartyl protease family protein), AT1G49630 (ATPREP2 metalloendopeptidase). Figure 1.3.12 shows peptides MS/MS spectrum of proteases which identified by Q-TOF MS/MS. According to peptides’ ion count intensity and identified time, four proteases (AT1G76140, AT2G47390, AT3G14067 and AT1G47128) were selected for further study by using protease mutant lines.
Figure 1.3.11 SDS-PAGE profile of protease after steps purification.

M: Low molecular weight protein marker.

a: 2 μg protein of 50% ethanol precipitation of *Arabidopsis* crude extract.

b: 2 μg protein eluted from DEAE column.

c: 2 μg protein eluted from gel filtration column.

11,12: 2 μg protein eluted from MonoQ column in fraction 11,12.

(1), (2), (3): Gel range for protease validation with Q-TOF MS/MS analysis eluted from MonoQ column fraction 11, 12.
Table 1.3.1 Proteases identified by LC-MS/MS

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Description</th>
<th>Molecular weigh (Da)</th>
<th>Identified time</th>
<th>Ion count</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G76140</td>
<td>Prolyl oligopeptidase</td>
<td>89348</td>
<td>3</td>
<td>2260.4</td>
<td>3.01</td>
</tr>
<tr>
<td>AT2G47390</td>
<td>Prolyl oligopeptidase</td>
<td>106003</td>
<td>4</td>
<td>455.5</td>
<td>1.46</td>
</tr>
<tr>
<td>AT3G14067</td>
<td>Subtilase family protein</td>
<td>81766</td>
<td>3</td>
<td>9364.8</td>
<td>7.34</td>
</tr>
<tr>
<td>AT3G19400</td>
<td>Putative cysteine proteinase</td>
<td>40264</td>
<td>3</td>
<td>616.4</td>
<td>6.65</td>
</tr>
<tr>
<td>AT1G47128</td>
<td>RD21 responsive to dehydration 21 cysteine type peptidase</td>
<td>50993</td>
<td>2</td>
<td>8489.7</td>
<td>10.6</td>
</tr>
<tr>
<td>AT5G45120</td>
<td>Aspartyl protease family protein</td>
<td>53713</td>
<td>1</td>
<td>312.4</td>
<td>6.72</td>
</tr>
<tr>
<td>AT1G49630</td>
<td>ATPREP2 metalloendopeptidase</td>
<td>21055</td>
<td>1</td>
<td>3357.1</td>
<td>1.85</td>
</tr>
</tbody>
</table>
Figure 1.3.12 MS/MS spectrum of peptides of purified proteases identified by QTOF-MS.

(Continued)
Continuing of Figure 1.3.12

AT3G14067

AT3G14067

AT1G47128

AT1G47128

AT1G47128

AT1G47128

AT3G19400

AT1G49630
1.3.5 Protease homozygous single and double mutants screening

As T-DNA insertion in chromosome can cause the corresponding gene loss of function in plant. Based on peptides’ ion count intensity and identify time, we bought four proteases (AT1G76140, AT2G47390, AT3G14067 and AT1G47128) T-DNA insertion single mutant lines from Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) and screened homozygous loss of function lines for in vitro proteolytic assay experiment to validate protease activity in mutants. According to the information of the loss of function mutants on website (www.arabidopsis.org), T-DNA left border primer and gene primers on two sides of T-DNA insertion site were used in PCR validation. Figure 1.3.13 shows that by using genomic DNA of mutant plants as template in PCR validation revealed T-DNA in the four proteases single mutants respectively, so the protease gene can’t be translated to the corresponding protein. In order to further decrease protease activity, two prolyl oligopeptidase single mutants (at1g76140 and at2g47390) were used for crossing to get a double mutant line. Transcripts of at1g76140 and at2g47390 loss of function lines were validated by RT-PCR. RT-PCR results shows that the transcripts of AT1G76140 and AT2G47390 were absent in corresponding mutant plants (Figure 1.3.14). F1 generation of crossing result showed by Figure 1.3.15 that two protease heterozygous plants were obtained after crossing of two single mutants. In F2 generation plants after crossing, homozygous double mutant lines were screened out with PCR validation (Figure 1.3.16). Figure 1.3.16 shows that T-DNA in both of two genes to cause them loss of function at the same time. The four single mutants and one double mutant were used for in vitro proteolytic assay experiment to validate proteinase activity.
Figure 1.3.13 PCR validation of proteases loss of function lines.

A. PCR validation of four plants of \textit{at1g76140} mutant. M:100 bp DNA ladder; L1: forward primer specific for AT1G76140 and primer specific for the T-DNA left border; F1: gene forward and reverse primers.

B. PCR validation of five plants of \textit{at2g47390} mutant. M:1 kb DNA maker; L2: reverse primer specific for AT2G47390 and primer specific for the T-DNA left border; F2: gene forward and reverse primers.

C. PCR validation of four plants of \textit{at3g14067} mutant. M:100 bp DNA ladder; L3: reverse primer specific for AT3G14067 and primer specific for the T-DNA left border; F3: gene forward and reverse primers.

D. PCR validation of nine plants of \textit{at1g47128} mutant. M:100 bp DNA ladder; L: forward primer specific for AT1G47128 and primer specific for the T-DNA left border; F: gene forward and reverse primers.

WT: wild-type plant as negative control.
Figure 1.3.14 RT-PCR validation of *at1g76140* and *at2g47390* loss of function lines.

M: 1 kb DNA marker.

S1: RT-PCR result of full-length CDS of AT1G76140 gene in *at1g76140* mutant (~2.4 kb).

C1: RT-PCR result of full-length CDS of AT1G76140 gene in wild type (~2.4 kb).

S2: RT-PCR result of full-length CDS of AT2G47390 gene in *at2g47390* mutant (~2.9 kb).

C2: RT-PCR result of full-length CDS of AT2G47390 gene in wild type (~2.9 kb).

A1, A2, AC: RT-PCR result of ACTIN2 gene fragment in *at1g76140*, *at2g47390* and wild type respectively to check cDNA quality, PCR product is about 400 bp.
Figure 1.3.15 PCR to validate F1 generation plants of *at1g76140* and *at2g47390* mutants crossing.

M: 100 bp DNA ladder.

L1: forward primer specific for AT1G76140 and primer specific for the T-DNA left border.

F1: AT1G76140 gene forward and reverse primers.

L2: reverse primer specific for AT2G47390 and primer specific for the T-DNA left border.

F2: AT2G47390 gene forward and reverse primers.

WT: wild-type plant as negative control.
Figure 1.3.16 PCR to validation of F2 generation homozygous *at1g76140/at2g47390* double mutant.

M: 100 bp DNA ladder.

L1: forward primer specific for AT1G76140 and primer specific for the T-DNA left border.

F1: AT1G76140 gene forward and reverse primers.

L2: reverse primer specific for AT2G47390 and primer specific for the T-DNA left border.

F2: AT2G47390 gene forward and reverse primers.

WT: wild-type plant as negative control.
1.3.6 Protease activity validation in proteases mutants

The four proteases (at1g76140, at2g47390, at3g14067 and at1g47128) homozygous single mutants and one homozygous double mutant (at1g76140/at2g47390) were used in the in vitro assay experiment to validate protease activity in mutants for processing ACS2 C-terminus. SDS-PAGE gel was used to profile the in vitro assay result. Figure 1.3.17 shows that protease activity in three mutants (at1g76014, at2g47390 and at1g47128 mutants) for processing ACS2 C-terminal decrease by comparing with wild-type plant, only one mutant (at3g14067) still has high protease activity just link wild-type plant. The double mutant (at1g76014/at2g47390) also has lower protease activity than wild-type. Quantified data showed by Figure 1.3.18 shows 48.6% substrate was processed by wild-type plant protease in three hours assay; 30.0%, 29.2%, 22.3% and 28.0% substrate was processed by protease from at1g76140, at2g47390, at1g47128 and the double mutant respectively. At least, more than 18.6% substrate can’t be processed in mutant plants by comparison with wild-type plant. Therefore, these three proteases have the function to process ACS2 C-terminus in the in vitro assay experiments, maybe there also have the function to process AtACS2 C-terminus in vivo.
Figure 1.3.17 In-vitro proteolysis assay to validate protease activity in the proteases loss of function lines.

M: Low range protein marker.
C: Only substrate.
1: Substrate plus wild type proteins with DEAE column purification.
2: Substrate plus at3g14067 mutant proteins with DEAE column purification.
3: Substrate plus at1g47128 mutant proteins with DEAE column purification.
4: Substrate plus at2g47390 mutant proteins with DEAE column purification.
5: Substrate plus at1g76140 mutant proteins with DEAE column purification.
6: Substrate plus at1g76140/at2g47390 double mutant proteins with DEAE column purification.
Figure 1.3.18 Quantification analysis of substrate LeACS2-GST protein after time course in vitro proteolysis assay.

WT: wild-type.
Mutant 1: Cysteine type peptidase mutant (at1g47128).
Mutant 2: Prolyl oligopeptidase mutant (at2g47390).
Mutant 3: Prolyl oligopeptidase mutant (at1g76140)
DM: double mutant of mutant 2 and mutant 3.
1.3.7 Protease over-expression in *E.coli* and the activity validation

In order to further study the function of two prolyl oligopeptidase, wild-type RNA (Figure 1.3.19) was used to synthesize cDNA and full-length CDS of AT1G76140 and AT2G47390 genes were cloned to pET30a vector respectively. The recombinant plasmids were validated by enzyme digestion and PCR (Figure 1.3.20), transformed into bacterial *E.coli* strain BL21 and overexpression by IPTG inducible. Figure 1.3.21A shows that AT1G76140 protease can express in *E.coli* by 0.5 mM IPTG induce at 37 or 30°C. But AT2G47390 can’t express in *E.coli* with IPTG induce. Then over-expressed AT1G76140 proteins were purified with Ni-NTA beads, and Figure 1.3.21B shows that the *E.coli* expressed protein can be purified by using Ni-NTA beads. But the molecular weight of expression protein less than 89 kDa AT1G76140 protein, maybe the proteases can cleave itself. The purified protease was used for in vitro proteolytic assay to validate its activity. In vitro assay result as showed by Figure 1.3.22 that *E.coli* expressed AT1G76140 protease still has the function to process ACS2 protein C-terminus, but the protease activity is lower than from plants, maybe the structure of *E.coli* expressed protease has changed or been processed by itself. When we added protease amount increase to 2 and 3 μg from 1μg in assay experiment, more substrate can be processed by the protease in 3 h assay experiment.
Figure 1.3.19 Validation of RNA samples by electrophoresis.

1, 2: RNA from wild-type plants.
Figure 1.3.20 Validation of pET30a-AT1G76140 (A) and pET30a-AT2G47390 (B) constructs by PCR and enzyme digestion.

M: 1 kb DNA marker.
1, 2, 3, 4: Clone number of pET30a-AT1G76140 construct, enzyme digestion product and PCR product about 2.4 kb. NC: Negative control.
a, b: Clone numbers of pET30a-AT2G47390 construct, enzyme digestion product and PCR product about 2.9 kb.
Figure 1.3.21 Validation of *E. coli* over-expression proteases and purification by SDS-PAGE.

**A.** Protease over-expression validation. M: Low molecular weight protein marker; C1 and C2: Negative control without adding IPTG; 1 and 4: 37°C inducible expression with 0.5 mM IPTG for 5 h; 2 and 5: 30°C inducible expression with 0.5 mM IPTG for 5h; 3 and 6: 18°C inducible expression with 0.5 mM IPTG for 8h.

**B.** AT1G76140 protease purification from *E. coli*. M: Low molecular weight protein marker; E1: Purified protein by using Ni-NTA beads (Arrow head shows the protein 89 kDa).
Figure 1.3.22 SDS-PAGE profile of proteolysis assay to validate the *E.coli* expressed protease activity.

M: Low range protein marker.

Ctr: 5 µg LeACS2-GST at 37°C for 3 h.

a: 5 µg LeACS2-GST plus 1 µg protease at 37°C for 3 h.

b: 5 µg LeACS2-GST plus 2 µg protease at 37°C for 3 h.

c: 5 µg LeACS2-GST plus 3 µg protease at 37°C for 3 h.
1.3.8 Fusion protein CFP-AtACS2-YFP expression in transgenic plant

Based on in vitro proteolytic assay result that ACS2 C-terminus can be cleaved in vitro by protease from both wild-type *Arabidopsis* plant and express in *E.coli*, CFP-AtACS2-YFP fusion gene under the control of estrogen inducible promoter was transformed into wild-type plants to detect protease processes C-terminal of transgenic AtACS2 protein in vivo by using western blot analysis. As the protein structure prediction model showed by Figure 1.3.23, that the distance between N-terminus and C-terminus is about 5.687 nm, less than 10 nm to satisfy requirement of FRET observation, so it also can be used for in vivo validation of AtACS2 protein C-terminus cleavage detection by using CFP/YFP FRET technology. The fusion gene was validated by using PCR in transgenic lines. The PCR validation result shows that CFP/YFP gene is in transgenic plants (Figure 1.3.24). The fusion protein expression level was validated by using western blot with anti-GFP antibody. Figure 1.3.25 shows that the fusion protein CFP-AtACS2-YFP can over-express in transgenic plant by 10 μM β-estradiol inducible. Three weeks old transgenic plant after 24 h inducing, transgenic protein was detected by western blot, and it can increase to the highest expression level after 36 h inducing from 0 to 48 h. The molecular weight of fusion protein is about 110 kDa, three protein bands on western blot means that the fusion protein was processed, ~90 kDa protein band indicated that partial YFP be cleaved, ~80 kDa protein band indicated that C-terminal of AtACS2 be cleaved and YFP was cut off. According to later experiment result, the process is not produce in vivo and the fusion protein was cleaved during protein extraction when protease released from cells. The protein localization was detected with confocal microscopy. Figure 1.3.26 shows that CFP and YFP fluorescence indicated the transgenic protein localized in nucleus of root cells. FRET fluorescence also can be detected by using CFP excitation wavelength and YFP emission wavelength, and the intensity is lower than YFP fluorescence, maybe partial AtACS2 transgenic proteins C-terminus be processed. We used this transgenic line to try to find out a condition for AtACS2 protein C-terminus can be process in vivo by using western blot analysis.
Figure 1.3.23 Structure prediction model of AtACS2 protein. The distance of N-terminus and C-terminus is about 5.687 nm. The prediction was done on line: http://raptorx.uchicago.edu/StructurePrediction/predict/
Figure 1.3.24 PCR validation of CFP-AtACS2-YFP::WT transgenic plants.

M: 1 kb DNA marker.
1-15: CFP-AtACS2-YFP::WT transgenic lines.
WT: wild-type background as negative control.
The arrowheads show the size of CFP/YFP gene PCR product is about 0.7 kb.
Figure 1.3.25 Western blot analysis of the fusion protein CFP-AtACS2-YFP inducible expression. M: Low range protein marker; 0, 12, 24, 36, 48: 0-48h time course of 10 μM β-estradiol inducible expression of CFP-AtACS2-YFP fusion protein in CFP-AtACS2-YFP::WT transgenic plants; W48: wild-type plant by spraying 10 μM β-estradiol for 48 h.
Figure 1.3.26 AtACS2 localization observation and CFP-YFP FRET validation with confocal microscopy.

3-week-old *CFP-AtACS2-YFP::WT* transgenic plants treated with 10 μM β-estradiol for 36 h, root tissues were mounted on glass slides in the presence of PBS buffer, fluorescence was detected using the confocal microscope with 40x lense, bar = 10μm.
1.3.9 Different treatment of \textit{CFP-AtACS2-YFP::WT} transgenic plant to find out a condition for transgenic AtACS2 can be processed in vivo

Western blot was used to detect the fusion protein processed under development stages and different treatments of transgenic plants, including wounding, cold, salt, UV and ethylene treatment. In development stages experiment, from 2 to 7 weeks old transgenic plants were used for were western blot analysis respectively. The result showed by Figure 1.3.27 that C-terminal of transgenic AtACS2 protein can’t be processed in vivo in 2 to 7 weeks developmental stages. As endogenous LeACS2 C-terminus can be processed by wounding treatment of ripe tomato fruit (Li et al., 2005a), wounding treatment was used to detect transgenic AtACS2 C-terminus’ cleavage. Figure 1.3.28 shows that transgenic AtACS2 C-terminus can’t be cleaved by protease after wounding treatment up to 12 hours. Based on cold treatment of 2-week-old wild-type Col plants can rapidly decrease ethylene production (Shi et al., 2012), we used cold treatment to validate protease activity to process transgenic AtACS2 in vivo. Western blot analysis data showed by Figure 1.3.29 that -20°C cold treated 3-week-old \textit{CFP-AtACS2-YFP::WT} transgenic plants in 4 h still can’t let transgenic AtACS2 C-terminus be processed by protease. UV, NaCl infiltration and ethylene treatments were used to validate protease activity for processing transgenic AtACS2 C-terminus. The western blot result shows that transgenic AtACS2 protein’s C-terminus still can’t be processed by UV, NaCl infiltration and ethylene treatments of transgenic plants (Figure 3.30-32). Maybe transgenic protein’s localization affects its function in vivo.
Figure 1.3.27 Western blot analysis of AtACS2 in-vivo process in developmental stages.

M: Low range protein marker.

2w-7w: 2 to 7 weeks old CFP-AtACS2-YFP::WT transgenic plants.
Figure 1.3.28 Western blot analysis of AtACS2 in-vivo process after wounding treatment.

M: Low range protein marker.

0-12h: 0-12 hours wounding treatment of CFP-AtACS2-YFP::WT transgenic plants.
Figure 1.3.29 Western blot analysis of AtACS2 in-vivo process after cold treatment.

M: Low range protein marker.

0-4h: 0-4 hours -20°C cold treatment of CFP-AtACS2-YFP::WT transgenic plants.
Figure 1.3.30 Western blot analysis of AtACS2 in-vivo process after NaCl infiltration treatment.

M: Low range protein marker.
Ctr: None treatment $CFP$-$AtACS2$-$YFP$::$WT$ transgenic plants.
0-600: 0-600 mM NaCl and 15min infiltration treatment of $CFP$-$AtACS2$-$YFP$::$WT$ transgenic plants.
Figure 1.3.31 Western blot analysis of AtACS2 in-vivo process after UV treatment.

M: Low range protein marker.

0-12h: 0-12 hours UV-A and UV-B treatment of CFP-AtACS2-YFP::WT transgenic plants.
Figure 1.3.32 Western blot analysis of AtACS2 in-vivo process after air or ethylene treatment.

M: Low range protein marker.

0-12h: 0-12 hours air or 5ppm ethylene treatment of $CFP$-AtACS2-YFP::WT transgenic plants.
1.4 Discussion

1.4.1 LeACS2-GST fusion protein overexpression in E.coli

As much protein substrate need to be used for in vitro assay experiment, we used PET expression system to get enough LeACS2-GST fusion protein by GST beads purification. E.coli expression system is one of the most extensively used prokaryotic expression system. It's advantages including inexpensive growth medium, high cell density and rapid biomass accumulation (Lesley, 2001). In generally, protein highly overexpress in E.coli can form inclusion body, which increased the difficulty for protein purification (Mukhija et al., 1995), and maybe affect the protein activity in later in vitro assay experiment. In order to avoid inclusion body formation and increase the solubel yield of the target LeACS2-GST fusion protein, we carried out the expression at a low-temperature (30°C) and succeeded getting the target protein at high expression level. And the construct LeACS2-GST could potentially be purified by using glutathione-agarose beads. The purification procedure could be performed with native GST and without denature, then it can be used for later in vitro assay experiment.

1.4.2 In vitro validation and purification of the protease which can process of ACS2 C-terminus

Proteins in plants or animals which have high homology in the amino acid sequences usually have similar biological function in vivo and they usually belong to a family. As tomato LeACS2 protein and Arabidopsis AtACS2 protein have highly conserve domain, and both of them belong to plant ACS family protein, we predict they have similar function in plant physiology during plant growth, development and stress response. Based on previous studies that tomato ACS2 protein C-terminal can be cleaved in to some small pieces by protease and the protease was also can be purified by HPLC approach (Li et al., 2005a; Li et al., 2005b), we suppose the protease in Arabidopsis plant which can cleave AtACS2 C-terminus, and it also can cleave C-terminal of tomato LeACS2. In our in vitro proteolysis assay result showed
that the protease in wild-type *Arabidopsis* plant actually can process LeACS2 protein C-terminus. And the substrate LeACS2 protein also can cleave itself very slow in the in vitro assay without protease existence (Figure 1.3.3). Although the in vivo proteolysis of the C-terminus of the ACC synthase has been mentioned in many studies (Edelman and Kende, 1990; Hansen et al., 2009; Joo et al., 2008; Kende, 1993; Sato et al., 1991; Sato and Theologis, 1989; Vanderstraeten et al., 1990), this present study is the first time attempt to purify an ACC synthase-processing enzyme from *Arabidopsis* plant. One of important factors which help us to validate the protease existence in plant and can be isolated from wild-type *Arabidopsis* tissues is that the GST tag is resistant to proteolysis because of its compact structure. The GST tag fused to the C-terminal of LeACS2 protein was not only to facilitate the recombinant protein purified from an *E.coli* heterogenous expression system, but also mark the difference between about 80 kDa unprocessed and about 55 kDa processed substrate on SDS-PAGE gel for profiling in vitro assays result. Another important factor to help us purify the protease from *Arabidopsis* plant is that most of the abundance expression protein such as rubisco can be got rid of from plant crude extract after 50% ethanol precipitation, but the protease still can dissolve in native phosphate buffer without any factor to change the protease activity after the precipitation and still have the activity to process LeACS2 protein C-terminus in the in vitro assays. Thus the total protein can be concentrated to small volume for HPLC purification with not very high protein concentration, and it easy for separation of the protease on the column.

The *Arabidopsis* protease was isolated with three columns purification, via a week anion exchange column (DEAE column), a 30 cm length gel filtration column, and a strong anion exchange column. Based on differences in the physical properties of the three chromatography columns, the separation of the candidate protease from wild-type *Arabidopsis* crude extract was feasible. However, protein degradation during the purification procedure without adding protease inhibitors caused the low yield of the protease. After multi-step column chromatography, the *Arabidopsis* candidate proteases were detectable by SDS-PAGE gel with silver stain, the problem is that too many other proteins still in elution fractions and it’s difficult to differentiate the candidate protease from none function protease and some other proteins (Figure 1.3.11). After LC-MS/MS analysis of purified protease, seven
proteases and some other proteins were identified. Because we focus on studying protease, so the biological function study of these proteases is our research purpose. According to the procession pattern of in vivo proteolysis of LeACS2 protein by immunoblotting analysis (Li et al., 2005a), we predict that these purified proteases maybe responsible for the in vivo C-terminal truncation of ACC synthase in Arabidopsis plant. However, this prediction awaits more in vivo experimental evidence to support it. On the basis of the result and prediction, these proteases would be a step closer to reveal the in vivo ACC synthase-processing enzyme in response to stimulation during plant wounding or senescence (Jakubowicz, 2002). Therefore, study of proteases mutants and protein-protein interaction stimulate us to attempt to find out the proteases biological function for processing AtACS2 C-terminus in vivo.

1.4.3 Protease activity validation in T-DNA insertion loss of function mutant lines

The T-DNA of Agrobacterium tumefacians has been used as mutagens to created loss of function mutants by inserting on genomic DNA of the genes in plants (AzpirozLeehan and Feldmann, 1997). T-DNA insertion proteases mutants were guaranteed that the protease genes can’t express in plants. In the basis of this biotechnology, these mutant lines can be used for detect the protease activity for procession AtACS2 C-terminus. According to ion count intensity and identify time from LC-MS/MS result, we have bought four proteases single mutants (at1g76140, at2g47390, at1g47128 and at3g14067) from Arabidopsis Biological Resource Center, and we also have got a double mutant by crossing with two single mutants (at1g76140 and at2g47390). From the in vitro assay result, although one protease mutant still has high protease activity, three proteases T-DNA insertion single mutants and the double mutant have lower protease activity than wild-type plant, especially the at1g47128 (RD21, Responsive-to-Desiccation-21)) protease mutant line has the lowest protease activity than other two mutants. RD21-like proteases are ubiquitous, which carry a C-terminal granulin domain and is one type of plant-specific papain-like proteases (Koizumi et al., 1993; Yamada et al., 2001). Its function is relative to immunity, senescence, and various types of biotic and
abiotic stresses (Gu et al., 2012). RD21 is localized in the vacuole, which involved in protein degradation (Kikuchi et al., 2008), and its activity has been validated in Arabidopsis leaf extracts by using protease activity profiling (van der Hoorn et al., 2004). But the enzymatic properties of RD21 have not been clarified yet. Figure 1.4.1 shows our hypothetic model which based on our experimental data that RD21 could be induced by ROS or MPK6 kinase signal and then process ACS2 C-terminus in vivo (Hu et al., 2014; Liu and Zhang, 2004). Both of AT1G76140 and AT2G47390 are prolyl oligopeptidase family proteins, which have serine-type peptidase and endopeptidase activity in vivo. It’s report that AT1G76140 protein can be up-regulation by cold stress treatment in Arabidopsis thaliana (Kim and Kang, 2006). Proteome studied of the luminal and peripheral thylakoid in Arabidopsis revealed that AT2G47390 protein maybe involved in antioxidative response (Peltier et al., 2002). Therefore, biotic and abiotic stresses maybe can induce these three proteases to process ACS2 C-terminus in vivo. But this prediction needs further experimental in vivo validation.
Figure 1.4.1 Hypothetic model of ACC synthesis 2 processed by protease and promote ethylene production in plant. (Reference: Liu and Zhang., 2004; Qin Hu et al., 2014)
1.4.4 Transgenic protein CFP-AtACS2-YFP overexpression in wild-type plant for detecting in vivo processed by proteases

Gene transferred into plant for overexpression by using Agrobacterium transformation is a common approach to study biological function of a single gene. In order to find out C-terminal of ACS2 protein can be processed in vivo, CFP-AtACS2-YFP under control of estrogen inducible promoter was transferred into wild-type Arabidopsis plant for overexpression. In the basis of three proteases have high activity when stimulated by stress treatment from literature report, we used wounding, cold, UV, NaCl and ethylene treatment, then used western blot analysis to detect transgenic protein processed in vivo, then used FRET to detect YFP fluorescence protein can be cut off. FRET fluorescence can be detected with confocal microscopy and FRET fluorescence intensity is lower than YFP fluorescence intensity (Figure 1.3.26), maybe partial CFP-AtACS2-YFP proteins were processed, but it’s difficult to detect all proteins processed in vivo. According to our western blot analysis data, we still can’t find a good condition to let CFP-AtACS2-YFP protein processed in vivo, though some artificial factors such as denature plant proteins slowly during protein extraction with urea buffer can cause CFP-AtACS2-YFP can be processed by proteases which release from broken cells (Figure 1.4.2). Based on confocal microscopy data, we found the AtACS2 protein localize in cell nucleus (Figure 1.3.26), but literature report that ACS protein localization in the cytoplasm and can be stabilized by 14-3-3 proteins act through a direct interaction to decrease the abundance of the ubiquitin ligases that target a subset of ACS proteins for degradation (Yoon and Kieber, 2013), so maybe the proteases which localize in cytosome can’t process transgenic ACS2 protein which localize in nucleus. Maybe estrogen inducible promoter is not as good as native promoter to let ACS2 protein express and function in vivo.
Three-week-old CFP-AtACS2-YFP::WT transgenic plant in MS agar-based jars treated by spraying 10 μM β-estradiol for 36 h (spray every 12 h), then collect tissue by using liquid nitrogen. Tissues were divided into two groups, one was used for protein extraction with denature slowly and the other was used for protein extraction with denature quickly. 1, 2 indicate two times repeat for each experiment.
Chapter 2.

Function proteomic study of ethylene up-regulated phosphorylation protein in ein3-1/eil1-1 double mutant

2.1 Introduction

2.1.1 Over view of ethylene signaling pathway

Ethylene (C$_2$H$_4$) is a plant hormone which has been known to be a signaling molecule that can regulates various developmental stages and biotic or abiotic stress responses of plants (Abeles et al., 1992; Tsuchisaka et al., 2009). It is perceived by receptors (ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2 and EIN4) and represses a Raf-like mitogen-activated protein kinase CTR1 (Constitutive ethylene response 1) (Guo and Ecker, 2004; Kieber et al., 1993). Inactivation of CTR1 then let the activation of the downstream components such as the EIN2 (Ethylene insensitive 2) gene product. EIN2 is downstream of CTR1 as a substrate of CTR1 kinase (Alonso et al., 1999), and it is an essential positive regulator which is maintained in an inactive state when phosphorylated by CTR1 in ethylene signaling transduction pathway (Ju et al., 2012; Qiu et al., 2012). Inactive CTR1 cause EIN2 is proteolytically processed such its C-terminal domain and released to migrate to the nucleus (Ju et al., 2012; Qiu et al., 2012; Wen et al., 2012). It’s possible that a kinase cascade consisting of MEK and MAP kinase similar proteins between CTR1 and EIN2. EIN3 (Ethylene insensitive 3) and its closest homolog EIL1 (EIN3-LIKE 1) are two primary transcription factors downstream of EIN2 (Alonso et al., 2003; Chao et al., 1997; Solano et al., 1998), which are necessary for the majority of ethylene response genes induction (Alonso et al., 2003; An et al., 2010; Zhong et al., 2009). EIN3/EIL1 are quickly degraded without ethylene by EIN3-binding F-box protein 1 and 2 (EBF1 and EBF2), and EIN3/EIL1 can accumulate in presence of ethylene maybe by the repression of EBF1/2 (An et al., 2010;
Gagne et al., 2004; Guo and Ecker, 2003; Potuschak et al., 2003). Protein phosphorylation is one of the most important post-translational modifications (PTMs), and it can reversibly regulate almost all processes in the living cell (Reinders and Sickmann, 2005).

2.1.2 Ethylene signal perception

Hormone signal perception and transduction in microorganisms and animals have been studied for many years, but it was not until 1970s a putative receptor for the auxin transport inhibitor naphthylphthalamic acid (NPA) was identified in higher plants (Lembi et al., 1971). Plant can produce endogenous ethylene and it also has response to exogenous ethylene by receptors. The function of ethylene as a signal molecule depends on the ability of cells to monitor the variation of concentration and to transduce the information into physiological responses to the cell type. An interesting question has focused on how a proteinaceous receptor can perceive a gaseous signal consisting of two hydrogenated carbons with a double bond.

With the development of molecular genetic approaches in the model plant Arabidopsis, many of the biochemical components of ethylene perception and signal transduction reveal this secret that ethylene signaling is mediated by a family of copper-containing receptors which contains a metal binding domain for signal transduction through a pathway (Burg and Burg, 1967; Sanders et al., 1990). The P-type ATPase copper transporter, RAN1, provides the copper cofactor required for ethylene binding to receptors (Rodriguez et al., 1999), and is also important to the biogenesis of the receptors (Binder et al., 2010; Hirayama et al., 1999; Woeste and Kieber, 2000). Membrane-localized receptors which have homology to bacterial two-component histidine kinases were involved in sensing environmental changes and transmit their signal by His autophosphorylation on the His kinase domain, then followed by transfer of the phosphate to a conserved aspartate residue in the cognate receiver domain (Chang and Stadler, 2001; Pirrung, 1999; Wurgler-Murphy and Saito, 1997). The major breakthrough in defining an ethylene receptor came with studies on the etr mutant of Arabidopsis thaliana plant. Etiolated seedlings of etr mutant have no triple respond to
ethylene, and the plants show strongly reduced levels of ethylene binding (Bleecker et al., 1988a).

In Arabidopsis, there are eight two-component input regulators genes have been functionally identified as hormone receptors (Chow and McCourt, 2006). Three genes are belong to cytokinin perception, and five genes encode ethylene receptors: ETR1, ETR2, ERS1, ERS2 and EIN4 (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998), which share similarity with bacterial two-component regulators (Chang and Stadler, 2001), an amino-terminal ethylene-binding domain and the carboxyl-terminal portion similar to histidine protein kinases. On the basis of the structural similarities of the sensor domain, the receptor family can be divided into two subfamilies. Members of the type-I subfamily (ETR1-like) in Arabidopsis include ETR1 and ERS1, which contain an ethylene-binding domain (so called the sensor domain) in the N-terminus and a well-conserved histidine kinase domain in the C-terminus. The type-II subfamily (ETR2-like) receptors include ETR2, ERS2 and EIN4, which contain an amino-terminal ethylene-binding domain and a degenerate histidine kinase domain that lacks one or more elements that are necessary for catalytic activity. Although the differences between the five receptors, they all show redundancy with one another in that the single null mutant of each receptor appears as wild-type (Hall and Bleecker, 2003; Hua and Meyerowitz, 1998; Wang et al., 2003; Zhao et al., 2002). The identification of the ethylene receptor genes and even the proof of the ethylene binding activity of the corresponding proteins still can’t get conclusive evidences of the receptors’ action mode, because the dominant nature of the original alleles.

Some genetic and biochemical studies on ethylene receptors begin to focus on understanding the mechanism of regulation in planta. Mutant screening approach improves our understanding of biological functions of these receptors. etr1, ein4 and etr2 were firstly identified as dominant ethylene-insensitive plants (Bleecker et al., 1988a; Hua and Meyerowitz, 1998; Roman and Ecker, 1995; Sakai et al., 1998). Single and double loss-of-function (LOF) mutants have no significant ethylene response phenotypes, which revealing a high degree of functional overlap among the ethylene receptors. Evidence of the

86
receptors function as negative regulators in the signaling pathway comes from the observation that double and triple LOF receptor mutants demonstrate constitutive ethylene responses (Hua and Meyerowitz, 1998). Genetic studies are consistent with a model that the receptors actively repress responses in the absence of ethylene and dominant ethylene insensitivity results from gain-of-function mutations in the receptor genes, which allowing the receptors system to continue repressing response even in the presence of ethylene. Genetic and biochemical studies have a similar conclude that ethylene receptors are inactivated by ethylene binding. ETR1 and ETR2 subfamilies have also been identified in other plant species. In tomato, the Never Ripe (NR) gene encodes a receptor with no receiver domain similar to the ETR1-like, but LeETR4 is an ETR2-like member with a receiver domain. Decreasing expression of LeETR4 leads to enhanced ethylene responses, and overexpression of NR can decrease the ethylene sensitivity. These results revealed that mechanisms of ethylene perceptions possibly conserved in flowering plants (Tieman et al., 2000).

### 2.1.3 Ethylene signal transduction

An important breakthrough for our understanding the mechanism of ethylene signal transduction is the recent finding that ethylene perception and signaling occur at the endoplasmic reticulum (ER) (Chen et al., 2002; Gao et al., 2003). Acting downstream of the ethylene receptors is a putative MAPK kinase kinase CTR1, a negative regulator of ethylene responses with similarity to Raf protein kinases (Kieber et al., 1993). *ctr1* was originally identified as an *Arabidopsis* mutant that displayed constitutive ethylene triple response (Kieber et al., 1993). Critical for understanding of the role of CTR1 in the ethylene signaling pathway was the finding that CTR1 has a non-catalytic N-terminal region that physically interacts with type-I subfamily receptors and colocalizes with them in the ER, resulting in association of CTR1 with the ethylene receptor complex (Clark et al., 1998; Gao et al., 2003). In the absence of the ethylene, CTR1 is thought to form a complex with the receptors, at least those of the type-I subfamily, and negatively regulate downstream ethylene signaling events. Ethylene inactivation of the receptors-CTR1 complex could leads to the activation of a kinase
cascade comprised of MKK9 and MPK3/6 and finally changes the phosphorylation state of transcription factor EIN3 (ethylene insensitive 3), then controlling turnover of EIN3 protein (Yoo et al., 2008).

Ethylene response mutants with genetic epistasis analysis has reveal that EIN2 acts downstream of CTR1 and upstream of EIN3. EIN2 is a transmembrane protein located on ER membrane (Alonso et al., 1999). EIN2 gene null mutations lead to the complete loss of ethylene responsiveness during plant development, revealing that EIN2 is an essential positive regulator in the ethylene signaling pathway. Two different domains can be defied in EIN2 structure, an N-terminal hydrophobic region with sequence similar to the NPAMP family of metal ion transporters, and a novel C-terminal domain. Interestingly, overexpression of the C-terminus alone not the full-length protein can activate ethylene responses (Alonso et al., 1999; Qiao et al., 2009), suggesting that the N-terminal region negatively regulates the activity of the C-terminus.

Signals generated in the receptors and transduced trough CTR1, EIN2 and potentially the MKK9-Mpk3/6 MAP kinase cascade (Yoo et al., 2008) converge on the EIN3 and EIL1 (EIN3-Like 1) transcription factors (Figure 2.1.1). The cloning of EIN3 revealed direct evidence for nuclear regulation in the early ethylene signal transduction pathway, EIN3 and EIL1 function as positive regulators of ethylene signaling and maybe to control nucleus transcriptional events (Chao et al., 1997). ein3 and eill single mutants are incomplete ethylene insensitive phenotype, and eill is more sensitive to ethylene than ein3. The reason maybe EIL has the lower expression level compare with EIN3 (Guo and Ecker, 2004; Stepanova and Ecker, 2000). ein3 eill double mutant shows complete ethylene insensitive phenotype, revealing a primary role of downstream transcriptional regulation for these two proteins (Alonso et al., 2003).
2.1.4 Protein phosphorylation form in ethylene signal transduction

Phosphorylation is one of the most important post-translational modifications (PTMs) in the intricate regulation of biological events. Phosphorylation of protein is a very important regulator of intra-cellular biological processes and is a reversible modification to affect both the protein folding and function, and it can regulate protein complex formation, localization, degradation, enzymic activities and substrate specificities phosphorylate EIN3 (Reinders and Sickmann, 2005; Thingholm et al., 2009). It plays an important role in affecting on various cell functions, such as signal transduction, homeostasis, proliferation, cell differentiation, metabolic maintenance and cell division. Protein phosphorylation is very important in hormone signal transduction and developmental response of plant, such as controlling the activation (Kim et al., 2011) and affecting the key signaling components’ stability (Liu and Zhang, 2004). The tobacco plant pathogenesis response by the induction of pathogenesis-related (RP) genes revealed that ethylene-dependent signal transduction in the plant cell via putative phosphorylated intermediates that are regulated by specific kinases and phosphatases (Raz and Fluhr, 1993). MKK9a-acitvated MPK6 immunoprecipitated from protoplast extracts possible to phosphorylate EIN3 in vitro (Yoo et al., 2008). In microsomal membrane proteins which isolated from ethylene-treated and untreated etiolated Arabidopsis seedlings, ethylene signaling component EIN2 phosphorylation sites in the C-terminal domain were identified by mass spectrometry (Chen et al., 2011). Chuanli Ju et al. found that EIN2 is a direct target of the CTR1 kinase in the absence of ethylene by using alanine substitutions preventing phosphorylation approach (Ju et al., 2012). Ethylene regulated serine-62 phosphorylated isoform of Ethylene Response Factor 110 (ERF110) in transgenic plant was absolute quantified by using the absolute quantitation of isoforms of post-translationally modified proteins (AQUIP) was substantiated with a SILIA-based relative and accurate quantitative proteomic approach (Li et al., 2012). And the serine-62 has been found that is involved in the regulation of bolting time (Zhu et al., 2013). It’s demonstrated that ERF110 is a novel signaling component in ethylene singling transduction pathway (Figure 2.1.2). Differential and quantitative phosphoproteomics analyses of wild-type and ctr1-1 plants under
long-term ethylene treatment revealed that Ser-280 and Ser-283 of plasma membrane intrinsic protein 2A was regulated by CTR1 protein, and the prediction model (Figure 2.1.3) showed that PIP2A as a novel signaling component in ethylene signaling transduction pathway (Yang et al., 2013). Therefore, protein phosphorylation plays an important role in ethylene signaling transduction pathway, and quantitative and functional phosphoproteomics approach become more and more robust in signaling components discovery in hormone signaling transduction pathway.
Figure 2.1.1 Representation of the ethylene signal transduction pathway. Ethylene is perceived by a family of receptors which located in the ER membrane. Binding of ethylene to the receptors is mediated by a copper cofactor. The receptors interact with the Raf-like kinase CTR1. Binding of ethylene to the receptors results in the inactivation of both receptors and CTR1, which causing derepression of a positive regulatory molecule, EIN2. A MAPK cascade may be involved in the signal transduction between CTR1 and EIN2. A positive signal then transmitted to the transcription factors EIN3/EILs from EIN2, resulting in stabilization and accumulation of the EIN3/EIL proteins in the nucleus, where they induce transcription of ERF1, EDF1,2,3,4 and other ethylene-regulated genes as the first step in a transcriptional cascade that releases the downstream ethylene responses (Yoo et al., 2008).
Figure 2.1.2 The mode of action for ethylene in the regulation of phosphorylated ERF110 isoform during Arabidopsis bolting. Arrows indicate positive effects, whereas stop signs indicate inhibitory effects (Zhu et al., 2013).
Figure 2.1.3 Proposed model of ethylene dual-and-opposing effect on plasma membrane intrinsic protein 2;1 (Yang et al., 2013).

Arrows indicate positive effects, whereas stop ends indicate negative effects. Red lines indicate the regulation of protein phosphorylation, and blue lines indicate the regulation of gene expression. Black lines indicate the effects on the synthesis of hormones. Solid lines indicate direct regulation, and double solid lines indicate direct or indirect regulation. Dashed lines indicate putative interactions.
2.1.5 Quantitative phosphoproteomics study in plant signaling

Protein phosphorylation in plant cells is the most important type of reversible post-translational modification involved in the regulation of cellular signal-transduction processes. A lot of evidence demonstrates that phosphorylation is necessary for immune responses in animal and plants. About one-third proteins are phosphorylated in plant (Hubbard and Cohen, 1993; van Bentem and Hirt, 2007), which are involved in a signaling pathway provides the basis for complex signaling networks and regulatory processes in plants, such as photosynthesis, hormones (auxin, ethylene, cytokinins and abscisic acid), defense reaction, wounding and osmolarity (DeLong, 2006; DeLong et al., 2002; Sakakibara et al., 2000; Xing et al., 2002; Yang et al., 1997). There are more than 1000 predicted protein kinases in the Arabidopsis and other sequences plant genomes (Lukowitz et al., 2000), almost twice of the kinase genes predicted in humans (Manning et al., 2002), which indicate that a complex phosphorylation network in plant signaling. A large scale phosphorylation mapping study demonstrated that there are 1,346 phosphorylatable proteins in Arabidopsis thaliana and the relative abundances of phosphoserine, phosphothreonine and phosphotyrosine sites was 85.0, 10.7 and 4.3% (Sugiyama et al., 2008), respectively.

Phosphorylated peptides isolated from the total plant protein still a big challenge. A fundamental challenge in protein phosphorylation analysis by mass spectrometry is the low stoichiometry of phosphorylated proteins arising from their low abundance in a particular phosphorylated form (Olsen et al., 2010; Wu et al., 2011). Especially of signal transduction proteins have low copy number with pivotal roles in signaling cascades cause the challenge more and more severe. And the relatively low ionization efficiency compared with none phosphorylated peptides also decrease the detection of phosphopeptides by MS (Peck, 2006). Series of phosphopeptides enrichment approaches have been used for getting high-throughput identification of phosphopeptides, including phosphor-specific antibody purification, immobilized metal affinity purification (IMAC), titanium dioxide (TiO₂) and so on (Kersten et al., 2006). In various of phosphopeptide enrichment methods (Figure 2.1.4) of plant phosphoproteomics studies (Kersten et al., 2009), IMAC and TiO₂, alone or in combination
(SIMAC), have demonstrated powerful approaches for the selective phosphorylated peptides enrichment (Sugiyama et al., 2007). As IMAC prefers to combine multi-phosphorylated peptides while TiO$_2$ prefers to combine mono-phosphorylated peptides and is much more tolerant to detergents and salts used in purification buffers. 2172 unique phosphorylation sites were identified from *Arabidopsis* plant in large-scale phosphopeptides analysis by LTQ-Orbitrap machine (Sugiyama et al., 2008). To study the rapid signaling transduction in plant, some novel signaling components in several plant hormones including BR, ABA, cytokinins and ethylene were identified by functional phosphoproteomics studies (Chen et al., 2010; Deng et al., 2007; Heintz et al., 2006; Kim et al., 2009; Zhu et al., 2013). 42 BR-related phosphorylated proteins were found in *Arabidopsis* by functional phosphoproteomics study (Deng et al., 2007), and 112 phosphorylated proteins were reported to respond to cytokinins with short treatment for *Physcomitrella patens* (Heintz et al., 2006). Although little progress in ethylene signaling study by using phosphoproteomics analysis, our group have identified 224 phosphopeptides in the ethylene insensitive mutant *ein2-5*, and two phosphopeptides were ethylene-enhanced and three were ethylene-repressed (Li et al., 2009). Serine-62-phosphorylated isoform of a novel ethylene signaling component ethylene response factor110 (ERF110) is involved in *Arabidopsis* bolting by functional phosphoproteomics analysis (Zhu et al., 2013). Therefore, functional phosphoproteomics approach plays an important role in signaling transduction study in plants.

Although most proteomic experiments elucidated the protein constituents of a biological system eight years ago, quantitative measurements are at the heart of really every proteomic study today. Different quantitative proteomics approaches are discovered and both 2D gel electrophoresis (2DE) and liquid chromatography-tandem mass spectrometry serve as key quantitative methods. 2DE is a classical and powerful analytical method in proteomics study that can be separated complex protein mixtures based on charge (Gorg et al., 2004). 2DE delivers a map of intact proteins, which reflects changes in protein expression, isoform or PTMs (Oh et al., 2004). However, 2DE has the low reproducibility for quantitative analysis and the low frequency of phosphorylation limited its application in phosphoproteomic study. Recently invention of fluorescent 2D-DIGE overcame this problem by labeling proteins from
two to three samples with spectrally resolvable fluorescent dyes before running gel (Unlu et al., 1997; Viswanathan et al., 2006). Wang et al used 2D-DIGE analysis of phosphoprotein and plasma membrane fractions from brassinosteroid-treated Arabidopsis and identified 42 signaling protein in this steroid hormone signaling pathway (Tang et al., 2008b).

Liquid chromatography-tandem mass spectrometry based quantitative methods can be divided into label-free methods (Neilson et al., 2011) and labeling methods, such as metabolic incorporation of amino acids (SILAC) (Jiang and English, 2002; Ong et al., 2002), chemical modification with isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004; Thompson et al., 2003) and dimethyl labeling (Boersema et al., 2010). Label-free approaches to quantitation involve the integrated measurement of peptide peak areas in LC-MS runs (Chelius and Bondarenko, 2002). Samples must be analyzed separately, and LC retention times and ion counts are compared between treatments and controls. Although label-free methods have poor accuracy of the quantitation and the deviation could be low to 50%, the advantage of this methods are inexpensive, no or little limitation in biological samples and high coverage of protein for quantification, which is very useful in expected global protein level changes samples (Old et al., 2005). SnRK2 protein kinase substrates were identified with label-free quantitative phosphoproteomics study (Wang et al., 2013). By comparison, labeling quantitation methods have the advantage of relatively actual quantitation of peptides, but limited in proteome coverage, and number of sample could compare (Schulze and Usadel, 2010). According to different principles, more and more quantitation methods were developed, including enzymatic labeling with \[^{18}\text{O}\] water (Bonenfant et al., 2003) and ICAT (Ramus et al., 2006), SILIP (Schaff et al., 2008), SILAM (McClatchy et al., 2011), SILIA (Guo and Li, 2011) and standard peptides quantitation (AQUA (Gerber et al., 2003) and AQUIP (Li et al., 2012)). These quantitation methods are very powerful in plant phosphoproteomics study of signaling transduction.

Metabolic labeling of is one of important approaches in stable isotope labeling method. With SILAC metabolic labeling the label is supplied in the growth media and is incorporated into the proteins during cell growth. However, the first fully \(^{15}\text{N}\) labeled potato plant with
hydroponically growth (Ippel et al., 2004), the labeled plant material which was used for the first proteomics study was *Arabidopsis* cell culture (Engelsberger et al., 2006). Cells of *Arabidopsis* leaves were grown with the only nitrogen source K\(^{15}\)NO\(_3\), and the incorporation was estimated to be close to 98%. Nelson et al. demonstrated that \(^{15}\)N can be used for the metabolic labeling of intact plants by using \(^{15}\)NH\(_4\)\(^{15}\)NO\(_3\) and K\(^{15}\)NO\(_3\) in liquid cultures, and the incorporation rate also achieved to 98% (Nelson et al., 2007). A stable isotope labeling in *Arabidopsis* (SILIA) method has been established in our group by using modified agar-based medium supplemented with nitrogen source, 444 phosphorylation sites were identified and the \(^{15}\)N incorporation rate achieved to 97.44% (Guo and Li, 2011). Ser62-independent \(^{15}\)N-coded ERF110 protein from SILIA labeling tissue of transgenic plants was used as standard peptides in the Absolute Quantitation of Isoform of the Post-translationally modified recombinant protein (AQUIP) to quantitate the Ser-62 phosphorylation site occupancy (R(aqu)) (Li et al., 2012). Quantitative phosphoproteomics analysis of *Arabidopsis* ctr1-1 and rcn1-1 mutants reveals ethylene regulated time-dependent phosphoproteins and putative substrates of CTR1 kinase (Yang et al., 2013). Therefore, SILIA is a robust labeling method for quantitative phosphoproteomics study in plants signaling transduction.

Dimethyl labeling is a universal chemical labeling approach in quantitative proteomics study. Hsu et al explored the alternative labeling reagent (formaldehyde) for labeling the N-terminus and ε-amino group of Lys residues via reductive amination (Hsu et al., 2003). And quantitative protein profiling was found by analyzing nuclear protein abundance variation in an immortalized E7 cell with and without arsenic treatment. The advantage of dimethyl labeling method is the isotopic formaldehyde is cheap and commercially available, the labeling efficiency near 100% in around 5 min without the ionic state variation significantly (Hsu et al., 2006; Hsu et al., 2003). A modified N-terminal dimethyl labeling method was found by Ji et al. can facilitate protein identification and quantification in proteome analysis (Ji et al., 2005), in which the N-terminal of tryptic peptides are labeled with either \(^{12}\)C-formaldehyde or \(^{13}\)C-formaldehyde after lysing residues in peptides are blocked by guanidination. Based on stable isotope dimethyl labeling, triplex quantification method was constructed to label three samples at the same time with 4 Da mass difference between peaks
in the generated peptide triplets, and more than 600 proteins were quantified in 1300 identified proteins from 60 μg of starting MEL cells (Boersema et al., 2008). Therefore, the stable isotope dimethyl labeling is a robust approach in quantitative phosphoproteomics study.
Figure 2.1.4 An overview of methods used for phosphoproteins and phosphopeptides enrichment. Wildly accepted phosphoprotein detection strategies or phosphopeptides enrichment are highlighted in gray color.
2.1.6 Water uptake and transport in plant tissues

Water is the most abundant molecules in all plant cells and the maintenance of water status in suitable range is necessary to life. Plant growth relies on water absorption from the soil and the movement of water from the roots to other parts of the whole plant (Aharon et al., 2003). The plant water status is constantly changed by diurnal variations in environmental parameters, such as temperature and light, or water availability in soil or atmospheric humidity. Plants dynamically and considerably regulate their hydraulic efficiency by continue of different organs resistances along with the soil-plant-atmosphere supply (Tyerman et al., 2002). For example, 50% of the hydraulic resistance to the shoots contributed by the leaf of plants, which represents near 30% resistance of the whole plant (Nardini and Tyree, 1999; Sack et al., 2003; Tsuda and Tyree, 2000). There is evidence that a large part of the changes observed in leaf growth relies on water transport in the plant (Bouchabke et al., 2006; Sperry et al., 1998). Water movement is controlled by both apoplastic and symplastic pathways in plants (Aharon et al., 2003). The apoplastic pathway means water moves from cell to cell through spaces in the cellulose cell walls until it reaches the endodermis. Water movement through a living organ such as root or leaf also can by an apoplastic pathway or a transcellular route (Steudle, 2000). The symplastic pathway is responsible for transporting water pass through membranes when plants are encountering abiotic stress (Lian et al., 2004; Suga et al., 2002; Vera-Estrella et al., 2004), and is regulated mainly by the aquaporin family member proteins (Amodeo et al., 1999). Transpiration stream of most plants starts by the uptake of water from the soil and ends with water loss by vaporing from the leaves of the plant in daytime. Plants can control water potential by stomatal adjustment of transpiration in a short time (Sperry and Pockman, 1993). Several studies on the stomatal movements revealed different responses between the investigative species (Abeles et al., 1992; Gunderson and Taylor, 1991; Taylor and Gunderson, 1986; Woodrow et al., 1988). Ethylene greatly increased stomatal conductance, root hydraulic conductivity (Lp) and root oxygen uptake in hypoxic aspen seedlings (Kamaluddin and Zwiazek, 2002). In some case, ethylene can maintain stomata in the open state and counteracting ABA action (Wilkinson and Davies, 2010). It’s
report that exogenous ethylene can increase membrane permeability in petal cells (Mayak et al., 1977). Plasma membranes are thought to be the primary obstacle to water flow. Water transport across cell membranes of intact higher plant occurs mainly through water channels (Chrispeels et al., 1997). Ethylene down-regulated rose water channels PIP2;1 gene and significantly reduced petal size (Ma et al., 2008), and induced the transcription factor RhNAC100 expression also decreased petal size (Pei et al., 2013). Ethylene maybe played role in regulating root hydraulic conductivity by Ser-280 phosphorylation of PIP2 protein in mycorrhizal flooded tomato plants (Calvo-Polanco et al., 2014). Therefore, aquaporin proteins play an important role in water transport of plant cells.

### 2.1.7 Plant aquaporin proteins (AQPs)

Aquaporins are water channel protein of vacuolar and plasma membranes, and most of them are intracellular components of plant cells. They are relative to the maintenance of the whole plant water condition by mediating osmoregulation of every single cell and trans-cellular water transport in leaves and roots. In the early 1990s, the existence of water channels in plants had not been clearly hypothesized, although some aquaporins had been molecularly characterized due to they are highly abundance proteins in plant cells. Aquaporins (AQPs) transport water just like other small molecules such as CO\(_2\), glycerol, and boron through membranes (Kaldenhoff and Fischer, 2006; Sade et al., 2010; Uehlein et al., 2003). Biological activities which are relative to AQPs including seed germination, stomatal movement, cell elongation, reproductive growth, phloem loading/unloading and stress responses of plants (Eisenbarth and Weig, 2005; Gao et al., 2010). Aquaporins in the tonoplast can increases the effective cellular cross-section through which water flows freely after passing through the plasma membrane (Chrispeels and Maurel, 1994). The expression and biological function of aquaporins are affected by a numbers of signals, including abiotic stresses, light and plant hormones (Bienert et al., 2006; Chaumont et al., 2005; Horie et al., 2011; Johanson et al., 2001; Kaldenhoff and Fischer, 2006; North and Nobel, 2000). It’s report that most aquaporin genes were repressed by auxin during lateral root formation (Peret et al., 2012). Aquaporin 2
of Samanea saman expressed in Xenopus laevis oocytes can highly increase osmotic water permeability (Pf). (Moshelion et al., 2002).

In comparison to other living species, plants have a large number of genes that code for aquaporins. Arabidopsis, Zea mays and rice have more than 30 aquaporin-encoding genes respectively (Chaumont et al., 2005; Luu and Maurel, 2005; Sakurai et al., 2005). Four major subfamilies have been classified of plant aquaporins: tonoplast intrinsic proteins (TIPs), plasma membrane intrinsic proteins (PIPs), nodulin 26-like intrinsic protein (NIPs), and small basic intrinsic proteins (SIPs). These proteins have been reported in most of plant cell subcellular compartments, including plasma membrane, tonoplast, golgi apparatus, endoplasmic reticulum and chloroplast (Hachez et al., 2013; Luu and Maurel, 2013). It’s reported that $\text{H}_2\text{O}_2$ reduces the transcription of most Arabidopsis PIP2 genes in root (Hooijmaijers et al., 2012), and also affect their C-terminal phosphorylation or subcellular localization (Boursiac et al., 2008; Har et al., 2013; Prak et al., 2008). The plasma membrane intrinsic proteins constitute the largest plant aquaporin subfamily, thirteen members belong to the PIP subfamily which can be divided further into two sequence homology groups, PIP1 and PIP2 in Arabidopsis plant (Johanson et al., 2001). The PIP2 group in the PIP subfamily has been validated to play an important role in water flow across the plasma membrane (Santoni et al., 2006; Tornroth-Horsefield et al., 2006). And PIP2 subfamily proteins could be relative to cellular water transport in root, leaves (Javot et al., 2003; Lopez et al., 2003), reproduction organs (Bots et al., 2005a; Bots et al., 2005b) and seed germination (Schuurmans et al., 2003). Based on early structure-function analysis and more recently the description of several aquaporins structures at atomic resolution, aquaporins have a conserved fold and membrane topology (Fujiyoshi et al., 2002; Hedfalk et al., 2006). Aquaporin monomers exhibit six membrane-spanning $\alpha$-helices pitched along the plane of the membrane and interrupted by five connection loops (loops A-E) (Fujiyoshi et al., 2002; Tornroth-Horsefield et al., 2006) (Figure 2.1.5). The N-terminus, C-terminus, loops B and D are always dipping in the cytosol of cell. By contrast, loops A, C and E face the apoplasma in plasma membrane aquaporins.
2.1.8 Post-translational modifications (PTMs) of aquaporins

With the developments of phosphoproteomics technology and membrane proteomics, a number of studies reveal that the activity of aquaporins may be regulated by post-translational modifications, such as methylation (Kline et al., 2010; Sahr et al., 2010; Santoni et al., 2006), ubiquitination (Lee et al., 2009), deamidation (Har et al., 2013) and phosphorylation (Har et al., 2013; Kline et al., 2010; Prado et al., 2013; Prak et al., 2008; Wu et al., 2013). In general PIP2 aquaporins possess a shorter N-terminal extension and a longer C-terminal end. Phosphorylation of PIPs was found to play an important role in the mechanisms that determine their gating and subcellular dynamics activity. PIP2s have several conserved phosphorylation sites in their cytosolic loop B and C-terminal tail (Johansson et al., 1998b). X-ray crystallography structure of spinach PIP2;1 revealed that phosphorylation of these sites can play an important role in pore gating function (Tornroth-Horsefield et al., 2006). PIP phosphorylation also can affect their subcellular localization (Luu and Maurel, 2013). Ser-283 phosphorylation of AtPIP2;1 is required for targeting of newly synthesized proteins to the plasma membrane (Prak et al., 2008). Although recent phosphoproteomic studies have given out a good discovery of phosphorylation sites present in PIPs of Arabidopsis (di Pietro et al., 2013; Niittyla et al., 2007; Nuhse et al., 2004), only a few studies have established the function of PIP phosphorylation in regulating water transport in planta. Some studies confirmed that aquaporins in the Arabidopsis plasma membrane can be phosphorylated at multiple sites on PIP2;1/2/3 proteins’ C-terminal tail (Kline et al., 2010; Niittyla et al., 2007; Nuhse et al., 2004; Prado et al., 2013; Prak et al., 2008; Yang et al., 2013). Quantitative phosphoproteomics and function studies of AtPIP2;1 in transgenic Arabidopsis indicated that Ser-280 and Ser-283 phosphorylation was necessary for regulating leaf hydraulic conductivity enhancement under dark condition (Prado et al., 2013). The protein kinase(s) and protein phosphatase(s) that target plant aquaporins may provide an efficient link between water transport and the signaling cascades involved in plant cell osmoregulation (Johansson et al., 1996; Maurel et al., 1997a). It can undoubtedly help us understanding of regulation networks involved in aquaporin regulation. For example, a large scale analysis of
sucrose-induced protein phosphorylation revealed that C-terminal tail phosphorylation of several PIP2s by SIRK1 (a Leucine-Rich Repeat Receptor-Like Kinase) under sucrose resupply to starved plants (Wu et al., 2013). Large scale interactome studies are also expected to indicate novel regulation proteins which acting on aquaporin function (Jones et al., 2014). Therefore, how to find kinases which responsible for aquaporins phosphorylation by using functional phosphoproteomics approach become more and more important in plant system biological research.

2.1.9 Objectives of this study

Further confirm ethylene-regulated phosphosites on PIP2;1 protein C-terminus in ein3-1/eil1-1 double mutant by using SILIA-based and chemical labeling-based quantitative phosphoproteomic analysis, and study biological function of the phosphorylation sites by using point mutation transgenic plants.
AtPIP2;1 has six transmembrane domains (1-6), five connecting loops (A-E) and N- and C-terminal extremities. In AtPIP2;1 the initiating Met residue is co-translational cleaved (cross). Dimethylation (Met Met) of Lys3, monomethylation (Met) of Glu6, and phosphorylation (P) of Ser-277, Ser-280 and Ser-283 have been validated experimentally. The phosphorylation of loop B at Ser119 from spinach SoPIP2;1 (Li et al., 2014). Ser-277, Ser-280 and Ser-283 were also found in this work.
2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

The Arabidopsis thaliana ecotypes Columbia-0 (Col-0) and its mutants, ctrl-1, pip2;2 (GK-317A09-015858) and octuple acs mutant (CS16651) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). ein3-1/eil1-1 (abbreviated as ein3eil1) double mutant line was a gift from Dr. GUO Hongwei. ein2-5 and etr1-1 mutant lines were gifts from Dr. Joseph Ecker and Dr. Elliot Meyerowitz, respectively. Seeds of pip2;1-2 and pip2;1-2/pip2;2-3 (abbreviated as pip2;1/2) double mutant lines were gifts from Dr. Anton R. Schäffner. For planting Arabidopsis seeds with MS medium, seeds were surface-sterilized with 70% ethanol for 2 min, followed by 10-15 min in a solution containing 30% (v/v) commercial Clorox bleach and 0.1% Triton X-100, sterilized seeds were stored at 4 °C for 4 d under dark condition before planting. Seeds were sown onto the surface of 1×MS (Murashige and Skoog basal salt mixture, pH 5.7) and 0.8% agar-base medium inside jars about 1.5 cm apart from each other. For planting $^{15}$N labeling Arabidopsis, seeds were sown on 0.8% agar-based SILIA medium glass jars (either with K$^{14}$NO$_3$ or K$^{15}$NO$_3$) and grown 3 weeks at 22 ± 2°C with a 16-h-light/8-h-dark cycle under a light intensity of 150-250 μmol photons m$^{-2}$s$^{-1}$ for quantitative phosphoproteomics analysis as described previously (Yang et al., 2013). The plants which subjected to phosphorylation analysis by western-blot were grown on 1× MS medium supplemented with 10 g/L sucrose, 10 mg/L thiamine HCl; 1 mg/L pyridoxine; 1 mg/L nicotinic acid; 100 mg/L myo-inositol and 0.8% agar-base medium in glass jars for 3 weeks. 100 μM AOA (aminoxyacetic acid) is added into MS medium if plants are for ethylene treatment, as AOA is the ethylene precursor (ACC synthesis, 1-Aminocyclopropane-1-carboxylic acid synthesis) inhibitor and it can inhibit endogenous ethylene biosynthesis.

In the case of ethylene treatment, 3-week-old adult plants growth on 100 μM AOA-containing M/S solid medium were flushed with an air-flow for 5 h before 10-ppm ethylene was applied.
onto these plants for additional 12 h with flow rate of 110 mL per min under dim light inside a hood to remove ethylene leaked out from the fumigation containers. All air control plants were grown on 100 µM AOA-containing M/S solid medium in glass jars and kept under air-flow throughout the acrylic plastic containers in the experiment. For ethylene treatment of \(^{15}\text{N}\) and \(^{14}\text{N}\) labeling \textit{ein3eill} double mutant plant was also performed in the acrylic plastic containers. Two groups of 3-week-old ethylene response mutant plants, one labeled with \(^{14}\text{N}\) and the other with \(^{15}\text{N}\), were placed in the airflow plastic chamber for 5 h to reduce endogenous ethylene level. Afterwards, one group (either \(^{14}\text{N}\) or \(^{15}\text{N}\)-labeled plants) was treated with 10 ppm of ethylene flow for 12 h. In contrast, the other half was retained in air for the same amount of time. Both groups of plants were harvested in liquid nitrogen as described previously (Yang et al., 2013). In the case of ACC (aminocyclopropane-1-carboxylic acid, the immediate precursor for ethylene) treatment, both \textit{Col-0} and mutant plants were firstly grown on 100 µM AOA plus ACC–containing solid M/S medium in glass jars for 3 weeks (Zhu et al., 2013; Yang et al., 2013), then sprayed corresponding concentration of ACC on plants leaves once a day for 3 d before collecting

### 2.2.2 Preparation of genomic DNA for amplification of PIP2;1, EIN3 and EIL1 genes from \textit{Arabidopsis} plant

Wild-type plant tissues were frozen in liquid nitrogen and ground to powder with molten 1 mL blue tips in 1.5 mL eppendorf tubes. Add 0.7 ml of extraction buffer (2% CTAB, 1.4 M NaCl, 0.1M Tris-HCl pH8.0, 20 mM EDTA and 1% 2-mercaptoethanol) and mix well by vortex. Incubate as 65°C for 1 h and invert tubes to mix gently per 10 min, then chill on ice for 2 min. Add 0.5 mL of chloroform to each tube and mix gently, then centrifuge at 10,000 rpm for 10 min and transfer the supernatant to a new tube. Add equal volume of isopropanol and mix gently, precipitation at -20°C for 2 h. Spin down at 13,000 rpm for 10 min and discard supernatant. Add 0.75 mL of 75% ethanol and 0.15 mL 3 M NaAc (pH 5.3) and shake for 30 min at room temperature. Spin down at 10,000 rpm for 2 min and wash pellet with 75% ethanol for two times, then dry on air. Add 50 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA
pH 8.0) and add 20 ng/μL RNase I and incubate at 65°C for 30 min. Genomic DNA was used for PCR reaction. Primers for amplifying EIN3 and EIL1 genes as follows: ein3-1F: 5'-TACCAAGTATCAAGCGGAG-3', ein3-1R: 5'-AGGCCACCAATCTTCTTTC-3', eil1-1F: 5'-GGGAATGGTGAAAGATAAG-3', eil1-1R: 5'-CTTTCGCCGTCATCTTATCC-3'.

### 2.2.3 Overexpression of the wild-type and mutant PIP2;1 proteins

The 1.5 kb full-length *Arabidopsis* PIP2;1 genomic DNA (Genbank accession number: NC_003074) was amplified by PCR reaction with the following primers: PIP2A-WT-F: 5’-TTTGGCAGGCCATGGCAAAGGATGTGGAAGC-3’ (*AscI* underlined); PIP2A-WT-R: 5’-GGGAGAGCTCTTCTAGACGTGTCAGCAGCCTTCTGAATG-3’ (*SacI* underlined). Mutant versions of PIP2;1 genomic DNA at Ser-280 and Ser-283 were generated by PCR using mutagenic reverse primers to replace PIP2A-WT-R primer respectively in PCR reaction (mutation sites; bold characters):

- PIP2A-S280A-R: 5’-GGGAGAGCTCTTCTAGACGTGTCAGCAGCCTTCTGAATG-3’;
- PIP2A-S283A-R: 5’-GGGAGAGCTCTTCTAGACGTGTCAGCAGCCTTCTGAATG-3’;
- PIP2A-S280/3A-R: 5’-GGGAGAGCTCTTCTAGACGTGTCAGCAGCCTTCTGAATG-3’;

Cloning PCR reaction conditions with Pfx DNA polymerase (Invitogen.): 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 1 min, 68°C for 2min, followed by 68°C for 5 min. PCR products digested with *AscI* and *SacI*, and four DNA fragments were inserted into a modified binary vector pBI121 respectively after a double cauliflower mosaic virus 35S promoter and His₈-YFP tag. The resulting recombinant binary vectors harboring His₈-YFP-PIP2;1, His₈-YFP-PIP2;1S²⁸⁰A, His₈-YFP-PIP2;1S²⁸³A, His₈-YFP-PIP2;1S²⁸⁰A/S²⁸³A fusion genes were sequenced to confirm the altered sequence. Sequencing primers as follows:

- NOS-R: 5’-GTCATGCATTACATGTTAATTATTAATGC-3’;
- PIP2-seq-1R: 5’-AGACAGGGTATAGTCATGTC-3’;
- PIP2-seq-1F: 5’-GCTTGGTGACATTACTATTTAATC-3’;
- YFP-R: 5’-TTTGGCAGCGCCGCTTGTACAGCTCGTCCATGC-3’.

108
Prepared wild-type and mutant PIP2;1 constructs were introduced into an Agrobacterium GV3101 strain and used to transform pip2;1/2 double mutant line by the floral dip method (Clough and Bent, 1998). (His)_8-YFP-PIP2;1 fusion gene was also introduced into wild-type and ein3-1/eil1-1 backgrounds. Transgenic plants were selected from M/S agar-base medium supplemented with 1% sucrose and 25mgL^{-1} hygromycin B (Gold Biotechnology Inc. St. Louis, MO, USA). T2 and T3 transgenic lines were validated by western-blots analysis and used for physiological assay.

2.2.4 Microsomal protein extraction and PIP2;1 phosphorylation peptide quantitation analysis of ein3-1/eil1-1

The frozen $^{14}$N- and $^{15}$N-labeled 3-week-old ein3-1/eil1-1 tissues which treated with air or ethylene for 12 h before collection were ground to powder with -80°C pre-cooled mortar and pestle. In forward experiment, $^{15}$N-labeled plants treated with ethylene and $^{14}$N-labeled plant treated with air; in reciprocal experiment just reverse. Added mixed tissue powder (1:1 ratio of $^{14}$N- and $^{15}$N-labeling tissues) into 5 volumes (g/ml) of microsomal extraction buffer containing 150 mM Tris-HCl, pH 7.6, 8 M urea, 5 mM ascorbic acid, 5 mM DTT, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 1% glycerol 2-phosphate, 1 mM PMSF, 0.5% phosphatase inhibitor cocktail 2 (Sigma), 1× protease inhibitor (complete EDTA free; Roche), and 2% polyvinylpyrrolidone and centrifuged at 10,000 ×g for 20 min at 12°C to get rid of cell debris. The supernatant was centrifuged again at 110,000 ×g for 2 h to get microsomal. The microsomal was dissolved in urea buffer containing 150 mM Tris-HCl, pH 7.6, 8 M urea, 1% SDS, 1% Triton X-100, 5 mM ascorbic acid, 5 mM DTT, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 1% glycerol 2-phosphate, 1 mM PMSF, 0.5% phosphatase inhibitor cocktail 2 (Sigma), 1× protease inhibitor (complete EDTA free; Roche) and centrifuged at 110,000 ×g for 2 h. The microsomal protein in supernatant was precipitated with 3 volumes of -20°C pre-cooled acetone:methanol (12:1 v/v) for at least 2 h. Collected the protein pellet by centrifugation at 11,000 ×g for 20 min and re-suspended it in protein re-suspension buffer (50mM Tris-HCl, pH 6.8, 8 M urea, 1% SDS, 5 mM DTT, 10 mM EDTA). The microsomal
proteins from mixed tissues were quantified by protein DC assay method (Bio-Rad). Protein (1.5 mg) was loaded into a 7 cm × 8 cm 10% SDS-PAGE gel for running about 2 h to separate proteins according to different molecular weight. 3 mg protein was used in each biological experiment for one-dimension gel separation. The SDS-PAGE gel stain, destain and in-gel digestion methods were described previously (Li et al., 2009). About from 25-35 kDa protein size protein was cut for in-gel digestion to get peptides of PIP2;1/2/3 monomer protein. Fe$^{3+}$-IMAC and TiO$_2$ beads were used in phosphorylated peptide enrichment. The lyophilized peptide mixture (0.1 mg) dissolved in 0.5ml 0.1% trifluoroacetic acid (TFA) was desalted using a C18 reverse-phase column ( Oasis HLB, Waters) and re-suspend peptide with 200 µL immobilized metal-ion-affinity chromatography (IMAC) loading buffer containing 250 mM acetic acid, 30% acetonitrile (ACN). 20 µL Fe$^{3+}$-NTA beads (NTA beads charged with Fe$^{3+}$, Qiagen) were added into the peptide and incubated at room temperature for 45 min. Then beads were washed with 200 µL loading buffer for two times and 200 µL Milli-Q water for one time. The phosphopeptides were eluted from beads with 50 µL of 5% ammonium hydroxide, and by adding 8 µL formic acid to acidize phosphopeptides immediately. Added 5 mg TiO$_2$ beads (GL Science Inc., Tokyo, Japan) and 800 µL TiO$_2$ beads binding buffer (82.5% ACN, 1.25 M glycolic acid and 6.25% TFA) in flow-through from Fe$^{3+}$-NTA beads, and incubated at room temperature for 45 min. The TiO$_2$ beads were washed with 200 µL TiO$_2$ washing buffer (80% ACN, 1 M glycolic acid and 5% TFA) for two times and 200 µL Milli-Q water for one time. The phosphopeptides were eluted from TiO$_2$ beads with 50 µL of 5% ammonium hydroxide, and acidize phosphopeptides with 8 µL formic acid immediately. Phosphopeptides eluted from Fe$^{3+}$-NTA and TiO$_2$ beads were mixed together and desalted with zip-tip C18 pipette tips (Millipore) for LC-MS/MS analysis. LC-MS/MS data acquisition, $^{14}$N/$^{15}$N-coded phosphopeptides identification and quantitative analysis were described previously (Yang et al., 2013).
2.2.5 PIP2;1 antigen preparation by purification of recombinant protein from *E.coli* and used for production of polyclonal antibody

As PIP2;1 is a membrane protein, full-length of protein is difficult to express in *E.coli*. Seven coding regions (aa1-39, 63-81, 105-124, 148-166, 190-201, 225-247, 271-287) corresponding to inside or outside membrane residues (Predicted with TMHMM version 2.0) of PIP2;1 protein was synthesized by company (Tech Dragon Limited, Hong Kong) and combined together to pGEM-B1 vector. The combined fragment was amplified by PCR with the following primers: PIP2A-BamHI-F: 5’-CGGGATCCATGGCAAAAGGATGTGGAAGC-3’ and PIP2A-XhoI-1R: 5’-CCGCTCGAGACGTTGCAGCAGCCTCTTCTG-3’. PCR product and pET30a vector were digested with *BamHI* and *XhoI*, and digested PIP2;1 combined cDNA fragment was cloned to pET30a vector. Recombinant PIP2;1 cDNA fragment was sequenced to confirm without mutation. Primer used for sequencing as follows: pET30a-F: 5’-GCAGCCAACTCAGCTTC-3’. Recombinant plasmid was transformed into *E.coli* strain BL21, and single clone which screened out from kanamycin LB agar plate was inoculated into 10 mL 2×YT medium for overnight incubation in 37°C shaker. The bacterial culture was 1:100 diluted into 800 ml 2×YT medium and continue to incubation in 37°C shaker till to OD$_{600}$ reach 0.6. The expression of recombinant PIP2;1 was induced by 1 mM IPTG for 5 hours at 30°C. Cells were harvested by 5,000 rpm for 15 min centrifugation. Protein was extracted by sonication in lysis buffer pH 8.0 containing 100 mM NaH$_2$PO$_4$, 10 mM Tris•HCl and 8 M Urea.

The overexpression PIP2;1 protein was purified by Ni-NTA beads. In order to get rid of urea and get soluble PIP2;1 protein for making antibody, the purified recombinant proteins were then run into a 10% SDS-PAGE gel to separate the PIP2;1 protein with other co-eluted proteins. The gel was stained with 2 M KCl for 5 min. The corresponding gel band was cut and washed with Milli-Q water for 10 min of three times. The gel band was grinded into powder by using liquid nitrogen and soaked in PBS buffer (pH7.4) overnight re-nature the PIP2;1 protein. Centrifuged at 10,000 xg for 2 min to get rid of gel debris and detected
antigen concentration by DC assay. 2 μg purified PIP2;1 protein was digested with trypsin and validated by QTOF-MS/MS analysis. The purified and validated PIP2;1 antigen was used for injection into rabbit to generate anti-PIP2 antibody. Three rabbits were used for injection with 2 mg antigen respectively at day 1, and 1 mg antigen was used for injection respectively at day 31 and day 61. 1 mL serum was collected of each rabbit before the first injection as control, and the rabbits were sacrificed for collecting the antiserum at day 81.

For antibody purification experiment, the purified 1mg PIP2;1 antigen was firstly covalently linked to a 1 mL HiTrap NHS-activated HP column (Ge Healthcare, USA) by following the manufacture’s guideline. Washed the column and loaded 10 mL antiserum for antibody purification. Wash the column with 10 mL PBS buffer pH 7.4, and eluted the antibody by using 0.1 M glycine-HCl (pH3.0) and balanced by equal volume of 1 M Tris-HCl (pH 9.0). Purified anti-PIP2 antibody was use for western-blot analysis. Protein samples were extracted from Arabidopsis tissue with fully denature urea buffer, and separated by running 10% SDS-PAGE gel to blotted onto a PVDF membrane (GE Healthcare). The blot was probed with anti-PIP2 polyclonal antibody to confirm PIP2;1 protein band. Ser-280 and Ser-283 doubly phosphorylated polyclonal antibody (Anti-pS280/S283-PIP2) and Ser-283 single phosphorylated polyclonal antibody (Anti-pS283-PIP2) were made from company (ChinaPeptides Co., Ltd.) by using following corresponding phosphorylated peptides and control peptide. Peptide sequences for making anti-pS280/S283 and anti-pS283 polyclonal phosphorylated antibodies as follows: anti-pS280/283-PIP2 antibody: 277SLG(pS)FR(pS)AANV, non-phosphorylated synthetic peptide 277SLGSFRSAANV as control; anti-pS283-PIP2 antibody: 277SLGSFR(pS)AANV, non-phosphorylated synthetic peptide 277SLGSFRSAANV as control. For quantification of western-blot data, absolute intensity of protein bands were used for analysis, absolute intensity = mean×pixels. Photoshop 7.0 software was used for mean and pixels calculation.
2.2.6 In vivo phosphorylated protein quantitation with western blot analysis

The extracted microsomal proteins were used for SDS-PAGE gel separation directly and analyzed with western blot to quantify relative phosphorylation level of endogenous PIP2;1/2/3 protein with phosphorylation antibody after normalizing with none phosphorylation antibody. Overexpressed transgenic His8-YFP-PIP2;1 fusion protein from transgenic plants were purified from the microsomal proteins by using tandem affinity purification approach with Ni²⁺-NTA agarose beads (Qiagen) under fully denature condition as described previously (Li et al., 2012). Eluted protein was used for western-blots analysis to validate protein relative phosphorylation level in vivo with polyclone antibodies raised against both Ser-280 and Ser-283-phosphorylated peptide, and the single Ser-283-phosphorylated peptide. Total PIP2;1 protein relative expression level was quantified with homemade polyclone antibody raised against PIP2;1. The integrated intensity of the protein bands was measured using Photoshop 7.0 software. The relative abundance of phosphorylated protein from western-blots analysis was normalized with bands intensity of homemade anti-PIP2;1 polyclone antibody.

2.2.7 Peptides dimethyl labeling and analyzed by LC-MS/MS

Three weeks old His8-YFP-PIP2;1::ein3-1/eil1-1 transgenic plants were treated with air or ethylene described as before. Microsomal proteins were extracted from tissues for His-tag transgenic PIP2;1 protein purification. Transgenic proteins were on Ni²⁺-NTA beads trypsin digestion 12 h at 37°C in 50 mM Tris-HCl (pH8.0). Digested peptides from air and ethylene tissues were dimethyl labeled with ¹²CH₂O (Thermo Fisher Scientific) or ¹³CD₂O (Sigma) respectively in buffer containing 3 M NaBH₃CN (Sigma) in 1 M NaOH. In forward experiment, peptides from ethylene treatment tissue labeled with ¹³CD₂O, and peptides from air treatment tissue labeled with ¹²CH₂O; in reciprocal experiment just reverse. Labeling peptides from two treatment groups were mixed together by peptides ratio of 1:1 and desalted.
with zip-tip C18 pipette tips (Millipore) before LC-MS/MS (QE, Thermo Fisher Scientific) analysis.

### 2.2.8 Water loss measurement

In this water loss experiment, the wild type, mutants and transgenic plants were all grown in soil inside growth chambers at 22 ± 2 °C under 24-h light condition with a light intensity of 80-100 μmol photons m\(^{-2}\)s\(^{-1}\). These plants were water-irrigated once every 3 days with 90 mL of water per cup (200 cm\(^3\) soil per paper cup), and 3 plants were cultivated in a cup. Six days before tissues were harvested for water loss measurement, plants were sprayed on leaf surface either with 100 μM AOA or 100 μM AOA plus 5 μM ACC on leaves twice a day, and by three days before being harvested, plants were irrigated for the last time with 30 mL of 0.1× MS solution per cup, which was mixed either with 100 μM AOA or 100 μM AOA plus 5 μM ACC into soil. During this period of reduced water supply, plants were still surface-sprayed either with AOA or AOA plus ACC twice a day. On the day when plants being harvested for physiological experiments, AOA-treated plants and AOA + ACC-treated plants were placed in plastic containers and fumigated either with air or 10-ppm ethylene for additional 12 h, respectively. From these differentially treated two groups of plants (AOA-A and ACC-E treated plants), a total of 10 rosette leaves of each line were detached and placed onto aluminum foil in corresponding plastic containers at 22 ± 2°C with a humidity of 60-80% laboratory condition for water loss measurement according to a modified well-established method (Li et al., 2008). The total fresh weight of these leaves was measured at different time points during the period of being fumigated either with air or ethylene. Five biological replicates were performed for each genotype or transgenic line. The level of leaf water loss was determined as a percentage of the fresh weight measured at various time points over the initial fresh weight measured at the beginning of leaf detachment.
2.2.9 Protoplast generation

Leaf protoplasts from various transgenic plants and backgrouds plants were prepared according to a modified method (Wu et al., 2013), by which 0.1 gram of leaves from 3-week-old with AOA-A and ACC-E treated plants (described in the above section) were sliced into 2-mm strips using a razor blade and transferred to a Petri dish containing 3 mL of enzymatic digestion buffer, which contains 500 mM mannitol, 10 mM MES/KOH (pH 5.8), 10 mM CaCl2, 10 mM KCl, 1% (w/v) cellulase R10 (Gold Biotechnology Inc. St. Louis, MO, USA) and 1% macerozyme R10 (KARLAN RESEARCH PRODUCTS CORPORATION, Phoenix, AZ, USA) into each of these two groups of protoplasts, 100 μM AOA and 100 μM AOA plus 10μM ACC was added into the enzymatic digestion buffer for AOA-A and ACC-E pretreated plant leaf tissues, respectively. These leaf strips submerged in the hydrolytic enzyme digestion buffer were vacuum infiltrated for 30 min to deliver both AOA and ACC into cells. The protoplasts were produced with a gentle shaking of leaf strips in digestion buffer for 2 h at 50 rpm at room temperature. The resulting protoplasts were filtered through a 50 μm nylon filter, centrifuged at 100g at room temperature for 5 min and washed 3 times with enzyme-free digestion buffer. Protoplasts were stored at room temperature for 15 min before being used for assays and protein extraction. The time used for the entire protoplast generation is less than 3 h.

2.2.10 Protoplast swelling and shrinking assays

The protoplasts swelling and shrinking assay method was modified from the previously described (Wu et al., 2013). Five microliter of protoplast suspension was added into a small chamber on the poly-L-Lys (0.1%, Sigma)-treated glass slide and mounted on the stage of an inverted microscope. Some protoplasts were allowed to settle down for 2 - 3 min. The mannitol concentration of 500 mM in the chamber was diluted into 350 mM by adding 5 uL of 200 mM mannitol buffer and mix well immediately. The protoplast cell images were taken every 3 sec with a video camera-equipped microscope for a period of 4 min long. In the case
of the shrinking assay, a mannitol buffer of 650 mM was added to 350 mM mannitol buffer in the chamber at 1:1 ratio to get the final mannitol buffer of 500 mM. Photos of cells were taken again in the same laps of time. The size measurement was identical to that for the swelling assay. Each swelling and shrinking assay experiment was done within 1.5 h after the initial protoplast generation. The osmolality of a buffer was measured with a Vapor Pressure Osmometer (Wescor Vapro 5520, Wescor Inc., Logan, Utah, USA). The osmolality for 650 mM, 500 mM, 350 mM and 200 mM mannitol buffers were measured to be 752 mmol kg\(^{-1}\), 591 mmol kg\(^{-1}\), 419 mmol kg\(^{-1}\) and 262 mmol kg\(^{-1}\), respectively. The diameters of the protoplasts at 0, 1, 2, 3, 4 min of swelling and shrinking assays were measured with the imageJ software (fiji.sc/wiki/index.php/Fiji). Diameters were converted to μm for volume calculation. Student’s t-test analysis was applied onto percentage of volume increase and decrease in protoplast swelling and shrinking assays.

2.2.11 Confocal laser microscopy examination of subcellular localization of PIP2;1 fusion protein in transgenic plant

Detection of YFP fusion protein was performed using a Zeiss LSM 710 confocal microscopy. Imaging of the leaf cells was achieved with a Plan-Apochromat ×40/1.3 oil immersion objective. YFP was excited with the 488-nm argon laser line, and emitted light was collected through a dichroic mirror on detectors from 520 to 550 nm. A transmitted light detector was used for collecting bright-field images. Tissues for the confocal microscopy experiment were prepared from transgenic plants grown on M/S agar-base medium in glass jars supplemented with 100 μM AOA and 100 μM AOA plus 5 μM ACC, respectively. Epidermal layers of leaves from the 3-week-old transgenic plants were mounted on glass slides for the confocal microscope examination in the presence of PBS buffer supplemented either with 100 μM AOA, or 100 μM AOA plus 5 μM ACC for corresponding treated plants.
2.3 Result

2.3.1 Overexpression of PIP2;1 protein in *E.coli* and production of PIP2;1 polyclonal antibody

The pET30a-PIP2;1 construct was firstly validated by enzyme digestion and PCR. Figure 2.3.1 shows that the size of PIP2;1 DNA fragment of enzyme digestion product and PCR product is about 450 bp, and it was consistent with the design. Figure 2.3.2 shows the sequencing result of ORF of 6×His-PIP2;1-6×His fusion gene, the sequence is also correct. The recombinant plasmid was transformed into bacterial *E.coli* strain BL21 and overexpression by IPTG inducible. Figure 2.3.3 shows that His-tag PIP2;1 can overexpression by IPTG induce at 37°C, 30°C or 22°C, and it can has relatively high expression level at 30°C. Therefore, the fusion protein which used for Ni-NTA beads purification was inducible overexpression by 1 mM IPTG at 30°C for 5 h.

The induced PIP2;1 protein was firstly purified by using Ni-NTA beads, and then separated by a 10% SDS-PAGE gel, co-eluted proteins also were separated (Figure 2.3.4A). After re-nature the PIP2;1 protein from the SDS-PAGE gel by using PBS buffer, the protein purity was checked by using SDS-PAGE gel (Figure 2.3.4B), the protein in SDS-PAGE gel was used for in-gel trypsin digestion and analyzed with QTOF-MS machine. MS/MS spectrum result shows that peptides from the purified protein is PIP2;1 (Figure 2.3.5). The protein was then used as antigen to inject into rabbits to generate anti-PIP2 polyclone antibody.

Purified polyclonal antibody was firstly used for western blot analysis to check the quality for experiment. Both the E.coli expressed PIP2;1 protein and total protein of wild-type *Arabidopsis* plant were used to verified the polyclonal antibody which produced from rabbit. Figure 2.3.6 shows that the polyclonal antibody is able to identify both antigen and PIP2;1 protein in *Arabidopsis* plant.
Figure 2.3.1 PCR and enzyme digestion to validate pET30a-PIP2;1 construct for PIP2;1 overexpression in *E.coli*.

M: 1 kb DNA marker.

E: enzyme digestion product (*XhoI/BamHI*: ~450 bp).

P: PCR product (~450 bp).
Figure 2.3.2 Sequencing result of 6xHis-PIP2;1-6xHis fusion gene ORF in pET30a-PIP2;1 construct.
Figure 2.3.3 Overexpression of 6xHis-PIP2;1-6xHis fusion protein in *E.coli*.

**M:** Low molecular weight protein marker.

**C:** Negative control with no IPTG in *E.coli*.

1: Protein express by adding 1 mM IPTG for 5 h at 37°C.

2: Protein express by adding 1 mM IPTG for 5 h at 30°C.

3: Protein express by adding 1 mM IPTG for 5 h at 22°C

4: Protein express by adding 1 mM IPTG for 5 h at 18°C

Arrow indicate molecular weight of 6xHis-PIP2;1-6xHis fusion protein.
Figure 2.3.4 6xHis-PIP2;1-6xHis fusion protein validation after purification and re-nature.

(A): The fusion protein purification from *E.coli*. M: Low molecular weight protein marker. C: Negative control with no IPTG in *E.coli*, S: Protein extract before purification with Ni-NTA beads; E1-E3: Elution fractions from Ni-NTA beads.

(B): Re-natured fusion protein before injected into rabbits. P1, P2: two times repeat.

Arrow indicate size of 6xHis-PIP2;1-6xHis fusion protein.
Figure 2.3.5 MS/MS spectrum of peptides identified by QTOF-MS to validate PIP2;1 protein purified from *E.coli*. 
Figure 2.3.6 Western blot to validate the polyclonal antibody of PIP2;1.

P-Ctrl: 20 ng *E.coli* expressed PIP2;1.

WT: 5 μg microsomal protein of wild-type *Arabidopsis* plant.
2.3.2 Further validation of ethylene up-regulated phosphopeptide of endogenous PIP2;1/2/3 by quantitative phosphoproteomics analysis

ein3-1/eil1-1 is an ethylene insensitive double mutant line. Figure 2.3.7 shows that both of EIN3 and EIL1 genes loss of function in the double mutant plant. EIN3 gene has a point mutation (TGG mutate to TGA) and get a stop codon in the middle of the gene of the double mutant line, and EIL1 gene containing a transposon of the double mutant line. In previous study, some important ethylene regulated phosphorylated peptides were identified from ein3-1/eil1-1 ethylene-insensitive double mutant by SILIA-assisted PTM proteomic method (Table 2.3.1). The phosphorylated peptide with penultimate (Ser-280 of PIP2;1) and ultimate (Ser-283 of PIP2;1) has relatively higher up-regulation level in quantitative phosphopeptides which belongs to C-terminal tail of PIP2;1/2/3 protein, and it was also identified in previous studies (Hsu et al., 2009; Kline et al., 2010; Niittyla et al., 2007; Nuhse et al., 2004; Prado et al., 2013; Prak et al., 2008; Yang et al., 2013). Although PIP2;2 and PIP2;3 protein also have the same C-terminal sequence, PIP2;1 phosphorylation level was greatly higher than PIP2;2 and PIP2;3 under ethylene treatment or air treatment by western-blot analysis of pip2;1, pip2;2, and pip2;1/2 double mutant lines (Figure 2.3.8). pip2;1, pip2;2, and pip2;1/2 mutant double lines have been validated by PCR or RT-PCR. Figure 2.3.9A shows that PIP2;1 gene contains a transposon and can’t be amplified by PCR in pip2;1 mutant. As shown by Figure 2.3.9B that T-DNA insertion in PIP2;2 gene of pip2;2 mutant and also can’t be amplified by PCR. Both of PIP2;1 and PIP2;2 genes can’t be amplified by PCR in pip2;1/2 double mutant (Figure 2.3.9C). RT-PCR result shown by Figure 2.3.10 that PIP2;1 and PIP2;2 genes have no transcription in pip2;1/2 double mutant line. Endogenous PIP2;1 transcription level and protein level are also higher than PIP2;2 and PIP2;3 in Arabidopsis rosette leaves (Alexandersson et al., 2005; Monneuse et al., 2011; Prado et al., 2013). The doubly phosphorylated form on C-terminal of PIP2 was found down-regulated by H2O2 treatment and osmotic stress (Kline et al., 2010; Prak et al., 2008), but up-regulated by dark treatment (Prado et al., 2013) in previous studies. To further confirm the significantly changed phosphorylation levels discovered by quantitative phosphoproteomic analysis of double
phosphorylation sites of PIP2;1 C-terminal tail, liquid chromatography-tandem mass spectrometry analysis of 20~40 kDa microsomal protein in-gel digests from 1:1 ratio mix of 3-week-old $^{14}$N- and $^{15}$N-labeled tissues by air or ethylene treatment 12 hours respectively revealed Ser-280 and Ser-283 phosphorylation up-regulated by ethylene stimulation both in forward and reciprocal experiment (Figure 2.3.11). MS1 data was shown by Figure 2.3.11A, the phosphopeptide peak from ethylene treated ein3-1/eil1-1 double mutant plants is higher than from air treatment plants in both forward and reciprocal experiments, but non-phosphopeptides have the similar high peaks in both forward and reciprocal experiments. Quantitative MS1 data in Figure 2.3.11A also reveals that ethylene up-regulated the Ser-280 and Ser-283-doubly phosphorylation 2.04 ± 0.22-fold than air treatment tissue in ein3-1/eil1-1, and a non-phosphorylated peptide has no significant change, only 0.99 ± 0.01-fold in comparison. The peptide ions of 277, 275, 275SLG$_p$SFR$_p$SAANV, were found using MS/MS spectra (Figure 2.3.11B). Among the y ion series of $^{14}$N-code peptide shown in the MS/MS spectra, y6 ion was found to has a neutral loss of H$_3$PO$_4$ moiety ($\Delta m = 98$ Da) and y9 has a neutral loss of 2 H$_3$PO$_4$ moieties ($\Delta m = 196$ Da), respectively. In b ion series, as b5, b6 were found to have a neutral loss of H$_3$PO$_4$ moiety ($\Delta m = 98$ Da) and b8, b9 have a neutral loss of 2 H$_3$PO$_4$ moieties ($\Delta m = 196$ Da), respectively. For $^{15}$N-code peptide ions, y5 ion was found to has a neutral loss of H$_3$PO$_4$ moiety ($\Delta m = 98$ Da) and y9 has a neutral loss of 2 H$_3$PO$_4$ moieties, respectively. In b ion series, as b4, b6 were found to have a neutral loss of H$_3$PO$_4$ moiety ($\Delta m = 98$ Da) and b9, b10 have a neutral loss of 2 H$_3$PO$_4$ moieties ($\Delta m = 196$ Da) respectively. Therefore, further validation data by using quantitative phosphoproteomics method indicate Ser-280 and Ser-283 phosphorylation up-regulated by ethylene independent of EIN3 and EIL1.
Figure 2.3.7 Genotype validation of *ein3-1/eil1-1* double mutant line.

A: PCR validation of EIL gene mutation. PCR product is about 650 bp for mutant gene and 400 bp for wild-type gene.

B: Sequencing validation of EIN3 gene mutation from No.1 plant.
### Table 2.3.1 Phosphopeptides affected by 12 h ethylene treatment in *ein3-1/eil1-1*

<table>
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<tr>
<th>Accession No.</th>
<th>Fold-change$^a$</th>
<th>p-value$^b$</th>
<th>Phosphopeptide$^c$</th>
<th>Motif category</th>
<th>Protein</th>
<th>Subcellular location$^d$</th>
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<tbody>
<tr>
<td><strong>Ethylene enhanced</strong></td>
<td></td>
<td></td>
<td></td>
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<td>AT5G35220.</td>
<td>1.67</td>
<td>$4.0 \times 10^{-5}$</td>
<td>SYTSVGSPSAANV</td>
<td>Plasma membrane intrinsic protein 2,3 (FPP2,3)</td>
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<tr>
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<td>1.26</td>
<td>$5.2 \times 10^{-6}$</td>
<td>212KEQVSGYPYEEAVS</td>
<td>Ranp[6]</td>
<td></td>
<td>ENTH-VHS family protein</td>
</tr>
<tr>
<td><strong>Ethylene suppressed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2G20000</td>
<td>-1.55</td>
<td>$3.4 \times 10^{-5}$</td>
<td>QLVSNESADLQKQGTELDGK</td>
<td>[pS]xxD/E</td>
<td>Delta1-pyrrolin-5-carboxylate synthase 1 (PCS1)</td>
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<tr>
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<td>-0.49</td>
<td>$8.7 \times 10^{-5}$</td>
<td>50GLAVD5TSDQTDTR</td>
<td>[pS]xxD/E</td>
<td>Rubisco small subunit (RCA)</td>
<td>apoplast, cell wall, chloroplast, chloroplast envelope, chloroplast stroma, chloroplast thylakoid membrane, membrane, nucleus, plastoglobule, thylakoid</td>
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<td>$8.0 \times 10^{-5}$</td>
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<td>-</td>
<td>21LGDGAASLHVK</td>
<td>Fruuctose-1,6-bisphosphate aldolase E (FBAL)</td>
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$^a$ log2 fold changes were employed as the cutoff of log2 change in the selection of the altered phosphopeptides.
$^b$ Given by two-tailed Student's t-test. All phosphopeptides listed were selected using two-tailed Student's t-test followed by Benjamini-Hochberg correction (FDR<0.05).
$^c$ The phosphorylated amino acids (Ser or Thr) are marked by subscript p.
$^d$ The information is based on the annotation of TAIR (http://www.arabidopsis.org/).
$^e$ The result was not given from statistical test.
**Figure 2.3.8 Western blot to validate Ser-280 and Ser-283 phosphorylation in *pip2;1*, *pip2;2* and *pip2;1/2* double mutants under ethylene treatment.**

*Arabidopsis* seedlings grown to 3 weeks on M/S agar-base medium in glass jars supplemented with 100 μM AOA and treated with air or 10 ppm ethylene for 12 h before collecting tissues. The Microsomal proteins were extracted from each treatment tissue, and 5 μg protein was used for western-blot analysis. The arrowhead shows PIP2;1/2/3 protein monomer.
Figure 2.3.9 PCR validation of *pip2;1*, *pip2;2* and *pip2;1/2* double mutant lines.

A: PCR validation of *pip2;1* mutant. M: 1 kb DNA marker; length of PCR product is ~ 1.5 kb.

B: PCR validation of *pip2;2* mutant. M1: 100 bp DNA ladder; LB: left border primer and gene reverse primer; FR: gene forward and reverse primers; length of PCR product is ~1.0 kb.

C: PCR validation of *pip2;1/2* double mutant. M: 1kb DNA marker; a: PIP2;1 gene validation; b: PIP2;2 gene validation; length of PCR product of PIP2;1 gene is about 1.5 kb and PIP2;2 gene is ~1.0 kb. WT: wild-type plant as control.
Figure 2.3.10 RT-PCR validation *pip2;1/2* double mutant line.

**A:** RNA from *pip2;1/2* double mutant and wild-type plants.

**B:** RT-PCR result of PIP2;1 and PIP2;2 genes in *pip2;1/2* double mutant and wild-type plants. [M: 1 kb DNA marker; a: PIP2;1 gene validation (~850 bp); b: PIP2;2 gene validation (~750 bp).]

**C:** RT-PCR result of ACTIN2 gene to check cDNA quality. (M1: 100 bp DNA ladder; length of PCR product is about 400 bp.)
Figure 2.3.11 SILIA-based quantitative phosphoproteomic to validate PIP2;1/2/3 phosphorylation in ethylene-treated ein3-Ieil1-I double mutant.

(A) MS spectra and quantification of ethylene-up-regulated phosphopeptide and none-regulated none-phosphopeptide of PIP2;1/2/3 endogenous protein. “Forward” and “reciprocal” refer to the order of mixing of $^{15}$N-coded proteins with $^{14}$N-coded protein samples. In forward experiment, $^{15}$N labeling plants treated with ethylene and $^{14}$N labeling plants treated with air; in reciprocal experiment just reverse, bar = mean ± SE.

(B) MS/MS spectrum of 12 h ethylene up-regulated phosphopeptide of plasma membrane intrinsic protein 2;1/2/3. The upper and lower panel is the mass spectrogram of $^{14}$N- and $^{15}$N-coded peptide ions, respectively.
2.3.3 Validation of ethylene up-regulated phosphopeptide of endogenous PIP2;1/2/3 by western blot analysis

Western blot is another approach to validate protein expression level or phosphorylation level in cells. In order to further confirm the quantitative phosphoproteomics data, anti-pSer280/S283 phosphorylated polyclone antibody (anti-pS280/S283-PIP2) and anti-pSer283 phosphorylated polyclone antibody (anti-pS283-PIP2) were used in western blot analysis. Relative phosphorylation level was normalized by using anti-PIP2 protein polyclonal antibody. Data was shown by Figure 2.3.12 that doubly phosphorylated protein up-regulate 3.59 ± 0.15-fold and 1.66 ± 0.03-fold in wild type, ein3-1/eil1-1 background respectively; Ser-283 phosphorylated protein up-regulate 2.16 ± 0.16-fold and 1.58 ± 0.18-fold in wild-type, ein3-1/eil1-1 background respectively. It also reveals that the Ser-280 and Ser-283-doubly phosphorylated peptide on C-terminal tail of endogenous PIP2;1 still up-regulated by ethylene independent the ethylene-signaling components EIN3 and EIL1, which consistent with quantitative phosphoproteomics result.
Figure 2.3.12 Quantitative western-blot analysis of Ser-280 and Ser-283 phosphorylation of endogenous PIP2;1/2 in wild-type and ein3-1/eil1-1 double mutant lines. (A) and (B) shows the relative phosphorylation level of both doubly (A) and singly (B) phosphorylated endogenous PIP2;1/2 (30 kDa) proteins tested by anti-pS280/S283-PIP2 and anti-pS283-PIP2 antibodies, respectively, against that by anti-PIP2 antibody. All Arabidopsis seedlings were grown to 3 weeks old on M/S agar-base medium in glass jars supplemented with 100 μM AOA and treated with air or 10 ppm ethylene for 12 h before collecting tissues. The microsomal proteins were extracted from each treatment tissue and 5 μg protein was used for western-blot analysis. Three times western-blot analysis results were quantified. Error bars are ± SE (n=3). Statistical significance is calculated by student’s t-test. *, ** and *** represent p < 0.05, p < 0.01 and p < 0.001, respectively.
2.3.4 Wild-type and mutant PIP2;1 gene overexpression in transgenic lines

To study Ser-280 and Ser-283 phosphorylation and biological function of PIP2;1 isoform protein, prepared wild-type and mutant PIP2;1 constructs were introduced into pip2;1/2 double mutant line by the floral dip method; wild-type PIP2;1 construct also transformed into ein3-1/eil1-1 double mutant background (Constructs design showed by Figure 2.3.13). All constructs were validated firstly by PCR and enzyme digestion, and the point mutation sites were confirmed by sequencing. Figure 2.3.14 shows the PCR and enzyme digestion data that the size of PIP2;1 genomic DNA on recombinant plasmids is correct, about 1.7 kb. The ORF of the fusion gene include His₈-YFP tag is about 2.4 kb. And Figure 2.3.15 shows that Ser-280 and Ser-283 point mutant sites were also correct. Transgenic plants which screen out from MS agar plates containing hygromycin were firstly validate the fusion gene His₈-YFP-PIP2;1 (or mutant PIP2;1) by PCR. Figure 2.3.16 shows that the length of PCR product of His₈-YFP-PIP2;1(or mutant PIP2;1) fusion gene is about 2.4 kb, and it consistent with the design. In order to validate the fusion gene expression in transgenic plants, western blot was used to check protein expression. As shown by Figure 2.3.17 that wild-type PIP2;1 and mutant PIP2;1 can express in transgenic plants, the molecular weight of the fusion protein is about 59 kDa. And phenotype of wild-type and point mutation transgenic lines has no signification difference of the same background (Figure 2.3.18).
<table>
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<th>Double-35S promoter</th>
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<th>8xHis</th>
<th>EYFP</th>
<th>WT/Mutant PIP2;1</th>
<th>NOS</th>
</tr>
</thead>
</table>

**Figure 2.3.13 WT/Mutant PIP2;1 overexpression construct map.** Double-35S promoter: a dual cauliflower mosaic virus promoter region, and the first 35S promoter without TATA box; UTR: rubisco 5’-untranslated region; WT/Mutant PIP2;1: *Arabidopsis* full-length genomic DNA of PIP2;1 gene (none mutated gene and S280A, S283A, S280/3A mutated gene); NOS: Nos terminator.
Figure 2.3.14 Validation of pHUB10-UTR-His<sub>8</sub>-YFP-PIP2;1 (WT, S280A, S283A, S280/3A) constructs by PCR and enzyme digestion.

M: 1kb DNA marker.

D: Enzyme digestion product (AscI and SacI, ~1.7kb).

P: PCR product (~1.7kb).
Figure 2.3.15 Sequencing result of WT and mutant PIP2;1 genes on constructs.

S: Serine; A: Alanine; S280A: Ser-280 site mutates to alanine; S283A: Ser-283 site mutates to Alanine; S280/3A: both Ser-280 and Ser-283 sites mutate to alanine.
Figure 2.3.16 PCR validation of His$_8$-YFP-PIP2;1 (WT and mutant PIP2;1) fusion gene in transgenic plants.

M: 1kb DNA marker.

W: His$_8$-YFP-PIP2;1::WT.

E: His$_8$-YFP-PIP2;1::ein3-1/eil1-1.

P1: His$_8$-YFP-PIP2;1::pip2;1/2.

P2: His$_8$-YFP-PIP2;1::pip2;1/2.$^{S280A}$

P3: His$_8$-YFP-PIP2;1::pip2;1/2.$^{S283A}$

P4,5: His$_8$-YFP-PIP2;1::pip2;1/2.$^{S280A/S283A}$

NC: wild-type background as negative control.

The size of PCR product of the whole fusion gene is about 2.4 kb.
Figure 2.3.17 Western blot validation of WTPIP2;1 and mutant PIP2;1 transgenic plants.

M: Low molecular weight protein marker.

NC: pip2;1/2 double mutant as negative control.

The size of the fusion protein (Hist8-YFP-PIP2;1) is about 59 kDa with arrow indicate.
Figure 2.3.18 Phenotype of background plants and PIP2;1 transgenic plants.

A: Three weeks old ein3-1/eil1-1 double mutant, wild-type and pip2;1/2 double mutant plants.

B: wtPIP2;1 and point mutation transgenic plants.

T-ein3-1/eil1-1: HisGFP-PIP2;1::ein3-1/eil1-1 transgenic plant.

T-pip2;1/2: HisGFP-PIP2;1::pip2;1/2 transgenic plant.

280A: HisGFP-PIP2;1S280A::pip2;1/2 transgenic plant.

283A: HisGFP-PIP2;1S283A::pip2;1/2 transgenic plant.

280/3A: HisGFP-PIP2;1S280A/S283A::pip2;1/2 transgenic plant.
2.3.5 Ethylene up-regulated phosphorylation of PIP2;1 isoform protein in transgenic lines

To further confirm ethylene up-regulated Ser-280 and Ser-283-doubly phosphorylation sites of PIP2;1 isoform, we transformed *Arabidopsis* plants with a fusion gene (His<sub>8</sub>-YFP-PIP2;1) under the control of double cauliflower mosaic virus 35S promoter and overexpressed in the wild-type, *ein3-1/eil1-1* and *pip2;1/2* double mutant background lines (see “materials and methods”) respectively. Three weeks old transgenic plants were treated with air or ethylene for 12 h. Western-blot analysis shows that the relative phosphorylation level of Ser-280 and Ser-283-doubly phosphorylated transgenic fusion protein (His<sub>8</sub>-YFP-PIP2;1) which purified by Ni<sup>2+</sup>-NTA beads from microsomal proteins still up-regulated by 10 ppm ethylene treatment for 12 h in three different backgrounds transgenic lines (Figure 2.3.19). Figure 2.3.19 shows doubly phosphorylated protein and Ser-283 phosphorylated of transgenic PIP2;1 isoform protein up-regulated by ethylene in western-blot quantitative analysis, doubly phosphorylated protein up-regulate 4.86 ± 0.18-fold, 2.03 ± 0.11-fold and 3.82 ± 0.23-fold in wild type, *ein3-1/eil1-1* and *pip2;1/2* background respectively; Ser-283 phosphorylated protein up-regulate 2.73 ± 0.10-fold, 1.81 ± 0.06-fold and 2.57 ± 0.21-fold in wild-type, *ein3-1/eil1-1* and *pip2;1/2* background respectively.

In order to further validate transgenic PIP2;1 protein up-regulated by ethylene independent of EIN3/EIL1, dimethyl labeling phosphopeptides from 3-week-old 12 h air or ethylene treated *His<sub>8</sub>-YFP-PIP2;1::ein3-1/eil1-1* transgenic plants analysis with LC-MS/MS method. Both of the MS1 spectra quantification and MS2 spectrum results showed by Figure 2.3.20A, data shows that the phosphorylation peptide <sub>27</sub>SLG<sub>p</sub>SFR<sub>p</sub>SAANV up-regulated 1.28 ± 0.04-fold by ethylene in forward and reciprocal experiments. MS2 spectrums were showed by Figure 2.3.20B, among the y ion series of light formaldehyde labeling peptide shown in the MS/MS spectra, y7 ion was found to has a neutral loss of H<sub>3</sub>PO<sub>4</sub> moiety (Δm = 98 Da) and y9, y10 have a neutral loss of 2 H<sub>3</sub>PO<sub>4</sub> moieties (Δm = 196 Da), respectively. In b ion series, as b4, b5, b6 were found to have a neutral loss of H<sub>3</sub>PO<sub>4</sub> moiety (Δm = 98 Da) and b7, b8, b9, b10 have
neutral loss of 2 H$_3$PO$_4$ moieties (Δm = 196 Da). For heavy formaldehyde labeling peptide ions, y7 ion was found to have a neutral loss of H$_3$PO$_4$ moiety (Δm = 98 Da) and y9, y10 have a neutral loss of 2 H$_3$PO$_4$ moieties (Δm = 196 Da), respectively. In b ion series, as b4, b6 were found to have a neutral loss of H$_3$PO$_4$ moiety (Δm = 98 Da). On the basis of this MS quantitative result, PIP2;1 isoform is a novel signaling component of phosphorylation regulation by ethylene independent EIN3 and EIL1 in ethylene signaling transduction pathways. Except for PTM modification peptides, other peptide of transgenic PIP2;1 protein also were identified by LC-MS/MS analysis (Table 2.3.2). Therefore, PIP2;1 protein which transferred into Arabidopsis plant is successful and has the same regulation as endogenous PIP2;1 protein under ethylene condition in ein3-1/eil1-1 double mutant line.

A further detailed analysis of the MS/MS fragmentation spectra of endogenous PIP2;1/2/3 isolated from wild type plants revealed that Ser-277/Ser-283 double phosphorylation modification on 277,275,275pSLGSFRpSAANV peptide, which is of 2 charges (MS2 showed by Figure 2.3.20B). In the fragmentation ions of phosphopeptide 277,275,275pSLGSFRpSAANV, y5, y6 and y7 ions were found to have a neutral loss of H$_3$PO$_4$ moiety (Δm = 98 Da), whereas in the case of b ion series, b2, b3, b4, b5 and b6 ions were found to have a neutral loss of single H$_3$PO$_4$ moiety, and, in contrast, b7, b8 and b9 ions have a neutral loss of 2 H$_3$PO$_4$ moieties (Δm =196). Based on the MS/MS fragmentation spectra of these ions, S277 and S283 were both phosphorylated on this peptide. Because this doubly phosphorylated PIP2;1 peptide was only detected from $^{14}$N-coded peptide, it was not used for quantitation of ethylene-regulated phosphorylation.
Figure 2.3.19 Quantitative western-blot analysis of Ser-280 and Ser-283 phosphorylation of transgenic PIP2;1 isoform in wild-type, ein3-1/eil1-1 and pip2;1/2 backgrounds respectively. (A) and (B) shows the relative phosphorylation level of both doubly (A) and singly (B) phosphorylated transgenic protein His$_8$-YFP-PIP2;1 (59 kDa) illustrated by anti-pS280/S283-PIP2 and anti-pS283-PIP2 antibodies, respectively, against that by anti-PIP2 antibody. All *Arabidopsis* seedlings were grown to 3 weeks old on M/S agar-base medium in glass jars supplemented with 100 μM AOA and treated with air or 10 ppm ethylene for 12 hours before collecting tissues. His$_8$-YFP-PIP2;1 fusion protein was purified from microsomal proteins by Ni-NTA beads, eluted protein was used for western-blot analysis. Rabbit polyclonal antibody raised against both Ser-280 and Ser-283 phosphorylation sites of PIP2;1, rabbit polyclonal antibody raised against Ser-283 phosphorylation site of PIP2;1 and a homemade rabbit polyclonal antibody raised against PIP2;1 were used to calculate relative phosphorylation level. Three times western-blot analysis results were quantified. Error bars are ± SE (n=3). Statistical significance is calculated by student’s t-test. ** and *** represent $p < 0.01$ and $p < 0.001$, respectively.
Figure 2.3.20 Phosphopeptide quantitation and PTM analysis of transgenic PIP2;1 protein in ethylene-treated His_{8}-YFP-PIP2;1::ein3-1/eil1-1 transgenic plant.

(A) MS spectra and quantification of ethylene-up-regulated phosphopeptide and none phosphopeptide of transgenic PIP2;1 isoform protein. In forward experiment, $^{13}$CD$_2$O labeling peptides from ethylene treatment tissues mix with $^{12}$CH$_2$O labeling peptides from air treatment tissues at 1:1 ratio; in reciprocal experiment just reverse, bar = mean ± SE.

(B) MS/MS spectrum of 12 h ethylene up-regulated phosphopeptide from transgenic plants and S277/S283 doubly phosphorylated peptides detected from wild-type plants.
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2.3.6 Phosphorylated Ser-280 and Ser-283 of PIP2;1 isoform up-regulated patterns under time courses ethylene treatment

To further study phosphorylated PIP2;1 protein regulation under ethylene treatment condition. His$_8$-YFP-PIP2;1::ein3-1/eil1-1 and His$_8$-YFP-PIP2;1::pip2;1/2 transgenic lines were treated with 10 ppm ethylene for 0, 4, 8, 24 h respectively. His$_8$-YFP-PIP2;1 fusion proteins were purified from treated tissues and the phosphorylated proteins regulation were validated by western-blot analysis with anti-pS280/S283-PIP2 and anti-pS283-PIP2 phosphorylated polyclonal antibodies, then using fusion protein level detected by anti-PIP2 polyclonal antibody for normalization. PIP2;1 isoform relative phosphorylation level was quantified by signal intensity on western-blot films (Figure 2.3.21). Figure 2.3.21A shows that Ser-280 and Ser-283-doubly phosphorylated protein and Figure 2.2.21B shows that Ser-283 single phosphorylated protein up-regulated under 4, 8, 24 h ethylene treatment respectively in His$_8$-YFP-PIP2;1::pip2;1/2 and His$_8$-YFP-PIP2;1::ein3-1/eil1-1 transgenic lines. Data shows that C-terminal of PIP2;1 phosphorylation level was rapidly enhanced by 10 ppm ethylene treatment. After 4 h ethylene treatment, the double and single phosphorylation levels up-regulated 2.62 ± 0.15-fold and 2.00 ± 0.13-fold in His$_8$-YFP-PIP2;1::ein3-1/eil1-1 transgenic plants and 3.52 ± 0.36-fold and 4.67 ± 0.32-fold in His$_8$-YFP-PIP2;1::pip2;1/2 transgenic plants, respectively. And the phosphorylation level further raised by 8 h ethylene treatment to 4.08 ± 0.18-fold and 2.57 ± 0.21-fold in His$_8$-YFP-PIP2;1::ein3-1/eil1-1 transgenic plants and 4.29 ± 0.17-fold and 4.87 ± 0.17-fold in His$_8$-YFP-PIP2;1::pip2;1/2 transgenic plants, respectively. However, the double and single phosphorylation levels dropped back to 2.19 ± 0.11-fold and 1.51 ± 0.26-fold respectively in His$_8$-YFP-PIP2;1::ein3-1/eil1-1 transgenic plants, whereas kept increasing to 2.19 ± 0.11-fold and 1.51 ± 0.26-fold respectively in His$_8$-YFP-PIP2;1::pip2;1/2 transgenic plants. Maybe ein3-1/eil1-1 double mutant and wild-type have different kinases or phosphatases activity to regulate PIP2;1 phosphorylation in vivo when in a long time high concentration ethylene condition.
Figure 2.3.21 Phosphorylation of Ser-280 and Ser-283 of His$_8$-YFP-PIP2;1 regulated by ethylene treatment of different periods of time. Transgenic fusion proteins His$_8$-YFP-PIP2;1 (59 kDa) from His$_8$-YFP-PIP2;1::ein3-1/eil1-1 plants (A) and His$_8$-YFP-PIP2;1::pip2;1/2 plants (B) treated by 10 ppm ethylene for 0, 4, 8 and 24 h were tested using western-blot analysis with anti-pS280/S283-PIP2, anti-pS283-PIP2 and anti-PIP2 antibodies respectively. The relative phosphorylation level of is calculated from the relative level of anti-pS280/S283-PIP2 or anti-pS283-PIP2 antibodies against anti-PIP2 antibody. Three times western-blot analysis repeats were included. Error bars are ± SE (n=3). Statistical test employing student’s $t$-test was performed between sample of 4, 8 and 24 h treatment and that of 0 h treatment. * , ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$. 
2.3.7 Phosphorylated Ser-280 and Ser-283 of PIP2;1 isoform up-regulated patterns under dose ACC treatment

Based on phosphorylated PIP2;1 protein up-regulation under ethylene treatment condition, dose of ethylene precursor (ACC) treatment was used to study Ser-280 and Ser-283 phosphorylation regulation of PIP2;1 isoform by ethylene which produced from plant cells. In doses ACC treatment experiment, 100 uM AOA plus different concentration of ACC (0, 1, 5 and 10 uM) were added into MS agar-base medium respectively, and 3 weeks old plant tissue was used for western blot analysis. Figure 2.3.2 shows that the relative levels of both double and singly phosphorylated protein increased linearly. Ser-280 and Ser-283 two sites phosphorylation level of PIP2;1 isoform increase 1.93 ± 0.25-fold and 1.96 ± 0.28-fold in *His*<sub>8</sub>*YFP-PIP2;1::pip2;1/2* and *His*<sub>8</sub>*YFP-PIP2;1::ein3-1/eil1-1* respectively when ACC concentration increase to 10 μM. And Ser-283 phosphorylation level also increase, although has only 1.45 ± 0.04-fold and 1.55 ± 0.24-fold under 10 μM ACC treatment in *His*<sub>8</sub>*YFP-PIP2;1::pip2;1/2* and *His*<sub>8</sub>*YFP-PIP2;1::ein3-1/eil1-1* respectively. After fitting the quantitative results from western blot analysis, the slope of double phosphorylation protein were 0.10 and 0.09 for *His*<sub>8</sub>*YFP-PIP2;1::ein3-1/eil1-1* and *His*<sub>8</sub>*YFP-PIP2;1::pip2;1/2*, respectively, whereas the slope of single phosphorylation protein were 0.05 and 0.04 for *His*<sub>8</sub>*YFP-PIP2;1::ein3-1/eil1-1* and *His*<sub>8</sub>*YFP-PIP2;1::pip2;1/2*, respectively. Therefore, ACC also can up-regulate PIP2;1 phosphorylation in *His*<sub>8</sub>*YFP-PIP2;1::pip2;1/2* and *His*<sub>8</sub>*YFP-PIP2;1::ein3-1/eil1-1* transgenic plants just like ethylene treatment, and this result also indicate that the Ser-280 and Ser-283 of PIP2;1 isoform phosphorylation up-regulation under ACC treatment independent of EIN3/EIL1 signaling component.
Figure 2.3.22 Phosphorylation of Ser-280 and Ser-283 of His$_8$-YFP-PIP2;1 regulated by different concentration of ACC. The dose-dependence of the double (A) and single (B) phosphorylation level of the transgenic fusion proteins His$_8$-YFP-PIP2;1 (~58 kDa) from *His$_8$-YFP-PIP2;1::ein3-1/eil1-1* (diamond) and *His$_8$-YFP-PIP2;1::pip2;1/2* (circular) treated by 0, 1, 5 and 10 μM ACC were analyzed using western-blot. Transgenic seedlings were grown to three weeks old on M/S agar-base medium in glass jars supplemented with 100 μM AOA plus 0, 1, 5 and 10 μM ACC. Purified His$_8$-YFP-PIP2;1 fusion protein was used for western-blot analysis with anti-pS280/S283-PIP2, anti-pS283-PIP2 and anti-PIP2 antibodies respectively. The relative phosphorylation level of is calculated from the relative level of anti-pS280/S283-PIP2 or anti-pS283-PIP2 antibodies against anti-PIP2 antibody. Three times western-blot analysis repeats were included. Error bars are ± SE (n=3). Experimental data were fitted by linear function and the results were illustrated in dash (*His$_8$-YFP-PIP2;1::ein3-1/eil1-1*) and solid (*His$_8$-YFP-PIP2;1::pip2;1/2*) lines, respectively.
2.3.8 Ethylene up-regulated Ser-280 and Ser-283 phosphorylation of PIP2;1 isoform increase the cellular water transport activity independent of EIN3/EIL1

Phosphorylation modification of plant aquaporins has function of affecting water channel activity directly (Johnson and Chrispeels, 1992; Miao et al., 1992; Weaver and Roberts, 1991, 1992). Ser-280 and Ser-283 of PIP2;1 phosphorylation enhanced under dark condition can increase water permeability of leaf protoplasts (Prado et al., 2013). In this study, based on ethylene up-regulated PIP2;1 phosphorylation in plant leaf tissue, both of protoplasts swelling and shrinking assay were used to study Ser-280 and Ser-283 phosphorylation sites biological function in single cell. We prepared protoplasts from leaves of 3-week-old transgenic lines (His8-YFP-PIP2;1::ein3/eil1, His8-YFP-PIP2;1::pip2;1/2, His8-YFP-PIP2;1S280A::pip2;1/2, His8-YFP-PIP2;1S283A::pip2;1/2 and His8-YFP-PIP2;1S280A/S283A::pip2;1/2) pretreated by spraying 3-day 100 μM AOA or 100 μM AOA plus 10 μM ACC, then treated by 12 h air or 10 ppm ethylene, and compared protoplasts swelling and shrinking rate in 240 seconds. Western blot analysis data shows that protoplasts from ACC/ethylene treated His8-YFP-PIP2;1::ein3/eil1-1 and His8-YFP-PIP2;1::pip2;1/2 transgenic plants have relatively high phosphorylation level of His8-YFP-PIP2;1 fusion protein (Figure 2.3.23). And fluorescence protoplasts from transgenic plants were used in swelling and shrinking assay experiment. Figure 2.3.24 shows that fluorescence protoplasts from ACC/ethylene treated wild-type PIP2;1 transgenic plants of pip2;1/2 and ein3-1/eil1-1 backgrounds have more quickly swelling and shrinking rate in assay than from AOA/air treated transgenic plants. Figure 2.3.24A shows in 240 seconds protoplasts swelling assay experiment from 500 mM mannitol change to 350 mM mannitol buffer, protoplasts volumes can increase 23.84 ± 1.18% and 24.70 ± 1.00% of initial volume from ACC/ethylene treated His8-YFP-PIP2;1::ein3-1/eil1-1 and His8-YFP-PIP2;1::pip2;1/2 transgenic plants respectively; but only increase 12.77 ± 0.73% and 13.17 ± 0.73% from AOA/air treated transgenic plants respectively. Figure 2.3.24B shows that in 240 seconds protoplasts shrinking assay experiment, when 350 mM mannitol change to 500 mM mannitol buffer, protoplasts
volumes can shrink to $75.74 \pm 0.66\%$ and $77.87 \pm 0.76\%$ of initial volume from ACC/ethylene treated $\text{His}_8$-$\text{YFP}$-PIP2;1::$\text{ein3-1/eil1-1}$ and $\text{His}_8$-$\text{YFP}$-PIP2;1::pip2;1/2 transgenic plants respectively; but only shrink to $84.82 \pm 0.58\%$ and $86.58 \pm 0.50\%$ from AOA/air treated transgenic plants respectively. For Ser-280 or Ser-283 mutated to alanine of PIP2;1 transgenic lines, protoplasts from these two lines which treated with ACC/ethylene also have relatively higher swelling and shrinking rate than from AOA/air treated plants, but the increasing ratio is less than from wild-type PIP2;1 transgenic lines in 4 min assay. S280A and S283A transgenic plants can increase $19.79 \pm 0.78\%$ and $19.55 \pm 0.78\%$ respectively in swelling experiments, and decrease to $80.58 \pm 0.77\%$ and $80.54 \pm 0.73\%$ respectively in shrinking experiments of ACC/ethylene treated transgenic plants. It also have significant difference to swelling and shrinking rate of protoplast from correspond of AOA/air treated transgenic plants ($p<0.001$), volume of protoplasts only increase $13.33 \pm 0.52\%$ and $13.13 \pm 0.56\%$ respectively in swelling assay, and decrease to $85.67 \pm 0.73\%$ and $86.68 \pm 0.85\%$ respectively in shrinking assay. When both Ser-280 and Ser-280 phosphorylation sites were abolished in pip2;1/2 background transgenic line, swelling or shrinking rate of protoplast has no significant difference between from ethylene and air treatment plants. At 4 min assay, volume of protoplasts from ACC/ethylene and AOA/air treated $\text{His}_8$-$\text{YFP}$-PIP2;1$^{\text{S280A/S283A}}$::pip2;1/2 transgenic plants only increase $12.16 \pm 0.79\%$ and $14.24 \pm 1.02\%$ respectively in swelling experiments, and decrease to $89.18 \pm 0.97\%$ and $88.85 \pm 0.95\%$ respectively in shrinking experiments. Figure 2.3.24C shows the morphology of transgenic plants protoplasts before and after swelling/shrinking assay.

Protoplast swelling and shrinking assay also were used in $\text{ein3-1/eil1-1}$, wild-type and pip2;1/2 background, the result shows protoplasts from ethylene treated $\text{ein3-1/eil1-1}$ and wild-type have more quickly swelling and shrinking rate than air treated plants as endogenous PIP2;1 phosphorylation increase by ethylene; but no significant difference for pip2;1/2 background (Figure 2.3.25) as there are no PIP2;1 and PIP2;2 protein in mutant plant. Figure 2.3.25A shows in 240 seconds protoplasts swelling assay experiment from 500 mM mannitol change to 350 mM mannitol buffer, protoplasts volumes can increase $19.02 \pm 0.61\%$ and $17.28 \pm 0.81\%$ of initial volume from ACC/ethylene treated wild-type and $\text{ein3-1/eil1-1}$
double mutant plants respectively; but only increase $12.03 \pm 0.45\%$ and $10.39 \pm 0.54\%$ from AOA/air treated transgenic plants respectively. About $6.99\%$ and $6.89\%$ of initial volume increase from after ethylene treated wild-type and $ein3-1/eil1-1$ double mutant plants respectively. Figure 2.3.25B shows that in 240 seconds protoplasts shrinking assay experiment, when $350$ mM mannitol change to $500$ mM mannitol buffer, protoplasts volumes can shrink to $79.87 \pm 0.70\%$ and $81.16 \pm 0.87\%$ of initial volume from ACC/ethylene treated wild-type and $ein3-1/eil1-1$ double mutant plants respectively; but only shrink to $86.35 \pm 0.56\%$ and $87.98 \pm 0.69\%$ from AOA/air treated wild-type and $ein3-1/eil1-1$ double mutant plants respectively. And about $6.48\%$ and $6.82\%$ of initial volume decrease from after ethylene treated wild-type and $ein3-1/eil1-1$ double mutant plants respectively. For $pip2;1/2$ double mutant, protoplasts volumes only increase $11.31 \pm 0.20\%$ for ACC/ethylene treated plants, and has small difference to from AOA/air treated plants ($10.68 \pm 0.63\%$) in swelling assay; In shrinking assay, protoplasts volumes only decrease to $87.68 \pm 0.80\%$ for ACC/ethylene treated plants, and has small difference to from AOA/air treated plants ($87.56 \pm 0.96\%$). Figure 2.3.25C shows the morphology of protoplasts before and after swelling/shrinking assay. Base of protoplast swelling and shrinking assay result, Ser-280 and Ser-283 phosphorylation of PIP2;1 isoform increase cellular water transport activity also independent of EIN3/EIL1.
Western-blot validation of Ser-280 and Ser-283 phosphorylation in protoplast of His8-YFP-PIP2;1::ein3-1/eil1-1 and His8-YFP-PIP2;1::pip2;1/2 transgenic lines.

**A0:** 3-week-old transgenic plants by spraying 100 μM AOA for 3 days, then 12hr air treatment before generating protoplast; freshly prepared protoplast mixed with 2×SDS loading buffer and boiled at 95 °C for 10 min, then it was used for western-blot analysis.

**E0:** 3-week-old transgenic plants by spraying 100 μM AOA plus 10 μM ACC for 3 days, then 10 ppm ethylene treatment for 12 h before generating protoplast; freshly prepared protoplast mixed with 2×SDS loading buffer and boiled at 95 °C for 10 min, then it was used for western-blot analysis.

**A2:** 3-week-old transgenic plants by spraying 100 μM AOA for 3 days, then 12 h air treatment before generating protoplast; prepared protoplast stayed at room temperature for 2 h, mixed with 2 × SDS loading buffer and boiled at 95 °C for 10 min, then it was used for western-blot analysis.

**E2:** Three weeks old transgenic plants by spraying 100 μM AOA plus 10 μM ACC for 3 days, then 10 ppm ethylene treatment for 12 h before generating protoplast; prepared protoplast stayed at room temperature for 2 h, mixed with 2 × SDS loading buffer and boiled at 95 °C for 10 min, then it was used for western-blot analysis.

Arrow indicates ~58 kDa His8-YFP-PIP2;1 protein.
Figure 2.3.24 PIP2;1 phosphorylation increase protoplast swelling/shrinking rate in transgenic lines.

A: Transgenic plants protoplast swelling assay result. B: Transgenic plants protoplast shrinking assay result. C: Morphology of protoplasts before and after swelling/shrinking assay. Protoplasts were generated from 3-week-old His8-YFP-PIP2;1::pip2;1/2, His8-YFP-PIP2;1::ein3-1/eil1-1, mutant PIP2;1 transgenic lines respectively. Transgenic plants treated by spraying 100 μM AOA or 100μM AOA plus 10μM ACC for three days, then treated with 12 h air or10ppm ethylene before generating protoplasts. Fluorescent protoplasts were used for size measurement in swelling and shrinking experiment. Mean ± SE from the indicated number of protoplasts are obtained from at least 5 independent preparations and four plant leaves tissue for each experiment, bar = 20 μm on microscope pictures. (** and *** represent p<0.01 and p<0.001 respectively in student t-test analysis; V/V₀ = swelling or shrinking volume/initial volume)
Figure 2.3.25 Protoplast swelling and shrinking assay of *ein3-1/eil1-1*, WT and *pip2;1/2* background lines.

Protoplasts were generated from 3-week-old plants. Plants treated by spraying 100 μM AOA or 100 μM AOA plus 10 μM ACC for three days, then treated with 12 h air or 10ppm ethylene before generating protoplasts. Protoplasts were used for size measurement in swelling and shrinking experiment. Mean ± SE from the indicated number of protoplasts are obtained from at least three independent preparations and three plant leaves tissue for each experiment, bar = 20 μm on microscope pictures. (*** and **** represent p<0.01 and p<0.001 respectively in student t-test analysis; V/V₀ = swelling or shrinking volume/initial volume)
2.3.9 Ethylene up-regulated Ser-280 and Ser-283 phosphorylation sites of PIP2;1 isoform increase water loss

Aquaporins many posttranslational regulation mechanisms have been demonstrated to affect the channel abundance and activity in their target membrane. We used mutant PIP2;1 transgenic plants to validate the Ser-280 and Ser-283 phosphorylation sites biological function under ACC/ethylene regulation. Figure 2.3.26A shows detached rosette leaves of 3-4 weeks old ACC/ethylene treated transgenic plants’ water loss result from 0 to 8 h with 1 h interval for calculating water loss rate. From the statistic data of Figure 2.3.26A that His\textsubscript{8}-YFP-PIP2;1::pip2;1/2 and His\textsubscript{8}-YFP-PIP2;1::ein3-1/eil1-1 transgenic lines’ 8 h water loss percentage of initial fresh weight increase about 9.00% (26.64 ± 1.40% to 35.64 ± 2.70%) and 11.18% (30.62 ± 3.85% to 41.80 ± 3.17%) respectively after ACC/ethylene treatment. But for mutant PIP2;1 transgenic lines, only increase about 4.08% (27.84 ± 1.54% to 31.92 ± 2.48%), 3.07% (30.50 ± 1.84% to 33.57 ± 3.51%) in His\textsubscript{8}-YFP-PIP2;1\textsuperscript{S280A}::pip2;1/2, His\textsubscript{8}-YFP-PIP2;1\textsuperscript{S283A}::pip2;1/2 mutant transgenic lines respectively. Specially for His\textsubscript{8}-YFP-PIP2;1\textsuperscript{S280A/S283A}::pip2;1/2 transgenic line with abolish both of Ser-280 and Ser-280 phosphorylation sites, 8 h water loss rate only increase 2.35% (26.90 ± 1.32% to 29.25 ± 0.91) by ACC/ethylene treatment. We also study endogenous PIP2;1/2 protein’s biological function by ACC/ethylene treatment in wild-type and ein3-1/eil1-1 double mutant. 8 h water loss of ACC/ethylene treated wild-type and ein3-1/eil1-1 double mutant lines can increase about 9.09% (27.31 ± 2.32% to 36.40 ± 3.21%) and 8.62% (35.30 ± 4.25% to 43.92 ± 2.27) respectively (Figure 2.3.28). But for pip2;1/2 double mutant, about 6.15% (24.48 ± 1.00% to 30.63 ± 2.94%) increase after ACC/ethylene treatment, maybe ethylene can affect other PIP proteins posttranslational modification or expression to complement PIP2;1 protein to increase water loss. We also detect PIP2;1 protein localization in plant guard cells which relative to control water transport and transpiration. Figure 2.3.26B shows all wild type and mutant PIP2;1 proteins localize on plasma membrane of guard cells from AOA treated transgenic plants, maybe involve in controlling water loss through affecting transpiration. In ACC treated plants, all wild type and mutant PIP2;1 proteins still localize on plasma membrane of guard cells.
(Figure 2.3.27). Figure 2.3.29 shows the morphology of leaves before and after 8 h water loss. After 8 h water loss, ethylene treated plants and leaves looks wilting quickly than air treated plants and leaves except for *His*-YFP-*PIP2;1*<sup>S280A/S283A</sup>::*pip2;1/2* transgenic line. The results reveal ethylene enhance Ser-280 and Ser-283 phosphorylation of *PIP2;1* isoform can increase plant water loss also independent of EIN3/EIL1 signaling component.
Figure 2.3.26 Water loss increase with Ser-280 and Ser-283 phosphorylation level enhanced by ethylene treatment.

Ten rosette leaves from the first and second pair of true leaves were excised from three plants of each line, and their total fresh weight was measured at different time points during incubation with air or ethylene treatment at ambient laboratory conditions, five times biological repeats. (A) is the percentage of water loss weights over the initial fresh weight of detached leaves. Error bars are ± SE (n = 5). (B) is the localization of wild type and mutant PIP2;1 proteins in guard cells. For confocal microscopy experiment, leaves of each transgenic line were mounted on glass slides in the presence of PBS buffer supplemented with 100 μM AOA to localize the fusion proteins containing either wild type or mutant PIP2;1 proteins using YFP fluorescence detected with the confocal microscope (scale bars = 5 μm).
Figure 2.3.27 Subcellular localization of wild type and mutant PIP2;1 proteins in guard cells of 5 different ACC-treated transgenic plants. Transgenic Arabidopsis plants were grown to 3 weeks on M/S agar-base medium in glass jars supplemented with 100 μM AOA plus 5 μM ACC. The epidermal cell layer of leaves was mounted on a glass slide in the presence of PBS buffer supplemented with 100 μM AOA plus 5μM ACC. YFP fluorescence in plant guard cells of 5 different transgenic plants was detected using a confocal microscope (scale bar = 5 μm).
Figure 2.3.28 Water loss increase in *Arabidopsis* wild type and *ein3-1/eil1-1* double mutant by ethylene treatment. Ten rosette leaves from the first and second pair of true leaves were excised from three plants of each line, and their total fresh weight was measured at different time points during incubation with air or ethylene treatment at ambient laboratory conditions, five times biological repeats. The percentage of water loss was calculated from water loss weights over the initial fresh weight of detached leaves. Error bars are ± SE (n = 5).
Figure 2.3.29 Leaves morphology of plants before and after water loss.

Water loss measurement from detached leaves of $\text{His}_8$-$\text{YFP}$-$\text{PIP}_2;1$::$\text{ein3-1/eil1-1}$ (PE), $\text{His}_8$-$\text{YFP}$-$\text{PIP}_2;1$::$\text{pip2;1/2}$ (PP), $\text{His}_8$-$\text{YFP}$-$\text{PIP}_2;1$::$\text{pip}_2;1/2^{S280A}$ (280A), $\text{His}_8$-$\text{YFP}$-$\text{PIP}_2A^{S283A}$::$\text{pip2;1/2}$ (283A), $\text{His}_8$-$\text{YFP}$-$\text{PIP}_2A^{S280A/S283A}$::$\text{pip2;1/2}$ (AA), wild-type, $\text{ein3-1/eil1-1}$ and $\text{pip2;1/2}$. **A0** and **E0**: Photographs were taken just after detachment from air and ethylene treatment plants respectively; **A8** and **E8**: Photographs were taken after water loss 8 hours from air and ethylene treatment plants respectively, bar = 1 cm.
2.3.10 Ser-280 and Ser-283 phosphorylation regulation in ethylene mutants under ethylene treatment

Ethylene regulated C-terminal phosphorylation protein of PIP2;1 were also validated in others ethylene mutants (ein2-5, ctr1-1, etr1-1 and octuple acs mutant). 3-week-old plants in MS agar base medium (additional 100 uM AOA in medium) treated with 10 ppm ethylene or air for 12 h, and Ser-280 and Ser-283 doubly phosphorylation protein and Ser-283 single phosphorylation protein regulation were validated with western-blot analysis (Figure 2.3.30). Figure 2.3.30 shows that doubly Ser-280 and Ser-283 phosphorylation also up-regulated 1.84 ± 0.20-fold and 2.35 ± 0.35-fold by ethylene in ein2-5 and acs octuple mutants, respectively, but no significant regulation in ctr1-1 and etr1-1 ethylene mutants, only 0.96 ± 0.09-fold and 1.10 ± 0.17-fold respectively. Ser-283 also up-regulated by ethylene in ein2-5 and acs octuple mutants, 1.78 ± 0.16-fold and 1.91 ± 0.37-fold respectively. It’s reveals PIP2;1 protein C-terminal phosphorylation is also EIN2 independent, but relative to CTR1 and ETR1. Phosphorylation level is very high in ctr1-1 mutant as endogenous ethylene is high in this mutant to stimulate PIP2;1 protein phosphorylation. Maybe the ethylene receptor-CTR1 complex regulates the phosphorylation of aquaporin C-terminal mediated via some unknown kinases or phosphatases. In the absence of ethylene, the Ser280- and Ser283-phosphorylation of PIP2;1 are suppressed by the complex, and the negative interaction is partially released once ethylene binds to the receptor and modulated the activity of CTR1. Maybe the kinase(s) which are responsible for PIP2;1 protein phosphorylation under ethylene response can interrelation with ETR1 and CTR1 proteins on membrane and then phosphorylate PIP2;1 protein in the presence of ethylene, but the kinase move away from membrane in etr1-1 mutant, so PIP2;1 protein phosphorylation level regulation has no significant difference between air and ethylene treatment in these mutant lines. The result of ethylene precursor ACC treatment of wild-type and ethylene mutants also obtained with western-blot analysis (Figure 2.3.31). The data consistent with ethylene treatment result and reveals PIP2;1 C-terminus phosphorylation independent of EIN2 and EIN3/EIL1.
Figure 2.3.30 Ser-280 and Ser-283 phosphorylation regulation under ethylene treatment in wild type and ethylene mutants. Arabidopsis seedlings were grown to 3 weeks on M/S agar-base medium in glass jars supplemented with 100 μM AOA and treated with air or 10 ppm ethylene for 12 h before collecting tissues. The Microsomal proteins were extracted from each tissue sample and 5 μg protein was used for western-blot analysis with phosphorylation and protein antibodies respectively. The relative phosphorylation level of is calculated from the relative level of anti-pS280/S283-PIP2 or anti-pS283-PIP2 antibodies against anti-PIP2 antibody. Two biological repeat experiments plus one additional technical repeat experiment were performed. The Error bars are ± SE (n = 3). *, ** and n.s. represent $p < 0.05$, $p < 0.01$ and $p > 0.05$ given by student’s t-test, respectively.
Figure 2.3.31 Ser-280 and Ser-283 phosphorylation regulation under ACC treatment in wild-type and ethylene mutants. Arabidopsis seedlings were grown to 3 weeks on M/S agar-base medium in glass jars supplemented with 100 μM AOA or 100 μM AOA plus 5 μM ACC. The Microsomal proteins were extracted from each tissue sample and 5 μg protein was used for western-blot analysis with phosphorylation and protein antibodies respectively. The relative phosphorylation level of is calculated from the relative level of anti-pS280/S283-PIP2 antibody against anti-PIP2 antibody. Two biological repeat experiments plus one additional technical repeat experiment were performed. The Error bars are ± SE (n = 3). * and n.s. represent $p < 0.05$ and $p > 0.05$ given by student’s $t$-test, respectively.
2.4 Discussion

2.4.1 Over view of function study of ethylene up-regulated PIP2;1 phosphorylation independent of EIN3/EIL1

SILIA-based of quantitative phosphoproteomics approach is a robust way to discover novel signaling components in the ethylene signaling transduction pathway. As EIN3 and EIL1 are important signaling components in ethylene signaling pathway, we used the double loss of function mutant in study to get novel signaling components which phosphorylation regulated by ethylene of them independent of EIN3/EIL1. We recently showed (Yang et al., 2013) that Ser-280 and Ser-283 phosphorylation of PIP2;1 was regulated by CTR1 kinase by studying of ctr1-1 mutant with quantitative and differential phosphoproteomics approach. In this study, we present evidence that in vivo Ser-280 and Ser-283 phosphorylation of AtPIP2;1 isoform up-regulated by gaseous plant hormone stimulation independent of EIN3/EIL1 in ethylene signaling transduction pathway. As PIP aquaporins are key proteins to facilitate water movement through regulating the plasma membrane permeability. We also showed that Ser-280 and Ser-283 phosphorylation by ethylene can increase water channel activity in plant cells and enhance water loss of detached leaves of intact plants. The biological function of AtPIP2;1 C-terminus phosphorylation also independent of EIN3/EIL1 signaling component.

2.4.2 Quantitative phosphoproteomics study of ein3-1/eil1-1 double mutant

Phosphoproteomic studies have been used to discover functional information on plant signaling (Hoehenwarter et al., 2013; Lassowskat et al., 2014), and following SILIA approach efficiently elucidated a protein complex including ethylene receptor ETR1 and constitutive triple response 1 (CTR1) (Yang et al., 2013). A prediction model of PIP2;1 phosphorylation regulation by ethylene was found, PIP2;1 PTM regulation independent of EIN2 and relative to
ETR-CTR1 complex under a dual-and-opposing effect of ethylene. The unphosphorylated form of the peptide with sequence 277, 275, 275SLGSFSAANV of PIP2;1 protein could not be detected in any of our MS analyses without Fe$^{3+}$-NTA and TiO$_2$ enrichment. This could be due to the unphosphorylated peptide has low ionization efficiency for MS/MS detection or, more probably, to the unphosphorylated C-terminus of PIP2;1 protein is less stabilized than phosphorylation C-terminus, and it could be more easy to be processed in vivo. Based on our identified phosphorylation sites (Ser-280 and Ser-283) of endogenous and transgenic PIP2;1 protein from microsomal proteins, we constructed a phosphorylation motif of these highly conserved phosphorylation sites, maybe other proteins containing similar sequence in the motif also could be phosphorylated by kinases in vivo (Figure 2.4.1).
Figure 2.4.1 Bioinformatics prediction motif of Ser-280 and Ser-283 phosphosites on PIP2;1 C-terminus. Alignment of the two phosphosites motif LGSFRS between the authentic mass spectrometry-derived phosphopeptide and those of Arabidopsis gene accessions by using the BLAST P program, and sequences homology more than 55% were used for constructing the motif. The phosphorylation sites are marked with black asterisk. The conserved motif sequence among these members is shown at the bottom of the alignment. The conservation percentage and the sequence logo are also shown at the bottom of the alignment result, representing frequencies of the conserved amino acids present in the motif.
2.4.3 AtPIP2;1 isoform phosphorylation regulation in ethylene signaling pathway

Phosphorylation of ethylene signaling components play an important role in ethylene response regulation of plant. Five ethylene receptors can bind ethylene (Hall et al., 2000; O'Malley et al., 2005; Schaller and Bleecker, 1995) and all have kinase activity. ETR1 has histidine kinase activity (Gamble et al., 1998), ETS1 has both histidine and Ser/Thr kinase activity, and other three only has Ser/Thr kinase activity (Moussatche and Klee, 2004). Downstream of receptors signaling component CTR1 phosphorylates EIN2 (Ju et al., 2012) and stabilize its C-terminus to block response to ethylene signal. EIN3 can be phosphorylated by MPK3 and increase its stability to prohibit protein degradation (Yoo et al., 2008). ERF110 can be dephosphorylated by ethylene in signaling transduction and regulate plant bolting time (Zhu et al., 2013). We found than PIP2;1 was a novel signaling component which its phosphorylation independent of EIN3 and EIL1 by quantitative phosphoproteomics approach. And western-blot analysis result also indicated that PIP2;1 phosphorylation independent of EIN3, EIL1 and EIN2, but it relative to CTR1 and ETR1 (Figure 2.3.29 and 2.3.30). ETR1 plays a role in promote C-terminal of PIP2;1 phosphorylation under ethylene condition maybe by controlling kinase activity, but CTR1 as a negative regulator to PIP2;1 phosphorylation by ethylene, it maybe play a role in inhibiting its phosphorylation by controlling kinase activity or promoting dephosphorylation by controlling phosphatases activity.

In order to find out the kinase which responsible for PIP2;1 phosphorylation under ethylene treatment to further complement ethylene signaling pathway, some kinases were predicted based on kinases function which reported in literatures. These candidate kinases proposed to phosphorylate PIP2;1 with GPS software prediction (Yang et al., 2013) are: Phototropins (PHOT1-At3g45780, PHOT2-At5g58140) contains two Light Oxygen Voltage domains (LOV1 and LOV2) and a C-terminal protein kinase (AGC class) domain (Christie, 2007). It’s reported that the auxin transporter ABCB19 could be phosphorylated by phot1 in vitro (Christie et al., 2011). Phototropin-mediated phytochrome kinase substrate phosphorylation is
necessary for *Arabidopsis* phot responses (Inoue et al., 2008; Inoue et al., 2011). AtNIK1, AtNIK2 and AtNIK3 (At1g60800) belong to *Arabidopsis* LRRII subfamily and can interact with the begomovirus nuclear shuttle protein (NSP) (Sakamoto et al., 2012). ALTERED SEED GERMINATION 5 (ASG5, At1g20650) is a protein kinase superfamily protein on plasma membrane, involved in gibberellic acid mediated signaling pathway and seed dormancy process ([www.arabidopsis.org](http://www.arabidopsis.org)). D6PK (At5g55910) is one of *Arabidopsis* AGD VIII kinase and phosphorylates PIN proteins in vitro and phosphorylates PIN1 protein in vivo, regulate the auxin transport activity of PINs by phosphorylation (Zourelidou et al., 2009). (D6PKL1; At4g26610, D6PK2; At5g47750, D6PKL3, At3g27580) D6PKs-mediated phosphorylation of PIN3, PIN4 and PIN7 is necessary for regulation auxin transport in responses of phototropic growth (Willige et al., 2013). According to the kinases prediction result, five kinases homozygous mutants were used for PIP2;1 phosphorylation validation with ethylene treatment. But western blot analysis data shows that these kinases are not play role in phosphorylating of PIP2;1 under ethylene condition (Figure 2.4.2). Maybe two or more kinases which phosphorylate PIP2;1 protein at the same time, or other kinase really phosphorylate PIP2;1 under ethylene condition.

According to transcriptome study of PIP2 gens, gene expression of rose (*Rosa hybrid*) aquaporin gene *RhPIP2;1* was down-regulated by ethylene in petal (Ma et al., 2008; Chen et al., 2013). Similarly, in *Arabidopsis* Col-0 plant, transcripts of *AtPIP2;1*, *AtPIP2;6* (AT2G39010) and *AtPIP1;4* (AT4G00430) aquaporin genes were all suppressed by 4 h of ethylene treatment (Qiao et al., 2009). A significant reduction in PIP2;1 protein level was observed in *ctl* mutant as compared to those in *etr1-1* or *octuple acs* deletion lines after 12-h ethylene treatment (Figure 2.3.30 and Figure 2.3.31). The ethylene-triggered decrease of *AtPIP2;1* transcription may be achieved by up-regulation of both synthesis and perception of auxin (Swarup et al., 2007), which was reported to repress the expression of PIP2;1 (Peret et al., 2012). But based on PIP2;1 phosphorylation and its corresponsive biological function studying results, we constructed a model of PIP2;1 PTM regulation in ethylene signaling pathway (Figure 2.4.3). It demonstrated that PIP2;1 maybe regulate plant physiological response to ethylene by its phosphorylation regulation in signaling transduction pathway.
Figure 2.4.2 Western-blot analysis of Ser-280 and Ser-283 phosphorylation in kinase mutant lines after ethylene treatment. *Arabidopsis* plants growth in MS agar-base jars with addition of 100 μM AOA, 3-week-old plants treated with air and 10 ppm ethylene for 12 h respectively before harvesting tissues with liquid nitrogen. 30 μg total protein or 5 μg microsomal protein was used for western-blot analysis.
Figure 2.4.3 The mechanistic action model for ethylene-regulated and EIN3/EIL1-independent S280 and S283 phosphorylation of PIP2;1 and their roles in regulation of water movement in Arabidopsis cell. Under ambient air condition, ethylene receptor-CTR1 complex-regulated phosphatase activity is higher than kinase activity, leading to a lower phosphorylation level on PIP2;1 and thus a lower water channel activity. However, under a higher level of ethylene fumigation condition, ethylene receptor- and CTR1-mediated kinase activity dominates over that of phosphatase, leading to an increase in S280 and S283 phosphorylation level on PIP2;1 and followed by an increased water channel activity in leaf cells of Arabidopsis. The ethylene-triggered phosphor-relay and enhanced S280 and S283 phosphorylation on PIP2;1 is independent to the function of EIN3 and EIL1 transcription factors. Black and red lines represent regulation of protein abundance and phosphorylation/dephosphorylation, respectively, whereas blue lines represent the movements of molecules. The relative strength of a particular regulation is indicated by the thickness of the line. Solid lines indicate effects that occur through direct interaction, whereas dashed lines indicate possible regulation by which the exact players are uncharacterized. Arrows and stop ends represent positive and inhibitory effects, respectively.
2.4.4 Ethylene up-regulate phosphorylation of AtPIP2;1 isoform increase water transport activity

To further study C-terminal phosphorylation function of PIP2;1 isoform in leaves, we demonstrated the molecular mechanism of phosphorylation regulate plant physiology by the ethylene stimulation. Quantitative phosphoproteomics and western-blot analysis were pivotal to showing that this regulation is intrinsic PTM. Plant plasma membrane aquaporin proteins have been identified in Arabidopsis plant for about two decades (Daniels et al., 1994; Kaldenhoff et al., 1995; Kammerloher et al., 1994). The physiological function of aquaporins in higher plants has yet to be resolved, though previous study indicated that they may have functions in cell expansion (Ludevid et al., 1992), solute transport (Opperman et al., 1994), water uptake (Maggio and Joly, 1995), drought tolerance (Yamaguchishinozaki et al., 1992), and salt tolerance (Yamada et al., 1995). AQPs play a major role in regulation of water transport through membrane in plant cells (Maurel et al., 2008). The plasma membrane PIPs appear to function in water transport of plant cells in aquaporins families. Although some experiments reveals that the cell reacts to osmotic stress by reducing water channel number on the plasma membrane, the PTM modification studies of affecting water channel activity remain elusive in plants. In vivo phosphorylation of putative aquaporins (PM28A and PM28B) of spinach leaf plasma membranes plays a role in regulation of cell turgor (Johansson et al., 1996), and Ser-274 of PM28A phosphorylated in vivo in response to increasing apoplastic water potential (Johansson et al., 1998a). C-terminal phosphorylation of PIP2 proteins can regulate its abundance and activity on the plasma membrane (Prado et al., 2013; Prak et al., 2008; Van Wilder et al., 2008). Li et al demonstrated that internalization of AtPIP2;1 involves two pathways: a methyl-cyclodextrin-sensitive, membrane raft-associated pathway and a tyrphostin A23-senstive clathrin-dependent pathway (Li et al., 2011). Phosphorylation of the vacuolar membrane aquaporin α-TIP was shown to increase greatly its water transport activity, suggested that cells regulate the water permeability of their membranes by in situ phosphorylation of water channel proteins (Maurel et al., 1995). AQP2 is phosphorylated at the C-terminus (Kuwahara et al., 1995), but whether it can increases water channel activity by
promoting fusion of AQP2-containing vesicles with the plasma membrane is still being debated (Lande et al., 1996). Recently study demonstrated that diphosphorylation of PIP2;1 at Ser-280 and Ser-283 under dark condition increase rosette hydraulic conductivity ($K_{\text{ros}}$) and membrane permeability ($P_i$) (Prado et al., 2013). In our work, we confirmed that Ser-280 and Ser-283 of PIP2;1 protein phosphorylation under ethylene condition can also increase water-channel activity, protoplast swell or shrink more quickly by changing osmotic potential and detach leaves loss water more quickly. Ser-280 and Ser-283 phosphorylation play a vital role on water transport and transpiration. O.Da Ines et al demonstrated that wild type and pip2;1 mutant have no significant differences in water loss (Da Ines et al., 2010), as PIP2;1 has low phosphorylation level without stimulation (Figure 2.3.12) and apoplastic pathway also let water loss increase from leave tissue without PIP2;1/2 under ethylene treatment (Figure 2.3.27).

PIP aquaporins have been reported to be post-translationally regulated by several factors or modifications, such as H+, free Ca$^{2+}$ or serine phosphorylation, which acting on the cytosolic side of the protein (Alleva et al., 2006; Gerbeau et al., 2002; Karlsson et al., 2003; Tournaire-Roux et al., 2003). Physiological and environmental stimulations were demonstrated to alter PIP phosphorylation in plants (Engelsberger and Schulze, 2012; Hsu et al., 2009; Johansson et al., 1996; Kline et al., 2010; Niittyla et al., 2007; Prado et al., 2013; Prak et al., 2008). PIP2s protein phosphorylation in loop B and the C-terminal tail may also control pore water channel opening and closing (Johansson et al., 1998b; Johansson et al., 1996). Ser-274 of SoPIP2;1 and Ser-262 of GmNod26 were phosphorylated by a membrane-associated CDPK can affect water channel regulation (Guenther et al., 2003; Johansson et al., 1996). Phosphorylation of SoPIP2;1, PvTIP3;1 and GmNod26 occurs on the protein termini, which one can predict to be reasonably mobile if these domains are disorder in the aquaporin crystal structures (Gonen et al., 2004b; Murata et al., 2000; Sui et al., 2001). There may be a common mechanism for channel gating with physical occlusion of the pore by the unphosphorylated amino- or carboxy-terminal domain (Daniels and Yeager, 2005). Positively charged residues in these domains maybe interact electrostatically with acidic residues around the pore vestibule and block the water channel; on the contrary, phosphate
group with negative charge would disrupt this interaction and help the water channel open.

Based on aquaporins structural studies (Fischer et al., 2009; Gonen et al., 2004a; Murata et al., 2000; Sui et al., 2001), in general, N- and C-terminal tails of mammalian, yeast and plant aquaporins have been predicted to be fairly mobile. Thus, C- and N-tail of PIPs have been proposed to be involved in the protein channel gating which, in turn, regulates water flow (Azad et al., 2008; Han and Schey, 2004; Nyblom et al., 2009; Tornroth-Horsefield et al., 2006). It has been proposed that the C-terminal tails phosphorylation of PIPs probably regulated its channel gating in some case of plants (Han and Schey, 2004; Nyblom et al., 2009; Tournaire-Roux et al., 2003). The fact that site-directed mutagenesis experiments have demonstrated that serine phosphorylation may be involved in the regulation of PIPs water transport activity in plant cells supported the hypothesis of C-terminal phosphorylation regulation of plant PIPs (Guenther et al., 2003; Han and Schey, 2004; Maurel et al., 1995; Maurel et al., 1997b; Prado et al., 2013; Suga and Maeshima, 2004). In our study, it also reveals that Ser-280 and Ser-283 of PIP2;1 isoform mutant to Alanine to abolish the phosphorylation sites can block water channel activity and water transport through membrane more slowly than phosphorylation status by ethylene stimulation (Figure 2.3.24).

Gating and trafficking regulation of PIP2;1 by phosphorylation or dephosphorylation events in plant cell would be considerable regulation of the membrane osmotic water permeability. PIP2;1 phosphorylation by ethylene can cause plant water loss more quickly, suggest it may be involved in stimulation of plant senescence.
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