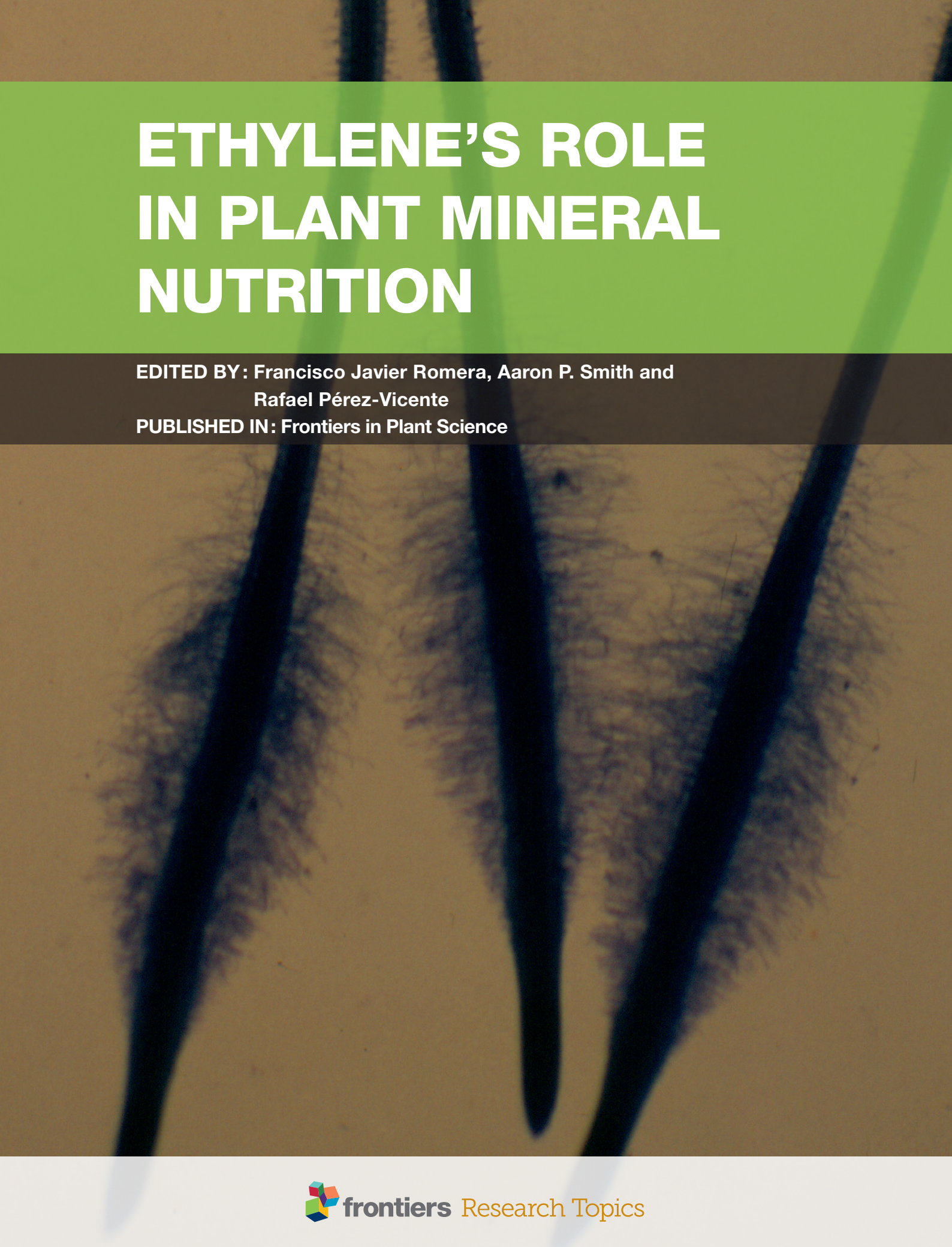

ETHYLENE'S ROLE IN PLANT MINERAL NUTRITION

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ETHYLENE'S ROLE IN PLANT MINERAL NUTRITION

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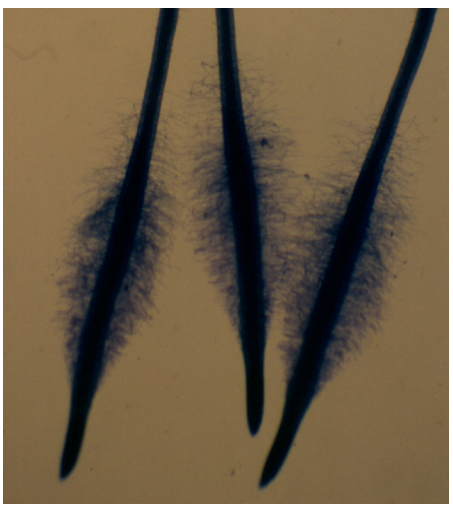

Ethylene has been implicated in the formation of root hairs induced by plants under different nutrient deficiencies, such as P deficiency, Fe deficiency, K deficiency or B deficiency. Figure by F. J. Romera.

Terrestrial plants are sessile organisms that, differently from animals, can not move in searching of the nutrients and water they need. Instead, they have to change continuously their physiology and morphology to adapt to the environmental changes. When plants suffer from a nutrient deficiency, they develop physiological and morphological responses (mainly in their roots) aimed to facilitate the acquisition and mobilization of such a nutrient. Physiological responses include some ones like acidification of the rizhosphere and release of chelating agents into the medium; and morphological responses include others, like changes in root architecture and development of root hairs. The regulation of these responses is not totally known but in the last years different plant hormones and signaling substances, such as auxin, ethylene, cytokinins and nitric oxide, have been involved in their control. Besides hormones, oxidative stress has also been related with most of the nutrient deficiencies.

The relationship of ethylene with the regulation of responses to nutrient deficiencies came from the nineties, when some works presented data suggesting its involvement in the regulation of responses to Fe and P deficiency. In the last years, the role of ethylene has been extended to many other nutrient deficiencies, such as K deficiency, Mg deficiency, S deficiency, N deficiency, and others. In most of the cases, it has been found that ethylene production, as well as the expression of ethylene synthesis genes, increases under these nutrient deficiencies. Furthermore, it has also been found that ethylene controls the expression of genes related to responses to different deficiencies. The involvement of ethylene in so many deficiencies suggests that it should act in conjunction with other signals that would confer nutrient-specificity to the distinct nutrient responses. These other signals could be plant hormones (auxin, cytokinins, etc) as well as other substances (nitric oxide, microRNAs, peptides, glutathione, etc), either originated in the roots or coming from the shoots through the phloem.

The role of ethylene in the mineral nutrition of plants is even more complex that the one related to its role in the responses to nutrient deficiencies. Ethylene has also been implicated in the N2 fixation of legume plants; in salt tolerance responses; and in responses to heavy metals, such as Cd toxicity. All these processes are related to ion uptake and, consequently, are related to plant mineral nutrition. We consider a good opportunity to review all this information in a coordinated way. This Research Topic will provide an overview about the role of the plant hormone ethylene on the regulation of physiological and morphological responses to different nutrient deficiencies. In addition, it will cover other aspects of ethylene related to plant nutrition such as its role on salinity, N2 fixation and tolerance to heavy metals.

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EDITORIAL
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Editorial: Ethylene's Role in Plant Mineral Nutrition

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Keywords: ethylene, heavy metals, mineral nutrition, nodulation, nutrient deficiency responses, salinity

The Editorial on the Research Topic

Ethylene's Role in Plant Mineral Nutrition

Ethylene is a gaseous plant hormone involved in many aspects of plant life, including seed germination, flower senescence, abscission, and fruit ripening (Abeles et al., 1992). It also plays a very important role in the responses of plants to both biotic and abiotic stresses (Abeles et al., 1992; Shakeel et al., 2013; Kazan, 2015). The production of ethylene is tightly regulated by internal signals, and usually increases in response to biotic (e.g., pathogen attack) and abiotic stresses, such as mechanical stress, hypoxia, chilling, and nutritional disorders (Abeles et al., 1992; Lynch and Brown, 1997; Concellon et al., 2005; Zheng et al., 2008; Geisler-Lee et al., 2010; Iqbal et al., 2013; García et al., 2015).

In processes related to mineral nutrition, ethylene has been implicated in the regulation of physiological and morphological responses to nutrient deficiencies; in nodulation of legume plants; in salt tolerance responses; and in responses to heavy metals (Abeles et al., 1992; Lynch and Brown, 1997; García et al., 2015).

This research topic updates recent results relating ethylene to different aspects of plant mineral nutrition. It includes 10 reviews and 2 original articles: 7 reviews are related to nutrient deficiencies (Khan et al.; Lucena et al.; Schachtman; Song and Liu; Wawrzynska et al.; González-Fontes et al.; Neumann), 1 to nodulation (Guinel), 1 to salt tolerance (Tao et al.), and 1 to heavy metals (Keunen et al.); 1 original article is related to Fe (iron) deficiency (Ye et al.), and the other one to N (nitrogen) deficiency (De Gernier et al.).

The role of ethylene in the regulation of responses to nutrient deficiencies was introduced in the nineties, when some studies showed an implication of ethylene in the regulation of physiological and/or morphological responses to Fe and P (phosphorus) deficiency (Romera and Alcántara, 1994; Lynch and Brown, 1997). In the last years, the role of ethylene has been extended to other nutrient deficiencies, such as K (potassium) deficiency, S (sulfur) deficiency, and others (Iqbal et al., 2013; García et al., 2015). The relationship between ethylene and other processes related to mineral nutrition (nodulation, salinity and heavy metals) has also been known for many years (Abeles et al., 1992; Lynch and Brown, 1997).

Since nutrient deficiencies cause stress to plants and stress promotes ethylene synthesis (Abeles et al., 1992), most plant species increase ethylene production under different deficiencies (Lucena et al.; Schachtman; Song and Liu; Wawrzynska et al.). This higher ethylene production is generally associated with increased transcript abundance for genes involved in ethylene biosynthesis and signaling (Lucena et al.; Schachtman; Song and Liu; Wawrzynska et al.; Neumann). Moreover, the mitogen-activated protein kinases 3 and 6 (MPK3/MPK6), that can regulate ethylene production, increase under Fe deficiency (Ye et al.) or under heavy metal stress (Keunen et al.). In the case of N, the relationship between its deficiency and ethylene production seems to be complex, affected by the degree of the deficiency and the plant genotype (Khan et al.; De Gernier et al.).

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Similarly to nutrient deficiencies, both salinity stress and heavy metal stress also cause higher ethylene production and increased transcription of ethylene biosynthesis and signaling genes (Tao et al.; Keunen et al.). In the nodulation process, ethylene production increases early in the symbiosis (Guinel).

In general, ethylene plays positive roles in the activation of responses of plants to nutrient deficiencies (Khan et al.; Lucena et al.; Schachtman; Song and Liu; Wawrzynska et al.; Ye et al.; De Gernier et al.; González-Fontes et al.; Neumann), to salinity stress (Tao et al.), and to heavy metal stress (Keunen et al.), while it is considered a negative regulator of the nodulation process (Guinel). Despite these generally accepted roles, conflicting results have also been reported. As examples, some research has shown that ethylene insensitive plants are more tolerant to salinity (Tao et al.) or to heavy metal stress (Keunen et al.) than corresponding wild types. To explain these contradictory results, it should be taken into account the different experimental conditions used, including plant material, dosage, days of treatments, etc. (Keunen et al.). Additionally, it should be considered that excessive ethylene could inhibit plant growth and development (Tao et al.; Keunen et al.).

In relation to nutrient deficiencies, ethylene has been implicated in the activation of both physiological and morphological responses, such as enhanced ferric reductase activity under Fe deficiency (Lucena et al.), enhanced acid phosphatase activity under P deficiency (Song and Liu), upregulation of the HAK5 potassium transporter under K deficiency (Schachtman), development of root hairs under Fe, K, B (boron) or P deficiency (Lucena et al.; Schachtman; González-Fontes et al.; Neumann), and development of cluster roots under Fe or P deficiency (Lucena et al.; Neumann). In relation to salinity stress, ethylene has been implicated in the regulation of Na^+/K^+ homeostasis (Tao et al.); and in relation to heavy metal stress, in the network leading to glutathione and phytochelatin synthesis (Keunen et al.). In the nodulation process, ethylene has been implicated in most of the steps leading to a mature nodule and even in nodule senescence (Guinel).

The participation of ethylene in all the processes described above suggests it should act in conjunction with other signals, and/or perhaps through different transduction pathways, to confer specificity to the different responses. Both possibilities are reflected in the reviews included in this research topic. As examples, ethylene interacts with auxin and phloem signals to regulate Fe deficiency (Lucena et al.) and P deficiency responses (Song and Liu; Neumann); with ABA to regulate responses to salinity (Tao et al.); and with ROS to regulate responses to K deficiency (Schachtman) and heavy metals (Keunen et al.). On the other hand, Lucena et al. present evidence suggesting that ethylene regulates different responses to Fe deficiency through distinct transduction pathways, which is in agreement with recent proposals about ethylene signaling (Shakeel et al., 2013; Zhang et al.).

Despite the specificity conferred by different signals, the common participation of ethylene in different processes related to plant mineral nutrition could partly explain the frequent cross talks among nutrient deficiency responses (Lucena et al.) and between salinity and nutrient deficiencies (Tao et al.).

In conclusion, this research topic, by putting together different nutritional aspects affected by ethylene, tries to pave the way for future research about the role of this simple but fascinating hormone on plant mineral nutrition.

AUTHOR CONTRIBUTIONS

For the Editorial, AS reviewed the works about P and K; RP reviewed the works about N and B; and FR reviewed the rest of works. A draft of the Editorial was first written by Dr. Romera and then Dr. Smith and Dr. Pérez-Vicente revised and modified it to get the final version.

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REVIEW
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Role of ethylene in responses of plants to nitrogen availability

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Ethylene is a plant hormone involved in several physiological processes and regulates the plant development during the whole life. Stressful conditions usually activate ethylene biosynthesis and signaling in plants. The availability of nutrients, shortage or excess, influences plant metabolism and ethylene plays an important role in plant adaptation under suboptimal conditions. Among the plant nutrients, the nitrogen (N) is one the most important mineral element required for plant growth and development. The availability of N significantly influences plant metabolism, including ethylene biology. The interaction between ethylene and N affects several physiological processes such as leaf gas exchanges, roots architecture, leaf, fruits, and flowers development. Low plant N use efficiency (NUE) leads to N loss and N deprivation, which affect ethylene biosynthesis and tissues sensitivity, inducing cell damage and ultimately lysis. Plants may respond differently to N availability balancing ethylene production through its signaling network. This review discusses the recent advances in the interaction between N availability and ethylene at whole plant and different organ levels, and explores how N availability induces ethylene biology and plant responses. Exogenously applied ethylene seems to cope the stress conditions and improves plant physiological performance. This can be explained considering the expression of ethylene biosynthesis and signaling genes under different N availability. A greater understanding of the regulation of N by means of ethylene modulation may help to increase NUE and directly influence crop productivity under conditions of limited N availability, leading to positive effects on the environment. Moreover, efforts should be focused on the effect of N deficiency or excess in fruit trees, where ethylene can have detrimental effects especially during postharvest.

Keywords: ethylene, mineral nutrients, nitrogen availability, N use efficiency, phytohormones

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INTRODUCTION

The classical plant hormone, ethylene has emerged as a potent molecule to regulate numerous physiological and morphological responses in plants by interacting with other signaling molecules (Iqbal et al., 2012; Khan and Khan, 2014; Fiebig and Dodd, 2015; Khan et al., 2015). Ethylene plays an important regulatory roles in plant responses to mineral nutrients availability, such as nitrogen (N; Iqbal et al., 2015), phosphorous (P; Li et al., 2009), potassium (K; Jung et al., 2009), calcium (Ca; Xu et al., 2010), magnesium (Mg), manganese (Mn; Dorling et al., 2011), copper (Cu; Arteca and Arteca, 2007), zinc (Zn; Khan and Khan, 2014) and controls plant responses under both optimal

and stressful conditions (Iqbal et al., 2013). The ethylene biosynthesis and plant responses vary with the availability of mineral nutrients (Iqbal et al., 2013).

Nitrogen is an important nutrient required for plant growth and development as it is a core constituent of a plant's nucleic acid, proteins, enzymes, and cell wall and pigment system (Krapp, 2015). Plants are frequently exposed to N stressed conditions, excess N due to application of N fertilizers or deficiency. While low N limits the growth of crop plants (Iqbal et al., 2015), the loss of excess N fertilizers contributes to environmental pollution (Gastal and Lemaire, 2002). The availability of N is of agricultural concern because plant metabolism is differently affected by excess, optimal and deficient levels (Iqbal et al., 2015). In maintaining the physiological status of plants under these conditions, the role of ethylene in responding to N status in plants has been identified (Tian et al., 2009; Fiebig and Dodd, 2015; Iqbal et al., 2015). The availability of N concentrations modify the effect of ethylene and plant responses, like other mineral nutrients such as phosphate (Li et al., 2009), sulfate (Zuchi et al., 2009), potassium (Shin and Schachtman, 2004), iron (Romera and Alcantara, 1994). Fiebig and Dodd (2015) have recently reported that N supplementation of 10 mM returned ethylene concentrations in over-irrigated *Solanum lycopersicum* plants to the levels of well-drained plants, leading to an increase in shoot fresh weight that correlated with decreased ethylene levels. This can be explained considering that over-irrigation induces nitrate leakage and subsequently N deficiency. Similarly, N differentially regulates proline and ethylene biosynthesis in order to alleviate salt-induced photosynthetic inhibition in mustard plants (Iqbal et al., 2015). It has been also shown that exogenous ethylene (applied as ethephon, an ethylene releasing compound) increases N assimilation and photosynthesis in *Brassica juncea* plants subjected to different levels of N (Khan et al., 2008; Iqbal et al., 2011). In *B. juncea*, Iqbal et al. (2015) have shown that plants exhibited lesser photosynthesis and growth when treated with 5 mM N than 10 mM N, whereas 20 mM N was inhibitory under no-stress condition. This indicated that these levels were low, sufficient and excess, respectively. The inhibitory effect of excess-N was related to high ethylene production, but under salt stress, as the demand for N increased the excess-S optimized ethylene and led to higher proline production and promoted photosynthesis and growth (Iqbal et al., 2015). Similarly, it has been found that a high (10 mM) concentration of N inhibits the lateral root growth of *Arabidopsis thaliana*, although the number and length of lateral roots of the *etr1-3* and *ein2-1* mutants were less affected than wild-type plants. The leaf longevity in *Agropyron cristatum* was affected by ethylene at different N levels (Ren et al., 2013). Plants under low N conditions accelerate the development and usually show early transition to reproductive stage, reaching earlier to senescence stage. Plants grown to high N availability have longer vegetative stage and delayed senescence. In both cases, ethylene has a pivotal role, since it is also known as senescence hormone.

This review explored the state of the art of the information available on the role of N in modulating ethylene responses in whole plant and different plant organs. The information related to ethylene and N availability has been critically discussed arising due to the contrary results obtained in different works. Moreover, the lack of information has been highlighted indicating where further investigations should be addressed.

N AVAILABILITY AND ETHYLENE BIOSYNTHESIS AND SIGNALING

The literature has only recently started to explore the nature of the relationships between plant hormones and macronutrient signaling. The following pages describe recent advances in the study of the ethylene signaling pathway in the presence of N perturbation and provide new information based on *in silico* analyses.

The availability of N is one of the main factors limiting plant growth and development. Ammonium (NH_4^+) and nitrate (NO_3^-) are the predominated inorganic forms of nitrogen taken up from the soil. In particularly, nitrates are the most readily available form of N for root absorption because it is not absorbed by colloids. Nitrate is assimilated by higher plants after being reduced to nitrite and then ammonium as a result of the sequential action of nitrate and nitrite reductases, and the NH_4^+ can be subsequently assimilated into glutamate and glutamine via the glutamine synthase (GS)/glutamate oxyglutarate aminotransferase cycle (GOGAT) (Crawford, 1995). These metabolic intermediates act as important signaling molecules or as the major amino donors for the synthesis of other amino acids and N-containing compounds, thus sustaining plant growth and development, and plant responses to biotic and abiotic stresses (Stitt, 1999; Forde and Lea, 2007; Vidal and Gutiérrez, 2008; Mur et al., 2012; Renault et al., 2013). The assimilation of N by plants, or its incorporation in plants, depends on the availability of light and activities of photosynthesis because N can only be incorporated if there are enough carbon (C) skeletons.

It is thought that N acts as a signaling element in plants, but very little is known about how this occurs (Lea and Miflin, 2003) or how N interacts with the ethylene biosynthesis and signaling pathway that is closely associated with complex environmental stresses. Ethylene is essential for regulating plant responses to biotic and abiotic stresses, and plays a key role in regulating growth and senescence (Lin et al., 2009). Ethylene production rapidly increases in plants subjected to wounding, flooding, drought, osmotic shock, senescence, ozone, and pathogen/insect invasion (Wang et al., 2002; van Loon et al., 2006; Di Baccio et al., 2012), and this leads to the activation of cell responses through the ethylene signaling pathway and its interactions with the signaling pathways of other plant hormones (Overmyer et al., 2000; Wang et al., 2002; Trivellini et al., 2014). Ethylene is synthesized by two enzymes encoded by small gene families: 1 aminocyclopropane 1 carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO). The reaction is first catalyzed by ACS, which converts *S*-adenosyl-L-methionine (SAM) to ACC, and then ACC oxidase catalyzes the conversion of ACC to ethylene with the release of CO_2 and cyanide (Wang et al., 2002). ACS is the rate-limiting step in ethylene biosynthesis, and controls the main step in stress-induced ethylene regulation (Tsuchisaka

et al., 2009), whereas ACO activity is constitutively present in most vegetative tissues. The ethylene biosynthetic pathway is relatively simple, but its production is strictly regulated at various levels. In addition to transcriptional regulation (Tsuchisaka and Theologis, 2004a,b), post-translational regulation is pivotal for developmental and stress-induced ethylene production (Christians et al., 2009; Han et al., 2010; Skottke et al., 2011; Lyzenga et al., 2012).

In order to investigate the role of ethylene depending on N availability, we first listed the genes involved in ethylene biosynthesis, signaling and responses by searching The *Arabidopsis* Information Resource (TAIR[1]) (Supplementary Table S1) and then analyzed the publicly available microarray data on the Affymetrix ATH1 microarray platform (as of June 2015) using Genevestigator (Hruz et al., 2008). A similarity search subsequently enabled the determination of lists of the same genes regulated upon a given N perturbation (Supplementary Table S1). The analysis considered the expression profiles of the genes that showed a >2-fold change in transcription level ($P < 0.01$) under conditions of nitrate starvation and low or high N content, and the fold-change values were hierarchically clustered by genes and experiments using Euclidean distances.

This meta-profiling showed that the ethylene biosynthetic pathway is regulated by N conditions (**Figure 1A**), and that the genes involved in ethylene biosynthesis appeared to be transcriptionally active under these conditions. In the case of nitrate starvation, ACC synthase *ACS7* and a putative *ACO* (and *ACS10*) were strongly repressed in seedlings, but both were induced in rosette samples treated with low and high N levels, whereas ACC synthases *ACS8* and *ACS4*, and ACC oxidase *ACO1*, *ACO5 and ACO2* were negatively regulated under both conditions. However, *ETO1* (*OVERPRODUCER1*), *SAM1*, *EOL1*-like (ETO-like) and other putative *ACO* were induced in response to N deprivation and low/high N conditions. It is tempting to hypothesize that the multi-gene *ACS* and *ACO* families are both temporally and spatially differentially expressed under low N environmental conditions, as has previously been shown in the case of stresses such as Pi-deprivation (Kim et al., 2008; Roldan et al., 2013), and depend on the species, tissue and developmental stage of the plants (Inaba et al., 2007; Trivellini et al., 2011). A large-scale transcriptome analysis has detected an *ACO6* homolog involved in ethylene synthesis during the early response of cucumber seedlings to N deficiency (Zhao et al., 2015), and the induction of an *ACO4* homolog and *ACO*-like transcript has been observed in response to N starvation in studies of chronic low N conditions (Bi et al., 2007; Peng et al., 2007).

It is also worth noting that suboptimal nutrient supply promotes leaf senescence (Mei and Thimann, 1984; Jibran et al., 2013). Balazadeh et al. (2014) have recently reported that plants undergoing senescence retain the capacity to sense and respond to the availability of N nutrition by reversing the senescence phenotype induced by N starvation. In this study, the expression of *ACS2*, *ACS6,* and *ACS7*, and *ACO2*, *ACO3,* and *ACO4* was increased during senescence, but only *ACS6* was first induced after 4 days of N deficiency and then reduced 3 h after N resupply. *ACO2* and *ACO4* transcript levels were also increased by N deprivation and then significantly down-regulated after 3 days of N resupply, once again highlighting the complexity of ACS and ACO regulation by various stresses signals.

Ethylene production (particularly the rapid breakdown of ACS proteins) is also tightly controlled by means of protein degradation (Christians et al., 2009). The recently characterized *Arabidopsis* mutant *hps3* (Wang et al., 2012), which is hypersensitive to Pi starvation, was previously identified as an allele of the *ETO1* gene that negatively regulates ethylene biosynthesis by producing 10–50 times more ethylene than the wild type (Wang et al., 2004), and our Genevestigator analysis showed that *ETO1* and *EOL1* are weakly expressed under low N conditions. Although these findings potentially define the role of ethylene in regulating multiple plant responses to conditions including N starvation, there is a need for further experimental analyses aimed at identifying the molecular components that interact with ethylene signaling in regulating plant responses to N.

A meta-analyses of the ethylene receptors and mitogen-activated protein kinases (MAPK, MPK, or MKK) linking upstream sensors to the downstream processes of hormonal responses under conditions of N deprivation shows that *ERS2* and *EIL1* are down-regulated, whereas the MPKs involved in ethylene signaling are all induced (**Figure 1B**). Previous studies have shown that MPK3 can be activated by various MKKs that participate in specific signaling pathways: for example the MKK4/MKK5/MKK9 pathway activates MPK3/MPK6 to promote ethylene production (Liu et al., 2008), and MKK9 activates MPK3/MPK6 to regulate leaf senescence (Zhou et al., 2009) and ethylene signaling (Yoo et al., 2008). However, it is not yet known whether MAPK signaling cascades are directly involved in regulating plant responses to various N conditions.

Nitrogen deficiency may play a positive role in ethylene biosynthesis and signaling as *in silico* analysis reveals the slight down-regulation of *CTR1* and up-regulation of *EIN3* under conditions of N starvation and low/high N levels. Zheng et al. (2013) have similarly found that low-level nitrate treatment induces rapid bursts of ethylene production and regulates the expression of the ethylene signaling components *CTR1*, *EIN3* and *EIL1,* and *NRT2*.1 in wild-type plants. The authors used NO_3^- transporter mutants *nrt1.1* and *nrt2.1* and the ethylene mutants *ctr1-1* and *ein3-1eil1-1*, and elegantly proposed that NO_3^- deficiency induces a negative feedback loop between the transcription of *NRT2.1* and ethylene biosynthesis and signaling that allows plants to fine tune nitrate acquisition during the exploration of dynamic soil conditions.

The gene sets specified in **Figure 1C** were further classified into gene ontology (GO) categories in order to help the identification of over-representation. Sixty-nine genes were initially uploaded to the DAVID Bioinformatics Resources 6.7 platform (Huang et al., 2009[2]) in order to identify significantly enriched biological themes by examining enrichment in more than 40 publicly available annotation categories (Trivellini et al.,

[1]https://www.arabidopsis.org/

[2]http://david.abcc.ncifcrf.gov/

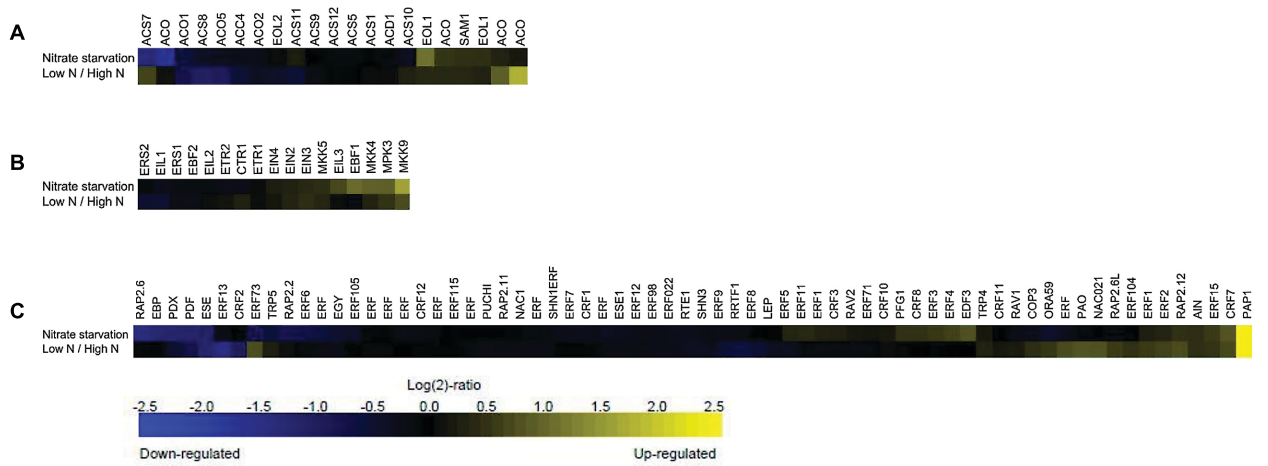

FIGURE 1 | Meta-profiling hierarchical average of ethylene genes induced in response to different nitrogen (N) perturbations. The expression profiles of three ethylene gene lists following N conditions were analyzed using the similarity search tool: **(A)** ethylene biosynthesis; **(B)** ethylene signaling; and **(C)** ethylene response. The data consist of the ethylene-related genes represented in the publicly available Affymetrix ATH1 microarrays obtained using the Genevestigator toolbox. Blue and yellow respectively indicate down- and up-regulation; black indicates no change in expression. The values indicate the fold-change in expression in nitrogen starved and untreated rosette samples, and in low- and high-nitrate seedlings.

2012). The analysis identified six clusters that showed significant enrichment, with enrichment scores (ES) ranging from 69.95–1.37 (**Table 1**). The most enriched annotation cluster (ES 69.95) was not surprisingly associated with the genes belong to the Apetala2/ethylene response factor (AP2/ERF-TF) super family; the second cluster contained genes with transcription repressor activity (ES 8.68); the third consisted of genes related the biological processes of root and lateral root development (ES 3.45); the fourth included genes involved in the negative regulation of the ethylene-mediated signaling pathway (ES 2.33); the fifth included genes involved in the response to cytokinin (CK) stimuli (ES 1.49); and the sixth the genes involved in the response to jasmonic acid stimuli (ES 1.39).

The AP2/ERF-TF family is involved in signaling processes and the responses to environmental stresses (Vogel et al., 2012). Most importantly, there is increasing evidence that AP2/ERF proteins are components of multiple signaling pathways as they control the expression of downstream genes and tune cross-talk between the signaling pathways involving macronutrients deficiency (Kim et al., 2012; Cai et al., 2013; Takehisa et al., 2013). Genome-scale transcriptional profiling of cucumber seedlings has shown that seven ERF genes are regulated under conditions of N starvation (Zhao et al., 2015), and comprehensive expression profiling of N starvation-responsive miRNAs has identified miR829.2, which is predicted to target an AP2 domain ethylene response factor required for morphogenesis in the early lateral root primordium of *Arabidopsis* (Liang et al., 2012), thus highlighting the important role of this transcription family in N-starved root development.

Furthermore, as was done in the case of the genes involved in the ethylene biosynthesis and perception machinery, the ethylene response gene list was compared with the publicly available microarray data using the Perturbations tool in the Condition Search toolset of Genevestigator (Hruz et al., 2008). All of the ethylene response genes seemed to be transcriptionally active under condition of N starvation and/or low/high N levels, with the *PURPLE ACID PHOSPHATASE 1 (PAP1)*, *PYRIDOXINE BIOSYNTHESIS 1.1 (PDX1.1)*, *PLANT DEFENSIN 1.2B (PDF1.2B)* and *ETHYLENE AND SALT INDUCIBLE 3 (ESE3)* responding strongly to both types of N perturbation (**Figure 1C**). *PAP1* encodes transcription factors regulating the expression of anthocyanin biosynthetic genes in vegetative tissues. *PAP1* expression is a frequent plant response to stress conditions such as drought, heat, chilling, N deficiency and in response to abscisic acid (ABA), and the sugars in which anthocyanin is accumulated (Luo et al., 2012). The availability of N represses anthocyanin biosynthesis-related gene expression (Rubin et al., 2009) whereas N deficiency stimulates it (Peng et al., 2008). As it is known that ethylene stimulates the expression of the genes related to anthocyanin biosynthesis (Afifi et al., 2003; El-Kereamy et al., 2003), N starvation could induce a transient rise in ethylene production and signaling (Zheng et al., 2013). A new allele of ROOT HAIR DEFECTIVE3 (RHD3) with an anthocyanin over-accumulation phenotype under conditions of N starvation has recently been identified (Wang et al., 2015), and the authors speculate that RHD3 achieves its negative effect on anthocyanin biosynthesis via an ethylene-regulating pathway involving the ETR1, EIN2, and EIN3/EIL1-mediated signaling cascade. Further investigations are needed to clarify the molecular mechanism of RDH3 underlying ethylene signal transduction.

Interestingly, *RAP 2.6*, *RAP 2.3 (EBP)*, and *RAP 2.2* were significantly down-regulated in the N starvation experiment, whereas *RAP2.12* and *RAP2.6L* were weakly up-regulated under both conditions, and *ERF73* seemed to be differentially regulated (**Figure 1C**). These genes of the AP2/ERF family are responsible for modulating tolerance to the hypoxic stresses encountered by plants: *AtRAP2.2* and *RAP2.3* are important for

TABLE 1 | Functional annotation clustering using DAVID bioinformatics resources 6.7.

Cluster number	Enrichment score	Category	Term	Gene count
1	69.95	GOTERM_BP_FAT	Response to ethylene stimulus	61
2	8.68	GOTERM_MF_FAT	Transcription repressor activity	9
3	3.45	GOTERM_BP_FAT	Root and lateral development	6
4	2.33	GOTERM_BP_FAT	Negative regulation of ethylene mediated signaling pathway	3
5	1.49	GOTERM_BP_FAT	Response to cytokinin stimulus	4
6	1.39	GOTERM_BP_FAT	Response to jasmonic acid stimulus	6

Cluster enrichment scores for selected significantly enriched functional clusters containing terms showing a significant change ($P < 0.05$) after Benjamini–Hochberg correction for multiple testing, and the term(s) most representative of each cluster. The genes associated with the most represented terms are shown in Supplementary Table S2.

ethylene-mediated tolerance to hypoxia in *Arabidopsis* seedlings (Hinz et al., 2010; Limami et al., 2014), and the same is true of *ERF73*, the hypoxia-responsive *ERF1* gene (*HRE1*) (Licausi et al., 2010); the overexpression of *RAP2.6L* delays the waterlogging induced by premature senescence and may function through the ABI1-mediated ABA signaling pathway (Liu et al., 2012); *RAP2.12* is involved in the activation of hypoxic gene expression and ethylene responses (Licausi et al., 2011; Zhao et al., 2012); and *RAP2.6* is involved in the response to ABA, wounding, jasmonic acid, salt, cold, and osmotic stresses (Fowler and Thomashow, 2002; Zhu et al., 2010). A number of studies have shown that hypoxic stress can be mitigated by nitrate fertilization (Morard et al., 2004; Horchani et al., 2010), but also that nitrate uptake and assimilation can be affected by oxygen-limiting conditions (Oliveira and Sodek, 2013; Oliveira et al., 2013). It therefore seems to be clear that there is a link between N-limiting conditions and the regulation of the genes associated with hypoxia. The ethylene-responsive genes typically involved in hypoxia are potential connectors in the gene/metabolite/hormone-related network of response to N starvation, but further studies are necessary in order to verify their possible role in the N assimilation and signaling pathway.

ETHYLENE RESPONSES TO VARYING LEVELS OF N AVAILABILITY

The N status of a plant influences its metabolism and growth, and can affect the synthesis of the building block metabolites and the distribution of growth substances. This interdependence is due to the fact that nutrient deficiency or excess affect the concentrations of specific hormones capable of directing the translocation and accumulation of nutrients (Kuiper, 1988; Kuiper et al., 1989), and a similar relationship has also been reported in the case of nutrient and ethylene interactions. Ren et al. (2013) have recently found that the addition of N reduces leaf longevity mainly by altering leaf ethylene production: this result was substantiated by the fact that the cobalt chloride-induced inhibition of ethylene biosynthesis reduced leaf N concentration and leaf longevity, presumably because a high N concentration stimulates the activities of the enzymes associated with ethylene synthesis (Tian et al., 2009). Increased ethylene production may also be involved in modulating nitrate transporters (Tian et al., 2009) and nitrate metabolism (Leblanc et al., 2008) at high nitrate levels. A nitrate concentration of 10 mM increases the expression of genes encoding *ACS* and *ACO* (*AtACS* and *AtACO*), and leads to a sudden increase in ethylene production in *A. thaliana* plants. Furthermore, the upregulation and downregulation of nitrate transporters (AtNRT1.1 and AtNRT2.1) was observed by exogenously applying the ethylene synthesis precursor ACC and AVG in low and high nitrate concentration, respectively, whereas the *etr1-3* and *ein2-1* mutants were insensitive to high nitrate concentrations (Tian et al., 2009). A very interesting study by Misyura et al. (2014) found that ethylene may act as a plant–plant communication signal in rice under conditions of high-density stress, when the expression of ethylene-associated genes was related to ethylene homeostasis. The authors showed that N availability can influence the growth of rice plants dependent on ethylene homeostasis, and that the developmental characteristics of plants were negatively affected under high density conditions when N was limited (3 mM NO_3^-) or sufficient (10 mM NO_3^-). The availability of N influences the evolution of ethylene and affects photosynthesis, stomatal conductance and growth in *B. juncea* plants (Khan et al., 2008; Iqbal et al., 2011). Field experiments demonstrated that the application of ethephon (ethylene-releasing compound) to plants grown with N levels of 40 and 80 mg N kg^{-1} increased ethylene production and photosynthesis (Iqbal et al., 2011). It has also been suggested that the application of ethephon induces stomatal and carboxylation efficiency and the Calvin cycle enzymes in mustard plants grown at various N levels, with significant interaction between ethylene, N availability, and photosynthetic characteristics.

Improving the acquisition of macronutrients such as N, phosphorus (P) and potassium (K) in poorly fertile soils is one of the main objectives of program aimed at reducing the use of fertilizers and increasing the efficiency of nutrient use (Hirel et al., 2007; Lynch, 2007). The efficient use of N fertilizer is essential to ensure a better return on investment and minimize the adverse effects of accumulated reactive N species on the environment. It is therefore important to increase the N use efficiency (NUE) of plants in order to avoid N wastage and accumulation. NUE, the efficiency of carboxylation and water use, and the dry mass of mustard plants increased at different levels of N in combination with ethephon (Iqbal et al., 2011). Exogenously sourced ethylene enhances photosynthetic NUE and promotes photosynthesis in various types of mustard plant with differing photosynthesising capacity (Iqbal et al., 2012). The application of

ethrel at basal 80 kg N ha^{-1} increased the efficiency of N uptake and use in mustard plants, and that the exogenous application of ethephon increased stomatal conductance, photosynthesis and growth under conditions of N deficiency and optimization as a result of increased NUE (Mir, 2002). It has also been found that, under N-deficient conditions, greater endogenous ethylene evolution decreases NUE, photosynthesis and growth in mustard plants (Iqbal et al., 2011). However, high levels of ethylene can also have a negative impact on plant growth and photosynthesis. Under certain conditions the increase of the ethylene sensitivity and ethylene action overcomes N deficiency by increasing photosynthesis and growth in plants with sufficient or deficient N availability (Iqbal et al., 2011). Recently has been reported that N availability regulates ethylene formation, which regulates plant N content and nitrate reductase (NR) activity (Iqbal et al., 2015). Subsequently ethylene increases the proline content and salt tolerance of *B. juncea* plants, and improves photosynthesis and growth.

Nitrogen deficiency leads to strong synergistic interactions between volicitin and ethylene, indicated by the induction of volatile sesquiterpene and indole emissions. Whereas volicitin-induced volatiles are greatly reduced in plants with medium N levels, and there are virtually no interactions with ethylene. The altered volicitin–ethylene interaction due to changes in the magnitude of induced volatile emissions observed in plants with low and medium levels of N availability is consistent with the known increase in ethylene sensitivity that occurs during N deficiency (Schmelz et al., 2003). N deprivation enhances the sensitivity of ethylene-responsive cells in root cortex, thus leading to cell lysis and aerenchyma formation, and that the exogenous application of ethylene (1.0 $\mu L\ L^{-1}$) further promoted aerenchyma formation in N-starved roots (He et al., 1992). N starvation increases the number or affinity of root receptors, thus allowing roots to responds to lower concentrations of ethylene than those found in unstressed roots. Plants supplied with high nitrate levels (30 mM) increased their aerial ACC content by translocating it from the roots to the shoot in order to induce ethylene synthesis in the leaves by means of ACC oxidase (Saiz-Fernández et al., 2015). Ethylene plays a role in the regulation of fully developed and expanding leaves by reducing leaf area when ethylene accumulates in developing tissues (Young et al., 2004; He et al., 2009). The interaction between ethylene and N may also increase the synthesis of amino acids, proteins and enzymes. The production of ethylene by soluble solids could be due to increased synthesis of the amino acid cysteine, a precursor of ethylene that may be extended to synthesize other amino acids (Kaack and Pedersen, 2014). Zhao et al. (2015) studied changes in the expression of transcriptional factor and kinase genes at transcriptional level during the early stage of the N deficiency response, and observed seven ERF and three MYB transcription factors, five NAC domain-containing proteins, and four zinc finger proteins. Bi et al. (2007) and Peng et al. (2007) have found that ACO4 and another ACO homologue showed responses to N deficiency: ethylene production generally increases upon N deprivation but, in comparison with explants in standard MS medium, ethylene production by rhizome explants in low N medium was reduced after 1–3 months of culture. Zhang et al. (2013) found low nitrate treatment-induced rapid bursts of ethylene production and the regulated expression of the ethylene signaling components *CTR1,EIN3* and *EIL1* in wild-type *A. thaliana* (Col-0) seedlings, and enhanced ethylene response reporter EBS:GUS activity in Col-0 and the ethylene mutants *ein3-1, eil1-1* and *ctr1-1*. The treatment also caused the up-regulation of *NRT2.1* expression, which was responsible for enhanced high-affinity nitrate uptake, and had a positive effect on ethylene biosynthesis and signaling. However, ethylene down-regulated *NRT2.1* expression and reduced high-affinity nitrate uptake, thus suggesting that nitrate deficiency gives rise to a negative feedback loop between *NRT2.1* expression and ethylene biosynthesis and signaling, which may contribute to the fine tuning of plant nitrate acquisition during the dynamic exploration of soil conditions.

ETHYLENE PRODUCTION IN DIFFERENT PLANT ORGANS AT DIFFERENT LEVELS OF N AVAILABILITY

Ethylene can be produced in any plant tissue and is modulated by various internal and external factors. The responses of different organs to ethylene vary, depending on tissue sensitivity and the stage of plant development.

ROOT RESPONSES

The efficient absorption of macronutrients such as N, and developing the traits involved in remodeling root system architecture in order to acquire N more efficiently, are important targets of modern plant breeding program (Forde, 2014). Phytohormones are involved in controlling root development and architecture by means of N-mediated signals, and recent transcriptomic studies have shown that auxin, ethylene and CK are involved in root architectural responses to nitrates (Tian et al., 2009; Ruffel et al., 2011; Jin et al., 2012). Lemaire et al. (2013) found that ethylene signaling affects nitrate uptake and the expression of *BnNRT* nitrate transporter genes depending on changes in the length of exploratory and root hair systems. Different species, and even the same species under different growing conditions, may have opposite behaviors. In comparison with the wild type, *Never Ripe* (NR) ethylene-insensitive tomato mutants have more below-ground roots and fewer above-ground adventitious roots. Interactions and cross-talk with other plant hormones can lead to different responses. The application of exogenous auxin leads to different behavior (Clark et al., 1999), thus indicating that the effects of ethylene depend on its interaction with auxins as well as abiotic stresses such as nutrient deficiency.

Ethylene deficiency generally induces root development in order to increase the root biomass necessary for exploring a wide area of soil in search of the deficient nutrient. Ethylene can modulate root waving, and the direction and length of root growth (Buer et al., 2003), but the response can be affected

by interactions with nutrients. More studies should be carried out in order to investigate root architecture under conditions of N deficiency or excess using ethylene inhibitors. It has been found that N starvation simultaneously increases ethylene evolution and induced aerenchyma formation in the roots of *Zea mays* plants (Drew et al., 2000). Basal roots are more sensitive to ethylene than apical roots (Takahashi et al., 2015). The induction of aerenchyma is also a means of adapting to flooding, and oxygen shortage can initiate programmed cell death (PCD) in roots. Hypoxia associated with N deficiency enhances aerenchyma development, whereas anoxia inhibits or reduces it because the complete lack of oxygen blocks the ACC oxidase enzyme, which catalyzes the last step in ethylene biosynthesis. The use of ethylene biosynthesis and action inhibitors has shown that ethylene is directly involved in PCD in roots (He et al., 1992). High N (especially nitrate) availability in soils induces ethylene biosynthesis in roots. A number of studies have investigated the effects of ethylene and high nitrate content on legumes, in which ethylene biosynthesis inhibits the nodules necessary for N fixation and lateral root development (Caba et al., 1998; Okushima et al., 2011). The effect of the interaction of ethylene and high nitrate concentration on nodule formation has been elegantly demonstrated in various experiments using ethylene activators and biosynthesis inhibitors such as AVG and silver (Peters and Crist-Estes, 1989; Caba et al., 1998; Nukui et al., 2000). The inhibition of nodule formation is negative because it reduces N fixation in leguminous plants; however, from an ecological point of view, plants do not need to develop nodules for gaseous N fixation in soils that are rich in N, particularly nitrates.

Ethylene causes a triple response in *Arabidopsis* roots: the rapid down-regulation of cell elongation, the induction of ectopic root hairs, and an increase in root width. Le et al. (2001) found that slight changes in the concentration of environmental ethylene in *Arabidopsis* modulate the elongation of target cells in root epidermis, and suggested that ethylene is a means of fine and fast tuning root elongation in nature. It has also been demonstrated that C/N balance is involved in root morphogenesis (Malamy and Ryan, 2001; Martin et al., 2002), and that C and N interact with the major plant hormones (Sheen et al., 1999). Le et al. (2001) reported that ethylene inhibits the elongation of root cells, but does not affect root length in the root regions in which cell wall formation occurs before an increase in ethylene level. Increased ethylene synthesis with low concentrations of ACC promotes the initiation of lateral root primordial; however, treatment with higher ACC doses inhibits the formation of new primordia, but promotes the emergence of those already existing (Ivanchenko et al., 2008). N deficiency increases root sensitivity to ethylene and subsequent aerenchyma formation in maize seedlings (He et al., 1992), although ethylene production is reduced (Drew et al., 1989). Tari and Csiszár (2003) have found that, at pH 4.0, nitrite treatment decreases the evolution of ethylene from the root apex but not from the base. Yang et al. (2011) reported that ethylene inhibits NH_4-stimulated root hair branching, and that ACC 0.04 mM antagonized the effect of NH_4 by reducing hair branching from the 24% caused by NH_4NO_3 to only 5%.

Nitrate can act as both a nutrient and a signal that regulates global gene expression in plant organs. Tian et al. (2009) found that, in the presence of high nitrate levels, roots ethylene production increases from roots as a result of an increase in the expression of the genes encoding *ACS* and *ACO*. They also showed that ethylene regulated nitrate-dependent root development by modulating the expression of nitrate transporters *NRT1.1* and *NRT2.1*, thus demonstrating that ethylene signaling is involved in regulating nitrate uptake on the basis of changes in root elongation. The *etr1-3* and *ein2-1* mutants of ethylene signaling were insensitive to high nitrate concentrations. Lemaire et al. (2013) demonstrated that treatment with the ethylene precursor ACC induces a partial compensatory increase in N uptake, associated with the over-expression of the nitrate transporter genes, *BnNRT2.1* and *BnNRT1.1*. Similar results were obtained by Leblanc et al. (2008) and Le Ny et al. (2013), who suggested that there is a linear correlation between root length and *BnNRT2.1* expression levels in response to 10 μM AVG or changes in nitrate availability. However, Leblanc et al. (2013) reported a decrease in *BnNrt2.1* expression with an increase in ACC concentrations from 0.1 to 10 μM, thus suggesting that *BnNrt2.1* expression may adapt to changes in the absorbing surface of whole mature root by means of a still unknown regulatory mechanism. Leblanc et al. (2008) found that the rapid modulation of root elongation is more dependent on ethylene than on the nitrate signal: ACC treatment reduced C allocation and aspartate content in roots, thus showing that aspartate content correlates with changes in root length and shoot surface area. Canales et al. (2014) reported that up to 10% of the *Arabidopsis* genomes are N responsive, and approximately 7% in maize transcriptomes (Yang et al., 2011). N-induced root developmental plasticity is highly cell specific and finely regulated within the root (Gifford et al., 2008). Among the N responsive gene, five nitrate-responsive genes encoding *NRT2.1*, *NR*, *HB2*, *NiR*, and *HB1* are specifically regulated in the transition zone (Manoli et al., 2014; Trevisan et al., 2014). Trevisan et al. (2015) also reported that the transition zone is critical in sensing nitrate, which directly influences the transcript levels of a few genes and acts indirectly through NR.

Ethylene is also involved in regulating legume–rhizobial interactions: it influences the initial response of root hairs to *Rhizobia* bacteria exposure and the progression of infection into the cortex. Penmetsa and Cook (1997) found that the sickle mutant in *Medicago truncatula* is ethylene-insensitive and hyper-nodulated, and provided genetic support for the involvement of ethylene in regulating rhizobial symbiosis by encoding an ortholog of *EIN2*. Exogenous ethylene severely inhibits the formation and function of N-fixation nodules on legume roots (Peters and Crist-Estes, 1989), possibly because the developmental effects of ethylene include the inhibition of cell division, DNA synthesis, and hook expansion (Apelbaum and Burg, 1972), and the induction of phytoalexin and extension biosynthesis (Ecker and Davis, 1987). Ethylene may act as a secondary signal regulating nodulation on the basis of the N status of the plant and as a negative feedback regulator of rhizobial infection (Oldroyd et al., 2001). Malamy and Ryan

(2001) have suggested that the number of lateral roots is reduced in older root regions under conditions of N starvation.

LEAF RESPONSES

N deficiency also increases ethylene evolution in leaves as a consequence of stress (Legé et al., 1997). Over-irrigation of tomato plants induces N deficiency in leaves and greater ethylene biosynthesis, whereas the use of calcium nitrate to restore adequate N levels reduces ethylene evolution to control levels (Fiebig and Dodd, 2015). N starvation or deficiency also induces leaf senescence (particularly leaf yellowing), promotes the re-mobilization of nutrients from leaves to storage organs, and increases tissue sensitivity to ethylene. Many plants react to N starvation by activating the phenylpropanoid pathway and accumulating anthocyanins (Lea et al., 2007). In particular, low N levels in soils or growing media activates phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the key enzyme of phenylpropanoid compounds. Various post-harvest studies have demonstrated that ethylene stimulates PAL activity, and it is known that the exposure of lettuce to ethylene generates russet spotting (i.e., brown spots on the mid-rib of head lettuce) (Hyodo et al., 1978; Ke and Saltveit, 1988).

There are no specific studies of the effects of the ethylene induced by N deficiency and the consequent PAL activity, but it is reasonable to assume that ethylene plays a pivotal role in this physiological behavior. Studies of *Medicago sativa* cell suspensions treated with 2-aminoindan-2-phosphonic acid (AIP), a potent inhibitor of PAL, have shown enhanced ethylene biosynthesis (Cvikrová et al., 1999), and similar results have been obtained *in vitro* callus cultures of red-fleshed apples with or without N supply in growth media. The absence of N strongly induces anthocyanin accumulation, thus demonstrating the direct role of N on phenylpropanoid activation. Under conditions of N deficiency or starvation, plant leaves activate a N-recycling system in which N is recycled from phenylalanine by means of deamination to cinnamic acid, a reaction that is catalyzed by the PAL enzyme. Although it has not yet been established, it is likely that this futile cycle is under the control of ethylene. Ethylene biosynthesis is also affected by excess N. It is well known that the availability of high N levels induces vegetative growth, makes shoots more susceptible to insect attack (Davies et al., 2004), and causes greater damage due to abiotic stresses. The availability of 20 mM N in *B. juncea* plants increases ACS activity and ethylene evolution (Iqbal et al., 2015).

Plant photosynthesis and sugar biosynthesis have to balance any reduction in nitrates. As uptaken nitrate is reduced to ammonia under conditions of high N levels, plants cannot incorporate all of the reduced ammonia in amino acids, and this can generate stress leading to ethylene biosynthesis.

FLOWER LIFE

There are no specific studies linking N deficiency to ethylene evolution in flowers, but it has been reported that N deficiency increases ethylene biosynthesis and tissue sensitivity. Pre-harvest N deficiency affects the photosynthetic activity of plants, and the life of both growing and cut flowers (Druege, 2000). Under conditions of stress such as N efficiency, plants accelerate all of the physiological processes related to species dispersal: these usually include the induction of flowering in order to ensure dissemination.

No studies of flower senescence under conditions of N deficiency or excess have yet been carried out, but this would be interesting to investigate further.

FRUIT RESPONSES

N availability affects fruit development and quality, in particular low nitrogen content delay ripening and fruits are poor of sugars affecting negatively the overall quality. High nitrogen content usually induces rapid growth and fruits after harvest have faster senescence. Melons harvested from soil with deficient (0 or 50 kg ha^{-1}) or excess N (165 kg ha^{-1}) levels have the same rate of ethylene biosynthesis. Ethylene production is lowest at the optimal fertilization dose of 110 kg ha^{-1} at harvest, after 8 days of post-harvest storage at 10°C, ethylene production decreased and no significant differences were found among treatments (Ferrante et al., 2008).

It has been found that the use of high, medium or low N fertilization does not affect ethylene production rates in peach trees, but high N levels delay the ripening of the fruit (Okamoto et al., 2001). No differences in ethylene production have been found in apples receiving soil and foliar N, and controls with no supply of N (Wargo et al., 2004). There are few published information concerning N availability and ethylene production in horticultural crops, and so further studies are required in order to establish how N soil content affects ethylene production and the subsequent post-harvest performance of fruit.

Figure 2 shows a general view of how ethylene concentrations are affected by N levels in the different parts of plants.

ETHYLENE INTERACTIONS WITH OTHER PHYTOHORMONES AT DIFFERENT LEVELS OF N AVAILABILITY

Nitrogen levels considerably influence root architecture and crop production (Mohd-Radzman et al., 2013), and plants have efficient internal execution points to control N uptake, reduction and assimilation, and environmental NUE (Xu et al., 2012). Some recently published studies have begun to elucidate the link between ethylene signaling and N availability. Nitrate assimilation can take place directly in roots, and the assimilated nitrate can be stored in vacuoles or transferred to the aerial parts of a plant (Krapp, 2015) but, in many species, it preferentially occurs in shoots, where photosynthesis takes place and energy is easily available (Searles and Bloom, 2003).

However, a number of stresses can separate nitrate assimilation and photosynthesis by triggering nitrate allocation to roots. As hormonal action is an interdependent process, the

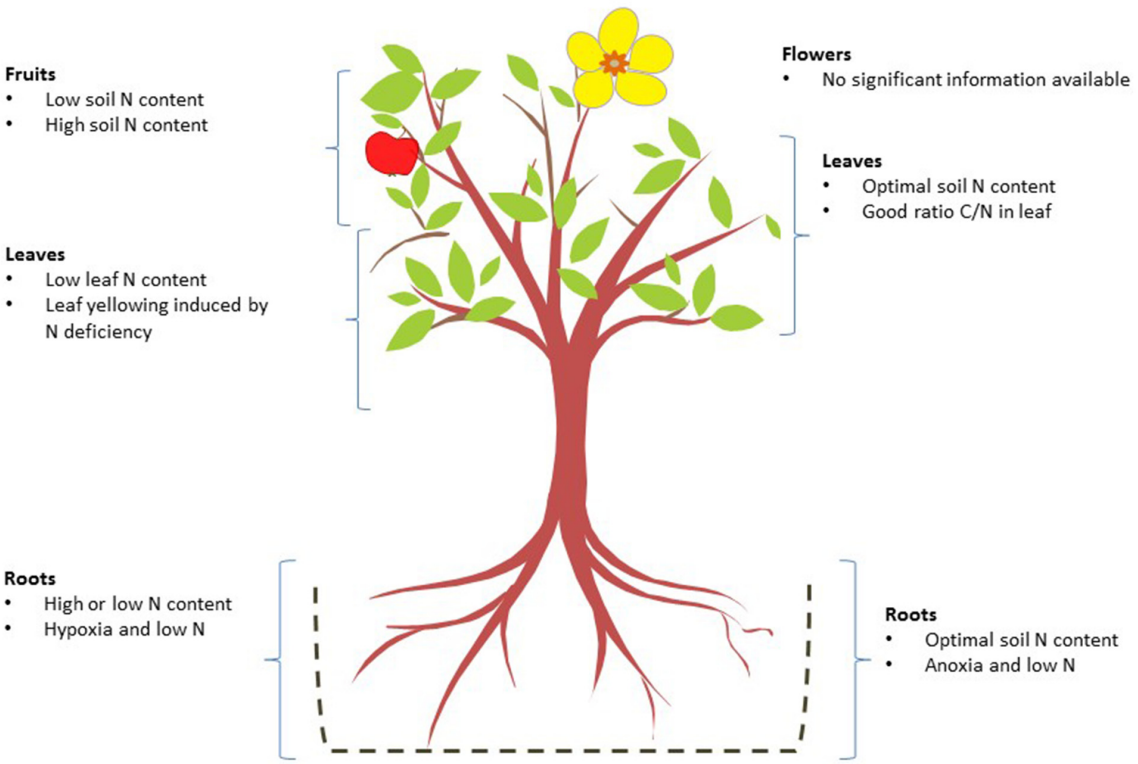

FIGURE 2 | Effects of nitrogen deficiency and excess on ethylene biosynthesis in different organs. The nitrogen levels that, alone or in combination with other factors, increase (left) or reduce ethylene biosynthesis (right) are shown for each organ.

action of ethylene at different N levels may be influenced by regulatory interactions between ethylene and other phytohormones. Genetic studies of *A. thaliana* by Swarup et al. (2007) have shown that ethylene-induced inhibition of root growth involves auxin, the presence of which significantly enhanced the inhibition of root cell elongation induced by the ethylene precursor ACC. It has also been reported the mutations in auxin transport or signaling components cause aberrant responses to ethylene, thus indicating the existence of cross-talk between the two phytohormones (Luschnig et al., 1998; Stepanova et al., 2005). Alonso et al. (2003) found that mutations in the auxin receptor TIR1 lead to ethylene-insensitive root growth phenotypes. Ethylene inhibits cell elongation by locally stimulating auxin biosynthesis and basipetal auxin transport toward the elongation zone; in mutants deficient in auxin perception or basipetal auxin transport, ethylene cannot activate the auxin response or regulate root growth (Růžička et al., 2007).

Stress-initiated nitrate allocation to roots (SINAR) improves stress tolerance and decreases plant growth under non-stressed conditions via an *ET/JA-NRT1.5/NRT1.8* signaling module (Zhang et al., 2014), which allows the regulation of nitrate assimilation at the level of the organ at which stresses initiate ET and JA signaling, which converges to *EIN3/EIN3-Like1 (EIL1)* in order to modulate *ERF* expression and up-regulate *NRT1.8*; ET and JA signaling mediates the down-regulation of *NRT1.5* via *EIN3/EIL1* and other unknown component(s). Krouk et al. (2010) demonstrated that, at low nitrate levels, mutations in the *NRT1.1* nitrate transporter enhance auxin accumulation in lateral roots and lateral root growth. At low (but not high) nitrate levels, *NRT1.1* represses lateral root growth by promoting the outward transport of basipetal auxin. Ma et al. (2014) showed that low N induces lateral root growth in *Arabidopsis*, and that this growth was dependent on the function of the auxin biosynthesis gene tryptophan aminotransferase related 2 (*TAR2*), which is induced under low N conditions. It has also been shown that molecules mediating auxin influx (AUX1, LAX2, LAX3) and efflux (PIN1, PIN2, PIN4, and PIN7) are transcriptionally regulated by N and/or C (Gutierrez et al., 2007; Li et al., 2011), and that most of these carriers are required for root development (Petrášek and Friml, 2009). It has been reported that jasmonic acid is a negative regulator of nodulation. Mi et al. (2008) reported that, in the presence of low N levels, the auxin, CK and nitric oxide (NO) signaling pathways are involved in regulating root elongation: an abundant N supply increases CK levels, but decreases auxin and NO levels in the roots of maize. The exogenous supply of CK increases ethylene production (Stenlid, 1982; Bertell et al., 1990). Nitrate-induced inhibition of root elongation in maize

is significantly reversed by treating the roots with a NO donor (SNP) and IAA (Zhao et al., 2007). In the presence of high nitrate levels, endogenous levels of NO in the root apices of maize seedlings are much lower than those in apices grown in the presence of low nitrate levels. The inhibition of NO synthesis reduces root elongation in maize plants grown in a low-nitrate medium (Mi et al., 2008).

It has been reported that N supplementation induces CK accumulation in detached *Helianthus annuus* and *Nicotiana tabacum* leaves (Salama and Wareing, 1979; Singh et al., 1992). *AtIPT3* (a gene involved in CK biosynthesis) is nitrate inducible, and *atipt3* mutants reduce CK levels, thus indicating that *AtIPT3* is a key determinant of nitrate-dependent CK biosynthesis (Miyawaki et al., 2004; Takei et al., 2004). The nitrate transporter *NRT1.1* mediates the nitrate inducible expression of *AtIPT3* (Liu et al., 1999; Ho et al., 2009), and Kiba et al. (2011) have found that CK represses the nitrate transporter gene and nitrate uptake regardless of plant N status. However, Rubio et al. (2009) and Krouk et al. (2011) have shown that CK also regulates the expression of N uptake- and assimilation-related genes, as well as root architecture (Werner et al., 2003; Higuchi et al., 2004). CK may function as a long-distance "root-to-shoot" signal related to NO_3^- supply (Takei et al., 2004; Sakakibara, 2006), and Ruffel et al. (2011) found that it is a crucial component of a root-shoot-root signaling mechanism that is involved in conveying a plant's NO_3^- status, thus enabling a compensatory increase in lateral root growth in NO_3^--rich zones of a root system foraging for N resources in a heterogeneous N environment.

Signora et al. (2001) showed that both ABA-insensitive mutants (*abi4-1*, *abi4-2*, and *abi5-1*) and ABA-deficient mutants (*aba1-1*, *aba2-3*, *aba2-4*, and *aba3-2*) are less sensitive to the inhibitory effects of high nitrate levels, and the study of the *Medicago truncatula latd* mutant by Yendrek et al. (2010) provided another line of evidence supporting a link between ABA and N signaling. The *latd* mutant is characterized by severe defects in root meristem maintenance and root growth, and its primary root growth is insensitive to nitrate; the *LATD* gene encodes a transporter belonging to the NRT1 (PTR) family, and is rescued by exogenous ABA (Bright et al., 2005; Liang et al., 2007).

Sun et al. (2015) have found that the NR pathway in *Oryza sativa* generates NO, which improves N acquisition capacity by increasing the initiation of lateral roots and the uptake of inorganic N, a strategy that allows the plants to adapt to a fluctuating nitrate supply and increase NUE. Liu et al. (2010) reported that ethylene induces NO formation in *A. thaliana*. N availability affects ethylene biosynthesis and signaling, which further increases N uptake and transport to enhance plant growth. The effect of ethylene on root architecture increases N absorption and influences N transport-related genes. It has been shown that the action of ethylene on N uptake or root growth is not independent of other phytohormones as low nitrate levels also increase CK, auxin, ABA and NO. CK increases the production of ethylene, which acts in coordination with auxin in order to ensure root growth and lateral root formation. These hormones are influenced by N and affect root growth but, as their cross-talk allows them to acquire N in the case of a limited supply, there is a need to verify whether ethylene functions in coordination with these hormones under the same conditions. Inhibiting these phytohormones under condition of limited N availability could provide insights into their mechanism of action, and enable the use of ethylene as a means of increasing plant NUE, avoiding N wastage, and preventing environmental pollution.

It is widely recognized that a high ethylene concentration is a potent inhibitor of nodule development in plants (Lee and LaRue, 1992; Sun et al., 2006; Gresshoff et al., 2009). Ferguson et al. (2011) have recently reported that there is a close relationship between gibberellin, ethylene and nodulation in *Pisum sativum*: the application of the ethylene precursor ACC significantly reduces the number of nodules and root and shoot length in wild-type NA plants, whereas treatment with the ethylene biosynthesis inhibitor AVG increases the number of nodules to 36 times the number formed on gibberellin-deficient mutant *na-1* plants. They also suggested that ethylene biosynthesis genes (*PsACS1* and *PsACO1*) were decreased in *na-1* roots, but the fact that there was no significant change in *PsACO1* in the roots of *na-1* plants during nodule formation indicates that ethylene plays a role in nodulation. However, there is a need for further experiments aimed at investigations the relationships between ethylene and other hormones in nodule formation.

The use of methyl jasmonate restrains nodulation in *Lotus japonicas*, including infection thread formation and *NIN* gene expression in wild-type plants and the hyper-nodulated *har1* mutant (Nakagawa and Kawaguchi, 2006). It has also been found that nodulation is inhibited in *Medicago truncatula* cultured in a growth medium containing JA, and that the number of *NIN* transcripts is larger in transgenic than in wild-type plants. Ethylene signaling negatively regulates the early stage of nodule development, including infection thread formation and the emergence of nodule primordia (Penmetsa and Cook, 1997).

CONCLUSION AND FUTURE PROSPECTS

Nitrogen availability has a strong influence on ethylene biosynthesis and signaling, and plants have different metabolic responses to optimal and stressful conditions. As N is a core mineral nutrient, larger amounts of N improve crop productivity, increasing NUE. Modulating ethylene availability should have a positive effect on sustainable development by reducing the wastage of applied N and increasing environmental protection. In order to maximize the genetic potential of N use, it is important to focus research on clarifying the interactions of N, ethylene and other hormones in order to be able to use more N under N-deficient conditions without affecting environmental safety.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00927

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A Comparative Study of Ethylene Emanation upon Nitrogen Deficiency in Natural Accessions of *Arabidopsis thaliana*

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An original approach to develop sustainable agriculture with less nitrogen fertilizer inputs is to tackle the cross-talk between nitrogen nutrition and plant growth regulators. In particular the gaseous hormone, ethylene, is a prime target for that purpose. The variation of ethylene production in natural accessions of the model species *Arabidopsis thaliana* was explored in response to the nitrate supply. Ethylene was measured with a laser-based photoacoustic detector. First, experimental conditions were established with Columbia-0 (Col-0) accession, which was grown *in vitro* on horizontal plates across a range of five nitrate concentrations (0.5, 1, 2.5, 5, or 10 mM). The concentrations of 1 and 10 mM nitrate were retained for further characterization. Along with a decrease of total dry biomass and higher biomass allocation to the roots, the ethylene production was 50% more important at 1 mM than at 10 mM nitrate. The total transcript levels of *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASES* (*ACS*) in roots and those of *ACC OXIDASES* (*ACO*) in shoots increased by 100% between the same treatments. This was mainly due to higher transcript levels of *ACS6* and of *ACO2* and *ACO4* respectively. The assumption was that during nitrogen deficiency, the greater biomass allocation in favor of the roots was controlled by ethylene being released in the shoots after conversion of ACC originating from the roots. Second, biomass and ethylene productions were measured in 20 additional accessions. Across all accessions, the total dry biomass and ethylene production were correlated negatively at 1 mM but positively at 10 mM nitrate. Furthermore, polymorphism was surveyed in ACC and ethylene biosynthesis genes and gene products among accessions. Very few substitutions modifying the amino acids properties in conserved motifs of the enzymes were found in the accessions. Natural variation of ethylene production could be further explored to improve Nitrogen Use Efficiency (NUE), in particular by manipulating features like the biomass production and the timing of senescence upon nitrogen limitation.

Keywords: Arabidopsis, biomass, ethylene, natural variation, nitrogen

INTRODUCTION

Plant growth requires profuse amount of nitrogen, since that element constitutes to nearby two percent of plant dry matter and is a component of key biological molecules like nucleic and amino acids, chlorophyll, and various metabolites. In agriculture, breeding strategies are urgently required to ameliorate Nitrogen Use Efficiency (NUE), in order to sustain the galloping world population growth and to preserve the environment from nitrogen (N) fertilizer overuse (Giles, 2005; Robertson and Vitousek, 2009; Godfray et al., 2010; Good and Beatty, 2011; Davidson et al., 2015). An innovative approach to enhance crop adaptability to nitrogen limitation is to tackle the cross-talk between mineral nutrition and biosynthetic pathways together with signaling cascades of plant growth regulators. Particularly the gaseous hormone, ethylene, is a prime target. Indeed, ethylene appears to regulate a wide range of morphological and developmental processes such as biomass production (Grichko and Glick, 2001; Khan, 2005), leaf cell expansion (Kieber et al., 1993; Rodrigues-Pousada et al., 1993), and lateral root formation (Swarup et al., 2007; Ivanchenko et al., 2008; Negi et al., 2008; Street et al., 2015). Moreover, ethylene is known to influence various physiological processes such as senescence timing (Jing et al., 2005; Ueda and Kusaba, 2015) and nutrient recycling (Nagarajan and Smith, 2012). The relevance of those biological processes in response to N availability is reviewed in this special issue (Khan et al., 2015). The depletion or excess of N on ethylene biosynthesis and signaling has already been examined by some authors (Mir et al., 2010; Iqbal et al., 2011, 2015). A transient and rapid increase of ethylene production was detected 1 h after transferring plants from low to high nitrate conditions (Tian et al., 2009) and reciprocally (Zheng et al., 2013). Effect of N deficiency on ethylene production has however not been characterized over a longer growth period yet. Besides, it is reported that ethylene modulates the expression of genes encoding major nitrate systems like *AtNITRATE TRANSPORTER 1.1 /NITRATE PEPTIDE TRANSPORTER FAMILY 6.3* (*NRT1.1*/*NPF6.3*) and *AtNRT2.1* (Tian et al., 2009; Zheng et al., 2013).

More generally, a large body of evidence indicates that ethylene is not solely associated with N but also with other mineral element availability (García et al., 2015; Thao et al., 2015; Song and Liu, 2015). For instance, ethylene production is elicited by the depletion of major essential elements like potassium (K) (Shin and Schachtman, 2004; Jung et al., 2009; Benlloch-Gonzàlez et al., 2010), phosphorus (P) (Borch et al., 1999), and magnesium (Mg) (Hermans et al., 2010b). The depletion of microelements such as iron (Fe) (Romera et al., 1999; Waters and Blevins, 2000; Romera and Alcántara, 2004; Molassiotis et al., 2005) and manganese (Mn) (Dorling et al., 2011) also enhances ethylene production. Likewise, the exposure to high concentrations of other essential trace elements like copper (Cu) (Arteca and Arteca, 2007) or non-essential ones like cadmium (Cd) (Rodríguez-Serrano et al., 2006; Arteca and Arteca, 2007; Schellingen et al., 2014) and selenium (Se) (Tamaoki et al., 2008) increases ethylene production in plants.

Enzymes in the ethylene biosynthetic pathway are crucial to modulate hormone production (Van de Poel and Van Der Straeten, 2014). Ethylene is synthesized from the amino acid methionine (Met) in successive steps, with the most limiting one being the transformation of the intermediate S-adenosyl-methionine (S-AdoMet) in 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthases (ACS) (Yang and Hoffman, 1984; De Paepe and Van Der Straeten, 2005). Ethylene is finally produced from ACC through the action of ACC oxidases (ACO) with the reduction of oxygen and oxidation of a reducing agent, possibly ascorbate (Chae and Kieber, 2005; Lin et al., 2009). The Arabidopsis genome contains 12 *ACS* genes which encode eight functional ACC synthase proteins relatively similar in their polypeptidic sequences (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004) and five *ACO* genes which belong to the 2-oxoglutarate dioxygenases family (Gómez-Lim et al., 1993; Raz and Ecker, 1999; Lin et al., 2009). The *ACS* and *ACO* genes are subjected to transcriptional and post-transcriptional regulations during plant development and stress conditions (Van Der Straeten et al., 2001; Wang et al., 2002; Tsuchisaka and Theologis, 2004; Lin et al., 2009; Yuan et al., 2010; Van de Poel and Van Der Straeten, 2014; Booker and DeLong, 2015). In Arabidopsis, mineral constraints are known to modulate the expression of *ACS* genes and mainly of *ACS2* and *ACS6*. For instance, up-regulation of *ACS* genes was observed in response to Mg deficiency (Hermans et al., 2010a,b), exposure to Cd (Schellingen et al., 2014) and Se (Van Hoewyk et al., 2008). However, reports about N supply effect on *ACS* gene expression are sparse and observations depend on the organ and developmental stage of the plants (Khan et al., 2015). Transferring plants from low to high nitrate conditions resulted in higher transcript levels of all *ACS* genes with the exception of *ACS9* (Tian et al., 2009). Resupplying nitrate to starved plants slightly increased *ACS6* transcript levels (Wang et al., 2002). Finally, the expression patterns and the functional redundancy of the ACO proteins are much less understood compared to ACS enzymes.

Arabidopsis thaliana is native to Europe and Central Asia and has a widespread geographical area, exposing it to various environmental selective pressures (Koornneef et al., 2004). Some examples of adaptive traits are the tolerance to drought, salinity, and frost (Rus et al., 2006; Bouchabke et al., 2008; McKhann et al., 2008; Des Marais et al., 2012; Kesari et al., 2012; Wollenberg and Amasino, 2012). The natural variation offered by that species permitted to gain insights into adaptive metabolic and morphological strategies to low N conditions (Loudet et al., 2003; Chardon et al., 2010, 2012; De Pessemier et al., 2013).

Arabidopsis is a model species with small genome but a weed without any agronomic value. However, it is closely related to Brassica crops having complex genomes, resulting from multiple rounds of polyploidy in their ancestry (Trick et al., 2009). In that context, the biodiversity offered by Arabidopsis could help deciphering mechanisms controlling plant developmental processes dependent on ethylene. This can be important for improving NUE in Brassica crops (Mir et al., 2010; Iqbal et al., 2011).

This study describes an *in vitro* approach to screen for variation in the ethylene emanation in response to the nitrate supply in a core set of Arabidopsis natural populations with diverse geographic origins. The experiment setup was first tested on Columbia-0 (Col-0) and then applied to other accessions. Furthermore, Col-0 accession was subjected to detailed physiological and molecular investigations in order to characterize the interaction between nitrate supply and ethylene production. How such research can help the breeding of future high-NUE cultivars is further discussed.

MATERIALS AND METHODS

In vitro Culture

Seeds of *A. thaliana* accessions were obtained from the *A. thaliana* Resource Centre for Genomics, INRA, Versailles, France. A panel of 24 accessions that maximizes the genetic diversity of the species (McKhann et al., 2004) and the reference Columbia-0 (Col-0) accession was composed. The information collected on the accessions used in this study is presented in Table S1. Batches of seeds simultaneously generated from the original stocks were used for *in vitro* growth experiments. The following four accessions were discarded due to poor germination rate or insufficient number of seeds for carrying experiments: Alcalá de Hernares (Alc-0), Canary Island-0 (Can-0), Greenville-0 (Gre-0), and Sakata. The following 20 accessions and Col-0 were retained for further phenotyping procedure: Akita, Bologna-1 (Bl-1), Bulhary-1 (Blh-1), Burren-0 (Bur-0), Catania-1 (Ct-1), Cape Verde Islands-0 (Cvi-0), Edinburgh-0 (Edi-0), Geneva-0 (Ge-0), Ibel Tazekka-0 (Ita-0), St Jean Cap Ferrat (JEA), Kaunas-0 (Kn-0), Mulhen-1 (Mh-1), Martuba-0 (Mt-0), Konchezero (N13), Oystese-0 (Oy-0), le Pyla-1 (Pyl-1), Shahdara River (Sha), Stockholm-0 (St-0), Stobowa-0 (Stw-0), and Tsu-0. Seeds were sterilized with ethanol 70% (v/v) for 10 min and hypochlorite 20% (v/v) solution for 5 min. Hundred seeds were plated on 1x Murashige and Skoog medium modified with nitrate as the sole N source, 1% sucrose, 0.6% agar, and pH = 5.7 (Hermans et al., 2010c). In the initial screen with Col-0 accession, nitrate concentrations were 0.5, 1, 2.5, or 10 mM (fully supplied condition). The nitrate concentrations of 1 and 10 mM were retained for screening 20 additional Arabidopsis accessions. In order to avoid inducing K depletion in media with lower nitrate concentrations (<10 mM), KCl salt was added as a replacement for KNO_3, as described in De Pessemier et al. (2013). Seeds were stratified at 4°C for two days in darkness, and the plates were horizontally incubated in a culture chamber at a temperature of 22°C and a constant light regime of 75 µmol photons m^{-2} s^{-1}. Thirteen days after germination, physiological parameters and ethylene production of the seedlings were measured.

Nitrogen and Carbon Determination

Sample dry weights of 10–50 mg were analyzed with a vario MAX cube (Elementar, Germany) for simultaneous C and N determination at Centre pour l'Agronomie et l'Agro-Industrie de la province de Hainaut, Belgium (CARAH).

Pigments Determination

Pigments were extracted from frozen shoot organs (∼20 mg fresh weight) according to the procedures described in Misyura et al. (2013) and Teng et al. (2005).

Ethylene Production Measurement

Headspace samples were analyzed with a laser-based photoacoustic ethylene detector (ETD-300, Sensor-Sense, Nijmegen, the Netherlands). A valve control box allowed automated sampling of ethylene production under a stop-and-flow routine as described in Hermans et al. (2011) (Figure S1). Ethylene production of six Petri dishes was measured sequentially. Gas accumulated during 1 h and then was flushed to the detector during 12 min. Each sample was measured during at least two sequences and the average ethylene production was calculated. Values were corrected by the signal recorded for an empty agar plate. The experiment was run in at least four replicates for each accession and nitrate concentration.

Gene Expression Analysis

Total RNA was extracted from shoot and root tissues seedlings grown for 13 days after germination, using the Maxwell LEV Plant RNA kit with the Maxwell 16 Research Instrument (Promega Fitchburg). The first-strand cDNA was synthesized using Promega GoScript Reverse Transcription System. Quantitative PCR analyses were carried with the Takyon qPCR Kit (Eurogentec) using the PikoReal™ Real-Time PCR System (Thermo Scientific). Gene-specific forward and reverse primers are listed in Table S2. PikoReal™ software was used for analyzing and quantifying qPCR curves. The two stably expressed reference genes *ACTINE 2* (*ACT2*) and *POLYUBIQUITIN 10* (*UBQ10*) were used for the normalization of all target gene expression.

Genomic Analysis and Association Tests

Genomic sequences were retrieved from the 250k SNP data published by Atwell et al. (2010) and Li et al. (2010) for Alc-0, Blh-1, Bur-0, Can-0, Ct-1, Cvi-0, Edi-0, Ge-0, Jea, Kn-0, Mh-1, Mt-0, N13, Oy-0, Sha, St-0, Stw-0 and Tsu-0, and from the Salk Arabidopsis 1001 Genomes database (http://signal.salk.edu/atg1001/3.0/gebrowser.php) for Bl-1, Gre-0, and Sakata. Sequences were missing for Akita, Ita-0 and Pyl-1. TAIR10 gene models were plotted with the R package *genoPlotR* (Guy et al., 2010). The density of single nucleotide polymorphisms (SNPs) in *ACS* and *ACO* genes was defined by the SNP number divided by the length of the corresponding exon. Association tests between SNPs (retrieved from 250k SNP data set) and ethylene production were performed with GEMMA (Zhou and Stephens, 2014).

Statistical Treatment

All statistical analyses were conducted using the R software (R Core Team, 2014). Mean comparisons were performed by analysis of variance (ANOVA). For association tests between paired variables (phenotype traits or SNPs), Pearson's product moment correlation coefficient (r) was used. Linear regression models were subsequently designed when needed. Adequate model fits were ensured through residual investigation with the help of the diagnostic plots implemented in the software.

RESULTS

Influence of Nitrate Supply on Biomass Production, Nitrogen, Carbon, and Pigment Concentrations in Columbia-0 Accession

Experimental conditions were first established with the reference Col-0 accession, which was challenged to a range of five nitrate concentrations (0.5, 1, 2.5, 5, or 10 mM) during *in vitro* culture for 13 days after germination (**Figure 1**). The total dry biomass of hundred pooled seedlings was slightly different [$F_{(5,\ 18)} = 2.24$, $p = 0.095$] between nitrate treatments. It gradually increased with the nitrate supply rising and was almost double between 0.5 mM and 10 mM nitrate (**Figure 1B**). The nitrate availability significantly [$F_{(5,\ 16)} = 33.63$, $p < 0.001$] affected the root to shoot dry biomass ratio. The ratio decreased from 0.91 to 0.25 with the nitrate supply augmenting (**Figure 1C**). Furthermore, N and carbon (C) tissue concentrations were measured in root and shoot organs. The N concentrations rose with increasing nitrate supply (**Figure 1D**). They were up to four times lower in shoots and two times lower in roots at 0.5 mM compared to 10 mM nitrate. Such important differences in C concentrations were not observed between the nitrate treatments. The C concentrations slightly decreased with increasing nitrate supply (**Figure 1E**). The difference of C concentrations was more prononced in shoots (−15%) than in roots between 0.5 and 10 mM nitrate supplies.

Because the leaves of seedlings grown at low nitrate supply (<1 mM) showed chloroses and purple shades (**Figure 1A**), the chlorophyll, carotenoid, and anthocyanin concentrations were quantified, together with a leaf senescence molecular marker. The total chlorophyll concentrations severely decreased at concentrations lower than 2.5 mM nitrate (**Figure 1F**). Chlorophyll concentrations were five or two times lower in seedlings grown respectively at 0.5 or 1 than at 10 mM nitrate. Along with a reduction in chlorophyll concentration, the expression of *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*) was massively triggered in N-deficient shoot tissues (Table S3). Carotenoid concentrations decreased at low nitrate supplies (**Figure 1G**). A decrease of one half or one third of their level was observed respectively at 0.5 or 1 mM compared to 10 mM nitrate. Anthocyanin concentrations increased by a factor 16 in seedlings grown at 0.5 mM and by a factor three at 1 mM compared to 10 mM nitrate (**Figure 1H**).

Influence of Nitrate Supply on Ethylene Production and Expression of Genes in the Ethylene Biosynthetic Pathway in Columbia-0 Accession

The ethylene production was measured 13 days after germination and normalized by the total dry biomass. The ethylene production was low at 0.5 mM, reached its highest value at 1 mM and then slowly decreased with the nitrate concentration increasing (**Figure 1I**). However, only one significant [$p < 0.1$, Tukey's HSD test] difference was found between 1 and 10 mM nitrate treatments. Therefore, those two nitrate concentrations were retained for screening the other accessions (see below).

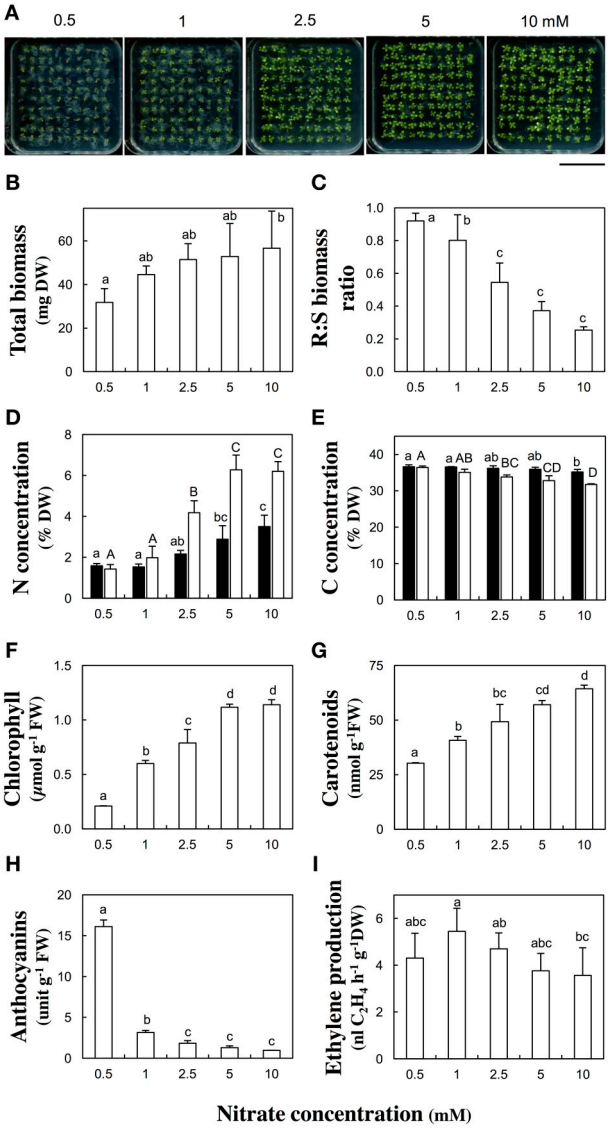

FIGURE 1 | Biomass production, nitrogen and carbon tissue concentration, pigment levels, and ethylene emanation in response to nitrate supply in Columbia-0 accession. *Arabidopsis thaliana* Col-0 seedlings were grown across a range of nitrate concentrations (0.5, 1, 2.5, 5, or 10 mM) and harvested 13 days after germination. **(A)** Representative pictures of petri plates containing 100 seedlings. Scale bar: 5 cm, **(B)** Total dry biomass, **(C)** Root to shoot dry biomass ratio, **(D,E)** Total nitrogen **(D)** and carbon **(E)** concentrations per dry weight of root (black columns) or shoot (white columns) tissues. **(F–H)** Pigment concentrations per fresh weight of shoot tissues: total chlorophyll (Chl*a*+*b*) **(F)**, carotenoids **(G)** and anthocyanins **(H)**, **(I)** Ethylene production per hour and per dry weight of total seedlings. Mean of four samples (100 pooled seedlings) ± SD. Letters indicate significant differences (Tukey's HSD test, $p < 0.1$).

That bell-shaped response was also observed upon normalization of the ethylene production by seedling number. The transcript abundance of *ACC SYNTHASES* and *ACC OXYDASES* multigene families was monitored in plant organs. The relative contribution of the most highly expressed genes of each family is shown

in **Figure 2** and the quantification of all isoform transcripts in Table S3. The total *ACS* transcript abundance in roots at 1 mM was twice as high as at 10 mM nitrate (**Figure 2A**). That increase was due in a large proportion to the induction of *ACS6* transcript levels, and in a lesser proportion to the induction of *ACS10*, *ACS11,* and *ASC12*. By contrast, the total *ACS* transcript abundance in shoots was relatively unchanged (**Figure 2A**). The total *ACO* transcript abundance in shoots at 1 mM was twice as high as at 10 mM nitrate (**Figure 2B**). Induction of transcript levels of *ACO2* and *ACO4* predominantly contributed to the total *ACO* expression level increase. In roots, all *ACO* isoforms were highly expressed but generally contributed to a slight increase of the total *ACO* expression level at 1 mM compared to 10 mM nitrate (**Figure 2B**).

Influence of Nitrate Supply on Biomass and Ethylene Production in a Core Set of Arabidopsis Accessions

Biomass parameters and ethylene production were measured in 20 additional accessions (Akita, Bl-1, Blh-1, Bur-0, Ct-1, Cvi-0, Edi-0, Ge-0, Ita-0, JEA, Kn-0, Mh-1, Mt-0, N13, Oy-0, Pyl-1, Sha, St-0, Stw-0, and Tsu-0) grown in the same conditions as described above (**Figure 3**). An analysis of variance was conducted for each measured variable in order to investigate individual effects of treatment (nitrate supply) and genotype (accession) as well as their interaction *treatment* × *genotype*. Across all accessions, the total dry biomass normalized to one-hundred seedlings was significantly [$F_{(1,\ 113)} = 1.84$, $p < 0.001$] lower at 1 mM (42.1 ± 9.3 mg) than at 10 mM (54.4 ± 22.8 mg) (**Figures 3A,B**). Moreover, the total dry biomasses were significantly [$F_{(20,\ 113)} = 1.84$, $p = 0.024$] different between accessions regardless of the nitrate supply. Finally, there was no marked biomass changes in response to nitrate supply between the accessions [no significant *treatment* × *genotype* effect: $F_{(20,\ 113)} = 0.927$, $p = 0.555$]. The biomasses were invariably lower at 1 mM than at 10 mM. As expected, most accessions significantly [$F_{(1,\ 113)} = 41.552$, $p < 0.001$] allocated more biomass to the roots at 1 mM (root to shoot dry biomass ratio = 0.47 ± 0.22) than at 10 mM (0.3 ± 0.09) but some accessions did not have that behavior [significant *treatment* × *genotype* effect: $F_{(20,\ 113)} = 2.829$, $p < 0.001$] (**Figures 3C,D**). Strikingly distinct nitrate-response occurred between accessions while considering the amounts of ethylene emanated per hour and per hundred seedlings [significant *treatment* × *genotype* effect: $F_{(20,\ 113)} = 1.778$, $p = 0.031$]. Nevertheless, on the average of all accessions, the ethylene production did not significantly [$F_{(1,\ 113)} = 0.584$, $p = 0.447$] differ between the nitrate treatments (0.39 ± 0.13 vs. 0.39 ± 0.18 nl C_2H_4 h^{-1} respectively at 1 and 10 mM nitrate; **Figures 3E,F**). Consequently, accessions with low biomass emanated similar amounts of ethylene per hour than those with high biomass, so that the ethylene production normalized by the total dry biomass was significantly [$F_{(1,\ 113)} = 14.706$, $p < 0.001$] 1.3 times higher at 1 mM than at 10 mM nitrate (10 ± 5.3 vs. 7.8 ± 3.7 nl C_2H_4 h^{-1} g^{-1} DW respectively; **Figures 3G,H**). Thirteen accessions showed such behavior in response to nitrate supply (Ita-0, Mt-0, N13, and Ge-0 presenting the largest differences between1 mM and 10 mM) and the other ones displayed an opposite trend (St-0, Pyl-1, Stw-0, Cvi-0, Edi-0, Sha, JEA, and Ct-1, ordered by decreasing differences).

The likely associations between the amount of ethylene emanated per hour and total dry biomass were investigated for both nitrate supplies. The linear regression models revealed a significant [$R^2 = 0.15$, $p < 0.001$] negative association between the two parameters at 1 mM but a significant [$R^2 = 0.15$, $p < 0.001$] positive association at 10 mM nitrate (**Figure 4**).

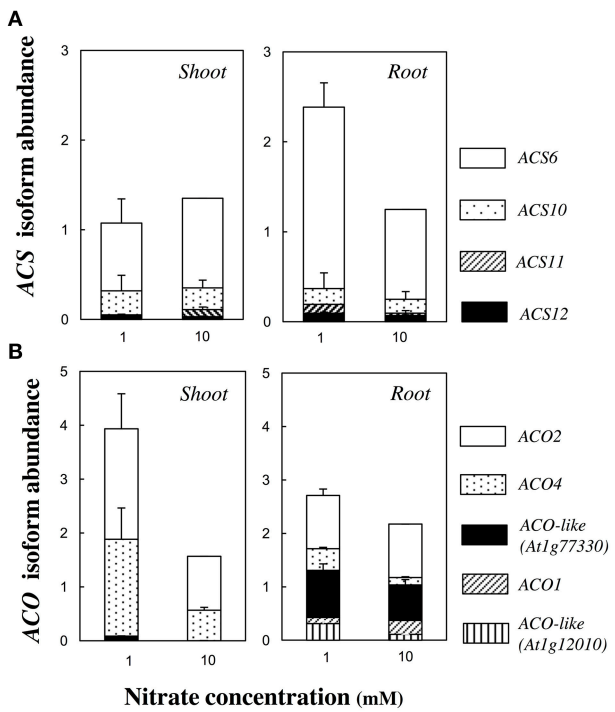

FIGURE 2 | Transcript abundance of *ACC SYNTHASE* and *ACC OXIDASE* multigene family. Transcript levels of the most highly expressed *ACS* **(A)** and *ACO* **(B)** members are presented in root and shoot tissues of Col-0 seedlings grown at 1 or 10 mM nitrate, 13 days after germination. Data represent mean abundance with the abundance of the most highly expressed (normalized by *ACT2* and *UBQ10* levels) family member in one organ, set as one under 10 mM nitrate condition (*ACS6* = 1 and *ACO2* = 1, respectively for *ACS* and *ACO* families). Mean of two or three pools of 100 organs ± SD (each sample assessed by three technical replicates).

Influence of Nitrate Supply on Pigment Concentration in a Core Set of Arabidopsis Accessions

Pigment concentrations were measured in accessions grown at 1 or 10 mM nitrate (Table S4). For the three pigment classes, highly significant [$p < 0.001$] treatment, genotype and *treatment* × *genotype* interaction effects were found. Across all accessions, the chlorophyll *a*+*b* concentration was two times lower at 1 mM (0.54 ± 0.12 μmol g^{-1} FW) than at 10 mM (1.06 ± 0.21 μmol g^{-1} FW) nitrate treatment [$F_{(1,\ 80)} = 3509.46$, $p < 0.001$]. Only Cvi-0 showed a slightly higher chlorophyll concentration at 1 mM than at 10 mM. The responses of the accessions to nitrate supply were significantly different [*treatment* × *genotype* interaction:

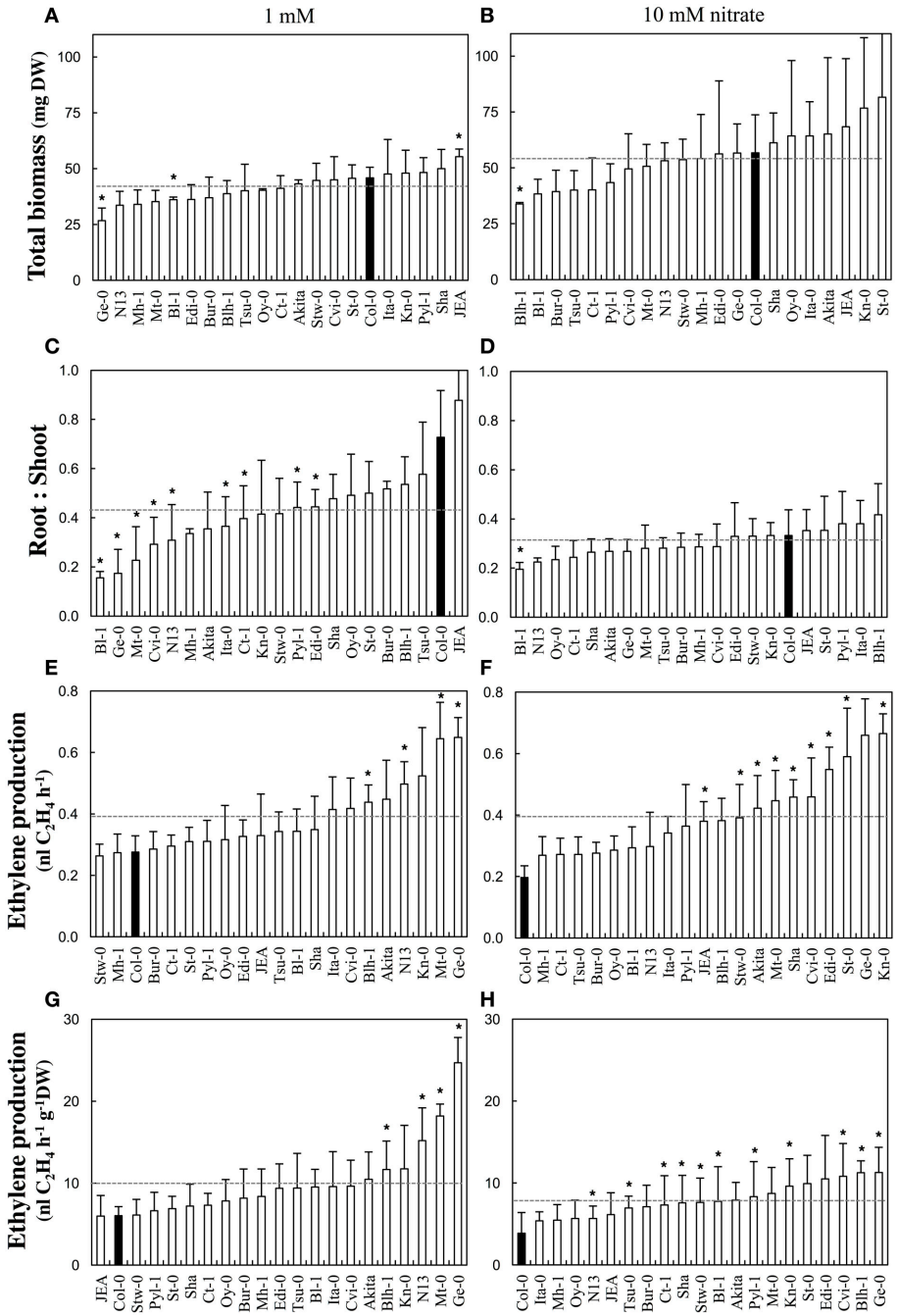

FIGURE 3 | Biomass production and ethylene emanation in response to nitrate supply in Arabidopsis accessions. Accessions were grown at 1 or 10 mM nitrate supplies and harvested 13 days after germination. **(A,B)** Total dry biomass, **(C,D)** Root to shoot dry biomass ratio, **(E,F)** Ethylene emanated per hour and per hundred seedlings, **(G,H)** Ethylene emanated per hour and per dry weight. Parameters were measured at 1 mM **(A,C,E,G)** and 10 mM **(B,D,F,H)** nitrate. Mean of four samples (100 pooled seedlings) ± SD. Asterisks represent significant ($p < 0.1$, Kolmogorov-Smirnov two-sided test) differences between one accession and Col-0.

$F_{(19,\ 80)} = 33.05$, $p < 0.001$]. In the same way, the carotenoid concentration at 1 mM (46.75 ± 5.95 nmol g^{-1} FW) was 1.3 times lower at 1 mM than at 10 mM [61.53 ± 12.31 nmol g^{-1} FW; $F_{(1,\ 80)} = 491.49$, $p < 0.001$]. The *treatment* × *genotype* effect was also significant [$F_{(19,\ 80)} = 16.99$, $p < 0.001$] and five accessions (Cvi-0, N13, Akita and Blh-1, Mh-1) showed either higher carotenoid concentrations at 1 mM than at 10 mM, either no difference between treatments. Finally, the anthocyanin concentration was 12 times more important at 1 mM (13.8 unit g^{-1} FW) than at 10 mM nitrate [1.18 unit g^{-1} FW; $F_{(1,\ 80)} =$

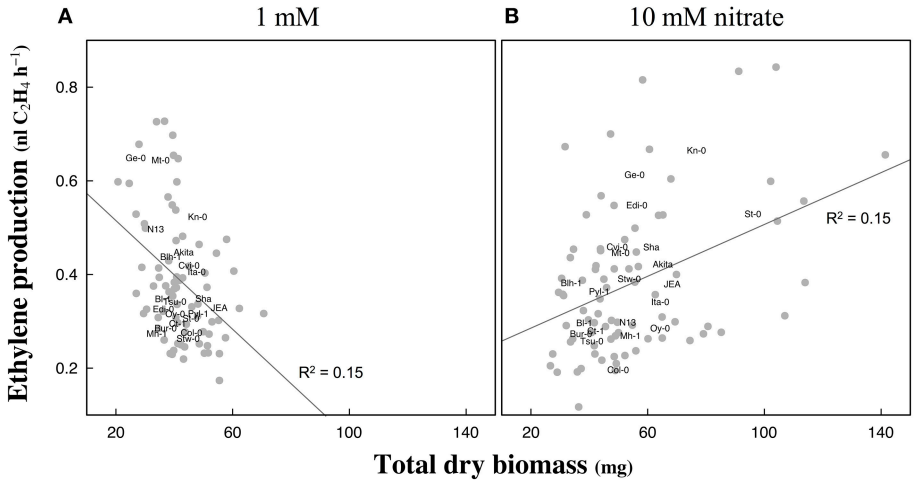

FIGURE 4 | Correlation between biomass production and ethylene emanation in response to nitrate availability in Arabidopsis accessions. Hundred seedlings of each accession were grown at 1 mM **(A)** or 10 mM **(B)** nitrate supplies and harvested 13 days after germination. Total dry weight biomass and ethylene emanation normalized to hundred seedlings were measured in four replicates. Accession names indicate means of four replicates. Linear regression models indicated a significant negative association between the total dry biomass and ethylene emanation at 1 mM and a positive association at 10 mM (slopes different from zero: $p < 0.001$). Model details at 1 mM: $p < 0.001$, adjusted $R^2 = 0.15$, F-statistic = 14.01 on 1 DF, residual SE = 0.124 on 72 DF; and 10 mM: $p < 0.001$, adjusted $R^2 = 0.15$, F-statistic = 14.82 on 1 DF, residual SE = 0.146 on 76 DF.

4951.34, $p < 0.001$]. The *treatment* × *genotype* effect was significant [$F_{(19, 80)} = 35.56$, $p < 0.001$] and Cvi-0 presented the lowest difference between the two nitrate treatments among the accession panel.

Eventually, the average pigment concentrations were computed for each treatment-genotype pair and combined with the corresponding average ethylene productions (normalized by fresh weight). Correlation tests were conducted after a paired resampling procedure was applied to increase the number of replicates (associated methods and results are in Table S5). They revealed a negative correlation between chlorophyll $a+b$ concentration and ethylene production, and a positive correlation between anthocyanin concentration and ethylene production for both nitrate treatments. Finally, the carotenoid concentration was positively correlated with the ethylene production at 1 mM but negatively at 10 mM nitrate (Table S5).

Polymorphism Analysis of Enzymes in the Ethylene Biosynthetic Pathway

Polymorphism variation present in *ACS* and *ACO* genes and proteins was compared along 21 accessions from the original McKhann et al. (2004) diversity panel. That initial survey was meant to highlight some naturally-occurring genetic differences which could be responsible for ethylene production differences observed between genotypes. First, available DNA coding sequences were compared to the corresponding Col-0 sequences and the SNP density was calculated in a given exon (**Figure 5**). The survey identified the first exon of *ACS4* and third one of *ACS12* with the highest SNP densities (**Figure 5A**). Overall, *ACS6* and *ACS12* were the most polymorphic genes with height SNPs (total exon length 1753 bp) and 17 SNPs (total exon length 3179 bp) respectively among *ACS* genes and *ACO4* with five SNPs (total exon length 1364 bp; Table S6). Second, particular attention was given to nucleotide substitutions leading to the same amino acid (synonymous or neutral) or resulting in amino acid variants (non-synonymous) in protein sequences (**Figure 6**, Figures S2, S3). Furthermore, non-synonymous substitutions were distinguished between conservative and non-conservative ones, if they respectively maintained or modified the amino acid property. In ACS proteins, seven conserved boxes and one phosphorylation site (Yamagami et al., 2003) were closely surveyed (**Figure 6A**). Within those boxes, non-conservative substitutions were found in a rather small number of accessions: in box 1 of ACS2 for Can-0, Edi and Kn-0, box 6 of ACS4 for Bur-0, box 4 of ACS6 for Ge-0, box 3 of ACS10 for Can-0, box 4 of ACS10 for Edi-0, Oy-0 and Sha, box 5 of ACS10 for all accessions and box 7 of ACS12 for Can-0, Cvi-0, Ge-0, Mt-0, St-0, and Stw-0 (Figure S2). In addition, only one of the 11 invariant amino acids (Yamagami et al., 2003) among ACS isozymes found synonymous substitution in ACS8 for Alc-0, Blh-1, Edi-0, and Sakata (Figure S2). No substitution originated from the putative phosphorylation site at the end of ACS1, 6, 8, 9, and 11 sequences. In ACO proteins, two conserved motifs involved in co-factor (Fe^{2+}) and co-substrate (ascorbate) binding pockets (Seo et al., 2004; Yuan et al., 2010) were surveyed (**Figure 6B**). Only one non-conservative substitution was observed in the co-factor binding pocket in ACO2 sequence of Alc-0 (Figure S3).

DISCUSSION

Mineral nutrient constraints can affect hormonal level and signaling, and in turn, hormones can alter mineral homeostasis and biomass production. This study illustrated such intricate interplay between biomass production, biomass allocation

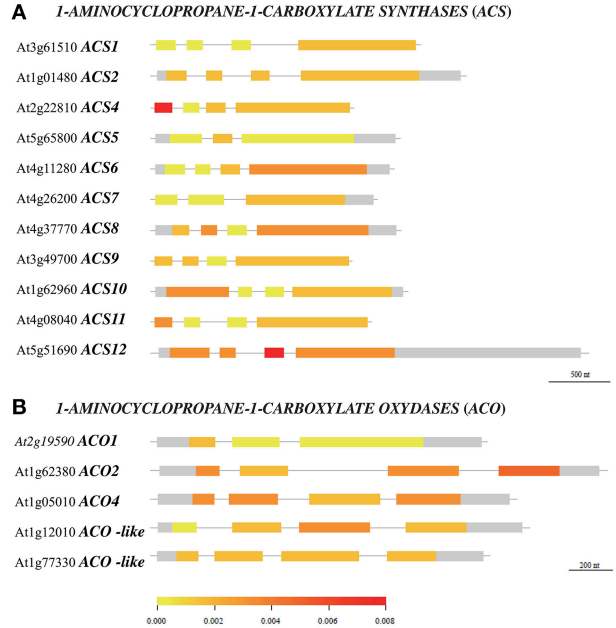

FIGURE 5 | Polymorphism density in DNA coding sequences of *ACC SYNTHASES* and *ACC OXYDASES* genes. Aivailable coding gene sequences of *ACS* **(A)** and *ACO* **(B)** for 21 accessions from the McKhann et al. (2004) core set were compared to the corresponding Col-0 sequences retrieved from 250k SNP data published in Atwell et al. (2010) and Li et al. (2010). The density of single nucleotide polymorphism, defined as the number of SNPs per base pair in a given exon, is indicated by the color scale.

to organs, and ethylene production in response to nitrate supply.

Columbia-0 Response to Low Nitrate Supply

Our morphological observations on Col-0 accession during N deficiency were consistent with previous reports (Hermans et al., 2006, 2010c, 2011; De Pessemier et al., 2013). Low nitrate supply reduced biomass production and increased the root to shoot biomass ratio (**Figures 1B,C**). Higher transcript levels of *ACS6* in roots and of *ACO2* and *ACO4* in shoots (**Figure 2**) were mirrored by higher ethylene production (**Figure 1I**) in N-deficient seedlings. That tissue-specific transcription increase of genes encoding the ACC synthesizing vs. ethylene synthesizing enzymes, may indicate that ACC accumulated in roots in response to low nitrate and was converted to ethylene by ACO in roots. Long distance ACC transport was previously observed upon hypoxia and salinity exposure (reviewed in Van de Poel and Van Der Straeten, 2014). Moreover, Lynch and Brown (1997) speculated that ethylene may play an important role in mediating plasticity of plant responses to nutrient stress, especially root responses to P deficiency. Our data indicated that the nitrate supply largely modulated ethylene biosynthesis with a strong influence on plant morphology and biomass allocation (**Figures 1**, **4**). The increase in biomass allocation to the roots at the expense of the shoots during N deficiency might be controlled by ethylene, released in the shoot upon conversion of ACC originating from roots. In Arabidopsis, ethylene inhibits leaf expansion (Van de Poel et al., 2015). Ethylene could therefore steer the alteration in root-shoot biomass ratio, with restriction of shoot expansion when N supply is low. However, that effect is probably primarily controlled by auxin or cytokinin, known to modulate ethylene biosynthesis and signaling, particularly at higher concentrations (Van de Poel et al., 2015). Thus, the alteration of biomass allocation between organs might be fine-tuned by ethylene as part of an intricate network in cross talk with other hormones (Khan et al., 2015).

Our physiological observations may reflect an ethylene-mediated senescence program (Qiu et al., 2015). Decreased chlorophyll concentrations (**Figure 1F**) and high transcript levels of *SAG12* (Figure S3) in N-deficient seedlings are obvious symptoms of senescence (Lohman et al., 1994; Qiu et al., 2015). Such severe yellowing was probably due to chloroplast degradation and nutrient recycling (Ueda and Kusaba, 2015). Low carotenoid concentration (**Figure 1G**) indicated that the color of N-deficient shoots was due to unmasking of yellow pigments rather than higher synthesis. Finally, the high anthocyanin concentration (**Figure 1H**) was consistent with earlier observations during N deficiency (Peng et al., 2008) and could reflect the activation of the phenylpropanoid pathway possibly by ethylene, as suggested by Khan et al. (2015). It is noteworthy that P deficiency can also cause anthocyanin accumulation but in that case, ethylene signaling plays a negative regulatory role in anthocyanin biosynthesis (Lei et al., 2011). To determine if ethylene action actually triggers chlorophyll degradation and/or anthocyanin production, ethylene signaling or biosynthesis mutants must be challenged to low N supply. That question urges further research in the area.

Genetic Variation Exists for Ethylene Production in Arabidopsis Natural Accessions

A core set of Arabidopsis accessions was screened for the ethylene production. Up to now, the assessment of natural variability for that trait is very limited. A two-fold variation of ethylene production upon exposure to abiotic stresses is reported between some foremost studied Arabidopsis accessions: Col-0, Landsberg-0 (Ler-0), and Wassilewskija-2 (Ws-2) (Tamaoki et al., 2008; van Zanten et al., 2010). Here, a six-fold variation of ethylene production was observed between JEA and Ge-0, which were positioned at opposite ends under low nitrate supply (**Figure 3G**). This clearly illustrates that natural genetic variation does exist for ethylene production within the *A. thaliana* species. Polymorphism in the ethylene biosynthetic enzymes could be responsible for phenotypic differences observed between Arabidopsis accessions. Our analysis showed that the genomic sequences of *ACS6*, *ACS12*, and *ACO4* have higher divergence than any other family members (**Figure 5**, Table S6). It is noteworthy that *ACS6* and *ACO4* are the most up-regulated genes during low nitrate treatment in Col-0 genotype (**Figure 2**). Despite the limited number of accessions analyzed in the present study, an association test was

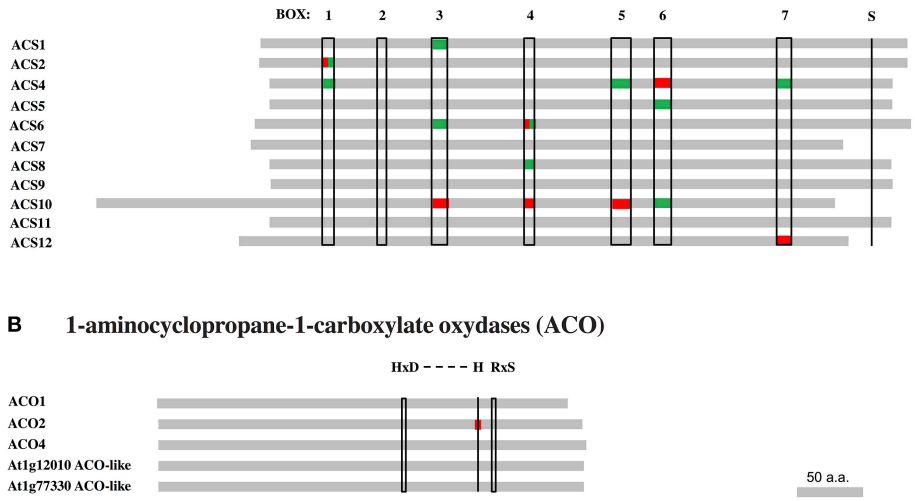

FIGURE 6 | Amino acid sequence alignment of ACC synthase and ACO oxydase proteins. Available protein sequences of 21 accessions from the McKhann et al. (2004) core set were compared to the corresponding Col-0 sequences retrieved from 250k SNP data published in Atwell et al. (2010) and Li et al. (2010). Amino acid substitutions are indicated for conserved domains and motifs of ACS and ACO protein families. **(A)** The sequences of 11 ACS isozymes present seven conserved domains marked as boxes 1–7. The S residue marked at the sequence end of ACS1-6, 8, 9, and 11 is a putative phosphorylation site. **(B)** In ACO proteins, the so-called "2-His-1-carboylate facial triad" (HxD…H) motif is involved in co-factor (Fe^{2+}) binding pocket, while the RxS motif is critical for substrate (ascorbate) binding pocket. The presence of one or more synonymous (green) or non-synonymous (red) amino acid substitutions in a given item is indicated.

performed between the retrieved genomic sequences of *ACS* and *ACO* genes (**Figure 5**) and ethylene production (**Figure 3**). Only *ACS4* showed significant association [$p < 0.001$] with ethylene production measured in 17 accessions grown at 10 mM nitrate (Table S7). It is worth mentioning that the statistical power of the analysis is low and the false discovery rate high.

Non-synonymous substitutions of amino acids can potentially change the protein function, although very little divergence was found in catalytic and phosphorylation sites of the ACC and ethylene biosynthetic enzymes (**Figure 6**). Though, a nucleotide substitution that does not modify the amino acid sequence, can yet affect the translation efficiency, disrupt splicing and modify protein structure, abundance and even substrate specificity (Kimchi-Sarfaty et al., 2007; Parmley and Hurst, 2007; Kesari et al., 2012). Our study only focused on the genetic variation of the coding sequences but further work can integrate regulatory elements in 5′ and 3′ regulatory sequences of those encoding genes. The identification of protein variants within the diversity panel will need to be further explored as they may represent adaption to cope with low nutrient availability in natural habitats.

Strategies to Improve Nitrogen Use Efficiency Could Come up from the Identification of the Genetic Determinism Implicated in Ethylene Production

A significant negative correlation was found between total biomass and ethylene production in Arabidopsis seedlings grown at low nitrate supply, while being opposite in a high nitrate medium (**Figure 4**). This once more underlines the influence of nutrient supply on ethylene responses, as previously reported by Smalle et al. (1997). Particularly, the effects of mineral nutrient deficiencies or excess on ethylene plant responses must be critically examined. The concept of reducing ethylene production to limit the damages to plants upon biotic and abiotic stresses was already considered, for example in the presence of microbial inoculants with ACC deaminase activity (Gamalero and Glick, 2015). However, manipulating ethylene biosynthetic pathway to improve NUE must be carefully considered and the effectiveness of such improvement must be evaluated (Khan et al., 2015). For instance, ethylene generally down-regulate photosynthetic genes (Van Zhong et al., 2003) but ethylene insensitivity can also lower photosynthetic activity (Tholen et al., 2007). Therefore, ethylene has not to be exclusively seen as a biomass negative regulator and senescence inducer *per se*, since it can also have a positive action on NUE. For instance, delay of leaf senescence may prolong photosynthesis, but rapid senescence increases N remobilization from vegetative parts to sink organs (reviewed in Xu et al., 2012). Furthermore, spraying ethephon- a molecule inducing ethylene release- on *Brassica juncea* can ameliorate photosynthesis rate and NUE (Khan et al., 2008; Iqbal et al., 2011).

Conclusion and Perspectives

This work highlighted the natural variation of ethylene production offered by a small panel of Arabidopsis accessions in response to N supply. The development of analytical procedures allowing the simultaneous measurement of

much larger number of gaseous samples will be a key step for successful genome wide association strategies (Atwell et al., 2010) with a higher number of accessions. The output of such studies may support the nomination of *loci* and alleles in the cross-talk between ethylene and mineral nutrition.

AUTHOR CONTRIBUTIONS

HDG, JDP, CH, and JX performed research and drew figures. HDG and CH wrote the paper and carried additional experiments during the revision. All authors edited the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00070

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REVIEW
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Ethylene, a Hormone at the Center-Stage of Nodulation

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Nodulation is the result of a beneficial interaction between legumes and rhizobia. It is a sophisticated process leading to nutrient exchange between the two types of symbionts. In this association, within a nodule, the rhizobia, using energy provided as photosynthates, fix atmospheric nitrogen and convert it to ammonium which is available to the plant. Nodulation is recognized as an essential process in nitrogen cycling and legume crops are known to enrich agricultural soils in nitrogenous compounds. Furthermore, as they are rich in nitrogen, legumes are considered important as staple foods for humans and fodder for animals. To tightly control this association and keep it mutualistic, the plant uses several means, including hormones. The hormone ethylene has been known as a negative regulator of nodulation for almost four decades. Since then, much progress has been made in the understanding of both the ethylene signaling pathway and the nodulation process. Here I have taken a large view, using recently obtained knowledge, to describe in some detail the major stages of the process. I have not only reviewed the steps most commonly covered (the common signaling transduction pathway, and the epidermal and cortical programs), but I have also looked into steps less understood (the pre-infection step with the plant defense response, the bacterial release and the formation of the symbiosome, and nodule functioning and senescence). After a succinct review of the ethylene signaling pathway, I have used the knowledge obtained from nodulation- and ethylene-related mutants to paint a more complete picture of the role played by the hormone in nodule organogenesis, functioning, and senescence. It transpires that ethylene is at the center of this effective symbiosis. It has not only been involved in most of the steps leading to a mature nodule, but it has also been implicated in host immunity and nodule senescence. It is likely responsible for the activation of other hormonal signaling pathways. I have completed the review by citing three studies which makes one wonder whether knowledge gained on nodulation in the last decades is ready to be transferred to agricultural fields.

Keywords: model legumes, rhizobia, *sickle*, host immunity, nodule organogenesis, nodule senescence, ethylene signaling, hormones

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INTRODUCTION

Symbiotic nitrogen fixation is essential to agriculture. Graham and Vance (2003) estimated that about 50 million metric tons of atmospheric nitrogen was fixed by agriculturally relevant legumes annually. This nitrogen fuels much of the earth's nitrogen cycle. Today, many farmers are moving to a more sustainable agriculture (as defined by Vance, 2001) as many of our soils are impoverished

because of abuse. In an ideal world, we should bank more on symbiotic nitrogen fixation to remediate some of the detrimental effects our intensive agriculture has had on the environment. Vance (2001) outlined five recommendations toward which scientists have worked. Much has been done since: dozens of genes involved in the rhizobial symbiosis have been identified and mutants have been created to unravel both the roles played by these genes and the order in which they act. Now, an integrated approach must be taken to understand how the gene products fit together in a plant physiology context to make the mutualistic interaction as effective as possible.

Many reviews, varying in their approach and focus, have appeared recently on the roles played by plant hormones during nodulation (Desbrosses and Stougaard, 2011; Mukherjee and Ané, 2011; Murray, 2011; Ferguson and Mathesius, 2014). In general, all reviews underline that all known hormones are involved in the process as they tightly regulate every step from bacterial recognition to nodule senescence. Auxin, cytokinin, and ethylene, are thought to be essential actors and as such their roles have been studied in depth. To assign a specific role to any of these three hormones is nearly impossible because each hormone acts differently in space and time. However, it is generally accepted that cytokinin and auxin act positively (e.g., Mortier et al., 2014 and Mathesius, 2008, respectively), and ethylene negatively (Penmetsa et al., 2008), in the development of a nodule primordium (NP) and that cytokinin and ethylene have a negative effect on the progression of infection threads (ITs) (e.g., Murray et al., 2007 and Guinel and LaRue, 1992, respectively). Besides, to separate individual hormonal actions in a process such as nodulation is hardly possible because hormonal signaling pathways cross multiple times and in multiple places. For example, the three hormones cited above all have an effect on nodule positioning (Ferguson and Mathesius, 2014) and it is likely that the nodule position on the root is determined by an integration of their signaling pathways.

In this review, I have focussed on ethylene and its effect on nodule organogenesis, functioning, and senescence. Because of space constraints, I have restricted the review to the plant side of the mutualism, although I recognize this is a rather narrow view. I have assumed that legumes follow a similar blueprint in creating a nodule and in making it functional. However, this is likely incorrect since at least four different structural types of nodules are known (Guinel, 2009a). I have mentioned here only events occurring in indeterminate nodules, i.e., with a long-living meristem, and in determinate nodules, the meristem of which stops functioning early in the life-span of the nodule (Guinel, 2009a). I see this review as a foundation from which refinements can be made.

A REVIEW OF NODULE FORMATION, FUNCTIONING, AND SENESCENCE

To describe effectively the effect ethylene has on nodulation, a review in some detail of the processes leading to a mature nodule is necessary. For reasons of space, I have simplified these processes as much as possible and I have mentioned only those genes which I thought could be involved directly or indirectly in an ethylene response. Readers interested in more in-depth reviews of nodule organogenesis *per se* or of specific steps in the process are invited to read Oldroyd and Downie (2008), Oldroyd et al. (2011), or Kondorosi et al. (2013).

Pre-infection Events

The rhizobium-legume interaction is initiated by the release of plant exudates such as flavonoids which attract rhizobia chemotactically toward the root. By binding to the rhizobial NodD1 protein, the flavonoids promote its affinity for the *nod* box (Peck et al., 2006), and thus initiate Nod Factor (NF) biosynthesis. NFs are recognized by the LysM receptor kinases Nod Factor Receptor1 (NFR1) and NFR5 (e.g., Desbrosses and Stougaard, 2011). Proper perception of NFs activates the common signaling transduction pathway (CSTP), the name of which alludes to the fact that this pathway is involved in the initiation of both rhizobial and arbuscular mycorrhizal symbioses (Kistner et al., 2005). In the symbiosis leading to nodulation, the CSTP (**Figure 1**, green box) initiates two distinct programs, the epidermal and the cortical programs of nodule organogenesis (Guinel and Geil, 2002). Recently, many reviews have been published on and around the CSTP (e.g., Desbrosses and Stougaard, 2011; Murray, 2011; Oldroyd, 2013).

Less often discussed in the pre-infection events are the defense responses that the legume must put in place when challenged with rhizobia. Recent studies have unraveled that a fine-balancing act is being played between the bacteria with their microbe-associated molecular patterns (MAMPs) production and the plant with its immune response elicitation (**Figure 1**; pink box) referred to as MAMP-triggered immunity (MTI; Gourion et al., 2015). Rhizobia produce not only NFs but also flagellin-like molecules (flg22) which are recognized by FLS2 (FLagellin-Sensing) receptors located in the epidermis plasmalemma (Khatabi and Schäfer, 2012). In response to these molecules, the host cell prompts a cascade of effects, such as calcium influx and production of reactive oxygen species (ROS). Furthermore, many genes such as those encoding peroxidases, chitinases, or ERFs (ethylene response factors) are up-regulated. This transient defense response is dependent on LjNFR1 (Nakagawa et al., 2011). Genes coding for Pathogenesis-Related (PR) proteins and a biosynthetic enzyme of the phytoalexin medicarpin are also up-regulated at the site of infection, the former transiently but the latter persistently (Breakspear et al., 2014). Bacteria have evolved to counteract these effects by secreting exopolysaccharides (EPS) and lipopolysaccharides (LPS); LPS inhibit ROS production whereas EPS are thought to chelate extracellular calcium ions preventing their cell entry (Gourion et al., 2015). To complement MTI, plants use another type of immunity known as effector-triggered immunity (ETI), which is set to respond to the direct injection of bacterial proteins, such as Nop proteins, in the cell cytoplasm via an effector (e.g., T3ss; Gourion et al., 2015). These bacterial proteins are known to inhibit MTI (**Figure 1**). The plant counter-attacks by encoding nucleotide-binding site/leucine-rich repeat proteins able to recognize the bacterial proteins (Gourion et al., 2015). Of interest for this review, hormones especially salicylic acid (SA), jasmonic acid

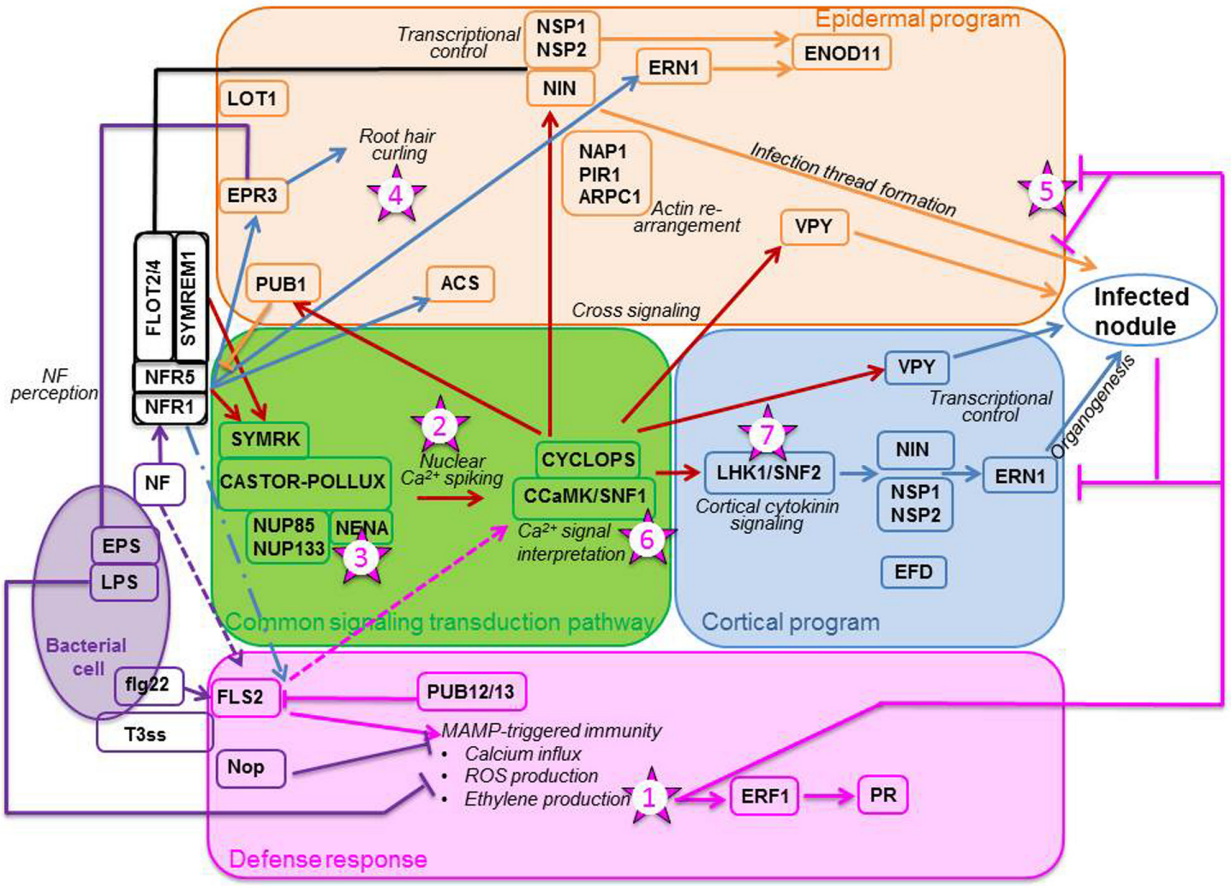

FIGURE 1 | Plant responses to the presence of rhizobia. The bacterium (purple oval) triggers a defense response (pink box) by producing exopolysaccharides (EPS) and lipopolysaccharides (LPS), flagellin-like molecules (flg22), and type III-effector molecules (T3ss) used to inject Nop proteins in the plant cell. As the plant senses these molecules, especially flg22 with the FLS2 receptor, it mounts a set of defense responses. Among the outcomes are the production of ethylene and the up-regulation of pathogenesis-related (PR) proteins. Simultaneously, the rhizobium secretes Nod factors (NFs) which are perceived by the plant receptors NFR1 and NFR5, which may be recruited to membrane micro-domains by remorins (SYMREM1) and flotillins (FLOT2/4). Perception of NFs initiate the CSTP (green box) composed of eight genes: SYMRK, CASTOR/POLLUX, NUP85 and NUP133, NENA, CCaMK and CYCLOPS. CCAMK decrypts the calcium signal, triggering an epidermal program (orange box) and a cortical program (blue box). Epidermal program: Signaling, via CCaMK, triggers the ubiquitin ligase PUB1, considered a negative regulator of NFR1, and the transcription factor NIN which, with NSP1 and NSP2, and the vapyrin (VPY), affects the formation of the infection thread. For this event to occur, proteins important in the layout of the cytoskeleton, such as NAP1, PIR1, and ARPC1, are likely recruited. NF perception may also directly induce transcription of specific genes, such as the EPS receptor EPR3, the ethylene biosynthetic enzyme ACS, and an ethylene response factor required for nodulation ERN1. Cortical program: CCaMK triggers the cytokinin receptor LHK1 and the downstream transcriptions factors NIN, NSP1 and NSP2. In this program, in contrast to the epidermal program, ERN1 induction appears to be done through NIN and the NSPs. VPY and EFD, an ethylene response factor required for nodule differentiation, are also implicated in the program. The proper decoding of the calcium signal leads to nodule organogenesis. For a nodule to become infected and functioning, all steps must be impeccably orchestrated. Pointed arrows denote stimulation, flat arrows reflect inhibition, and broken arrows indicate speculative action or contradiction in the literature. The numbered stars represent potential location of ethylene signaling or action. The numbers correspond to the order in which these actions are reported in the text. Schematics adapted from Desbrosses and Stougaard (2011). Most of the genes mentioned on these diagrams are those which have been designated for *Lotus japonicus*; their orthologs for *Medicago truncatula* are mentioned in the text.

(JA), and ethylene have been implicated in the setting of the response; these defense-related hormones likely cross-talk with DELLA proteins (Limpens et al., 2015) and hormones such as cytokinin and auxin (Zamioudis and Pieterse, 2012). Thus, there is an overlap between the defense and symbiotic pathways, with the defense reactions set up by the plant quickly suppressed (Gourion et al., 2015), allowing microbial entry and the potential successful rhizobial establishment in plant roots.

Nodule Organogenesis

The Nod Factor Receptors

How the NFs mediate rhizobial entry whilst modulating defense is still not understood. As well, the mechanism behind the dual function of the NFRs to adjust to the dual action of the NFs, i.e., on the epidermal and cortical programs, has thus far not been uncovered, although some progress is being made (Limpens et al., 2015). Furthermore, the role played by each of the NFRs in activating the CSTP is still obscure.

Mbengue et al. (2010) proposed that the entry receptor, of which LjNFR1 would be a component, sets off the epidermal program, whereas the signaling receptor, comprising LjNFR5, triggers the cortical program. How the two receptors work together to allow nodulation to proceed is difficult to envision. Mbengue et al. (2010) suggested that when NF binds to MtNFP, the ortholog of LjNFR5, PUB1 (Plant U-box protein), a U box-dependent E3 ubiquitin ligase, is phosphorylated by MtLYK3 (**Figure 1**), the ortholog of LjNFR1; this leads to its modulating the MtLYK3 downstream components by ubiquination. PUB1, considered a negative regulator of MtLYK3, is expressed early and transiently and its expression apparently requires the CSTP (Mbengue et al., 2010).

The Common Signal Transduction Pathway

The epidermal program encompasses all steps involving bacterial action, i.e., root hair (RH) curling, IT formation, and IT progression through the cortex, whereas the cortical program is responsible for the formation of the nodule infrastructure. For an efficient nodule to develop, both epidermal and cortical programs must not only be tightly regulated, but also accurately orchestrated (Guan et al., 2013). If the NFs are properly perceived and the Ca^{2+} signal correctly interpreted, then genes, the products of which regulate the two nodulation programs, **Figure 1**; Hayashi et al., 2010), are expressed downstream the CSTP. The correct expression of the genes comprised in the CSTP (**Figure 1**, green box) is required for nodulation success and if one of these genes is mutated, the nodulation process aborts (Oldroyd, 2013). It is possible for the two nodulation programs to be uncoupled since pseudo-nodules can form in the absence of bacteria; in such cases, the cortical program is activated on its own, independently of the epidermal program (for a review, see Guinel, 2009b). Such nodules form spontaneously on *Lotus japonicus* roots when the *CCaMK/SNF1*gene, coding for a calcium- and calmodulin-dependent kinase, is mutated (Gleason et al., 2006; Tirichine et al., 2006a) or when a phosphomimetic version of the *CYCLOPS* gene, coding for a phosphorylation substrate of CCaMK, is used (Singh et al., 2014). Evidence of the possible uncoupling of the two programs is also given by the mutant *Ljnena* (**Table 1**) which does not form ITs but exhibits nodules, albeit mostly empty (Groth et al., 2010). *NENA*, also of the CSTP (**Figure 1**), encodes a nucleoporin thought to work in concert with NUP85 as a scaffold protein within the nuclear pore complex (Groth et al., 2010).

The Epidermal Program

Much has been learned recently about the epidermal program (**Figure 1**; orange box). For example, within 24 hours of inoculation (hai), rhizobia induce the expression of remorin (MtSYMREM1 for SYMbiotic REMorin1; Lefebvre et al., 2010) and flotillins (MtFLOT2 and MtFLOT4; Haney and Long, 2010), two types of scaffolding proteins forming micro-domains in the plasmalemma. These proteins are thought to interact with MtLYK3 and MtNFP, maybe as a means to recruit them to their micro-domains (Lefebvre et al., 2010). MtSYMREM1 interacts with MtDMI2 (Does not Make Infections 2), an ortholog of LjSYMRK (SYMbiosis Receptor Kinase), located upstream of the CSTP CCaMK. *MtFLOT2* and *MtFLOT4* up-regulation requires the presence of nodule inception (NIN) and NSP2 (Haney and Long, 2010). Furthermore, MtFLOT4 is apparently required for proper IT elongation (Haney and Long, 2010). Recently, the plant receptor exopolysaccharide receptor 3 (EPR3) involved in epidermal bacterial entry was proposed to distinguish between EPS of compatible and incompatible rhizobia (Kawaharada et al., 2015). Its epidermal expression is triggered by NF perception and leads to RH curling (**Figure 1**). The importance of the actin cytoskeleton in the RH response is highlighted by three *L. japonicus* mutants, *Ljnap1* and *Ljpir1* (Nick-Associated Protein 1 and 121F-specific p53 Inducible RNA, respectively), and *Ljarpc1* (Actin-Related Protein Component 1) (Yokota et al., 2009; Hossain et al., 2012). NAP1, PIR1, and ARPC1 must play a role in the formation, maintenance and progression of the ITs because the mutants display aborted ITs in the epidermis and form non-colonized nodule primordia (Yokota et al., 2009; Hossain et al., 2012). An interesting mutant is *Ljlot1* (**Table 1**) since it is ethylene-insensitive. *Ljlot1* forms much less ITs than WT and thus displays few nodules; all are, however, functional (Ooki et al., 2005). The *Ljlot1* defect must be at the epidermal entry (**Figure 1**). In a recent RH transcriptomics study, Breakspear et al. (2014) showed that upon rhizobial infection the RH likely re-enters the cell cycle and that IT initiation is probably under auxin regulation.

The Cortical Program

Nodule organogenesis requires the dedifferentiation of the nodule progenitor cells, which are likely the target of the NF signal in the cortex. Once these cells have re-acquired the capability of dividing, they organize to form a NP and a nodule meristem (NM). As the NM grows outward toward the root surface, the IT grows inward toward the NP (Guinel and Geil, 2002) under the guidance of pre-ITs (for more details, see Murray, 2011). Nodule development necessitates the expression and regulation of many genes (**Figure 1**; blue box) and the involvement of many hormones. Cytokinin for example is known to play an essential role in nodule formation as *L. japonicus snf2* plants, which have a gain-of-function mutation in the *LHK1* cytokinin receptor gene, produce spontaneous nodules independently of CCaMK (Tirichine et al., 2007). That NF-induced cell reprogramming is dependent on a functional receptor was confirmed by van Zeijl et al. (2015) in a *Medicago truncatula* transcriptomics study; using a synthetic cytokinin reporter gene, these authors localized the cytokinin response in the cells known to be involved in NP formation. *LjLHK1* expression in the cortical cells increases as the NP enlarges until the nodule reaches the point of emergence (Held et al., 2014). A mutant of interest is *Mtefd-1*; it has its place in the cortical program because the expression of ERF required for nodule differentiation (*EFD*), in the central region of the nodule, can be triggered by a defective bacterial mutant (Vernié et al., 2008). As the *Mtefd-1* mutant forms many ITs and numerous NP which do not proceed correctly to maturity (Vernié et al., 2008), it is likely that *EFD-1* (**Table 1**) known to activate the expression of a cytokinin response regulator is a negative player

TABLE 1 | Characteristics of mutants displaying ethylene abnormality.

	*Ljnena-1**	*Ljlot1*	*Mtefd-1*	*Pssym16* (R50)	*Pssym15* (E151)
Mutation	Monogenic and recessive	Monogenic and recessive Unknown gene-product	Monogenic and recessive	Monogenic and recessive[1] Unknown gene-product	Monogenic and recessive[1] Unknown gene-product[2]
Nodule number	No infection threads Few pink nodules	Lower than WT by about 1/5th	Higher number of nodules	Significantly lower than WT[2]	Significantly lower than WT
Nodulation zone		No mention of it being atypical	Typical	Two, one close to the cotyledons, and one further down[3]	Two, one close to the cotyledons, and one further down[3]
Infection threads (IT)	Absent, infection resembles crack-entry	Normal in morphology but in low number	Numerous, especially in epidermis, and branched	Atypical, branched and convoluted[2]	Atypical, much branched and knobby[2]
Nodule primordia		All developed	Numerous and associated with ITs.	Consist of a single cell layer[2]	Consist of two cell layers[2]
Functional nodules	No	Yes	Broader and multi-lobed Defective in N_2 fixation	Multi-lobed With lower efficiency[4]	Multi-lobed With lower efficiency[4]
Organ controlling nodulation phenotype		Root		Root[2]	Root[2]
Nitrate-sensitive		Higher sensitivity than WT	Similar to WT		Similar to WT[2]
Classical Triple response		Typical		Partial etiolation phenotype[5]	Typical[4]
Ethylene sensitivity	Ethylene required for nodulation to occur	Insensitivity in terms of nodulation	Expression independent of ethylene	Roots sensitive as in WT[5] Shoots insensitive to ethylene[5]	Sensitive to silver[2] Insensitive to ACC or AVG[2]
Ethylene evolution				As WT[4]	
Cytokinin sensitivity			Likely targets the cytokinin response regulator MtRR4	Accumulator of cytokinin because of a defective cytokinin oxidase enzyme[6]	Accumulator of cytokinin[2]
ABA sensitivity					Hyper-sensitive to ABA when in the vegetative stage[2]
Root morphology of non-inoculated plants		Shorter roots			PR length similar to WT[2] Young: Fewer LR[2] Old: Many more than WT[3]
Other traits		Moderately dwarf Wavy trichomes on the calyx and abaxial side of leaflets		Short and thick epicotyl[5] Pale green leaves[2]	Shorter plants in nitrogen-limited conditions[4]
Response to mycorrhizal fungi	Low AM frequency as infection aborts after epidermal invasion	Typical, as in WT	Typical, as in WT	Typical, as in WT[4]	Hyper-mycorrhizal[2]
References	Groth et al., 2010 *Temperature-dependent	Ooki et al., 2005	Vernié et al., 2008	[1]Kneen et al., 1994 [2]Guinel and Sloetjes, 2000 [3]Remmler et al., 2014 [4]Unpublished data [5]Ferguson et al., 2005 [6]Held et al., 2008	[1]Kneen et al., 1994 [2]Jones et al., 2015 [3]Remmler et al., 2014 [4]Unpublished data

in nodule initiation and is required for the late stages of nodule development.

Coordination of the Two Programs

Few genes located downstream of *CCaMK* are known to be involved in both programs. First, the transcription factor (TF) NIN is thought to affect negatively the rhizobial infection but positively the cortical program (Yoro et al., 2014). Second, NSP2 and NSP1, two GRAS domain TFs thought to be indispensable in bridging CCaMK to downstream actors (Heckmann et al., 2006), act in a coordinated manner since their mutant phenotypes are similar. Despite RH deformation and calcium spiking, neither ITs nor NP form in these mutants (Heckmann et al., 2006). Third, the protein vapyrin (VPY), containing a Major Sperm Protein domain and a series of ankyrin repeats, is thought to be implicated in membrane biogenesis and trafficking because of its subcellular localization (Murray et al., 2011). In *vpy* mutants, there are more ITs and NP than in WT; however, the epidermis-arrested ITs are misshapen and the NP are not infected (Murray et al., 2011). Other mutants which should be placed in this group are *Mtbit1-1* and *Mtbit1-2*, now known as *MtERN1* (ERF Required for Nodulation). Both mutants display ITs not progressing beyond the epidermis and delayed NP not maturing (**Figure 1**; Middleton et al., 2007). In WT, *MtERN1* is not only expressed early in the nodule progenitor cells but also later in the cortical cells surrounding the IT (Cerri et al., 2012). As NP development is arrested in the mutant and *MtERN* expression requires *MtCRE1* (Plet et al., 2011), an ortholog of *LjLHK1*, Cerri et al. (2012) suggested that ERN1 is required for late nodule development. It is possible that the products of these TFs have different roles in the two nodulation programs, as for NIN.

When the cortical program is completed, the nodule structure is well organized. Depending on the types of legumes bearing them, the nodules are spherical (determinate) as on *L. japonicus* and soybean roots or oblong (indeterminate) as on *M. truncatula* or pea (*Pisum sativum*) roots (Guinel, 2009a). The former quickly loses NM activity whereas the latter with an active NM displays a characteristic zonation with six zones: the meristematic zone (zone I), the infection zone (zone II), the interzone II–III, the fixation zone (zone III), the senescing zone (zone IV) and the saprophytic zone (Zone V; see Guinel, 2009a for more details). In this indeterminate-type nodule, a specific cell borne in the meristematic zone passes through all the nodule zones and becomes infected or non-infected (Kondorosi et al., 2013). Recently, Xiao et al. (2014) demonstrated that not all nodule cells have a similar origin, whereas those located deep in the nodule originate from the NP, those at its distal end originate from the NM.

Via the dual action of several genes in both programs and a constant communication between the symbionts, likely through NF perception, nodule organogenesis proceeds according to a planned choreography. One of the controls relies on the constant perception and turnover of NFs within the developing and mature nodule. Thus, recently, Moling et al. (2014) localized the two NF receptors, MtLYK3 and MtNFP, to the cell periphery of two cellular layers located at the boundary between zones I and II. These receptors are also localized within the cell vacuoles. Moling et al. (2014) proposed that the periphery-located receptors, in addition to regulating the bacterial release in these cells, are involved in dampening plant defense responses whereas those receptors located in the vacuoles are targeted for degradation.

Internal Colonization of the Bacteria

Bacterial Release in the Infection Zone

If all the steps of early nodule development are performed correctly and the IT progresses without any mishap through the cortex, then the IT reaches the infection zone of the young nodule where it delivers un-walled infection droplets (**Figure 2**) in cells which are polyploid (Mergaert et al., 2006). Intracellular bacterial accommodation is linked to both high secretory activity and intense vesicle trafficking in the newly infected cells, as large amounts of endo-membranes are produced (Bapaume and Reinhardt, 2012). Bacteria surrounded by the peribacteroid membrane make up an organelle-like structure known as a symbiosome. The bacterial delivery is performed under tight genetic control (**Figure 2**) as demonstrated by the work of Ivanov et al. (2012) who prevented bacterial release into the cytoplasm of the infected cell by silencing two genes coding for soluble *N*-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins. Scaffolding proteins such as MtSYMREM1 and FLOT2/FLOT4 are also necessary for proper bacterial delivery (Lefebvre et al., 2010, and Haney and Long, 2010, respectively). MtSYMREM1 has been located at the plasmalemma lining the IT and onto the infection droplets in zone II, as well as to the symbiosome membrane in zone III (Lefebvre et al., 2010); as was mentioned earlier, the protein may act in recruiting the NF receptors into a microdomain. The two NF receptors are also important in rhizobial release (**Figure 2**). They are localized on the cell membrane lining the IT and removed from that membrane when the bacteria are released in infection droplets (Moling et al., 2014). MtIPD3 (Interacting Protein of DMI3), ortholog to LjCYCLOPS and known to interact with CCaMK (Ovchinnikova et al., 2011), NF-YA1 previously known as HAP2, a transcription regulator known to control both IT progression through the cortex (Laporte et al., 2014) and NM development in indeterminate nodules (Combier et al., 2006; Xiao et al., 2014), and MtEFD (Vernié et al., 2008), are all involved in bacterial release (**Figure 2**), because mutations in any of these proteins prevent the rhizobia from leaving the IT. In the bacterial discharge from ITs, MtIPD3 was shown to interact with MtDMI2 and MtDMI3 (Ovchinnikova et al., 2011). NSP1 has also been suggested to play a role in proper bacterial release (Heckmann et al., 2006).

This crucial step is subjected to the "scrutiny" of the plant which uses its defense system to assess whether or not the infecting rhizobia are welcome, as discussed by Lang and Long (2015) in their transcriptomics study of nitrogen fixation-defective mutants of *M. truncatula*. Thus, in nodules arrested early in development, one finds a high abundance of transcripts for ascorbate, glutathione, and proteins involved in ROS detoxification; these proteins if expressed would increase resistance against an inappropriate level of biotic

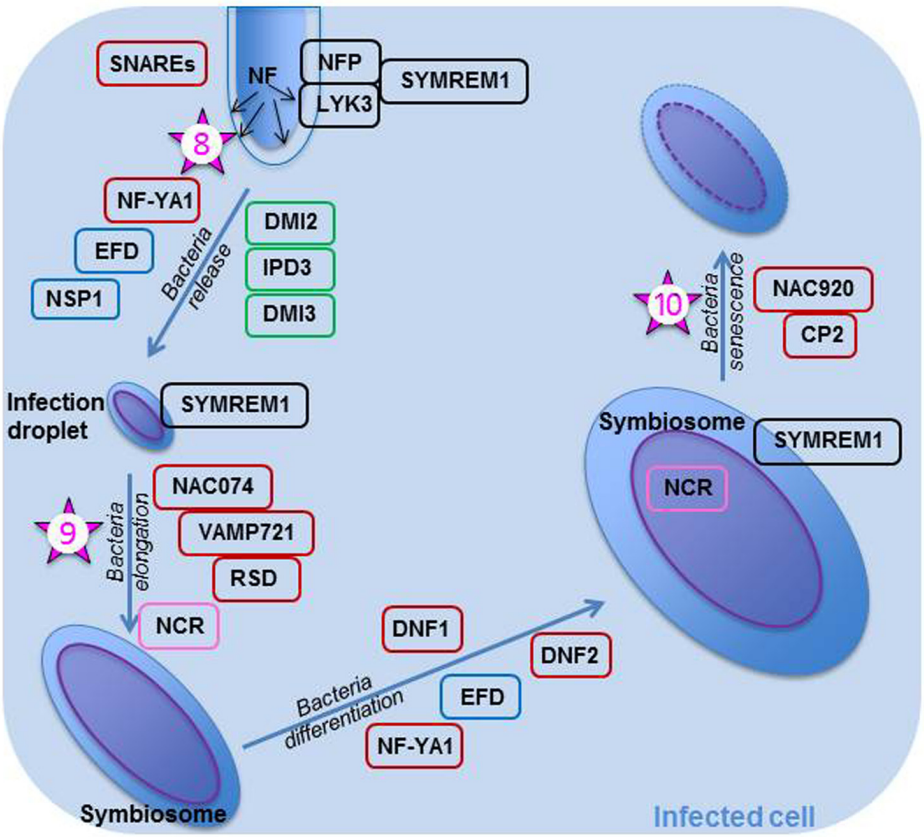

FIGURE 2 | Rhizobial release and bacteroid differentiation within an infected plant cell of *Medicago truncatula*. Once the rhizobia are released in the infection droplets, they divide and elongate. They later on differentiate into bacteroids; many bacterial genes, such as those necessary for division, are then turned off, whereas genes the products of which are necessary for bacteroid metabolism (nitrogenase, transporters, etc. . .) are turned on. As all these events are important steps in the symbiosis, they are controlled tightly and many genes and their products are implicated in their control. All proteins within a black box are known to be involved with the NF perception. Those in a green box are known to be part of the common symbiotic transduction pathway. Proteins in a blue box are thought to play a role in both the epidermal and cortical nodulation programs. Proteins in a brown box are not known to play a role early in the nodulation process; they may be specific for controlling these specific steps. NFs are continuously produced by rhizobia within the infection thread and when the rhizobia are released, the NFs are perceived by NFP and LYK3, the Nod factor receptors. These receptors are likely to be recruited in a micro-domain by SYMREM1 expressed in the plasmalemma of the infected cell. SNAREs and NF-YA1 are essential in bacterial release, whereas NAC074, VAMP721, and RSD are required for bacterial elongation. The bacteria differentiate once they have perceived the antimicrobial NCRs, which are found in the bacteroid after they crossed both symbiosome and bacterial membranes. DNF1 is necessary for the entry of NCRs in the symbiosome. Bacterial differentiation is also under the control of DNF2. Finally, MtNAC920 working in concert with CP2 trigger bacteroid senescence. The numbered stars represent potential location of ethylene signaling or action and the numbers correspond to the order in which these actions are reported in the text.

stress (Lang and Long, 2015). Recently, in a study designed to better understand nitrogen-induced senescence, Karmarkar (2014) found that nodules treated with inhibitory NH_4NO_3 concentrations specifically express *MtNAC074*, a TF that binds directly to the promoters of *MtVAMP721* genes, which are coding for members of the vesicle associated membrane protein (VAMP) family. Nodules over-expressing *NAC074* had symbiosomes delayed in development with an atypical cell arrangement and a lower nitrogenase activity than controls. Karmarkar (2014) proposed that MtNAC074 negatively affects symbiosome development because it represses VAMP721s which regulate many processes in plants, including delivery of cargo essential to symbiosome formation. Of interest is the recent characterization of *MtRDS* (Regulator of Symbiosome differentiation), a mutant exhibiting early senescence with incorrectly differentiated bacteroids (Sinharoy et al., 2013). *MtRSD* is expressed in zone II and interzone II–III; it codes for a TF belonging to the C_2H_2 family. RSD binds directly to the *VAMP721a* promoter and in doing so it represses VAMP721a production via its EAR domain (Sinharoy et al., 2013). EAR-repressors have been implicated in the suppression of defense and stress genes (Kazan, 2006) and may thus lower the plant defense responses during rhizobial release in the invasion zone.

It is becoming apparent that many genes involved in the initial stages of the nodulation process are also implicated in the release of rhizobia (Moreau et al., 2011). This may not be surprising knowing that NFs are being continuously produced and are active from the time of inoculation to that of rhizobial

release into the infected cell (Gourion et al., 2015). However, later during bacterial differentiation, when NFs are likely no longer synthesized, different plant genes come into play, suggesting different control strategies (Lang and Long, 2015). In views of Xiao et al.'s (2014) study, it is also likely that rhizobial release in NP cells is controlled differently from that in NM daughter cells because NF-YA1 is essential for bacterial release in the latter but not in the former.

Bacterial Symbiosome

The symbiosome is an enclosed space where the rhizobium, unable yet to fix nitrogen, differentiates into a bacteroid capable of nitrogen fixation. Bacteroid differentiation depends on the plant host; thus, the events occurring will differ in determinate and indeterminate nodules (Mergaert et al., 2006; Kondorosi et al., 2013). In plants such as *Medicago* or *Pisum*, which form indeterminate nodules, the trigger for bacteroid differentiation is the production by the infected cell of antimicrobial nodule-specific cysteine-rich (NCRs) which are targeted via a specific signal peptide to the symbiosome (**Figure 2**; Van de Velde et al., 2010). NCR proteins cross the symbiosome membrane and enter the bacterial cytosol where they modulate bacteroid maturation (Van de Velde et al., 2010). NCR peptides involvement in controlling bacterial release during the intermediate and late symbiotic stages was confirmed by the transcriptomics study of Lang and Long (2015). For these NCRs to be synthesized and sent to their target, the Mt*DNF1* (Defective in Nitrogen Fixation) gene encoding a subunit of a signal peptidase complex must be properly expressed in zone II (Wang et al., 2010). Additionally, *MtDNF2* which encodes a predicted phosphatidylinositol phospholipase C-like protein must be essential for bacteroid differentiation and maintenance, because its mutant exhibits early bacteroid senescence in zone III (Bourcy et al., 2013). Proteins important in bacterial release also play a role in bacteroid differentiation; thus, *MtNF-YA1* and *MtEFD* are implicated in this step, since their mutants display nodules with bacteria arrested in development (Combier et al., 2006 and Vernié et al., 2008, respectively).

Nodule Functioning in the Fixation Zone

As the nodule grows in size, the infected cells progress through the interzone II–III; it is in this zone that bacteroids differentiate and leghaemoglobin is synthesized (de Billy et al., 1991). The cells in this zone are metabolically active and contain many organelles, with the infected cells depending much on the metabolism of the non-infected cells. In the infected cells, the bacteroids fix nitrogen once their nitrogenase enzyme complex is turned on. The symbiosome has now become a compartment where a large amount of nutrient exchange occurs (Bapaume and Reinhardt, 2012; Clarke et al., 2014). Whereas ammonia, the product of nitrogenase, exits the symbiosome through ammonium transporters, carbohydrates required to fuel nitrogen fixation enter this space as dicarboxylic acids (Lodwig et al., 2003) via dicarboxylate transporters (e.g., Udvardi et al., 1988). For nitrogen fixation to occur, the bacteroids must be provided with a low amount of branched amino-acids (Prell et al., 2009), which are transported across the symbiosome compartment via ABC transporters (Prell et al., 2010). In some rhizobia-legume associations, the bacteroids in effect have become auxotrophic for these amino-acids, highlighting their metabolic dependence on the infected cell (Prell et al., 2009, 2010). The symbiosis has evolved in such a way that the rhizobial and plant requirements are all being met. As examples, to protect the bacterial nitrogenase enzyme from too high levels of oxygen, the plant hosts the bacteria in the center of the nodule, and to transport photosynthates to the nodule and nitrogeneous compounds to plant sinks, nodule vasculature develops in the nodule cortex (Guinel, 2009a,b). Nitrogen fixation is required for the bacteroids inside the nodule to remain alive; once nitrogen stops being fixed, a defense-like mechanism kicks in to degrade the ineffective bacteroids. According to Berrabah et al. (2015), each step involved from rhizobial release to bacteroid death is controlled but likely not in the same way.

Nodule Senescence

Nodule senescence is under tight control, genetic as well as hormonal. The events described below are likely linked to the cell-cycle arrest in the NM and to the cessation of bacterial release from the IT in the infection zone. In natural conditions, one of the first visible signs of nodule senescence is a shift of nodule color from pink to green, because plant leghemoglobin is no longer expressed once the nitrogenase activity stops (Puppo et al., 2005). In an indeterminate nodule, the steps making up the process are well orchestrated (Guerra et al., 2010). The same is likely true in a determinate nodule, but the means by which the control is taking place may be dissimilar temporally and spatially (Puppo et al., 2005). Also there may be differences in metabolism because indeterminate senescing nodules of pea have their superoxide and H_2O_2 levels decline while these levels increase in the determinate senescing nodules of soybean (Puppo et al., 2005). However, both nodule types go through the same physiological changes as they move from being a sink to become a source of nutrients for the plant (Guerra et al., 2010).

In *M. truncatula*, the first signs appear in the proximal area of zone III, and the event spreads in such a manner that the senescent tissue takes a cone-shape (Guerra et al., 2010). Bacteroids are first to degrade within the symbiosomes; then, once the plant cells have resorbed the symbiosome content, they start to deteriorate (Van de Velde et al., 2006). The infected cells collapse first while the non-infected cells mine the remobilized nutrients (Van de Velde et al., 2006) which are directed to the nodule vasculature. Cysteine proteases, likely active in mobilizing the nutrients from the degrading symbiosomes (Guerra et al., 2010), are the earliest molecular markers of the process (Van de Velde et al., 2006). Two genes, the expression of which is also up-regulated earlier, code for members of the AP2/ERF family usually expressed during stress responses and host immunity (Van de Velde et al., 2006). Later on, when the process is more advanced, genes coding for proteases, a vacuolar processing enzyme (VPE) -precursor, proteasome complexes, and catabolic enzymes are all up-regulated (Van de Velde et al., 2006). The expression of these genes reflects the high metabolic activity required for the recycling of the components of the symbiosomes and infected cells. Karmarkar (2014) in his nitrate-induced

senescence study demonstrated the importance of the TF MtNAC920 in the process (**Figure 2**). *MtNAC920* triggers nodule senescence by directly targeting the promoter region of *MtCP2*, a gene coding for a cysteine protease.

As for hormones, abscisic acid (ABA), ethylene, and JA have all been shown to be involved (Puppo et al., 2005; Hichri et al., 2015). Puppo et al. (2005) proposed a model whereby ABA plays a primary role in coordinating senescence. According to these authors, as the nodule ages, the ascorbate levels decrease and the carbon/nitrogen ratio increases in the nodular tissues; this results in ABA synthesis and its transduction pathway activation, leading to an increase in proteases and proteasome activities. The importance of ABA was not confirmed by the *Medicago* transcriptomic study of Van de Velde et al. (2006), who found gene tags suggesting the involvement of three other hormones. For these authors, ethylene and JA act positively during senescence while gibberellic acid (GA) acts negatively. JA positive action is suggested by the up-regulation of several lipoxygenase genes whereas GA negative role is indicated by the up-regulation of the gene coding for GA2-oxidase, an enzyme known to inactivate GA (Van de Velde et al., 2006). Recently, new evidence was provided toward ethylene having an active role in senescence, at least in stress- and nitrate-induced nodule senescence (Karmarkar, 2014); this will be elaborated upon further below.

A REVIEW OF ETHYLENE BIOSYNTHESIS AND SIGNALING

Ethylene Biosynthesis

The ethylene biosynthesis pathway is relatively simple and a good review on the subject was published by Lin et al. (2009). In short, there are three steps in the pathway (**Figure 3**). Methionine, its precursor, is provided by the Yang cycle which is one of the mechanisms used by plants to recycle sulfur. With the addition of an ATP molecule, methionine is converted into *S*-adenosyl methionine (SAM) by the enzyme SAM synthetase (SAMS). The rate-limiting step which commits SAM to ethylene synthesis is regulated by ACS (ACC synthase) which catalyzes the conversion of SAM into ACC (1-aminocyclopropane-1-carboxylate). In the presence of oxygen and ascorbate, ACC is converted into ethylene, cyanide, and CO_2 via the action of ACO (ACC oxidase). ACC can also be converted via ACC deaminase into ammonia and α-ketobutyrate (**Figure 3**). Bacteria as well as plants are known to possess that enzyme (Van de Poel and Van Der Straeten, 2014); the former may use it to decrease the ethylene levels of the latter (e.g., Klee et al., 1991; Gamalero and Glick, 2015).

ACS and ACO are both encoded by multigene families. The control of ethylene biosynthesis is thought to rest mostly on the regulation of the ACS isozymes. However, it is more than likely that control occurs too via ACO because (1) more than one ACO isozyme exist and these exhibit different temporal expressions (e.g., Jafari et al., 2013); (2) endogenous ethylene promotes its own synthesis during pea germination by stimulating ACO activity (**Figure 3**; Petruzzelli et al., 2000); (3) a feedback mechanism with ethylene inhibiting ACO has been suggested based on ACO transcript levels in *M. truncatula* inoculated by rhizobia (Prayitno et al., 2006a); and (4) Larrainzar et al. (2015) found in *M. truncatula* that the expression of one ACO is inhibited by ethylene whereas three ACOs require proper ethylene signaling to be induced.

ACS is under tight regulation, mainly via the control of its degradation rate (Rodrigues et al., 2014). In *Arabidopsis thaliana*, the stability of each ACS member depends on a specific domain located in the C-terminal end. Based on this domain, one can recognize three ACS classes (Types I–III) and each class exhibits distinct regulatory features (Rodrigues et al., 2014). Type II class-ACS members have a short C-terminus domain containing a putative calcium-dependent protein kinase target site (Rodrigues et al., 2014), which if altered prevents the degradation of the ACS enzyme as it is no longer targeted to the proteolytic machinery (Chae et al., 2003). This is seen in *Ateto2* and *Ateto3*, two mutants with mutations in the ACS5 C-terminal domain. Both mutants are ethylene over-producers, not because the mutation is stimulating the enzyme but rather because its degradation is prevented (Chae et al., 2003). An interesting mutant is *Ateto1*, also an overproducer of ethylene, but for a different reason. *Ateto1* is not mutated in ACS5 but in a protein involved in the proteasome-dependent degradation pathway. Upon binding of ETO1 to the ACS5 C-terminal domain, ACS5 is targeted for degradation (Yoshida et al., 2006). In *Ateto1*, because ETO1 is dysfunctional, ACS5 is stable and ethylene is overproduced (Chae et al., 2003).

It is worthwhile here to note that ethylene production, at least in *Arabidopsis*, is also under hormonal control. For example, cytokinin and auxin, two hormones known to play a role in nodulation, have been shown to promote its synthesis. The former promotes ethylene production by reducing the turnover rate of ACS5 (Chae et al., 2003; Hansen et al., 2009). The latter induces ethylene biosynthesis by promoting, in a cell-specific manner, all *ACS* expression, except for *ACS1* (Tsuchisaka and Theologis, 2004).

Ethylene Signaling

Recently, great progress has been made in this field as can be seen in many excellent reviews (e.g., for ethylene receptors, Lacey and Binder, 2014; and ethylene signaling, Merchante et al., 2013). In a nut-shell, ethylene is perceived by several receptor proteins, e.g., ETR1 (**Figure 3**, 1) or ETR2, which are located on the ER membrane (Chen et al., 2002); the receptors are negative regulators of signaling. Upon being triggered, the receptors are inactivated. This has two effects: (1) their de-phosphorylation which allows them to bind more efficiently to Ethylene INsensitive 2 (EIN2; Cho and Yoo, 2015), a protein also localized on the ER membrane (Bisson et al., 2009); and (2) the switching off of the protein Constitutive Triple Response 1 (CTR1) which is no longer capable of phosphorylating EIN2 (**Figure 3**, 2). Once removed from the CTR1 influence, EIN2 is subjected to a structural modification as its C-terminus is cleaved (**Figure 3**, 2). This fragment is moved physically to the nucleus where it stabilizes the TFs ethylene insensitive 3 (EIN3) and EIL1 (EIN3-Like 1), both responsible for the regulation of

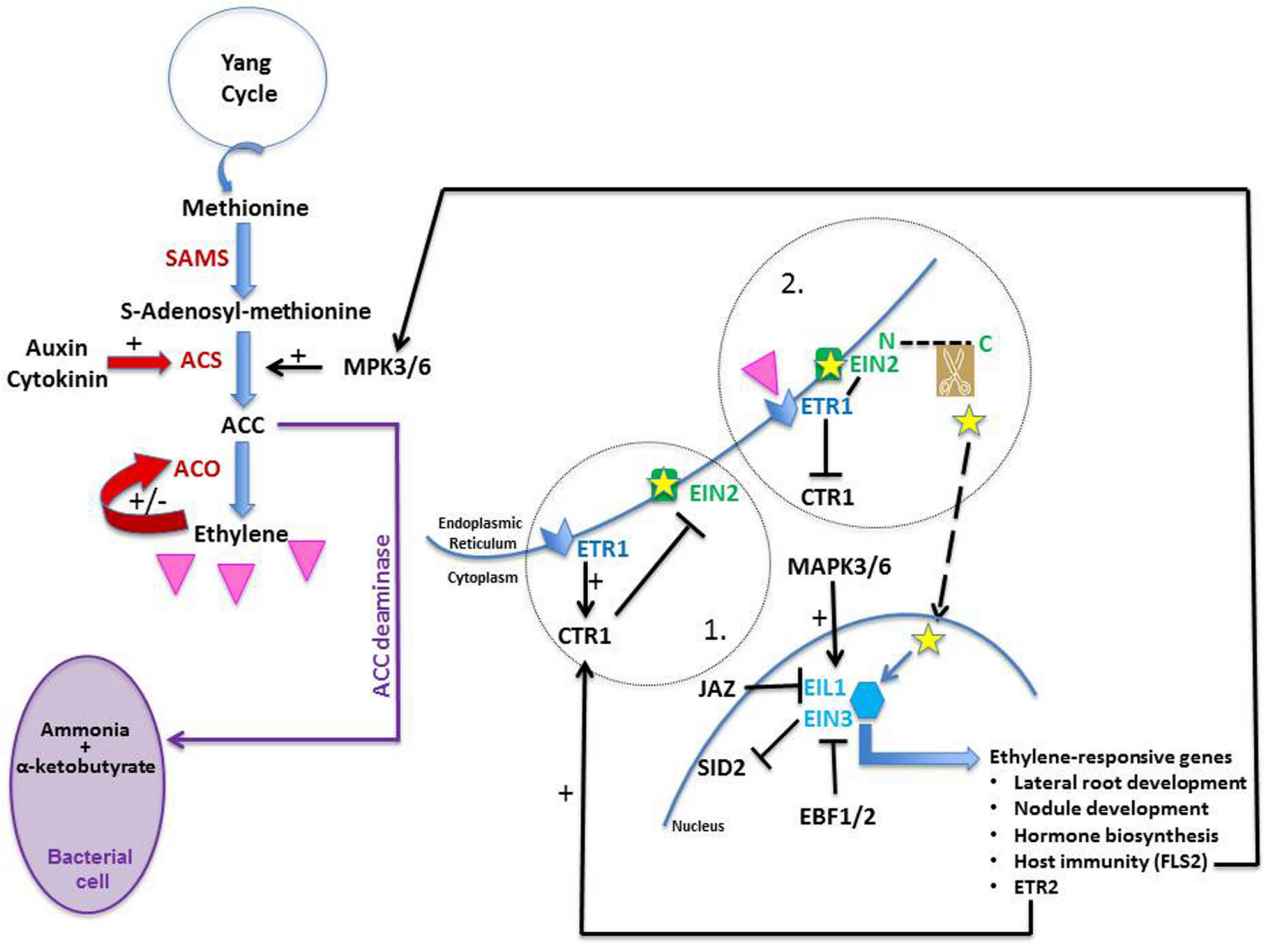

FIGURE 3 | Ethylene biosynthesis and transduction pathway. Methionine is the precursor of ethylene (pink triangle). It is converted into *S*-adenosyl-methionine (SAM) by SAM synthetase (SAMS); SAM is converted into 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). ACC is later converted into ethylene by ACC oxidase (ACO). Both auxin and cytokinin are known to promote ethylene production; the former up-regulates ACS expression whereas the latter prevents ACS degradation. Ethylene is known to modulate its own levels by stimulating (+) or inhibiting (–) ACO activity. ACC deaminase is an enzyme found in certain types of bacteria; it converts ACC into ammonia and α-ketobutyrate and both may be used as nutrients by the bacteria. When no ethylene is sensed in a cell (circle 1), ethylene receptors (such as ETR1) located on the ER membrane promote CTR1 activity; in doing so, they allow CTR1 to phosphorylate EIN2 which inactivates it. Upon ethylene perception (circle 2), ETR1 is dephosphorylated and CTR1 is switched off. ETR1 can now bind efficiently to EIN2 which is activated. Its C-end terminus (small yellow star) is cleaved and moves to the nucleus where it stabilizes the transcription factors EIN3 and EIL1, which are then able as dimers to trigger the expression of ethylene-responsive genes, inducing many developmental and biochemical events. When ethylene is not perceived, EIN3 and EIL1 are targeted to proteasomes via the action of EBF1/2. MAP kinases 3 and 6 (MAPK3/6) are known to activate ACS enzymes and to phosphorylate EIN3 in *Arabidopsis*, protecting it from degradation. Jasmonic acid is known to work synergistically with ethylene by promoting the degradation of JAZ2, a protein known to repress EIN3/EIL1. Finally, EIN3/EIL1 inhibits salicylic acid synthesis as they bind to the promoter of SID2, one of its biosynthetic genes, thus inactivating it.

ethylene-responsive genes (Ju et al., 2012); it does so likely by degrading EBF1/2, F-box proteins which would otherwise target EIN3 and EIL1 to proteasomes. Functioning as dimers, EIN3 and EIL1 trigger the expression of multiple ethylene-regulated genes, among which one can find the *ETR2* gene. Its gene-product, the ethylene receptor ETR2, activates the negative regulator CTR1 which as a result phosphorylates EIN2 which deactivates it. In this manner, ethylene signaling can be tuned down in the absence of additional ethylene (Merchante et al., 2013). Finally, some downstream targets of EIN3 are essential components of other hormonal signaling pathways, illustrating the intricacy of plant development regulation. Although understanding how EIN2 and CTR1 interact has filled a physical gap between the ER and the nucleus, there is still much to learn about ethylene signaling; for a recent review pointing to knowledge gaps in the field of ethylene signaling, see Cho and Yoo (2015). There may be yet surprises in the pathway as new CTR1-independent signaling routes are being proposed (Zhang et al., 2014).

NODULATION AND ETHYLENE

Ethylene has many roles in the development of a plant, from seed dormancy to fruit ripening (Abeles et al., 1992). In nodulation, its

effect was reported as early as four decades ago when rhizobia-inoculated root cultures of bean were treated with ethylene; as a result, not only nodule numbers decreased dramatically but the amount of nitrogen fixed was also reduced (Grobbelaar et al., 1971). Forty years later, we have a better understanding, although still far from being complete, of the effects the hormone has on the nodulation process. In the last decade or so, we have learned much from ethylene signaling pathway mutants and from transgenic plants carrying ethylene-related genes.

EIN2 in Model Legumes

The mutant *sickle* from *M. truncatula* proved to be especially useful; it has indeed been a determinant in getting a better grip on the role of ethylene in nodulation. *Mtskl,* first characterized by Penmetsa and Cook (1997) as a hyper-nodulation mutant, forms 10–30 times more nodules than the wild-type (WT) plants (**Table 2**). Because on younger plants the nodules it forms are all located in the typical nodulation zone, i.e., the most susceptible nodulation zone (Bhuvaneswari et al., 1981), they are tightly pressed one against the other. The gene *Mtskl* affects IT resilience; thus most ITs are associated with NP which develop, albeit with a delay, into nodules capable of typical nitrogen fixation (Penmetsa and Cook, 1997). The mutant is insensitive to ethylene as, for example, it failed to exhibit the classical "triple response" when treated with ACC or ethylene (Penmetsa and Cook, 1997). *Mtskl* ethylene-insensitivity was confirmed by Penmetsa et al. (2008) who demonstrated that MtSKL is an ortholog of AtEIN2. It is an integral membrane protein which comprises an N-terminal sequence similar to that seen in proteins belonging to the natural resistance-associated macrophage protein (NRAMP) family and a unique C-terminal sequence. Its hydrophobic core composed of 10 trans-membrane domains is located within its N-terminal end (Penmetsa et al., 2008). To date, a single EIN2 gene has been found in *M. truncatula* (Penmetsa et al., 2008) and in pea and chickpea (Weller et al., 2015), whereas two have been identified in common bean (Weller et al., 2015), in soybean (Miyata et al., 2013), and in *L. japonicus* (Chan et al., 2013). The two *L. japonicus* genes *LjEIN2a* and *LjEIN2b* are expressed in all organs examined, including roots and nodules (Miyata et al., 2013).

The presence of two *EIN2* genes in common bean, soybean and *L. japonicus*, all plants forming determinate nodules, may explain partly the conundrum exposed about 15 years ago when it was shown that in soybean nodules formed in the presence of ethylene (Lee and LaRue, 1992a) and on ethylene-insensitive mutants (Schmidt et al., 1999). These observations led to the hypothesis that the formation of indeterminate and determinate nodules may be regulated differently by ethylene. Yet, some legumes forming determinate nodules are known to respond to ethylene. For example, beans treated with ethephon (a compound which spontaneously releases ethylene) and *Macroptilium atropurpureum* treated with ACC exhibit a smaller number of nodules than non-treated plants, and either plant treated with amino-vinyl-glycine (AVG), an inhibitor of ACS, forms a larger number of nodules (Nukui et al., 2000; Tamimi and Timko, 2003). Furthermore, ethylene sensitivity, assessed by leaf senescence and chitinase activity assays, was shown to depend on soybean cultivars (Xie et al., 1996). These results led Nukui et al. (2000) to propose that recent breeding processes may have selected for soybean lines which were ethylene-insensitive. A differential in ethylene response is also seen in ethylene-insensitive *Lotus* transgenic plants carrying a vector with the mutated *etr1-1* from *Arabidopsis* (Lohar et al., 2009). Different lines classified according to their hypocotyl response to ACC were obtained: "hypo-insensitivity" lines bore nodule numbers similar to those of WT whereas "hyper-insensitivity" lines, with traits symptomatic of a lack of ethylene response, bore higher nodule numbers (Lohar et al., 2009).

Detailed Description of the *EIN2* Mutants and Transgenic Plants Altered in Ethylene Perception

Mtskl has been characterized in depth and used by many research groups to refine our understanding of the role(s) played by ethylene in nodulation. Typical of hormonal mutants (Karlowski and Hirsch, 2003), *Mtskl* is pleiotropic (Penmetsa et al., 2008; Prayitno, 2010) and the mutation has many effects on the plant (**Table 2**). For example, non-inoculated *Mtskl* exhibit longer primary roots than WT and a delay in lateral root growth in early developmental stage (Prayitno, 2010). Furthermore, *Mtskl* roots are thinner and have longer cortical cells (Prayitno and Mathesius, 2010) and shorter RHs (Oldroyd et al., 2001) than those of non-inoculated WT. *Mtskl* nodulation is root-controlled (Prayitno et al., 2006b) and the mutant roots respond to nitrate although not as much as those of WT (Prayitno, 2010). These two characteristics, root control and nitrate sensitivity, distinguish the hyper-nodulator *Mtskl* from super-nodulators which are affected in autoregulation of nodulation (Novak, 2010). The *Mtskl* mutation also affects nodule positioning because nodules on the mutant are no longer restricted to the cortical zone facing the xylem tissue (Penmetsa and Cook, 1997), where nodules are expected to form since ACO transcripts, indicative of ethylene biosynthesis, are located in phloem-facing cortical tissue (Heidstra et al., 1997). The *Mtskl* mutant is hypersensitive to NFs (Oldroyd et al., 2001) which makes it useful to researchers in the field of transcriptomics as the expression of genes induced by NFs will be up-regulated much more in *skl* than in WT (e.g., Breakspear et al., 2014; Larrainzar et al., 2015). Etiolated seedlings and leaves of *AtEIN2* mutants evolve more ethylene than those of WT (Guzmán and Ecker, 1990) and one would assume that *MtSkl* does the same. Penmetsa et al. (2008) postulated that *skl* is lacking ethylene-mediated ethylene production.

The nodulation phenotype of *Ljenigma-1*, mutated in LjEIN2, differs from that of *Mtskl* (**Table 2**). *Ljenigma-1* shares several traits with *Mtskl*, such as the lack of a typical triple response or a nitrate-sensitive nodulation, although it is more sensitive to nitrate than its WT (Chan et al., 2013). In contrast to *Mtskl*, *Ljenigma-1* displays a low nodule number, independently of the type of micro-symbionts used as inoculant (Chan et al., 2013). Other contrasting traits are the response of *Ljenigma-1* to mycorrhizal fungi and its nodulation phenotype being controlled

TABLE 2 | Characteristics of the mutants and transgenic plants altered in the protein EIN2.

	Mtskl	***Ljenigma-1***	***LjEIN2* (1 and 2)**	***etr1-1***	***ERS1***
Mutation	Single recessive mutation[1] of MtEIN2[2]	LjEIN2-2 (also named LjEIN2a) Recessive mutation	RNAi constructs targeting both genes	Transgenic plants containing a vector with *Atetr1-1**	Transgenic plants with a vector carrying a mutated *CmERS1**
Nodule number	10–30 × more [1 and 3]	3 × less	Together 3 × increase	Larger nodule number Smaller nodules	Higher number of nodule primordia but similar nodule number
Nodulation zone	Typical[1]		Clustered in a limited region	Typical	Typical
Infection threads	Persistent[1]	Typical and fewer		Increased number	Significantly higher
Nodule positioning	Atypical[1]	Atypical		Atypical	
Organ controlling nodulation phenotype	Root[6]	Root and shoot			
Nitrate-sensitive	Yes[4]	Increased sensitivity to high nitrate levels		Yes	
Classical Triple response	Lack of[2]	Lack of		Lack of	
Cytokinin sensitivity	Decreased[2]				
ABA sensitivity		Increased sensitivity Higher ABA production			
Root morphology of non-inoculated plants	Longer primary root[4] Delayed LR growth[4] Thin[5] Longer cortical cells[5] Shorter RH[3]	Increased root elongation Lightly increased LR number Greater root DW	Longer roots Shorter RH	Smaller LR number	
Other traits	Delayed leaf senescence[1] Delayed petal senescence[1] Decreased abscission of seed pod and leaves[1]	Slower plant growth Delayed flowering time Smaller seed pods Smaller leaves		Delayed flowering time Delayed petal senescence Delayed ripening of fruit	Delayed petal senescence
Ethylene sensitivity	Ethylene insensitive[1]			Ethylene-insensitive	Ethylene-sensitive as seen by macro-observations and growth of ACC-treated plants
Ethylene evolution	Absence of an ethylene-mediated ethylene production[2]	Seedling root levels five times as high as those of WT			
Response to mycorrhizal fungi	Increased[2]	As in WT			
Response to pathogens	More susceptible[2]				
References	[1]Penmetsa and Cook, 1997 [2]Penmetsa et al., 2008 [3]Oldroyd et al., 2001 [4]Prayitno, 2010 [5]Prayitno and Mathesius, 2010 [6]Prayitno et al., 2006b	Chan et al., 2013	Miyata et al., 2013	Lohar et al., 2009 *Constructs with a gene from *Arabidopsis thaliana*	Nukui et al., 2004 *Constructs with a gene from *Cucumis melo*

by both shoot and root (**Table 2**). In seedlings, *Ljenigma-1* roots evolve five times more ethylene than those of WT while the shoots evolve twice as much; this is likely a result of the ethylene-insensitivity of the plants: since the roots do not sense ethylene, they are making more of it (Chan et al., 2013). Furthermore, in contrast to those of *Mtskl* (Penmetsa et al., 2008), roots and shoots in *Ljenigma1* respond differently to ACC treatments. After an 18 h ACC-treatment of non-inoculated plants, *LjETR1* and *LjEIL3* transcript abundance is greatly increased in roots but not in shoots. This suggests that *Ljenigma-1* roots are ethylene-hypersensitive (Chan et al., 2013). The differences between the two mutants do not seem to lie on the type or location of the mutation. Both mutations are recessive and likely affect the C-domain activity of the EIN2 protein, thus altering its function. Thus, the disparities are difficult to reconcile, especially knowing that transgenic roots carrying RNA interference (RNAi) constructs targeting the two *Lotus EIN2* genes exhibit a hyper-nodulation phenotype (**Table 2**; Miyata et al., 2013). The two genes appear to work together as roots having both genes suppressed bear more nodules than either roots carrying an empty vector or roots carrying only one of the suppressed genes. The greatest density of nodules was also found on those roots with reduced expression of both genes (Miyata et al., 2013). Chan et al. (2013) proposed that in *Lotus*, *EIN2a* is responsible for the triple response and the nodulation phenotype, whereas *EIN2b* is involved in nodulation. The *Ljenigma-1* mutant being mutated in the former gene would not display the triple response phenotype and its roots would still be responsive to ethylene via the latter gene (Chan et al., 2013). Thus, the paradox of *Ljenigma-1* likely rests on EIN2 functional redundancy and the different expression patterns exhibited by the two *EIN2* genes.

Studies of transgenic plants have confirmed the results obtained with *Mtskl*, that ethylene inhibits nodulation. Thus, *L. japonicus* plants containing a vector with a mutated gene coding for an ethylene receptor, either *ETR1* of *Arabidopsis* (Lohar et al., 2009) or *ERS1* of *Cucumis melo* (Nukui et al., 2004), display symptoms of ethylene insensitivity (**Table 2**). For example, the ETR1 lines which were ethylene hyper-insensitive display a larger number of infection events, resulting in a larger number of nodules, than those which were hypo-insensitive. Their nodules were smaller and contained higher number of bacteroids per symbiosome than those of WT; furthermore, in these lines a larger number of nodules formed in between xylem poles (Lohar et al., 2009).

Sites of Action of Ethylene in Nodulation

In this section, I will describe in detail the roles played by ethylene during the different stages of the rhizobial symbiosis (**Figures 1** and **2**), as these were described in section A. Recently, ethylene has been placed at the forefront of the nodulation process by Larrainzar et al. (2015) who demonstrated that upon rhizobial inoculation, two ethylene-regulatory paths are set. The first path occurs fast (1 hai), is transient, independent of NF perception, and positively controlled by ethylene, while the second one occurs later (6 hai), is dependent on NF perception, and is controlled negatively by ethylene (Larrainzar et al., 2015). Whereas the former is likely part of the defense response, the latter corresponds to the activation of the nodulation programs and affects adversely several hormonal signaling pathways leading the authors to propose a master negative regulatory role for ethylene. Upon inoculation, ACS and ACO are up-regulated, and as a result ethylene is produced in the nodulation zone (Larrainzar et al., 2015). These results confirm the work of many who noted the induction of ethylene biosynthesis by NFs (e.g., Miyata et al., 2013; van Zeijl et al., 2015) and an ethylene production increase early in the symbiosis (Ligero et al., 1986; Suganuma et al., 1995; Lopez-Gomez et al., 2012). In fact, as early as 1986, Ligero et al. (1986) proposed that ethylene was likely to control nodule development, maintenance, and senescence as they measured three peaks of ethylene throughout the nodulation process.

Host Immunity

As mentioned earlier, the NF signaling cross-talks with the innate immune signaling early in the rhizobial symbiosis (Gourion et al., 2015; Limpens et al., 2015). When rhizobia are attempting to penetrate the plant epidermis, they are subjected to host immune responses, as are pathogens (Zamioudis and Pieterse, 2012; Gourion et al., 2015). The bacteria synthesize several molecules involved in warding off the MTI and ETI defense responses that the plant is putting into place. However, in a nodulating plant, it is not yet entirely understood which bacterial molecule is responsible for which event of the defense response. For example, there appears to be some discrepancy upon which symbiotic genes are expressed in response to the potent plant immune response elicitor flg22. Whereas Lopez-Gomez et al. (2012) did not observe an up-regulation of *NIN*, *NSP1* and *NSP2* upon flg22 perception by *L. japonicus*, Nakagawa et al. (2011) reported that their increased expression was done via the activation of CCaMK (**Figure 1**).

Although ROS production is a well-known defense response, in nodulation of *M. truncatula* it does not appear to be induced by ethylene signaling as abundance of transcripts involved in ROS production was similar in ACC-treated *Mtskl* and WT (Prayitno et al., 2006a). There is, nonetheless, agreement on the enhancement of ethylene production being one of the MTI events (star 1 in **Figure 1**). Earlier studies as well as recent ones have reported a transient evolution of the hormone in the early stages of the symbiosis (e.g., for indeterminate nodules, Ligero et al., 1987; for determinate nodules, Suganuma et al., 1995 and Lopez-Gomez et al., 2012). Furthermore, flg22-treated *Lotus* produces more ethylene, exhibits activated MAP kinases (MAPK) 3 and 6, and displays up-regulated expression of defense-related genes such as the TF *WRK33* (Lopez-Gomez et al., 2012), as flg22-challenged *Arabidopsis* does (Nicaise et al., 2009). If one draws parallels between the two model plants, one could suggest that flg22-treated *Lotus*, as flg22-challenged *Arabidopsis* (Nicaise et al., 2009), produces higher levels of ethylene because FLS2-perceived flg22 induces phosphorylation by MAPK3/6, and therefore activation, of ACS (**Figure 3**). The same MAP kinases phosphorylate EIN3 in *Arabidopsis*, protecting it from degradation and allowing it to bind to ethylene-responsive genes (Yoo et al., 2008). One of these genes is *FLS2* itself as

EIN3 binds to the primary ET response element in the *FLS2* promoter region (**Figure 3**; Khatabi and Schäfer, 2012). The parallel between defense responses of legumes and *Arabidopsis* can be extended to include *ein2* mutants. For example, *Atein2* mutants, incapable of sensing ethylene and impaired in MTI, exhibit an enhanced susceptibility to pathogens (Khatabi and Schäfer, 2012). As well, *Mtskl* is more susceptible to both *Rhizoctonia solani* and *Phytophthora medicaginis*, with only 10% of its seedlings surviving infection compared to 80% of the WT seedlings, likely because EIN2 regulates the pathogen progression through a positive feedback amplification of ethylene biosynthesis (Penmetsa et al., 2008). If EIN2 is mutated, then EIN3 is not protected from degradation by EBF1/2 (**Figure 3**) and thus cannot bind to the *FLS2* promoter. As a result, MAP kinases do not phosphorylate ACS and ethylene is not produced, allowing pathogen entry.

Ethylene does not act alone in this response; its action is integrated with the action of SA and JA, hormones known to be involved in plant defense responses. Ethylene, together with JA, can activate ERF1, a TF with an AP2-EREBP domain, responsible for triggering PR expression in *Arabidopsis* (Lorenzo et al., 2003). As well, upon rhizobial inoculation of *L. japonicus*, *LjERF1* expression is up-regulated within 24 hai by both ethylene and JA, triggering the transcription of PR10 (**Figure 1**; Asamizu et al., 2008). *LjERF1* is a positive regulator of nodulation since when it is RNAi-silenced, the plants respond to the rhizobia as if they were pathogens; they increase PR expression which leads to nodulation inhibition. Recently, EIN3/EIL1 has been considered as a key node where the three hormones' signaling pathways interact. Thus in *Arabidopsis*, ethylene stabilizes EIN3 and EIL1 (Merchante et al., 2013), and JA activates their transcription by promoting the degradation of the JAZ proteins known to repress them (**Figure 3**; Zhu et al., 2011). In *Mtskl*, JA is repressed by bacterial inoculation since the JA receptor JAZ2 and a JA biosynthetic gene are down-regulated (Breakspear et al., 2014). As for SA, EIN3/EIL1 inhibits its synthesis because it binds specifically to the promoter of *SID2* (SA Induction Deficient 2), which encodes a SA biosynthetic enzyme, and prevents the full activation of the defense responses (**Figure 3**; Chen et al., 2009).

The Common Signal Transduction Pathway

Ethylene by affecting the CSTP places itself at the heart of nodule development. It modulates calcium spikings (star 2, **Figure 1**) by decreasing their frequency as seen in *Mtskl* which exhibits longer periods between calcium spikes than the WT (Oldroyd et al., 2001). For this specific event, ethylene is thought to work antagonistically with JA inhibiting its action on calcium spiking through EIN2; the rapidity with which JA and ethylene interacts to affect calcium spiking suggests a direct crosstalk (Sun et al., 2006). The behavior of these two hormones is different here in the CSTP to that described earlier in the host immune response where ethylene and JA are working synergistically on EIN3/EIL1 (**Figure 3**). This suggests a very fine tuning (temporal and spatial) of each step of the nodulation process.

In interactions where the epidermis is bypassed by the rhizobia, ethylene appears to be promoting nodulation. In the semi-aquatic tropical legume *Sesbania rostrata* infected by *Azorhizobium caulinodans*, when the roots are flooded, ethylene is required for rhizobial infection (D'Haeze et al., 2003). Rhizobia enter these plants by crack-entry at the base of lateral roots, where the cortical cells are directly exposed to the environment. Once the rhizobia have colonized the exposed fissure, NFs trigger ethylene production and together NFs and hormone induce a programmed cell death allowing the rhizobia to progress intercellularly through the cortex. Simultaneously, nodule progenitor cells divide to form a NP toward which the bacterial colony grows (D'Haeze et al., 2003). The ethylene requirement was confirmed by pharmacological treatments, whereby no nodules formed with ethylene inhibitors. In this specific case, and in contrast to nodulation in *L. japonicus*, nodule initiation cannot be uncoupled from rhizobial invasion (D'Haeze et al., 2003). In non-flooded conditions, *Sesbania* develops RHs which are used for bacterial colonization, but in this case ethylene is inhibitory to nodulation (Goormachtig et al., 2004), reinforcing the idea of a specific epidermal control by the hormone. Nodulation in *Ljnena* (**Table 1**) is reminiscent to that seen in plants displaying crack-entry; in this mutant, no ITs form and yet some of the nodules are pink (Groth et al., 2010). Furthermore, nodule infection is promoted in flooded conditions, i.e., when ethylene is produced. As for *Sesbania*, *Ljnena* mutants treated with ethylene inhibitors are not infected (star 3, **Figure 1**). The crack-entry trait of *Ljnena* may be an ancient trait shared by common ancestors of *Lotus* and *Sesbania*, two species which belong to the same legume sub-clade (Groth et al., 2010). Before RH colonization evolved, ethylene may have played a stimulatory role in nodulation. Colonization via the RH would have added a check-point to the invasion process. In fact, ethylene is known to act negatively at the boundary epidermis-cortex in this type of infection (Guinel and LaRue, 1992). The question that posed Guinel and Geil (2002) is still of actuality. Is ethylene synthesized by epidermal cells of a higher plant organ? More specifically, is ACO present in epidermal cells? ACO activity is absent in the epidermal cells of mung bean stems and pea (var. Argenteum) leaves, which has led Osborne (1991) to question the existence of an alternative ethylene pathway in the epidermis of higher plants. Larrainzar et al. (2015) localized ACS transcripts in the epidermis of inoculated and non-inoculated roots; unfortunately, they did not elaborate on the localization of ACO transcripts. Similarly, no mention is made of ethylene biosynthesis genes in the RH "infectome" transcriptomics study of Breakspear et al. (2014). To put at rest this question, it is essential that we ascertain the existence of ACO activity in the legume epidermis.

The Epidermal Program

Ethylene negatively influences the epidermal program. Thus, infection events are inhibited by ACC but promoted by AVG in WT (Oldroyd et al., 2001), but they are promoted in ethylene-insensitive plants since these exhibit not only higher infection events but also more ITs than control plants (Penmetsa and Cook, 1997; Nukui et al., 2004; Lohar et al., 2009). ACS expression in RHs displaying aborted ITs suggests that ethylene synthesis may be directly linked to the infection arrest (Larrainzar et al., 2015).

However, if epidermal ACO activity is non-existent, then this would mean that ACC itself acts as a signal, in this case inhibitory, which is an interesting concept raised by Van de Poel and Van Der Straeten (2014). Ethylene plays a role as early as 6 hai when RHs are deforming and branching as it regulates the expression of NF-dependent genes involved in actin and tubulin reorganization (star 4, **Figure 1**; Larrainzar et al., 2015). To determine if there is a direct link between ethylene and the cytoskeleton in the process of nodulation, it would be useful to test the ethylene-sensitivity of cytoskeleton-altered mutants, i.e., *Ljnap1, Ljpir1* and *Ljarpc* (**Figure 1**), as these mutants form few ITs which do not enter the cortex. They are able to form nodules but these are empty and without any anatomical structures (Yokota et al., 2009; Hossain et al., 2012). As for *Ljlot1*, it is ethylene-insensitive in terms of nodulation (Ooki et al., 2005) but in contrast to *Mtskl* it forms very few ITs (**Table 1**). It would thus be interesting to cross *Ljlot1* with *Ljein2* mutants to determine whether the two mutations are epistatic.

As mentioned above, ethylene likely controls the IT entry into the cortex (star 5, **Figure 1**; Guinel and Geil, 2002). Ethylene-treated pea roots exhibit more ITs arrested at the interface epidermis-cortex than non-treated roots (Lee and LaRue, 1992a). Conversely, the low nodulator *Psbrz* treated with ethylene antagonists displays ITs breaching into the cortex when non-treated *Psbrz* has most of its ITs arrested in the epidermis (Guinel and LaRue, 1992). Several pea symbiotic mutants have their ITs halted within the epidermal cell base. *Pssym16* (Guinel and Sloetjes, 2000) and *Pssym15* (Jones et al., 2015) are two such mutants; they are also known to accumulate cytokinins (**Table 1**). Interestingly, *Mtcre1* (Gonzalez-Rizzo et al., 2006) and *Ljhit1-1* (Murray et al., 2007), two mutants defective in cytokinin sensing, have a majority of their ITs also blocked in the epidermis. *Mtcre1* (Plet et al., 2011), *Pssym15* (Jones et al., 2015), and *Pssym16* (Guinel and Sloetjes, 2000) are all ethylene-sensitive since they bear more nodules after AVG treatment; however, only *Pssym16* has its nodule number totally restored by AVG. As cytokinin is known to up-regulate ACS5 (**Figure 3**; Hansen et al., 2009), and as *Mtskl* also exhibits a reduced sensitivity to cytokinin (Penmetsa et al., 2008), the two hormones are likely involved in the IT progression across the inner periclinal wall of the infected epidermal cell. In an attempt to distinguish the effects of ethylene from those of cytokinin, Plet et al. (2011) created *skl cre1* mutants; these double mutants exhibited higher nodule number than *Mtcre1* but lower than WT, suggesting that the mutations are epistatic and that the two pathways, i.e., EIN2-dependent ethylene and CRE1-dependent cytokinin, run in parallel. Ethylene perception is likely at the outset of the cytokinin pathway activation because Larrainzar et al. (2015) demonstrated that within 48 hai, NF-dependent, ET-regulated biosynthetic genes of numerous hormones, including cytokinin, are expressed. For cytokinin biosynthesis, ethylene perception is required as transcripts of *MtIPT*, a cytokinin biosynthetic enzyme, are reduced in *Mtskl* (Larrainzar et al., 2015). These results are in agreement with Penmetsa et al. (2008) suggestion that ITs in the epidermis are negatively regulated by cytokinin-induced ethylene perception.

The Cortical Program

Because *Mtskl* bear numerous but small nodules, Xiao et al. (2014) proposed that in *M. truncatula* ethylene signaling has a different effect on NP and NM; whereas it would inhibit NP formation, it would strongly promote NM development. However, ethylene would likely not act alone. Thus ethylene is known to control negatively the cortical program by interfering with cytokinin signaling. This is seen with the *L. japonicus* spontaneous nodules which formed in the absence of rhizobia. Treating with AVG either *Ljsnf1* (star 6, **Figure 1**) or *Ljsnf2-2* (star 7, **Figure 1**), a mutant of *CCaMK* and a mutant of *LHK1*, respectively, increases pseudo-nodule number whereas treating the same mutants with ACC decreases that number dramatically (Tirichine et al., 2006b). The response of the ACC-treated mutants inoculated with *M. loti* suggests that their NF-induced ethylene signaling is turned on and that ethylene plays a role in nodule formation downstream of cytokinin perception (Tirichine et al., 2006b). However, this is likely not through MtEFD (**Table 1**), a TF known to target *MtARR4* (Response Regulator) and as such linked to the cytokinin signaling pathway, because (1) its gene expression does not differ between ACC-treated, AVG-treated and WT plants and (2) its transcripts are expressed in inoculated *Mtskl* (Vernié et al., 2008). Plet et al. (2011) suggested that ethylene may restrict cytokinin action to the cortical regions facing xylem poles; by doing so, it would have an indirect effect on positioning NP. NFs have been shown to induce local, MtCRE1-independent, cytokinin accumulation which promotes in a MtCRE1-dependent manner the expression of ACS, suggesting that cytokinin signaling promotes ethylene synthesis (star 7, **Figure 2**) in the cortical cells (van Zeijl et al., 2015). However, because *Mtskl* accumulated more cytokinin than WT, van Zeijl et al. (2015) suggested that a negative feedback loop is at play in the cortex, whereby ethylene would keep in check the cytokinin levels of the cortex to prevent further NP from forming.

Ethylene appears to control the position where nodules initiate, the number of nodule foci which initiate, and the NP growth because in ethylene-insensitive transformants numerous nodules formed in front of the phloem and the number of nodule foci and mature nodules increased (Lohar et al., 2009). Ethylene affects also the position of the nodules along the primary root length as Prayitno and Mathesius (2010), by testing the effect of AVG and silver, an antagonist of ethylene action, on the nodulation zone extent, found that AVG lengthens the zone but silver shortens it in WT, whereas in *Mtskl* AVG has no effect but silver reduces the length of that zone significantly. This effect may be mediated by auxin as ethylene was shown to mediate auxin transport from the shoot to the nodulation zone via EIN2. In *Mtskl*, auxin transport is enhanced resulting in high auxin concentrations in the zone where nodules would initiate, likely promoting NP formation; this would indicate an antagonistic action for the two hormones (Prayitno et al., 2006b).

In the mutant *Ljrel3* (for Reduced Leaflet 3), mutated in an ortholog of *AtAGO7* (ArGOnaute), rhizobial infections are reduced compared to WT and fewer nodules form (Li et al., 2014). *Ljrel3* is likely sensitive to ethylene and may be over-producing it because when *Ljrel3* is treated with AVG or

silver, nodulation is restored. Inoculated *Ljrel3* mutants exhibit decreased sensitivity to exogenous auxin, increased sensitivity to auxin transport inhibitors, and increased expression of *ARF3* and *ARF4* (Auxin Response Factor), the gene-products of which are known to be specific targets of REL3 (Li et al., 2014). Additionally, LjREL3 is localized in the root and nodule vascular tissues. As REL3 is a key player in the biogenesis of *TAS3* ta-siRNAs [trans-acting s(hort) i(nterfering) RNAs], Li et al. (2014) proposed that the REL3-derived *TAS3* tasiR-ARF pathway regulates auxin response and transport during nodulation, and that this control is mediated by ethylene. I would go further and propose that via the ta-siRNA pathway, auxin and ethylene are working together in NP positioning. This hypothesis is based on the following: (1) mature ta-siRNAs are mobile molecules involved in developmental patterning and thought to traffic for a short distance from the phloem to target cells where they silence gene expression (Chitwood and Timmermans, 2010), (2) the location of the auxin peak levels and that of ethylene production are both important in determining where nodules are formed, and (3) ethylene could mediate such an action because its low solubility in the aqueous environment of the cell would preclude its action over long distances (Penmetsa et al., 2008).

To complete this section on the ethylene effect on the cortical nodulation program, it is interesting to note that *Mtbit1-2*, an ERN1mutant (**Figure 1**), exhibits atypical root development as its cortical cells are shorter and wider than those of WT, with some cells apparently going through programmed cell death since sporadic intercellular spaces form in its cortex (Middleton et al., 2007), all characteristics of ethylene-hypersensitivity. As such, it may be worthy to characterize this mutant in more depth.

Nodule Functioning and Senescence

Ethylene is likely to play a role in bacterial release (star 8, **Figure 2**) as the bacteroid number per symbiosome was increased in ethylene-insensitive transgenic *Lotus* (Lohar et al., 2009). Furthermore the hormone must be important for bacteria elongation since for this event to occur, *MtNAC074* transcripts need to accumulate (star 9, **Figure 2**) and this cannot happen if ethylene biosynthesis and signaling are altered in any way (Karmarkar, 2014). Ethylene appears also essential for bacteroid senescence. For this step to take place, ethylene must activate *MtNAC920* (star 10, **Figure 2**) which is a positive senescence regulator; once the TF is up-regulated, it must bind to MtCP2 for the nodule to senesce (Karmarkar, 2014). It is worth mentioning here that several plant mutants exhibit early senescence (Serova and Tsyganov, 2014); however, to my knowledge, none of these mutants have been linked to ethylene.

Here again, ethylene appears to work in concert with other hormones. Puppo et al. (2005) proposed that ABA and ethylene work together to orchestrate this important step where nutrients from both plant cells and rhizobia are recycled. Whereas ABA would guarantee that the nodule defenses are strong to avoid disease and attack, ethylene would trigger remobilization processes (Puppo et al., 2005). However, in their transcriptomics study on nodule senescence, Van de Velde et al. (2006) did not mention any transcripts related to ABA. In that study, JA, GA, and ethylene are highlighted as playing a major role. The role of ethylene is considered as positive since SAMS, ACO, and several ERF TFs are up-regulated. Nevertheless, SAMS transcripts may also have indicated polyamine biosynthesis, especially since spermidine synthetase induction was noted (Van de Velde et al., 2006).

NODULATION, ETHYLENE, AND AGRICULTURE

Because of space constraint and despite the importance of this topic, I will not enter into detail here. Today, to alleviate the problem of feeding an ever-growing population, alternatives or supplementation to chemical fertilizers are continuously sought after. Thus, in the literature, one can see reports demonstrating the beneficial effect of adding compounds to the soils [e.g., Fe supplementation to soils to enhance symbiotic nitrogen fixation process (Arora et al., 2010); L-methionine addition to improve crop yield (Aziz et al., 2015)]. I would like to give below three examples illustrating that, from my point of view, our knowledge about ethylene and nodulation is still too limited to propose beneficial applications in agriculture, especially since we do not know the effects ethylene has on other rhizosphere microorganisms.

Because ethylene is a negative regulator of nodulation, scientists have used plant growth-promoting bacteria (PGPBs) possessing the enzyme ACC deaminase (**Figure 3**) as a mean of reducing plant ACC, thus preventing plant ethylene evolution. It is thought that ACC deaminase acts not on the ACC pool present in the plant at the time of inoculation but on any ACC molecules synthesized afterward (Gamalero and Glick, 2015). These two different pools of ACC reflect the different peaks of ethylene observed upon rhizobial inoculation of plants (Ligero et al., 1986). Thus ACC deaminase would decrease the later synthesized-ethylene and not the early transient peak of ethylene seen in the host immune response. This means that ACC deaminase would not interfere with the plant defense responses but may allow more nodules to form. Yet, not everyone agrees with this beneficial role of rhizobial ACC deaminase. For example, Murset et al. (2012) studying the *Bradyrhizobium japonicum*-soybean interaction questioned whether or not the true substrate of ACC deaminase is indeed ACC; in their study the bacterial protein uses ACC as well as D-serine, albeit less efficiently. The authors suggested another role for ACC deaminase as they observed that its gene was strongly up-regulated under micro-oxic conditions; they proposed that the enzyme acts in controlling anoxic metabolism and would be important in nodule functioning, rather than in alleviating plant ethylene levels (Murset et al., 2012).

Salt stress is detrimental to nodulation as it can reduce nitrogenase activity by up to 75% that of control plants. Plants respond to such stress by accumulating polyamines, recognized to stabilize cell structure and membranes, scavenge free radicals, and trigger antioxidant metabolism. Supplementation of soils with SA has been considered to help plants withstand such a

stress. Working on *M. sativa*, Palma et al. (2013) demonstrated that SA addition ahead of salt treatment greatly improves plant growth and allows full recovery of nitrogenase activity. Thus, no polyamines accumulated in the nodules and lipid peroxidation enzymes were up-regulated. Furthermore, nodular ACC levels were increased significantly, suggesting that ethylene production was increased. The authors suggested that the SA-treated plants alleviate the salt stress by increasing nodule antioxidant metabolism and rerouting SAM into ethylene biosynthesis rather than polyamine synthesis (Palma et al., 2013). Unfortunately, the authors did not elaborate on how the plant and its nodules dealt with the ethylene thus synthesized.

Finally, as the rhizosphere is a complex environment, supplementing fertilizers with compounds interfering with ethylene evolution may have a positive effect on the rhizobia but a negative effect on other microorganisms. For example, Jones et al. (2015) studying the mutant *Pssym15* demonstrated that mycorrhizal fungi and rhizobia appear to be controlled differently as they enter the root cortex. In that pea mutant, whereas bacterial entry is negatively affected at the epidermal-cortical interface, fungal entry appears to be promoted. As mentioned before, it is thought that ethylene interacts with cytokinin at that interface. So if we were to alter the ethylene levels of the plant in agricultural soils to improve nodulation, we may interfere in a detrimental manner with other microorganisms such as the mycorrhizal fungi. To transfer our knowledge to the field, we must make a shift in our way of thinking, so that we consider the entire ecosystem and not just one organism.

CONCLUSION

Here, I have described many studies which demonstrate that ethylene is crucial for the proper development of the rhizobia-legume mutualism. Yet this hormone does not act alone. NFs made by the rhizobia create an upheaval in the root; once they are perceived, ethylene biosynthesis and signaling are induced resulting in the activation of enzymes responsible for the synthesis of many hormones such as auxin (Breakspear et al., 2014), cytokinin, GA, ABA, and strigolactones (Larrainzar et al., 2015). Ethylene has been considered for several decades as a negative regulator for both early and late stages of nodulation. Now we are discovering that it plays positive parts in the symbiosis as it up-regulates transcription at certain steps of the process. Alone or in concert with other hormones, it is involved in host immune responses, nodule organogenesis, nodule positioning, bacterial differentiation, and nodule senescence.

As final remarks, I would like to highlight some of the directions which could be taken to advance this field of study. First and foremost, we must create more ethylene-related legume mutants. To my knowledge, apart from *Mtskl*, there are two legume mutants which overproduce ethylene. Both *Pssym17* and *Psna-1* mutants, displaying few and tiny nodules, produce twice as much ethylene as their WT (Lee and LaRue, 1992b, and Ferguson et al., 2011, respectively). With new genomic tools available, it may be time to look at these two mutants again. Second, it may be worthy to target specific ethylene-related genes, i.e., *ACS5*, *CTR1*, and *EIN3*, in model legumes, so that mutants could be created for in-depth characterization to extend our views beyond EIN2. Third, when phenotyping mutants, we should use a common "template" and measure the same traits so that useful comparisons can be made. Using Xiao et al.'s (2014) fate map as a tool would also be valuable. Fourth, transcriptomic studies should be accompanied whenever possible by proteomics studies as post-transcription controls are abundant. Although not related to ethylene, a good example for the necessity of performing protein tests (proteomics, enzyme activity, etc. . .) is given by Lang and Long (2015) who mentioned two mutants with white nodules exhibiting leghemoglobin transcript levels similar to those of WT. Finally, as many unsuspected hormonal cross-talks are being uncovered in transcriptomics studies, it is becoming essential that these are complemented by in-depth hormonal studies where the hormones in question, foremost ethylene, are measured. Each experiment should comprise well-designed controls so that it is ensured that the effects reported in the mutants/treated plants are only those directly linked to ethylene.

AUTHOR CONTRIBUTION

FG is the sole author of this manuscript.

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Ethylene and plant responses to phosphate deficiency

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Phosphorus is an essential macronutrient for plant growth and development. Phosphate (Pi), the major form of phosphorus that plants take up through roots, however, is limited in most soils. To cope with Pi deficiency, plants activate an array of adaptive responses to reprioritize internal Pi use and enhance external Pi acquisition. These responses are modulated by sophisticated regulatory networks through both local and systemic signaling, but the signaling mechanisms are poorly understood. Early studies suggested that the phytohormone ethylene plays a key role in Pi deficiency-induced remodeling of root system architecture. Recently, ethylene was also shown to be involved in the regulation of other signature responses of plants to Pi deficiency. In this article, we review how researchers have used pharmacological and genetic approaches to dissect the roles of ethylene in regulating Pi deficiency-induced developmental and physiological changes. The interactions between ethylene and other signaling molecules, such as sucrose, auxin, and microRNA399, in the control of plant Pi responses are also examined. Finally, we provide a perspective for the future research in this field.

Keywords: ethylene, phosphate responses, root architecture, transcriptional regulation, signaling, crosstalk

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Introduction

Plants are sessile organisms that acquire essential mineral nutrients from soils through their roots. Plants often encounter nutrient deficiency in natural ecosystems and in agricultural lands. P (Phosphorus) is an essential macronutrient for plant growth, development, and metabolism. Inorganic phosphate, the major form of P that plants assimilate, however, is highly immobile in most soils because it is converted to organophosphates by microorganisms or is fixed

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC OXIDASE; ACP5, ACID PHOSPHATASE TYPE 5; ACS, ACC SYNTHASE; AdoMet, *S*-adenosyl methionine; APase, acid phosphatase; AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR; At4, *Arabidopsis thaliana* cDNA 4; ARF19, AUXIN RESPONSE FACTOR 19; AVG, aminoethoxyvinyl glycine; CDPK, CALCIUM-DEPENDENT PROTEIN KINASE; CHIB, CHITINASE B; CR, cluster root; CTR1, CONSTITUTIVE TRIPLE RESPONSE 1; EBF, EIN3-BINDING F-BOX; EIL1, EIN3-LIKE 1; EIN2, ETHYLENE INSENSITIVE 2; EIN3, ETHYLENE INSENSITIVE 3; EIN4, ETHYLENE INSENSITIVE 4; FLS2, FLAGELLIN SENSITIVE 2; ER, endoplasmic reticulum; ERS1, ETHYLENE RESPONSE SENSOR 1; ETR1, ETHYLENE RESPONSE 1; ETR1, ETHYLENE RESPONSE 1; ETO1, ETHYLENE OVERPRODUCTION 1; hps, hypersensitive to phosphate starvation; IPS1, INDUCED BY PHOSPHATE STARVATION 1; LPR1, LOW PHOSPHATE ROOT 1; LUC, luciferase; MAPK, MITOGEN-ACTIVATED PROTEIN KINASE; MCP, 1-methylcyclopropene; PAP10, PURPLE ACID PHOSPHATASE 10; PDR2, PHOSPHATE DEFICIENCY RESPONSE 2; PHL1, PHR1-LIKE 1; PHO2, PHOSPHATE 2; PHR1, PHOSPHATE STARVATION RESPONSE 1; Pht1(AtPT1), PHOSPHATE TRANSPORTER 1; Pi, phosphate; PORA, PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A; PSI, phosphate starvation induced; RNS1, RIBONUCLEASE 1; RSA, root system architecture; SCR, SCARECROW; SHR, SHORT-ROOT; SUC2, SUCROSE TRANSPORTER 2; TIR1, TRANSPORT INHIBITOR RESPONSE 1.

with metals (Bieleski, 1973). Although P is abundant in most soils, the availability of Pi for plant uptake is quite low (Raghothama, 2000). Pi deficiency has become one of the most important constraints on agricultural productivity.

To cope with Pi deficiency, plants have evolved elaborate strategies to enhance acquisition and utilization of Pi from the environment and to conserve and reprioritize the internal use of Pi through recycling and redistribution processes. The major plant responses to Pi deficiency include: the active remodeling of RSA; the reduction in photosynthesis; the enhancement of high-affinity Pi transporter activities; the induction and secretion of APases, ribonucleases, and organic acids; the replacement of phospholipids in membranes with glycolipids and sulfolipids; and the accumulation of anthocyanin and starch (Vance et al., 2003; Yuan and Liu, 2008; **Figure 1**). These responses are modulated by sophisticated regulatory networks through both local and systemic signaling in which phytohormones play important roles (Chiou and Lin, 2011).

Ethylene, which is one of five classic phytohormones, regulates multiple aspects of plant development, such as seed germination, root growth, leaf abscission and senescence, and fruit ripening, as well as plant responses to biotic and abiotic stresses (Abeles et al., 1992). Ethylene was previously shown to be involved in Pi deficiency-induced remodeling of RSA. Recent research indicates that in addition to being a regulator of root growth, ethylene also participates in other plant responses to Pi deficiency (Nagarajan and Smith, 2012; Roldan et al., 2013). In this article, we will review the current knowledge about the roles of ethylene in plant responses to Pi deficiency. We will also provide a perspective about how research might increase our understanding of the molecular mechanisms by which ethylene regulates plant Pi responses.

Ethylene Biosynthetic and Signaling Pathways

The ethylene biosynthetic pathway has been well described in higher plants (Kende, 1993; Xu and Zhang, 2015; **Figure 2A**). Ethylene is produced from methionine, which is first converted to AdoMet by AdoMet synthetase. The next two steps are the conversion of AdoMet to ACC and the oxidative cleavage of ACC to form ethylene. The enzymes that catalyze these two reactions are ACS and ACO. Once ACC is formed in plant cells, it is automatically converted to ethylene by ACO in the presence of oxygen. The regulation of ethylene biosynthesis can be achieved by altering gene expression, protein stability, and enzymatic activity of ACS and ACO (McKeon et al., 1995; Bleecker and Kende, 2000).

The ethylene signaling pathway in *Arabidopsis* has provided a framework for the action mechanism of this hormone in higher plants (Zhao and Guo, 2011; Wen et al., 2015). In *Arabidopsis*, ethylene is perceived by its receptors, which are ETR1, ETR2, ERS1, ERS2, and EIN4 located in ER membrane. When ethylene levels are low, these five receptors activate the downstream component CTR1 through direct physical interaction. CTR1 belongs to the Raf-1 family of Ser/Thr protein kinases. The activated CTR1 suppresses the function of its target EIN2, an ER membrane-localized protein. When ethylene binds to the receptors, it disrupts the interaction between the receptors and CTR1, which inactivates CTR1. Therefore, the suppression of EIN2 by CTR1 is released. Next, a EIN2-C′ (C-terminal fragment of EIN2) is translocated to the nucleus. In the nucleus, EIN2-C′ enhances the levels of EIN3 and EIL1, two key transcription factors of the ethylene signaling pathway. EIN3 and EIL1 further activate the transcription of downstream target genes, such as ERFs, CHIB, PORA, and FLS2. Finally, the activation of these genes initiates a diverse array of plant responses to internal and external signals. In the absence of ethylene, EIN3 and EIL1 are degraded by the F-box proteins EBF1 (EIN3 BINDING F-BOX PROTEIN 1) and EBF2 through a 26S proteasome-mediated degradation pathway.

Ethylene's Role in Plant Responses to Pi Deficiency

Pi Deficiency Alters Ethylene Biosynthesis

It has long been observed that Pi deficiency alters ethylene biosynthesis in plants. Some reports indicated that ethylene production was decreased in Pi-starved maize and tomato (Drew et al., 1989; Kim et al., 2008). In contrast, the increase of ethylene production was found in the roots of common bean under Pi deficiency (*Phaseolus vulgaris*), white lupin (*Lupinus albus*), and *Medicago falcata* (Borch et al., 1999; Gilbert et al., 2000; Li et al., 2009). Using quantitative reverse-transcription PCR (RT-qPCR), Lei et al. (2011b) showed that the expression of three members of the *ACS* gene family, *ACS2*, *ACS4*, and *ACS6*, was enhanced in *Arabidopsis* seedlings grown on a Pi-deficient medium. The enhanced expression of ethylene biosynthetic genes, mainly *ACS* and *ACO*, in Pi-starved *Arabidopsis* plants has also been demonstrated in several microarray and RNA-seq analyses (Misson et al., 2005; Morcuende et al., 2007; Calderon-Vazquez et al., 2008; Thibaud et al., 2010; Chacón-López et al., 2011; O'Rourke et al., 2013; Kang et al., 2014; Wang et al., 2014b). Morcuende et al. (2007) further found that the enhanced expression of *ACS2* and *ACS6* genes was reversed when Pi-deficient plants were resupplied with an adequate amount of Pi, indicating a causal relationship between the expression of these genes and the levels of Pi in the environment. The up-regulation of *ACO* genes by Pi deficiency was also detected in a variety of plant species, including common bean (Graham et al., 2006; Hernández et al., 2007), white lupin (Uhde-Stone et al., 2003; Wang et al., 2014b), and white clover (*Trifolium repens*; Roldan et al., 2013). In the plants examined, not all members of *ACS* and *ACO* families respond to Pi deficiency in the same manner. Thus, it is important to know how the transcription of different members of *ACS* and *ACO* gene families is affected by Pi deficiency in specific tissues and at specific developmental stages. Such information will increase our understanding of how ethylene biosynthesis is regulated in a spatiotemporal manner by Pi deficiency.

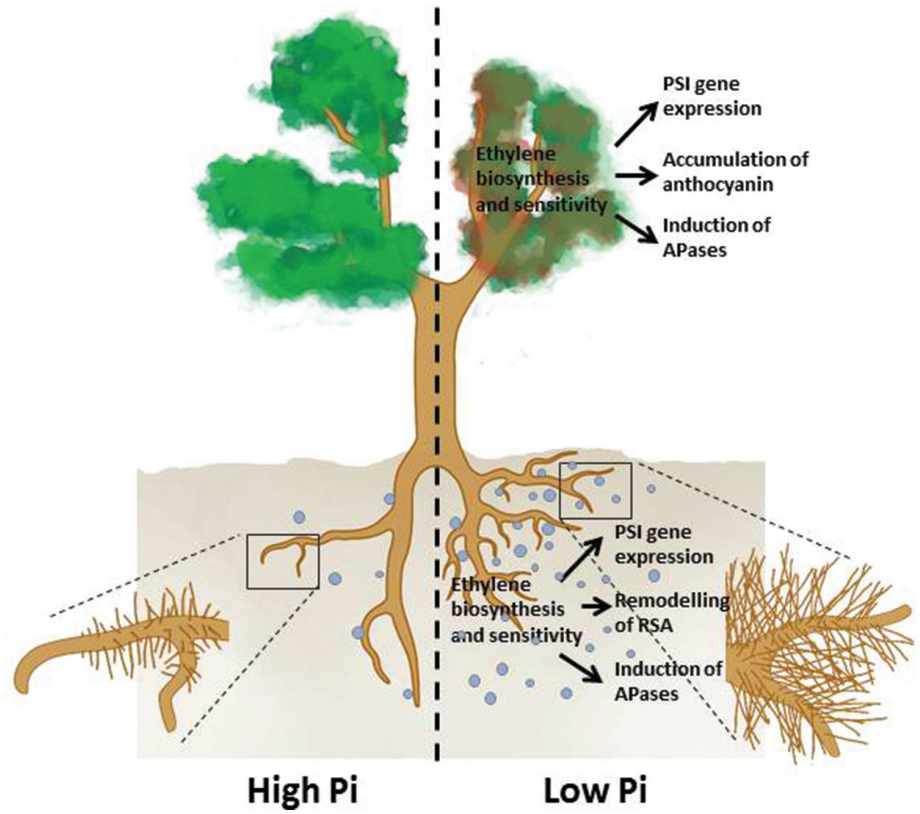

FIGURE 1 | An overview of the plant responses to Pi deficiency that involve ethylene. Under Pi deficiency, primary root growth is inhibited, production of lateral roots and root hairs is enhanced (remodeling of RSA) APases are induced, expression of PSI genes is increased in both roots and shoots, and shoots accumulate more anthocyanins. The light blue dots denote root exudates, including APases, RNases, organic acids, and protons.

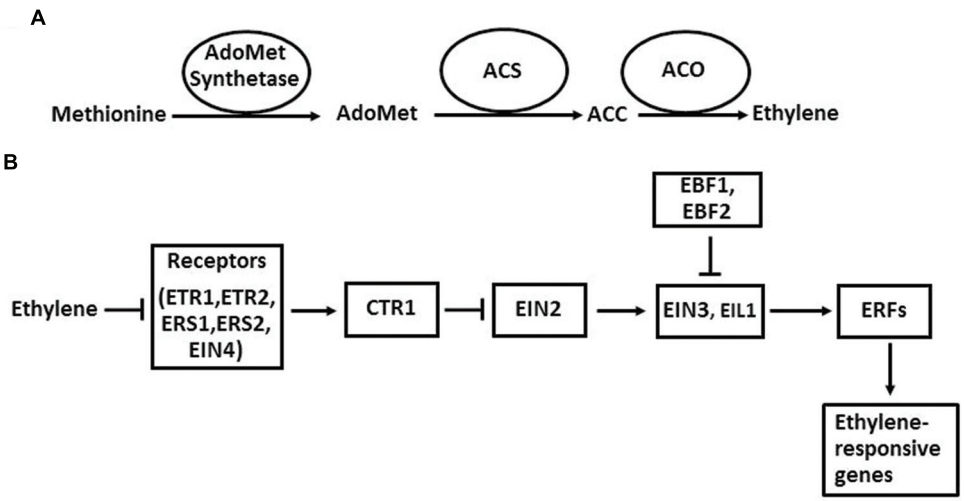

FIGURE 2 | The ethylene biosynthesis pathway in plants in general (A) and the ethylene signaling pathway in *Arabidopsis* (B). Arrows indicate promotion, and perpendicular lines indicate inhibition.

In *Arabidopsis*, PHR1 is a MYB-type transcription factor that binds to a *cis*-element with the imperfect palindromic sequence GNATATNC, a sequence that is prevalent in the promoters of PSI (Pi starvation-induced) genes (Rubio et al., 2001). PHR1 is regarded as a central regulator for transcriptional responses of plants to Pi starvation (Bustos et al., 2010). In *Arabidopsis*,

PHL1 is a close relative of PHR1. In the *phr1phl1* double mutant, the induction of most PSI genes, including *ACS6* and *ACS7*, is impaired to different extents (Bustos et al., 2010). These results indicate that the increased expression of these two *ACS* genes is modulated by the central regulatory pathway of Pi responses. Interestingly, the expression of another two PSI *ACS* genes, *ACS2* and *ACS4*, is independent of the PHR1 pathway (Bustos et al., 2010), suggesting that the regulation of different members of the *ACS* family is mediated by different signaling pathways.

Pi Deficiency Enhances Plant Sensitivity to Ethylene

In addition to altering ethylene biosynthesis, Pi deficiency also alters plant sensitivity to ethylene. Pi deficiency-enhanced ethylene sensitivity has been reported for adventitious roots of maize seedlings (He et al., 1992), basal roots of common bean (Basu et al., 2007), and lateral roots of white clover (Dinh et al., 2012). The enhanced ethylene sensitivity is also reflected in the induction of gene expression by Pi deficiency (Lei et al., 2011b) as discussed in more detail later in this article. The enhanced ethylene sensitivity in Pi-starved plants is probably achieved through the alteration of the expression of the genes that encode molecular components involved in the ethylene signaling pathway. ERFs are a group of AP2 (APETALA2) domain-containing transcription factors that bind to ethylene-responsive elements present in the promoters of many ethylene-responsive genes. These transcription factors serve either as activators or repressors of ethylene-mediated transcription. Some transcriptomic analyses have shown that the expression of several *ERF* genes, including *ERF1*, *ERF2*, and *ERF5*, is altered in Pi-starved *Arabidopsis* roots (Wang et al., 2002; Misson et al., 2005; Thibaud et al., 2010; Chacón-López et al., 2011; Kang et al., 2014). In the *phr1phl1* double mutant, the expression of at least eight *AP2/ERF* genes is attenuated (Bustos et al., 2010). Another mechanism for enhancing ethylene sensitivity may involve a change of protein abundance of some key components in the ethylene signaling pathway. When plants were grown under salinity conditions, the accumulation of EIN3 protein was increased while that of EBFs, which mediate EIN3 protein degradation, was decreased (Peng et al., 2014). Whether this is also the case for Pi-deficient plants requires investigation.

Ethylene and Root Responses to Pi Starvation

When plants are grown under Pi deficiency, their RSA undergoes a dramatic change, i.e., a remodeling. The Pi deficiency-induced remodeling of RSA includes a cessation of primary root growth and an enhanced production of root hairs and lateral roots (López-Bucio et al., 2003). For maize and some species in the *Proteaceae* and *Casuarinaceae* families, the remodeling of RSA involves a production of adventitious roots and cluster-roots (CRs; He et al., 1992; Wang et al., 2015). Such remodeling of RSA results in an increase in root surface area for Pi absorption. The remodeling starts with a reduction of cell elongation followed by the progressive loss of meristematic cells, i.e., "meristem exhaustion" (Sánchez-Calderón et al., 2005). At later stages, cell proliferation is arrested, and cell differentiation takes place at the former meristematic and elongation regions of the primary root. In some plant species, the angle between the basal root and primary root is increased, which enhances the capacity of roots to forage Pi in the top soil (Basu et al., 2007). The remodeling of RSA triggered by Pi deficiency is thought to be an active developmental response controlled by internal genetic programs because *Arabidopsis* mutants *lpi* and *lpr1* show normal growth of primary roots with an unexhausted meristem under Pi deficiency (Sánchez-Calderón et al., 2006; Svistoonoff et al., 2007).

Under normal growth conditions, application of ethylene (or ACC) to *Arabidopsis* plants inhibits primary root growth and enhances root hair production. This treatment results in meristem exhaustion of the primary root. *Arabidopsis* mutant that overproduces ethylene (*eto1*; Wang et al., 2004) or that has constitutive *ctr1* (Kieber et al., 1993) also exhibit reduced primary root growth and increased production of root hairs. These ethylene-induced root growth phenotypes mimic the plant root responses triggered by low Pi, suggesting that ethylene biosynthesis and signaling are involved in the Pi deficiency-triggered remodeling of RSA.

The roles of ethylene in Pi deficiency-induced remodeling of RSA have been investigated using both pharmacological and genetic approaches. Regarding the effect of ethylene on primary root growth under Pi deficiency, Chacón-López et al. (2011) found that inhibiting ethylene biosynthesis with AVG or ethylene perception with Ag^+ restricted the low Pi-induced meristem exhaustion of the primary root. This suggested that ethylene is involved in the Pi deficiency-induced inhibition of primary root growth. Our laboratory also found that application of Ag^+ reduces the inhibition of primary root growth triggered by Pi deficiency (Yu et al., 2012). The *Arabidopsis* mutant *hps4* (*hypersensitive to Pi starvation 4*) shows enhanced sensitivity to Pi deficiency in terms of Pi deficiency-induced inhibition of primary root growth and induction of APase activity. Under Pi sufficiency, the primary root of *hps4* was about 80% as long as WT (wild type) plants. Under Pi deficiency, the primary root growth was reduced for both the WT and *hps4*; however, this reduction was much greater for *hps4* than for the WT. *HPS4* encodes the SABRE protein. Although the precise biochemical function of SABRE is unknown, SABRE has been shown to antagonistically interact with ethylene to regulate root cell expansion (Aeschbacher et al., 1995). The hypersensitivity to Pi deficiency-induced inhibition of primary root growth is diminished when the *hps4* mutant is treated with Ag^+ but not with AVG. Nagarajan et al. (2011) showed that overexpression of an *Arabidopsis* high-affinity Pi transporter, Pht1:5, reduced primary root growth and increased root hair production under both Pi sufficiency and deficiency. These phenotypes could be reversed by application of AVG or Ag^+, indicating that ethylene biosynthesis had been altered in the *Pht1:5*-overexpressing lines. The *Pht1:5*-overexpressing lines also showed a disruption of the shoot to root Pi ratio, suggesting that an altered Pi homeostasis might enhance ethylene biosynthesis and/or signaling, which in turn, might modulate primary root growth. More experimental evidence is needed, however, to support this proposed link between altered Pi homeostasis and enhanced ethylene biosynthesis and/or signaling. Interestingly, when Ma et al. (2003) investigated the role of ethylene in Pi

starvation-induced inhibition of primary root growth, they found that inhibition of ethylene production by AVG or inhibition of ethylene action by MCP increased primary root growth under high Pi but decreased primary root growth under low Pi. This result differs from the observations of López-Bucio et al. (2002) and Yu et al. (2012). The discrepancy could be due to the differences in experimental conditions used by the research groups. For example, the degree of low Pi stress in the different media differed, and whether the effect of ethylene on primary root growth is stimulatory or inhibitory could depend on a subtle difference in Pi concentrations, even though all concentrations were low. In fact, ethylene can be as both a promoter or an inhibitor for root growth depending on its concentration (Pierik et al., 2006). Thus, after inhibitors were applied in the experiments of Ma et al. (2003), the concentration of ethylene in Pi-deficient roots may have been far below the optimal level for sustained primary root growth under Pi deficiency.

In young maize seedlings, Pi deficiency induces the formation of aerenchyma (tissue with large cortical gas spaces) in their adventitious roots (He et al., 1992). When Ag^+ or AVG was added to the nutrient solution, the formation of aerenchyma was blocked. Furthermore, when ethylene was added to the air of the growth chamber at a concentration as low as 1.0 μL/L, the aerenchyma formation was strongly promoted in Pi-starved roots relative to Pi-sufficient roots. Because the production of ethylene was decreased in Pi-starved maize seedlings in these experiments, it seemed that ethylene perception or sensitivity rather than ethylene production was involved in the formation of aerenchyma triggered by Pi deficiency. A similar case was found for tomato plants. Low Pi induced the formation of adventitious root in WT tomato plants but not in the ethylene-insensitive cultivar "Never-ripe" (Kim et al., 2008). Pi deficiency, however, reduced ethylene production in both tomato genotypes. This again indicated that it is ethylene perception rather than ethylene production that is involved in the response of roots to Pi availability.

The effects of ethylene on lateral root formation in Pi starved-plants have also been investigated. Pi deficiency stimulates the formation of lateral roots of white clover (Dinh et al., 2012). A low concentration of ACC had little effect on the development of lateral roots under Pi sufficiency but caused a super-stimulation of lateral roots under Pi deficiency. Unlike in white clover, Pi deficiency in common bean reduced lateral root number and did not inhibit primary root growth (Borch et al., 1999). This resulted in a reduction of lateral root density. AVG treatment increased lateral root density in Pi-deficient plants but reduced lateral root density in Pi-sufficient plants. These responses could be reversed by exogenous ethylene, suggesting an involvement of ethylene in modulating lateral root formation in common bean under Pi deficiency.

When grown under Pi deprivation, most members of the *Proteaceae* and *Casuarinaceae* form CR. The formation of CR densely covered with long root hairs dramatically increases the surface area for secretion of root exudates, including organic acids, protons, and APases. This root response helps plants mobilize Pi from organophosphates or Pi fixed with metals. Application of ethylene did not induce CR formation under Pi sufficiency; however, in Pi-deficient plants, the inhibition of ethylene production by Co^{2+} completely suppressed CR formation (Wang et al., 2015). Associated with this, the authors found a moderate increase in the expression of an *ACO* gene in the pre-emergent CR but a dramatic increase in *ACO* expression in the maturing CR.

In many soils, Pi availability is greatest in the upper layers and decreases with depth. Analyses of different genotypes of bean indicated that the degree of growth angle of basal roots was closely correlated with the extent of Pi acquisition of plants under low Pi availability (Basu et al., 2007). Ethylene sensitivity was higher for plants grown under Pi deficiency than under Pi sufficiency, and basal roots produced from the uppermost whorl were more sensitive to ethylene than those from the lower-most whorl. Thus, the growth angle of basal roots was strongly correlated with ethylene sensitivity but not with ethylene production.

The earliest visible change in the morphology of roots responding to Pi deficiency is the enhanced production of root hairs, including an increase in both root hair density and root hair length (Bates and Lynch, 1996). The increase in root hair length is due to an increase in both growth rate and growth duration. In *Arabidopsis*, root hairs are produced from H cells that are located over the intercellular space between two underlying adjacent cortical cells; however, not all H cells form root hairs. Under normal growth conditions, treatment with ACC or a mutation in the *CTR1* and *ETO1* genes causes a dramatic increase in root hair production that mimics the effect of Pi deficiency. Schmidt and Schikora (2001) found that low Pi could not fully restore the root hair density of *ein2* and *etr1* mutants to that of the WT. And, for the three ethylene biosynthesis inhibitors that they used (AVG, Co^{2+}, and aminooxyacetic acid), only Co^{2+} significantly blocked the Pi deficiency-induced increase in the root hair density of the WT plants. Thus, the authors concluded that the canonical ethylene signaling pathway was not involved in the development of extra root hairs in response to Pi deficiency. However, the research of Zhang et al. (2003) strongly suggested that ethylene is involved in the Pi deficiency-enhanced production of root hairs. These authors found that under Pi deficiency, treatment with inhibitors of either ethylene biosynthesis or ethylene signaling significantly reduced root hair density and root hair length. Although all ethylene-insensitive mutants still responded to Pi deficiency with increased root hair density and length, the extent of the increase was much lower than that of the WT. These results indicated that ethylene is indeed involved in the enhanced production of root hairs induced by Pi deficiency.

Anatomic examination, however, revealed some similarities and differences in the effects of ethylene and Pi deficiency on root hair formation (Zhang et al., 2003). The similarity is that both low Pi and ethylene shortened the length of trichoblast cells and that AVG added to Pi-deficient plants increased the length of trichoblast cells. The differences include: (1) Low Pi increases the number of cortical cells, but ethylene does not; (2) Ethylene increase the percentage of H cells that form root hairs, but low Pi does not. These differences suggested that low Pi and ethylene may use the different gene activation mechanisms to regulate root hair formation. It will be interest to compare

the transcriptomic changes in Pi-starved and ethylene-treated roots to identify the common and distinct targets of low Pi and ethylene. These information will help us further understand the molecular mechanisms of how ethylene mediates the root hair growth under Pi deficiency.

Further analysis demonstrated that there is an interaction between low Pi and ethylene in regulating both root hair length and root hair density (Zhang et al., 2003). The reduction in trichoblast length in *ein2* and *ein4* was stronger with low Pi than with high Pi, indicating that the degree to which ethylene affects extra root hair production depends on Pi availability. And, *ein2* and *ein4* mutants or the WT treated with AVG had greater reduction of H cells forming hairs under low Pi than under high Pi. In addition, under Pi deficiency, all ethylene-insensitive mutants clearly showed a reduction in root hair length, but the reduction varied for different mutants under high Pi. Cho and Cosgrove (2002) found that root hair elongation for ethylene-insensitive mutants or for plants treated with AVG was relatively normal under high Pi but was reduced under low Pi, providing additional evidence of an interaction between ethylene and Pi availability.

Ethylene and Pi Transcriptional Regulation

In searching for molecular components involved in transcriptional responses of plants to Pi starvation, Lei et al. (2011b) performed a screen for *Arabidopsis* mutants with altered transcriptional response. They used a transgenic line that carries a LUC gene fused to the promoter of the high-affinity Pi transporter AtPT2. The transcription of *AtPT2* is induced by Pi starvation. Using this marker line, the authors identified the *Arabidopsis* mutant *hps2* (*hypersensitive to Pi starvation2*), which showed hyper-induction of the *AtPT2::LUC* gene by Pi deficiency. *hps2* is a new allele of the *CTR1* gene (**Figure 2B**). Furthermore, under Pi deficiency, treatment of *Arabidopsis* plants with Ag^+ suppressed the induction of *AtPT2* whereas the addition of ACC dramatically enhanced its expression. Accordingly, the expression of *AtPT2* was partially blocked in *ein2* but was enhanced in *eto1*. A similar expression pattern was observed for several other PSI genes in the *hps2* and *ein2* mutants. These PSI genes included another high-affinity phosphate transporter, *AtPT1* (*Pht1; 1*; Muchhal et al., 1996); a non-coding transcript, *At4* (Burleigh and Harrison, 1999); an APase, *ACP5* (del Pozo et al., 1999); a ribonuclease, *RNS1* (Bariola et al., 1994; and *miR399d*, Fujii et al., 2005). The enhanced transcription of these PSI genes was also observed in the mutant *hps3*, which is another allele of *ETO1* (Wang et al., 2012), and in the mutant *hps4*, which has enhanced ethylene signaling (Yu et al., 2012). ETO1 protein is a member of the broad complex/tramtrack/bric-a-brac (BTB) protein superfamily that participates in substrate recognition during ubiquitin-mediated protein degradation (Christians et al., 2009). It directly binds to the C-terminal of ACS5 and mediates its degradation. When ETO1 is mutated, it causes an overproduction of ethylene in young seedlings (Wang et al., 2004). These results provided the first genetic evidence that ethylene signaling is involved in the transcriptional responses of plants to Pi deficiency. In another study using *M. falcata*, ACC induced the expression of the Pi transporter genes *MfPT1* and *MfPT5* under Pi-sufficient conditions, whereas both AVG and Co^{2+} blocked the low Pi-induced expression of these genes (Li et al., 2011). Taken together, these results indicate that ethylene positively regulates transcription of a subset of PSI genes. The incomplete blockage of PSI gene expression in the *ein2* mutant also indicates that ethylene is not the only mediator for the expression of these genes.

Interestingly, application of 25 μM ACC to young *Arabidopsis* seedlings under high Pi conditions barely induced the expression of *AtPT2*; under Pi deficiency, however, 0.5 μM ACC dramatically increased *AtPT2* expression (Lei et al., 2011b). These results suggested a synergistic interaction between low Pi and ethylene in mediating transcriptional Pi responses. This also provided another example of plant cells being sensitized to ethylene by low Pi.

Ethylene and Induction of Acid Phosphatases

Induction and secretion of APases is a universal response of plants to Pi deficiency (Tran et al., 2010). The intracellular APases are believed to be involved in the remobilization of Pi from senescing tissues to young growing tissues whereas secreted APases are thought to be important for releasing Pi from organophosphates in the rhizosphere and thus increasing Pi availability for root uptake. The secreted APases are further classified into two groups: one group is released into the environment, and the other is tightly associated with the root surface after secretion. Members of the second group are called root-associated APases. Lei et al. (2011b) found that *hps2* also had enhanced APase activity on the root surface. In contrast, *ein2* exhibited reduced root-associated APase activity under Pi deficiency, indicating that ethylene is a positive regulator of PSI APase activity. The role of ethylene in regulating APase activity was further confirmed by the analyses of mutants *hps3* and *hps4* (Wang et al., 2012; Yu et al., 2012). Treatment with Ag^+ suppressed the enhanced APase activity in *hps3* and *hps4*. Similarly, Li et al. (2011) showed that the induction of root APase activity in *M. falcata* was stimulated by ACC under Pi sufficiency but was blocked by AVG under Pi deficiency.

In *Arabidopsis*, AtPAP10 is a major PSI APase that is predominantly associated with the root surface after secretion (Wang et al., 2011, 2014a). Zhang et al. (2014) investigated how ethylene affects root-associated AtPAP10 activity at different regulatory steps. The transcription of *AtPAP10* was previously found to be increased by Pi starvation in the whole seedlings of *hps3* and *hps4* (Wang et al., 2012; Yu et al., 2012). The total root intracellular APase activity in *hps3* and *hps4*, however, did not significantly differ from that of the WT. When the transcription of *AtPAP10* was further analyzed using separated root and shoot tissues, Zhang et al. (2014) found that the transcription of *AtPAP10* did not significantly increase in ACC-treated seedlings or the *ctr1* mutant under Pi deficiency, nor did the accumulation of AtPAP10 proteins. Taken together, these results indicated that, in roots, ethylene mainly modulated the secretion of AtPAP10 protein or its enzymatic activity on the root surface. Some reports have shown that ethylene can increase H^+-ATPase activity by up-regulating the expression of H^+-ATPase genes (Waters et al., 2007; Wang et al., 2009; Staal et al., 2011),

which may decrease the cytosolic pH or the pH on the root surface. In contrast, application of MCP blocked the change of cytosolic pH (Sundaresan et al., 2015). In humans and animals, cytosolic pH levels affect protein secretion (Paroutis et al., 2004). In addition, APase activity is sensitive to the change of pH. Thus, it is possible that ethylene may increase secretion of AtPAP10 proteins and stabilize AtPAP10 enzymatic activity on the root surface by modulating the cytosolic and root surface pH. More experimental evidence, however, is required to support this hypothesis.

Ethylene and Anthocyanin Accumulation

Accumulation of anthocyanin is another hallmark response of plants to Pi starvation. The accumulation of anthocyanin is lower in *hps2*, *hps3,* and *hps4* mutants than in the WT under low Pi conditions (Lei et al., 2011b; Wang et al., 2012; Yu et al., 2012). In contrast, the Pi-starved *ein2* mutant shows increased anthocyanin content. Ag^{+}-treated *Arabidopsis* seedlings also displayed increased accumulation of anthocyanin under Pi deficiency. Furthermore, Lei et al. (2011b) showed that the expression of four genes that encode three enzymes involved in anthocyanin biosynthetic pathway and one transcription factor regulating anthocyanin biosynthesis was increased in *ein2* but reduced in *ctr1*. These results demonstrate that ethylene is a negative regulator of Pi starvation-induced anthocyanin accumulation and that this regulation is achieved at least partly through the regulation of the transcription of the genes involved in the biosynthesis of anthocyanin.

Ethylene's Role in Local Signaling

Plant responses to Pi deficiency are controlled by a complex regulatory network involving both local and systemic signaling. Whether a Pi response is controlled by local or systemic signaling can be determined by examining whether the degree of the response depends on the local Pi level or the Pi status of the whole plant. Using this approach, Ticconi et al. (2004) found that the remodeling of RSA triggered by Pi deficiency was regulated by the local, external Pi level. They first germinated *Arabidopsis* seeds on a Pi-sufficient medium for 5 days and then transferred the seedlings to an agar plate that contained a Pi-sufficient medium in the upper half and a Pi-deficient medium in the lower half, or *vice versa*. After the roots had grown for another 6 days, lateral root production had increased in the parts of the root that contacted the Pi-deficient medium but decreased in the parts that contacted the Pi-sufficient medium. The *Arabidopsis phf1* mutant has defects in the ER exit of high-affinity Pi transporters, which greatly impairs Pi uptake by roots (González et al., 2005). When these plants were grown on a Pi-sufficient medium, their internal Pi level was only 20% of the WT, but no remodeling of RSA was observed (Thibaud et al., 2010). Furthermore, Thibaud et al. (2010) found that injection of a high concentration of Pi into the shoots of *Arabidopsis* plants grown on a Pi-deficient medium could not suppress the Pi deficiency-induced remodeling of RSA. Together, these results demonstrated that it is the local, external Pi level rather than the internal Pi status of the whole plant that regulates the remodeling of RSA. The requirement for ethylene in the Pi deficiency-induced remodeling of RSA indicates that ethylene is involved in local Pi signaling.

The *Arabidopsis* mutant defective in LPR1 and its close paralog LPR2 are insensitive to the Pi deficiency-induced inhibition of the primary root growth (Svistoonoff et al., 2007). In contrast, the *Arabidopsis* mutant with functional disruption of PDR2, which encodes a P5-type ATPase, exhibits an exaggerated short-root phenotype under Pi deficiency owing to meristem exhaustion (Ticconi et al., 2004, 2009). SCR and SHR are two key regulators of root patterning. PDR2 is required for maintaining the levels of SCR protein and SHR trafficking from stele into endodermis. Based on genetic analysis, PDR2 was proposed to act upstream of LPR1/LPR2 to adjust meristem activity in an ER-resident pathway. The roots of WT plants accumulated more Fe under Pi deficiency than under Pi sufficiency (Svistoonoff et al., 2007; Ward et al., 2008; Zheng et al., 2009; Lei et al., 2011a). On a Fe-free medium, the inhibition of primary root growth was abolished. Thus, Ward et al. (2008) hypothesized that Pi deficiency triggers the inhibition of primary root growth by enhancing the accumulation of Fe in the root meristem, which results in severe damage to root cells. In a recent study, Müller et al. (2015) demonstrated that LPR1 is a ferroxidase. The root meristem of *lpr1* contains reduced levels of Fe^{3+} under Pi deficiency, which makes the root tip growth insensitive to inhibition caused by Pi deficiency. In contrast, *pdr2* accumulates increased levels of Fe^{3+}, which generates high levels of reactive oxygen species (ROS). The high level of ROS, in turn, causes the increased deposition of callose which impairs the trafficking of SHR, thus restricting root tip growth. Ethylene positively regulates Fe homeostasis in plants by up-regulating the expression of the genes involved in Fe acquisition (Waters et al., 2007; García et al., 2011). Thus, it is also possible that ethylene mediates Pi deficiency-induced inhibition of primary root growth by enhancing Fe accumulation in root tips.

As previously mentioned, AtPAP10 is a PSI APase that is predominantly associated with the root surface after secretion. Using split-root experiments, Zhang et al. (2014) demonstrated that although the transcription of *AtPAP10* is systemically controlled (i.e., affected by the Pi status of whole plant), AtPAP10 protein accumulation and enzymatic activity on the root surface are regulated by local Pi levels. Once the mRNA of *AtPAP10* is produced, the subsequent accumulation and secretion of AtPAP10 protein and perhaps also the protein's enzymatic activity on the root surface are controlled only by local signaling. Because ethylene mainly participates in the secretion of AtPAP10 proteins or stabilization of AtPAP10 enzymatic activity on the root surface during the induction of root-associated AtPAP10 activity, ethylene could be regarded as a local signal in regulating the induction of AtPAP10 activity. This represents another example that ethylene functions in local signaling besides being involved in the control of remodeling of RSA.

Although it is now evident that ethylene is involved in local Pi sensing and signaling, the mechanism by which ethylene regulates local signaling is largely unknown. Thibaud et al. (2010) dissected the transcriptional responses controlled by local and systemic signaling. They found that the transcription of the genes involved in development, stress responses, and hormonal

signaling is controlled by local signaling. The locally regulated genes include *ERF1* and *ERF2*. These results provide further support for the role of ethylene in local Pi signaling. The next important task will be to identify the direct downstream genes that are targets of ethylene and that participate in local Pi signaling.

Ethylene's Role in Systemic Signaling

Researchers have hypothesized that when the external Pi level drops, the root tissues sense the change in Pi availability and send warning signals through the xylem to the shoots (Chiou and Lin, 2011; **Figure 3**). Once these signals arrive in shoots, they trigger Pi-deficiency responses in the shoots including enhanced PSI gene expression, reduced photosynthetic activity, inhibition of shoot growth, increased anthocyanin accumulation, and induction of intracellular APase activity. At the same time, the shoots send signals to the roots through the phloem to regulate Pi responses in roots including enhanced high-affinity Pi transporter activity and induction of APase activity. Cytokinins, strigolactones, and Pi itself have been proposed to be root-to-shoot signals, whereas sucrose and miRNA399 are believed to be the shoot-to-root signals (Lin et al., 2014). Enhanced ethylene biosynthesis and expression of ethylene biosynthetic genes in shoots have been observed under Pi deficiency (Misson et al., 2005; Kim et al., 2008), suggesting that ethylene is involved in the Pi responses in shoots. Whether the increase of ethylene biosynthesis in shoots is triggered by local sensing due to the drop of Pi levels in the shoots or by the signals from the roots, however, is not known.

Thibaud et al. (2010) showed that the transcription of PSI genes that participate in Pi transport, signaling, and recycling is regulated by systemic signaling. The PSI genes whose expression is regulated by ethylene also belong to these functional categories (Lei et al., 2011b; Wang et al., 2012; Yu et al., 2012). The

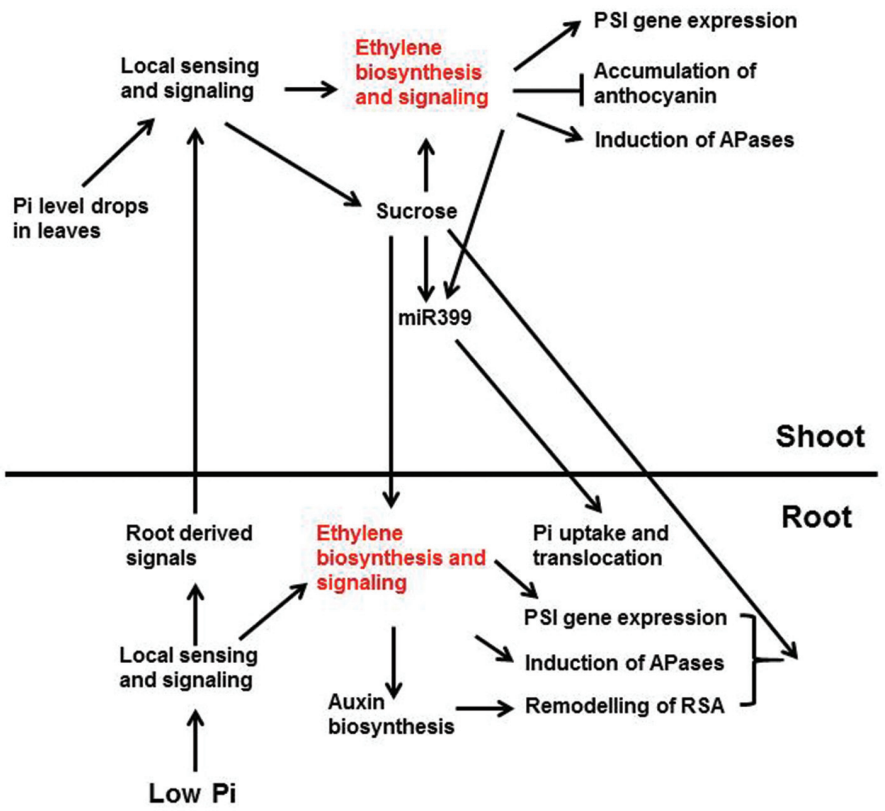

FIGURE 3 | A schematic model showing the interactions between ethylene and other Pi signaling components and Pi deficiency responses that these interactions are involved. Low Pi is perceived by unidentified intracellular and/or extracellular sensors in root, which activates local signaling pathways and the synthesis of systemic signals. The activated local signaling pathways then trigger the expression of PSI genes, the induction of APases, and the remodeling of RSA. These response are egulated partly through ethylene biosynthesis and signaling. The ethylene-mediated remodeling of RSA may also be achieved by enhancing auxin biosynthesis and signaling. The root-derived systemic signals are translocated to shoot via xylem. These signals, together with the drop of Pi level in shoots, elicit the local sensing and signaling there. The local signaling in shoots increases ethylene biosynthesis and signaling, as well as the synthesis of systemic signals, such as sucrose. The enhanced ethylene biosynthesis and signaling induce the expression of PSI genes and APase activity, but suppress the accumulation of anthocyanin. The increased sucrose level, in turn, can reinforce the ethylene biosynthesis and signaling and induces the production of miR399. The induction of miR399 is also modulated by ethylene and transcription factor PHR1 (not show in the figure). The shoot-derived sucrose and miR399 then move down to roots through phloem transport. In roots, sucrose is critical for the expression of some PSI genes and the induction of APase activity, perhaps also the remodeling of RSA (not discussed in this article), and the accumulation of miR399 enhances Pi uptake and translocation to shoots. Arrows indicate promotion, and the perpendicular line indicate inhibition (Lei et al., 2011b).

transcription of the Pi transporters Pht1;1 and Pht1;4, the APase ACP5, the RNase RNS1, and the Pi signaling molecules miRNA399, At4, and IPS1 is enhanced in *hps2*, *hps3*, and *hps4* but reduced in *ein2* or in the plants treated with Ag^{+}. Thibaud et al. (2010) further demonstrated that the promoters of most systemically regulated PSI genes contain the P1BS element, which is the binding site of the PHR1 transcription factor, indicating that PHR1 is an important component of the systemic signaling pathway. The reduced expression of one *ACS* gene and at least eight *AP2/ERF* genes in the *phr1phl1* mutant suggests that ethylene biosynthesis and signaling themselves are also controlled by systemic signaling. Thus, ethylene might be part of a regulatory loop involved in the systemic control of PSI gene expression. Once activated, however, how ethylene signaling functions in systemic control of PSI gene expression is unknown.

In the *phr1* mutant, anthocyanin accumulation is greatly attenuated, indicating that this response to low Pi is systemically controlled (Rubio et al., 2001). As discussed before, the accumulation of anthocyanin is enhanced in *ctr1* and *eto1* but reduced in *ein2*, indicating that ethylene is a negative regulator of this systemic response (Lei et al., 2011b; Wang et al., 2012). These results reveal another function of ethylene in the systemic control of plant responses to Pi deprivation.

Interaction between Ethylene and Other Signals

The past studies have indicated that the regulation of plant responses to Pi deficiency is complex and involves crosstalk among different signaling pathways. The precise control and coordination of these multi-faceted responses undoubtedly depends on an efficient interaction among the different signals. Here, we discuss how ethylene interacts with sucrose, auxin, and miRNA399 to regulate Pi-deficiency responses.

Ethylene and Sucrose

Growing evidence indicates that sucrose is a key systemic signal that globally regulates Pi-starvation responses. In several plant species, sucrose biosynthesis increases under low Pi availability (Hammond and White, 2008). Also, the expression of the genes involved in the synthesis, translocation, and degradation of sucrose is altered when plants are grown under Pi deficiency (Hammond et al., 2003; Wu et al., 2003; Misson et al., 2005; Muller et al., 2007). In addition to functioning as a carbon source, the sucrose delivered to the roots from leaves acts as a signal to initiate changes in gene expression, metabolism, and development in roots (Hammond and White, 2011). Karthikeyan et al. (2007) found that the level of PSI gene expression was positively correlated with the concentrations of sucrose in the growth medium under Pi deficiency. When plants were grown in the dark, the expression of PSI genes was greatly reduced; this reduction in PSI gene expression, however, was prevented by adding sucrose to the growth medium. Similarly, Liu et al. (2005, 2010) observed that application of sucrose stimulated accumulation of *LaPT1* (a Pi transporter), *LaSAP1* (an APase) and miR399 transcripts in dark-grown white lupin and common bean under Pi sufficiency. Furthermore, the use of stem-girdling to disrupt the phloem transport of photosynthates to P-deficient roots resulted in the suppression of the expression of these genes. Jain et al. (2007) reported that sucrose is required for Pi deficiency-induced lateral root proliferation and root hair formation. In addition, exogenous application of sucrose, like Pi deficiency, induced CR formation and PSI gene expression in Pi-sufficient white lupin (Zhou et al., 2008). Definitive evidence for the role of sucrose in plant responses to Pi starvation came from the study of the *Arabidopsis* mutant *hps1* (Lei et al., 2011a). *hps1* overexpresses the *SUC2* gene due to a T-DNA insertion in the promoter of the *SUC2* gene. SUC2 is the only transporter involved in the phloem loading of sucrose in mesophyll cells. *hps1* accumulates a high level of sucrose in both roots and shoots and is hypersensitive in almost all aspects of plant response to Pi starvation. Lei et al. (2011a) further showed that the *suc2-5* mutant had enhanced expression of the Pht1:4 Pi transporter in shoots but reduced expression in roots, as well as reduced root-associated APase activity. These results were consistent with the high accumulation of sucrose in shoots and low level of sucrose in roots in this mutant. The reduced root-associated APase activity was also observed in another mutant allele of *SUC2*, *pho3* (Zakhleniuk et al., 2001; Lloyd and Zakhleniuk, 2004).

In several plant species, sucrose increases ethylene production in a sucrose concentration-dependent manner (Philosoph-Hadas et al., 1985; Kobayashi and Saka, 2000; Jeong et al., 2010; **Figure 3**). The sucrose-induced formation of CR in white lupin was completely suppressed by application of Co^{2+} (Wang et al., 2015). In addition, Co^{2+} also suppressed Pi deficient-induced CR formation. Together, these results suggest that sucrose induces CR formation via the induction of ethylene biosynthesis (**Figure 3**).

Both sucrose and ethylene are positive regulators for the induction of AtPAP10 APase activity on the root surface (Lei et al., 2011a,b; Wang et al., 2012; Yu et al., 2012). Zhang et al. (2014) further investigated the relationship between sucrose and ethylene in the regulation of AtPAP10 activity. Under Pi deficiency, *hps1* had enhanced APase activity while *ein2* had reduced APase activity on the root surface. The double mutant *hps1ein2* displayed root-associated AtPAP10 activity that was intermediate between that of *hps1* and *ein2*. Also, when treated with Ag^{+}, the AtPAP10 activity in WT plants was partially reduced. When *ctr1* was grown in the dark on a sucrose-free Pi-deficient medium, however, the induction of the AtPAP10 activity on the root surface was completely blocked, although ethylene signaling was constitutively activated in the mutant. Thus, ethylene's induction of AtPAP10 activity depends on sucrose, but sucrose's function does not depend on ethylene. Further study indicated that sucrose was largely required for the induction of *AtPAP10* transcription while ethylene only modulated the secretion of AtPAP10 protein or AtPAP10 enzymatic activity on the root surface (Zhang et al., 2014). This is understandable because if *AtPAP10* mRNA is not transcribed due to the absence of sucrose, ethylene signaling, even if is constitutively activated, will not increase the AtPAP10 activity on the root surface. In addition, the above results also indicate that ethylene is not the only component that

controls the induction of APase activity; even if the ethylene pathway is completely blocked by the *ein2* mutation or by treatment with Ag^+, the induction of APase is only partially abolished.

Ethylene and Auxin

Auxin also plays an important role in controlling Pi deficiency-induced remodeling of RSA (López-Bucio et al., 2002; Nacry et al., 2005; Jain et al., 2007; Pérez-Torres et al., 2008). López-Bucio et al. (2002) showed that Pi-deprived plants were more sensitive to exogenously applied auxin than Pi-replete plants with respect to the arrest of primary root growth and enhanced formation of lateral roots. This enhanced auxin sensitivity was found to result from the enhanced expression of the auxin receptor TIR1 (Pérez-Torres et al., 2008). The enhanced TIR expression accelerates the degradation of AUX/IAA (AUXIN/INDOLE-3-ACETIC ACID) auxin response repressors, thus releasing repression of transcription factor ARF19 that is involved in the formation of lateral roots. Nacry et al. (2005) proposed that Pi deficiency causes: (1) an over-accumulation of auxin in the root apex of primary root and young lateral roots; (2) an over-accumulation of auxin or an increased auxin sensitivity in the lateral primordia; (3) a decrease in auxin concentration in the lateral primordia initiation zone of the primary roots and in old laterals. Using auxin transport inhibitor or the mutants with defects in auxin transport, the authors also showed that the changes in local auxin concentrations was achieved through the changes in auxin transport rather than auxin biosynthesis. Given that both ethylene and auxin are involved in the Pi deficiency-induced remodeling of RSA, ethylene may cooperate with auxin to regulate root growth. In fact, several lines of evidence have indicated that ethylene promotes auxin biosynthesis and transport to modulate root development (Osmont et al., 2007; Růzicka et al., 2007; Swarup et al., 2007; Stepanova and Alonso, 2009).

The *Arabidopsis* mutant *hps4* is hypersensitive to the Pi deficiency-induced inhibition of primary root growth (Yu et al., 2012). In this mutant, the expression of several genes related to auxin biosynthesis is increased. In addition, the *hps4* root tip produced twice as much auxin as that of the WT under Pi deficiency. The hypersensitivity of *hps4* to Pi deficiency was suppressed when the plants were treated with Ag^+ but not with AVG. These results suggested that the enhanced ethylene signaling in *hps4* might increase auxin biosynthesis in the root tip, thus enhancing the inhibition of the primary root growth (**Figure 3**). In white clover, Pi deficiency increases the ethylene sensitivity in roots (Dinh et al., 2012). ACC treatment induced a high expression of the auxin-responsive DR5::GUS marker gene in the root apex. To separate the effect of ACC on auxin biosynthesis from auxin transport, the authors applied auxin transport inhibitor to Pi deficient-roots. The results showed that the ACC-enhanced DR5::GUS expression could not be suppressed by the auxin transport inhibitor, suggesting that the effect of ACC is on auxin biosynthesis, although the role of ACC on auxin transport cannot be strictly excluded. This result is also consistent with what observed by Yu et al. (2012).

Ethylene and miR399

The first and also the best characterized miRNA involved in Pi-deficiency response is miR399. The expression of miR399 is highly induced in both shoots and roots by a decrease in external Pi or copper levels, but is reduced by iron deficiency (Fujii et al., 2005; Chiou et al., 2006; Buhtz et al., 2010). A detailed time course study indicated that the increase of miR399 in the shoot occurs prior to that in the root (Lin et al., 2008). Reciprocal grafting experiments further showed that miR399 could move from shoot to root (Lin et al., 2008; Pant et al., 2008). Observation of the increased accumulation of miR399 in the phloem sap in Pi starved-*Arabidopsis* and *Brassica* plants (Buhtz et al., 2008; Pant et al., 2008) also supported that miR399 is synthesized in shoots and is translocated to roots in order to act as a systemic signal that regulates Pi uptake. miR399 enhances Pi acquisition by direct cleavage of the mRNA that encodes the ubiquitin E2 conjugase PHO2, which is involved in the ubiquitin-mediated protein degradation pathway (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006). Overexpression of *miRNA399* or functional disruption of PHO2 leads to the over-accumulation of Pi in shoots. By screening for *pho2* suppressors and using quantitative membrane proteomics, Liu et al. (2012) and Huang et al. (2013) identified PHO1 and a group of high-affinity Pi transporters (PHT1) as the substrates of PHO2. PHO1 and PHT1 transporters are responsible for the translocation of Pi from roots to shoots and for the uptake of Pi from the external environment. When plants are exposed to Pi deficiency, miR399 is rapidly induced and degrades the mRNA of *PHO2*. The down-regulation of *PHO2* expression increases the stability of PHO1 and PHT1 proteins, thus enhancing Pi uptake in roots and Pi translocation from roots to shoots.

Transcription of the precursor of miR399d is enhanced in the *Arabidopsis* mutants *hps2*, *hps3*, and *hps4*, but the expression of miR399d is reduced in *ein2* (Lei et al., 2011b; Wang et al., 2012; Yu et al., 2012). These results demonstrated that ethylene positively regulates the expression of miR399d. Interestingly, Liu et al. (2010) found that the induction of miR399 was completely blocked in dark-grown or stem-girdled white lupin, suggesting that sucrose is required for miR399 expression (**Figure 3**). Given that an increase in sucrose level can induce ethylene biosynthesis, ethylene might act downstream of sucrose to affect the expression of miR399. Also, because the expression of miR399 is completely abolished in the absence of sucrose but is only partially blocked in the *ein2* mutant, it seems that ethylene functions in a branch of the pathway downstream of sucrose to modulate the expression of miR399 and thus to fine-tune Pi uptake by roots.

Conclusion and Perspectives

Based on genetic, pharmacological, biochemical, and physiological data, it is now evident that ethylene plays an important role in mediating plant responses to Pi deficiency. Pi deficiency increases ethylene biosynthesis and signaling in both roots and leaves. Early studies showed that ethylene is involved in Pi deficiency-induced inhibition of primary root growth and enhanced production of root hairs. Recent research

indicates that ethylene also regulates expression of PSI genes, induction of APases, and accumulation of anthocyanin under Pi deficiency. Ethylene participates in both transcriptional and post-transcriptional regulation of plant Pi responses. Moreover, ethylene interacts with other signals, such as sucrose, auxin, and miRNA399, to regulate both local and systemic signaling. Also, it should be noted that although ethylene clearly plays an important role in mediating multiple plant responses to Pi deficiency, there is no single Pi response that is completely under the control of ethylene.

Although the role of ethylene in mediating plant responses to Pi deficiency is well established, the following important questions remain regarding the underlying mechanisms: (1) How do plants sense the change in Pi availability to elicit the production of ethylene or to enhance ethylene sensitivity? (2) Which specific cells are the targets for ethylene action? (3) Through which downstream components does ethylene mediate plant responses to Pi starvation? To answer these questions, we need to identify the transcription factors that bind to the promoters of ethylene biosynthetic genes, such as *ACS* and *ACO*, and to understand how the expression of these transcription factors is regulated by Pi deficiency. The expression of *ACS* and *ACO* genes and the stability of these proteins are also regulated by CDPK and MAPK signaling pathways (Kim et al., 2003; Hernández Sebastià et al., 2004; Liu and Zhang, 2004). A recent report indicated that *Arabidopsis* MKK9-MPK3/MPK6 pathway is involved in the maintenance of Pi homeostasis by regulating transcription of Pi acquisition-related genes (Lei et al., 2014). It is not known, however, whether this pathway regulates Pi homeostasis by affecting the expression or activity of ACS and ACO. Thus, it may also worth to investigate the molecular link between this MAPK pathway and ethylene biosynthesis/signaling under Pi deficiency.

To identify the cells that are the targets for ethylene effects, researchers might use a cell type-specific promoter to drive a mutated ethylene receptor gene *etr1* to specifically block the action of ethylene in those cells; the change in Pi response would then indicate whether those cells are involved. This approach has been successfully used to dissect the role of ABA signaling in mediating the response of root growth to drought stress (Duan et al., 2013). To identify the downstream targets of ethylene signaling that are directly involved in plant Pi responses, researchers can combine genomic and genetic approaches. For example, EIN3 and EIL1 are two key transcription factors that regulate a suite of downstream ethylene-responsive genes. A transcriptomic analysis would indicate those genes whose expression is blocked in the Pi deficient-*ein3eil1* mutant. Using such an approach, researchers could identify a battery of genes that directly participate in plant responses to Pi deficiency, and these might include, for example, the genes of cell wall proteins that are directly involved in the elongation of root hairs. This approach could also reveal the gene regulatory network that ethylene uses to control specific Pi responses. In addition, by using a Pi starvation-responsive marker line (such as the plant carrying the *AtPT2::LUC* marker gene), researchers could screen for a mutant that uncouples the interaction between ethylene and Pi; thus identifying the molecular components involved in the regulation of ethylene sensitivity under Pi deficiency. With increased knowledge in these areas, we will better understand how ethylene functions in plant responses to Pi deficiency and how overall plant responses to Pi deficiency are regulated at the molecular level.

Author Contributions

LS drafted the manuscript. LS and DL revised the manuscript.

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REVIEW
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The Role of Ethylene in Plant Adaptations for Phosphate Acquisition in Soils – A Review

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Although a role of ethylene in the regulation of senescence and plant stress responses in general has a long history, a possible involvement in the regulation of adaptive responses to nutrient deficiencies has been mainly investigated since the last two decades. In the case of plant responses to phosphate (P_i) starvation, ethylene was identified as a modulator of adaptive responses in root growth and morphology. The molecular base of these adaptations has been elucidated in supplementation studies with ethylene precursors and antagonists, as well as analysis of mutants and transgenic plants with modified ethylene biosynthesis and responsiveness, using mainly *Arabidopsis thaliana* as a model plant. However, increasing evidence suggests that apart from root growth responses, ethylene may be involved in various additional plant adaptations to P_i limitation including P_i mobilization in the rhizosphere, P_i uptake and internal P_i recycling. The ethylene-mediated responses are frequently characterized by high genotypic variability and may partially share common pathways in different nutrient limitations.

Keywords: ethylene, root growth, root morphology, phosphate deficiency, phosphate acquistion

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INTRODUCTION

Among the wide range of phosphorus (P) forms in soils, inorganic phosphate anions (P_i) are taken up exclusively by plant roots. However, due to limited solubility, P is the macronutrient with the lowest plant availability in soils. Even in well-fertilized soils, on average only 20% of the fertilizer input are utilized by plants since the majority of fertilizer P_i is prone to P_i fixation and incorporation into organic P_i forms comprising 20–80% of total soil P. (Richardson, 1994; Holford, 1997). Therefore, higher plants are strongly dependent on specific adaptations to acquire P_i in sufficient amounts. Adaptive responses toward improved spatial P_i acquisition comprise stimulation of root growth, increased formation of fine root structures (lateral roots, root hairs) (**Figure 1**), preferential root development in the top soil with the highest P content (Lynch and Brown, 2001) or stimulation of lateral root growth in nutrient patches rich in P and also N (Forde and Lorenzo, 2001). Modifications of the rhizosphere chemistry, such as rhizosphere acidification, secretion of organic metal-chelators (carboxylates, phenolics) and phosphohydrolases (acid phosphatase, phytase) increase the solubility and plant availability of P_i in the rhizosphere (Neumann and Römheld, 2002, 2007). The formation of so-called cluster roots (CR; **Figure 2**) within the Proteaceae, Casuarinaceae, Myrtaceae, and Fabaceae (Dinkelaker et al., 1995; Neumann and Martinoia, 2002), or dauciform roots in Cyperaceae (Playsted et al., 2006) are among the most specialized root-morphological adaptations to promote the secretion of Pi-mobilizing root

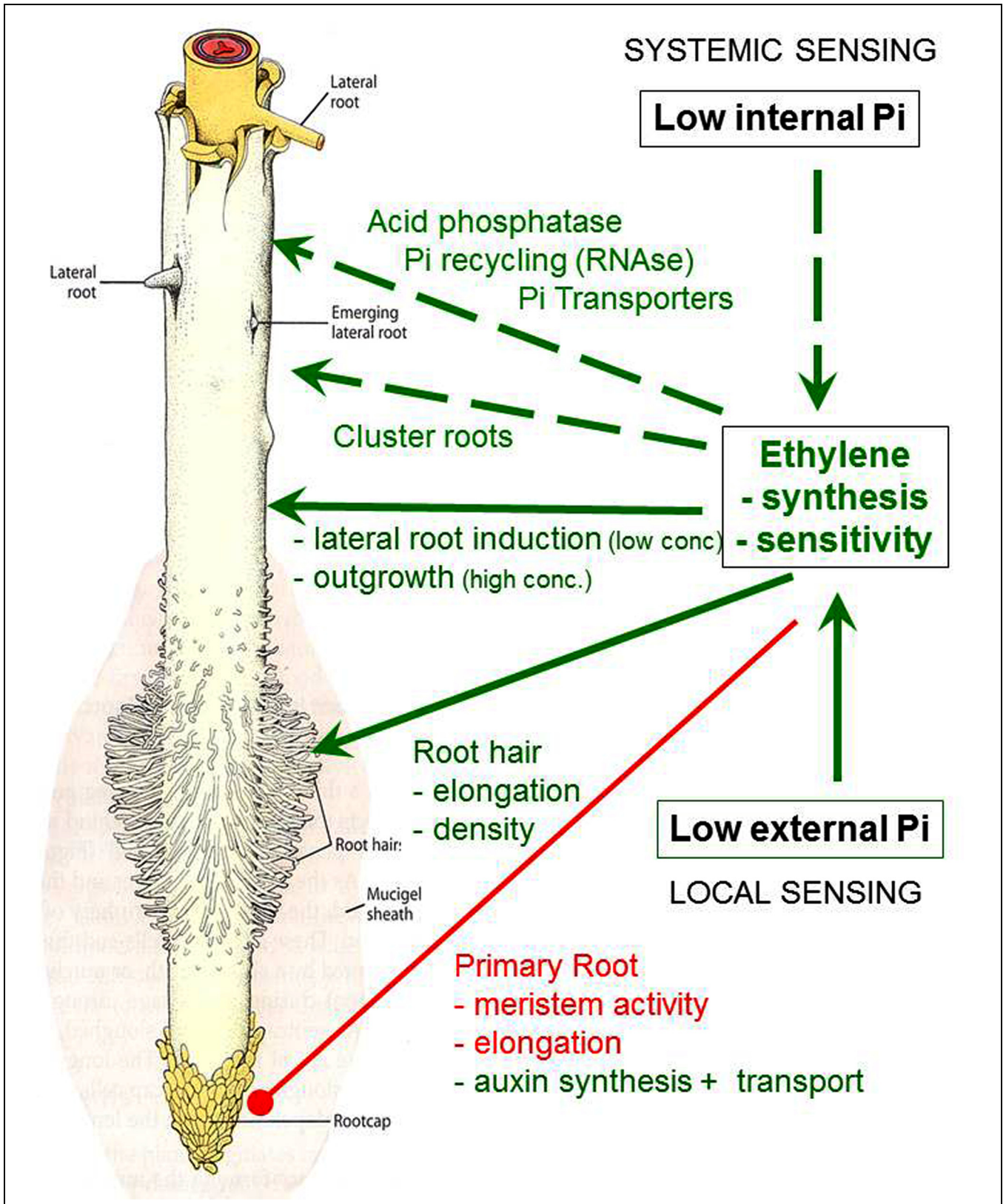

FIGURE 1 | Adaptive responses to P_i limitation in plant roots modulated by ethylene. Green: stimulation; red: inhibition.

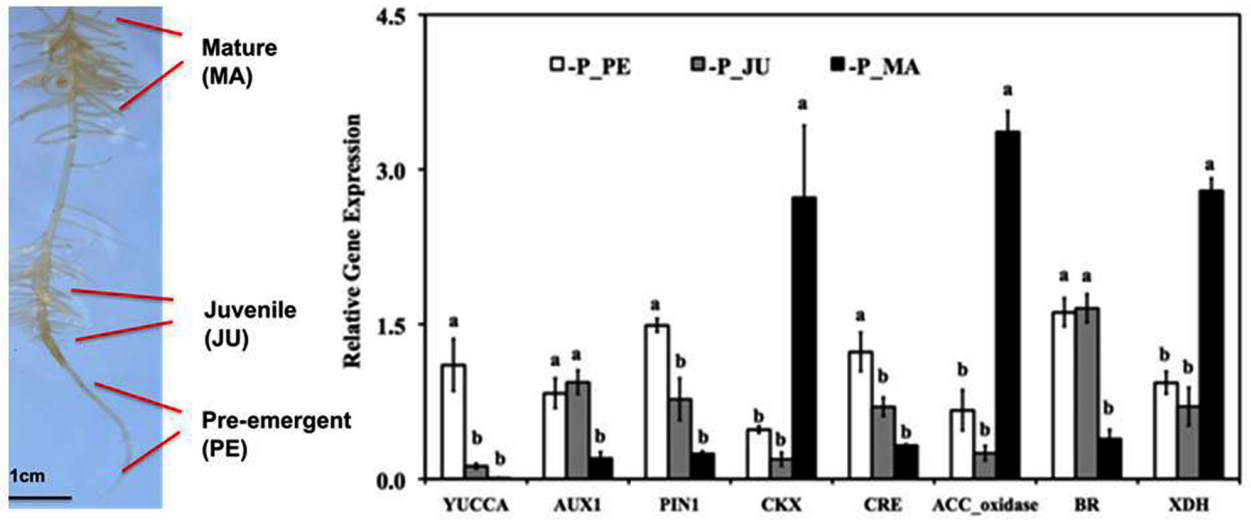

FIGURE 2 | Expression pattern of hormone-related genes during cluster root (CR) development in Lupinus albus grown for 20 days without P_i supply and evaluated by quantitative real time PCR (RT-qPCR). Genes are involved in auxin biosynthesis (YUCCA), auxin transport (AUX1, PIN1), cytokinin degradation (CKX), cytokinin receptors (CRE), ethylene biosynthesis (ACC_oxidase), brassinosteroid biosynthesis (BR), and RNA degradation associated with nitric oxide formation (XDH). The gene expression level is indicated relative to reference genes. Data represent means ± SE ($n = 2$–3). Different lowercase letters denote significant differences ($P < 0.05$). PE, pre-emergent stage; JU, juvenile root clusters; MA, mature root clusters (modified after Wang et al., 2014b). The photo shows the different developmental stages of CRs used for RNA isolation: PE, pre-emergent stage; JU, juvenile cluster; MA, mature cluster.

exudates. The expression of high affinity P_i uptake systems provides the ability for efficient exploitation of the rhizosphere solution even at low P_i levels, hardly exceeding concentrations of 10 μM (Bieleski, 1973) even in well-fertilized soils. Also symbiotic associations with mycorrhizal fungi are frequently established as adaptive responses for improved spatial (arbuscular and ectomycorrhizal fungi) and chemical acquisition (mainly ectomycorrhizal fungi) of soil P forms (Neumann and Römheld, 2002).

Among the wide range of regulatory factors involved in the induction of adaptive responses to P_i limitation, there is increasing evidence that these processes are modulated also by ethylene as important regulator. In many studies, the role of ethylene has been investigated by exogenous application of precursors and antagonists of ethylene synthesis and signal transduction and by expression analysis of genes involved in ethylene biosynthesis, signaling and ethylene responses. Other strategies comprise the analysis of mutants and transgenic plants with modified synthesis, signaling and reception of ethylene, most frequently using *Arabidopsis thaliana* as model plant (Nagarajan and Smith, 2011).

ETHYLENE AND ROOT GROWTH RESPONSES

The involvement of ethylene in regulation of root growth has been postulated already in early studies by Chadwick and Burg (1967) on root geotropism and Smith and Russell (1969) on root growth responses under oxygen limitation, including also interactions with auxin (Chadwick and Burg, 1967, 1970). Meanwhile it is generally accepted that ethylene influences root growth in a biphasic manner with stimulatory effects, e.g., on lateral root formation induced by low ethylene concentrations, triggering both, synthesis and signaling of auxins, as indicated by analysis of *Arabidopsis* mutants affected in auxin signaling and ethylene-induced auxin synthesis (Stepanova et al., 2005; Ivanchenko et al., 2008). The ethylene-induced modifications of auxin synthesis and transport contribute to the formation of auxin gradients necessary for the induction of lateral root primordia in the pericycle opposite the prototoxylem poles (Fukaki and Tasaka, 2009).

By contrast, high ethylene concentrations exert inhibitory effects on lateral root formation, as demonstrated by Negi et al. (2008), showing that both, overproduction of ethylene by high external application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or by the *eto1* mutation, inhibited lateral root formation in *Arabidopsis*. On the other hand, lateral root formation was stimulated in the *etr1* (ethylene triple response1) or *ein2* (ethylene insensitive2) mutations, blocking ethylene responses (Negi et al., 2008). Similar to lateral root formation promoted by low levels of ethylene, ethylene/auxin interactions seem to be involved also in the inhibitory effects on root growth induced by high ethylene concentrations, stimulating both, acropetal and basipetal auxin transport with involvement of the AUX1 influx carrier as indicated by an ethylene-insensitive *aux1-7* mutant of *Arabidopsis* (Negi et al., 2008), as well as PIN3 and PIN7 efflux transporters (Lewis et al., 2011). The ethylene-mediated stimulation of auxin transport may inhibit lateral root formation by a reduction of auxin accumulation in the protoxylem pericycle, required for initiation of lateral root primordia (Fukaki and

Tasaka, 2009). Interestingly, high ethylene concentrations exerted inhibitory effects on formation of new lateral root primordia but stimulated outgrowth of already existing primordia (Ivanchenko et al., 2008). In primary roots of *Arabidopsis,* also a massive ethylene-induced stimulation of auxin synthesis in the root tip has been observed (Ruzicka et al., 2007; Swarup et al., 2007) with inhibitory effects on root growth, which may at least partially be attributed to a reduced extensibility of the cell wall due to inhibition of the auxin-dependent plasmalemma H^+-ATPase and formation of reactive oxygen species (ROS), promoting cross-linking of cell walls by hydroxyproline-rich glycoproteins in response to high auxin concentrations. Increased ethylene levels are also able to affect the activity of the primary root meristem, probably by interaction with jasmonic acid (Chacon-Lopez et al., 2011), inducing a determinate developmental program with arrested cell division and promotion of cell differentiation.

ADAPTIVE RESPONSES TO P_i LIMITATION – SPATIAL P_i ACQUISITION

Measurements of ethylene production, inhibitor studies (Borch et al., 1999; Lynch and Brown, 2001; Li et al., 2009), analyses of mutants in gene expression of the ethylene bio-synthetic pathway (Tsuchisaka and Theologis, 2004; O'Rourke et al., 2013; Wang et al., 2014a) revealed promotion of ethylene synthesis and/or enhanced ethylene sensitivity (**Figure 1**), induced by P_i limitation in higher plants (He et al., 1992; Kim et al., 2008). These responses seem to be expressed in a highly tissue-specific and developmental stage-dependent manner. Accordingly, Kim et al. (2008) reported up-regulation of ethylene production in shoots but not in roots of P_i-deficient tomato and no effects in Petunia with the conclusion that modifications in ethylene sensitivity are more important in latter cases. As another example, Wang et al. (2014a) recorded up-regulation of the ethylene biosynthesis gene encoding ACC oxidase in 1–2 cm sub-apical lateral root zones just prior emergence of secondary laterals during CR development in Pi-deficient white lupin (*Lupinus albus* L.). Gene expression of ACC oxidase declined after outgrowth of the secondary laterals, followed by a massive increase again during further development of the root clusters (**Figure 3**).

The P_i deficiency-induced changes in ethylene accumulation and ethylene responsiveness are involved in adaptive modifications of root growth toward improved P acquisition. Typical patterns comprise a reduction of primary root growth, associated with an increase in lateral root development (**Figure 1**) promoting the development of a shallower root system for exploitation of top soil layers with the highest P availability (Linkohr et al., 2002; Lopez-Bucio et al., 2002; Sanchez-Calderon et al., 2006; Svistoonoff et al., 2007). Similarly, the angle of basal lateral roots in common bean can be modulated by increased sensitivity to ethylene in response to low P_i supply, to direct lateral root development into the upper soil layers (Basu et al., 2007). However, the described ethylene-mediated root growth responses to P limitation cannot be generalized and exhibit high genotypic variability. Testing 73 ecotypes of *Arabidopsis thaliana* revealed P deficiency-induced inhibition of primary root elongation only for 50% of the accessions (Chevalier et al., 2003). A survey of 14 dicots and monocots in hydroponics showed that all tested species had the same degree of primary root elongation independent of the P_i-nutritional status (Narayanan and Reddy, 1982), and many plant species even exhibit root elongation under low-P_i conditions (Niu et al., 2012). Also the formation of shallower root systems in common bean is a heritable trait with genotypic variability, which has already been exploited for breeding programs to promote top soil foraging for improved P_i acquisition (Lynch and Brown, 2012).

One of the earliest detectable modifications of root morphology in response to P_i starvation is an increased number and length of root hairs (Bates and Lynch, 2001; Ma et al., 2001; Jain et al., 2007) as an important adaptation for improved spatial acquisition of available P_i in the rhizosphere (**Figures 1** and **2**) with particular importance for plant species unable to form mycorrhizal associations. Accordingly, length of root hairs was inversely correlated with the degree mycotrophy in different plant species (Schweiger et al., 1995). However, due to secretory properties (protons, organic metal chelators, mucilage) and surface extension, the length and the density of root hairs also determines root soil contact and chemical modifications of the rhizosphere toward improved solubilization of nutrients (Playsted et al., 2006; Haling et al., 2013; Abrahão et al., 2014).

Based on the observation that in contrast to Fe deficiency, the number of root hairs in *Arabidopsis* under P_i limitation was not affected by application of ethylene anatagonists and also not in the ethylene-insensitive *ein2* and the ethylene-resitant *etr1* mutants, Schmidt and Schikora (2001) concluded that the development of extra root hairs in response to P_i limitation does not appear to require ethylene signaling. However, treatments with the ethylene precursor ACC promoted root hair elongation, which was inhibited by ethylene antagonists (Zhang et al., 2003). Moreover, root hair length was reduced in various ethylene-response mutants as compared with the wild type under P limitation but not with sufficient P_i supply (Cho and Cosgrove, 2002; Zhang et al., 2003), suggesting that ethylene is involved in the regulation of P_i deficiency-induced root hair elongation (**Figure 1**). Moreover, ethylene increased also the density of root hairs by shortening trichoblast cells to increase the number of H cells per unit root length (Zhang et al., 2003). Apart from the hormone-dependent metabolic regulation, root hair development in response to P_i limitation also shows marked genotypic variations and improved P acquisition in cultivars with long root hairs has been documented for barley (Gahoonia et al., 1997; Haling et al., 2013) and *Phaseolus vulgaris* where a combination of ethylene-modulated root traits, such as long root hairs and a shallow root system was particularly efficient (Miguel et al., 2015).

Interestingly, the ethylene mediated responses of root growth to P_i limitation as described so far, seem to be largely independent from a low P-nutritional status of the plant as a systemic signal,

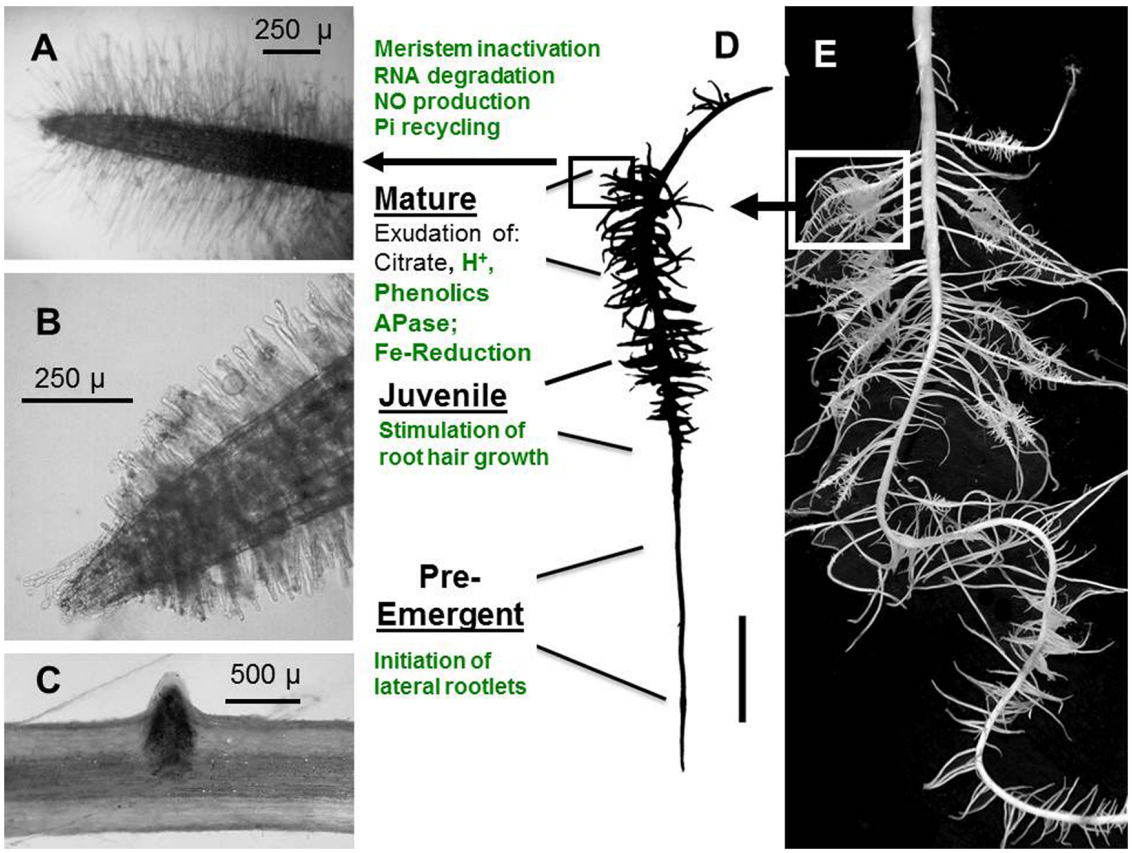

FIGURE 3 | Stages of CR development in P_i-deficient white lupin (*Lupinus albus* L.). Characteristic processes in the different root zones potentially modulated by ethylene are marked in green. **(A)** Single second order lateral rootlet of a MA root cluster, densely covered with root hairs. **(B)** Single lateral rootlet of an outgrowing juvenileJU root cluster with growing root hairs. **(C)** Outgrowth of a lateral rootlet primordium. **(D)** Root clusters in different developmental stages along a first-order lateral root; **(E)** Root system of P_i-deficient white lupin with CRs development. PE, pre-emergent stage; JU, juvenile cluster; MA, mature cluster. (Figure modified after Wang et al., 2014b).

and a low P_i level in the external rooting medium seems to be sufficient for the induction (Thibaud et al., 2010; Nagarajan and Smith, 2011). The local sensor is currently unknown but it seems to be plausible that high affinity P transporters, located in the plasma membrane of epidermal cells in roots and root hairs, could express a double function as transporters and receptors (transceptors) as already shown for the yeast Pho84p high-affinity P_i transporter (Popova et al., 2010) or the *Arabidopsis* nitrate transporter CHL1/NRT1.1 with functions as transporter and sensor for nitrate in the external medium (Ho et al., 2009). The low P_i status of the rooting medium is most probably sensed in the apoplast of the primary root tip (Svistoonoff et al., 2007) and a P5-type ATPase (PDR2) interacting with the SCARECROW transcription factor and multi-copper oxidases (LPR1/LPR2) in the ER of the root tip meristem have been characterized as components of the sensing system. After sensing the local P_i status at the primary root tip, the information of P_i depletion at the roots is translocated via xylem transport to the shoot and may involve P_i, strigolactones, and cytokinins as signal molecules (recently reviewed by Chiou and Lin (2011) and Zhang et al. (2014).

ADAPTIVE RESPONSES TO P_i LIMITATION – P_i MOBILIZATION

Apart from functions in adaptive modulation of root morphology and root architecture for improved spatial acquisition of available soil P_i, there is also increasing evidence for a role of ethylene in root-induced adaptations to increase the chemical availability of P_i in the rhizosphere. A large proportion of soil P_i (up to 80 %) is usually sequestered in organic binding forms, requiring mineralization by enzymatic hydrolysis prior to plant uptake (Richardson, 1994; Holford, 1997). Accordingly, both, soil microorganisms and plant roots are able release phosphohydrolases (e.g., acid phosphatases, alkaline phosphatases, phytases, nucleases) to acquire or recycle P_i from organic binding forms. Particularly root secretion of acid phosphatases is stimulated as a response to P_i limitation in many plant species (Neumann and Römheld, 2007) and ethylene signalling seems to be involved in the up-regulation of intracellular and secretory acid phosphatases (**Figure 1**), both, at the level of transcription and enzyme activity as indicated by precursor/inhibitor experiments and

analysis of the ethylene insensitive *ein2-5* and the ethylene-overproducing *hps2* mutant (Lei et al., 2011; Li et al., 2011).

In contrast to the adaptive responses in root growth, the up-regulation of acid phosphatases is induced systemically by a low internal P_i nutritional status of the plant. Other systemic, potentially ethylene-mediated responses comprise the up-regulation of ribonuclease genes (RNS1), intracellular acid phosphatases (ACP5) and P_i transporters (Pht1,4) involved in remobilization and re-translocation of P_i from RNA and other organic P compounds in senescing organs (**Figures 1** and **2**), during programmed cell death and also in response to P_i limitation (Thibaud et al., 2010; Nagarajan and Smith, 2011). Accordingly, increased ethylene responsiveness has been implicated also in the formation of lysigenic aerenchyma in P_i-deficient maize roots (He et al., 1992, 1994) as a strategy for P_i recycling.

CLUSTER ROOTS

The formation of cluster roots (CR) belongs to the most specialized adaptations for mobilization of sparingly soluble P_i sources in soils (**Figure 2**). Although CRs have been detected in various plant families such as Proteaceae, Casuarinaceae, Mytaceae, Fabaceae, and others, white lupin so far represents the best-characterized model plant with respect to regulatory aspects of CR development and CR function. CRs are bottlebrush like structures formed by short second-order laterals with determinate growth and densely covered with root hairs (Dinkelaker et al., 1995; Neumann and Martinoia, 2002). Thereby, the largely increased surface area enables a concentrated release of organic metal chelators (citrate, malate, phenolics), ectoenzymes (acid phosphatases), protons and reductive changes in the rhizosphere, mediating the mobilization of sparingly soluble soil phosphates but also other nutrients, such as Fe, Mn, Zn, and Mo (Gardner et al., 1983; Dinkelaker et al., 1997, Neumann and Römheld, 2007). In the past, only a few studies addressed a possible involvement of ethylene in CR development, mainly with inhibitor studies and measuring ethylene evolution from the whole root system (Gilbert et al., 2000; Zaid et al., 2003). More recently, transcriptomics and gene expression studies revealed considerable variation in the expression of genes encoding ethylene bio-synthetic enzymes (ACC oxidase, ACC synthase) during CR development (O'Rourke et al., 2013; Wang et al., 2014a,b).

In the 1.2 cm subapical root zones of first-order laterals, prior to the emergence of the second-order lateral cluster rootlets, ethylene biosynthesis genes are moderately up-regulated together with genes involved in auxin biosynthesis (YUCCA) and transport (AUX1, PIN1), synthesis of brassinosteroids, and cytokinin receptors (**Figure 3**; Wang et al., 2014a,b), in accordance with the postulated role of these hormonal factors in formation of auxin gradients required for priming of pericycle cells for induction of the lateral rootlet primordia (Fukaki and Tasaka, 2009). However, in contrast to ethylene-mediated modifications of root growth under P_i limitation discussed so far (including growth inhibition of the primary root and lateral root proliferation), CR formation is largely induced systemically determined mainly by the P_i-nutritional status of the shoot (Marschner et al., 1987; Shane et al., 2003). Accordingly, induction of CRs in P_i-deficient white lupin can be suppressed almost completely by foliar P_i application (Marschner et al., 1987). More recently, sucrose has been identified as important shoot-borne signal, triggering the formation of CRs (Zhou et al., 2008; Wang et al., 2015) mediated by the well-documented increased shoot-to root translocation of sucrose under P_i limitation (Hammond and White, 2008, 2011; Wang et al., 2015). Even in P_i-sufficient lupin plants cultivated with P_i concentrations suppressive for CR formation, external application of sucrose to the rooting medium induced the formation of CRs in a concentration dependent manner to a similar or even higher extent than in P-deficient plants (Wang et al., 2015). Both, P_i deficiency-induced CR formation and sucrose-induced formation of CRs under sufficient P_i supply are completely suppressed by the ethylene biosynthesis inhibitor $CoCl_2$ (Wang et al., 2014b). Moreover, in many other plant species it has been shown that external sucrose supply increases ethylene production in a concentration-dependent manner with effects on various processes, such as anthocyanin production, flowering, and fruit ripening (Philosoph-Hadas et al., 1985; Kobayashi and Saka, 2000; Jeong et al., 2010) and sucrose concentrations increased in the sub-apical root zones of first-order laterals in P-deficient white lupin (Wang et al., 2015). These findings raise the question whether sucrose as a shoot-borne signal exerts its stimulatory effects on CR formation via stimulation of ethylene biosynthesis. However, during outgrowth of the CR primordia in the juvenile (JU) stage of CR development, expression of transcripts involved in ethylene biosynthesis (ACC oxidase) and auxin synthesis and transport transiently declined, followed by a massive increase of ACC oxidase gene expression during cluster-root maturation (**Figure 3**; Wang et al., 2014a,b). This is associated with a range of metabolic and developmental modifications (Wang et al., 2014a) known to be mediated by ethylene signaling also in other plant species comprising: (i) initiation of determinate growth of the lateral rootlets by inactivation of the root tip meristem including interactions with jasmonic acid (Chacon-Lopez et al., 2011; Wang et al., 2014a); (ii) formation of long, densely spaced root hairs (Cho and Cosgrove, 2002; Neumann and Martinoia, 2002; Zhang et al., 2003); (iii) increased expression of root secretory acid phosphatase (Massonneau et al., 2001; Lei et al., 2011); (iv) a massive decline (80%) of total RNA contents (Massonneau et al., 2001) associated with up-regulation of ribonuclease genes and P_i transporters (**Figure 2**) involved in remobilization and re-translocation of P_i from RNA degradation to the young, actively growing root zones (Thibaud et al., 2010; Nagarajan and Smith, 2011; Wang et al., 2014a,b); (v) the massive RNA degradation during CR maturation results in the formation of NO as a side product.(Wang et al., 2010). Together with ethylene, NO may be involved in the induction of the FIT transcription factor as

a central regulator of the coordinated Fe deficiency responses in strategy I plants (Hindt and Guerinot, 2012), which surprisingly was similarly expressed in mature CRs of white lupin even under Fe-sufficient conditions (Wang et al., 2014a) including also the up-regulation of the plasma membrane ferric reductase system (FRO2) and the FeII transporter (IRT1). Interestingly many adaptations of CRs toward improved P_i acquisition, such as root hair proliferation, proton extrusion, exudation of phenolic compounds with metal-chelating properties and increased ferric reductase activity at the root surface are also part of the strategy I mechanism for Fe acquisition (Neumann and Römheld, 2007). Since lupins are naturally adapted to moderately acidic soils frequently characterized by P_i fixation on iron amd aluminum oxides/hydroxides, the expression of mechanisms for Fe acquisition may be beneficial also for mobilization of sparingly soöuble Fe-P forms even at low soil pH, where Fe availability is usually not a problem. Consequently, in white lupin responses to Fe deficiency and to P limitation may at least partially share the same ethylene-dependent signaling pathways. Also in *Arabidopsis,* an interplay of strategies for P and Fe acquisition is suggested by increased Fe accumulation in response to P limitation (Mission et al., 2005; Hirsch et al., 2006; Ward et al., 2008) However, in contrast to white lupin, this was associated with a down-regulation of the IRT1 tansporter and increased expression of FER1 encoding a Fe storage protein (Mission et al., 2005). This was interpreted as a protective mechanism to counteract Fe toxicity. In white lupin, despite up-regulation of the strategy I mechanism for Fe acquisition, no excessive Fe accumulation was observed in response to P_i limitation (Wang et al., 2014a) and the mechanism to counteract Fe toxicity is yet unknown.

CONCLUDING REMARKS

The recent knowledge on the role of ethylene in P_i acquisition of higher plants demonstrates that ethylene is much more than just a modulator of root growth for adaptations to facilitate spatial P_i acquisition. Increasing evidence points to numerous additional functions also in mechanisms for chemical P_i solubilization in the rhizosphere and internal P_i recycling. For future research activities in this context it will be important to demonstrate more in detail how ethylene is integrated into the signaling network mediating the respective P_i starvation responses, to identify receptors and how it interacts with other hormonal and non-hormonal regulators (e.g., auxin, jasmonic acid, brassinosteroids, cytokinins, GA3, abscisic acid, polyamines, NO, miRNAs, sucrose). Particularly interesting in this context are also interactions with mechanisms for acquisition of other nutrients as indicated, e.g., for a potential link between P_i acquisition and Fe acquisition in *Arabidopsis* and white lupin, which at least in case of white lupin share many similarities and even similar signaling pathways with ethyleäne as a modulator of both root growth responses and physiological adapatations for mobilization of Fe and P_i. Meanwhile it seems to be clear that ethylene-mediated P deficiency responses are not based on one general mechanism and considerable genotypic variation exists between plant species and cultivars, which needs to be characterized more in detail for potential exploitation in breeding programs.

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MINI REVIEW
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The Role of Ethylene in Plant Responses to K^+ Deficiency

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Potassium is an essential macronutrient that is involved in regulating turgor, in driving plant growth, and in modulating enzyme activation. The changes in root morphology, root function, as well as cellular and molecular responses to low potassium conditions have been studied in the model plant *Arabidopsis* and in other plant species. In *Arabidopsis* ethylene plays a key role in roots in the transduction of the low potassium signal, which results in altered root function and growth. The first clues regarding the role of ethylene were detected through transcriptional profiling experiments showing changes in the expression of genes related to ethylene biosynthesis. Later it was shown that ethylene plays a foundational early role in the many responses observed in *Arabidopsis*. One of the most striking findings is the link between ethylene and reactive oxygen species (ROS) production, which is part of the signal transduction pathway in K^+ deprived plants. This mini-review will summarize what is known about the role ethylene plays in response to low potassium in *Arabidopsis* and other plant species.

Keywords: potassium, deficiency, ethylene, reactive oxygen species, roots

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Ethylene is a plant hormone whose biosynthesis and signal transduction pathways have been well-elucidated (Merchante et al., 2013). The active ethylene substance was discovered as early as the turn of the 20th century and the manipulation of ethylene has been very important in the post-harvest physiology and management of climacteric fruit. Using mutants of *Arabidopsis* that are sensitive and insensitive to the action of the hormone or precursors of ethylene biosynthesis and blockers has led to the discovery of many molecules involved in ethylene perception and action. In *Arabidopsis* the ethylene receptors (*ETR*, *ENS,* and *EIN4*) have been identified along with many of the downstream molecules involved in the progression of the ethylene initiated signal such as *EIN2*, *CTR1*, *ELFs*, *EILs,* and several other molecules (Merchante et al., 2013). One of the most interesting features of studying the role of ethylene in signal transduction pathways is that both insensitive mutants and mutants where the ethylene response is constitutively active are available. In addition to this array of mutants there are also several chemicals that block ethylene responses, such as Ag^+, 2-aminoethoxyvinyl-glycine (AVG), as well as chemicals such as ethephon that stimulate ethylene production. Due to the excellent biological tools that have been developed in the model plant *Arabidopsis* and the array of different inhibitors, research is well-positioned to study ethylene's role in signal transduction pathways and to test hypotheses related to the role of ethylene in higher plants in the context of nutritional stresses.

About 10 years ago Shin and Schachtman (2004) embarked on studies to identify the signal transduction cascade that led from the perception of low levels of potassium to the long term adaptive responses that occur, such as changes in shoot growth and inhibition of lateral root growth. The first indication that ethylene was involved in this signal transduction cascade came from a microarray experiment in which whole *Arabidopsis* plants were deprived of potassium for

6 and 30 h followed by a microarray analysis of roots to identify up and down regulated genes (Shin and Schachtman, 2004). Before these experiments in *Arabidopsis* very little was known about the responses to low potassium that occur in plants, except for the well-known phenomenon of the induction of high affinity K^+ uptake that was described by Epstein (Epstein and Hagen, 1952) and formed the basis for the first studies on high vs. low affinity uptake mechanisms in roots. These early microarray experiments provided new information to generate multiple hypotheses about different hormones, small molecules, and proteins involved in the complex response to low potassium conditions (Shin and Schachtman, 2004). Two clear responses observed in these microarray experiments were the changes in expression of genes related to reactive oxygen species (ROS) production and ethylene (Shin and Schachtman, 2004; **Figure 1**). Two genes related to ethylene biosynthesis [1-aminocyclopropane-1-carboxylic acid (ACC)] were upregulated and one gene related to ethylene perception (*ETR2*; Sakai et al., 1998) was upregulated. From these findings the hypothesis was developed that ethylene production increased under K^+ deprived conditions. This was tested by placing *Arabidopsis* seedlings into vials containing nutrient solution with and without potassium and analyzing the headspace for ethylene. This biochemical test showed that increased amounts of ethylene were produced upon deprivation of K^+, validating the hypothesis developed from gene expression data (Shin and Schachtman, 2004).

To fully develop an understanding of ethylene's role in the low potassium signal transduction cascade, studies were initiated using both ethylene mutants and ethylene inhibitors. Experiments using ethylene insensitive mutants (*ETR* and *EIN2*) and a mutant in which the ethylene signaling pathway was constitutively active (*CTR1*) due to the mutation in a negative regulator showed that these lines responded differently from wild type to a low potassium stress (Jung et al., 2009). Key phenotypic changes in these mutants showed that ethylene is important to the changes to root length, shoot fresh weight, and chlorophyll content that occur upon K^+ deprivation. The changes in these phenotypes that were seen in wild type *Arabidopsis* were not observed in the two different ethylene insensitive mutants and were obvious in the constitutively active mutant in the absence of low K^+ stress.

Since ROS was previously shown to be involved in the signal transduction pathway in response to low K^+, experiments were designed to determine whether the ethylene response was up- or down-stream from the induction of ROS. Results showed that when ethephon was added to stimulate ethylene production, the ROS levels in *Arabidopsis* roots increased. Also, the constitutively active ethylene mutants *ctr1* and *eto* all had elevated levels of ROS whereas in several insensitive mutants (*etr*, *ein*) the levels of ROS induced by ethylene were reduced upon K^+ deprivation (Jung et al., 2009). In the case of the insensitive mutants a slight increase in ROS was detected in the roots, which was much smaller than the increases seen in the wild type. This suggested that in potassium-deprived *Arabidopsis* roots there are also ethylene-independent pathways in response to low potassium (Jung et al., 2009).

Ethylene and ROS are both early in the low K^+ signaling network. To study how ethylene modulates the downstream

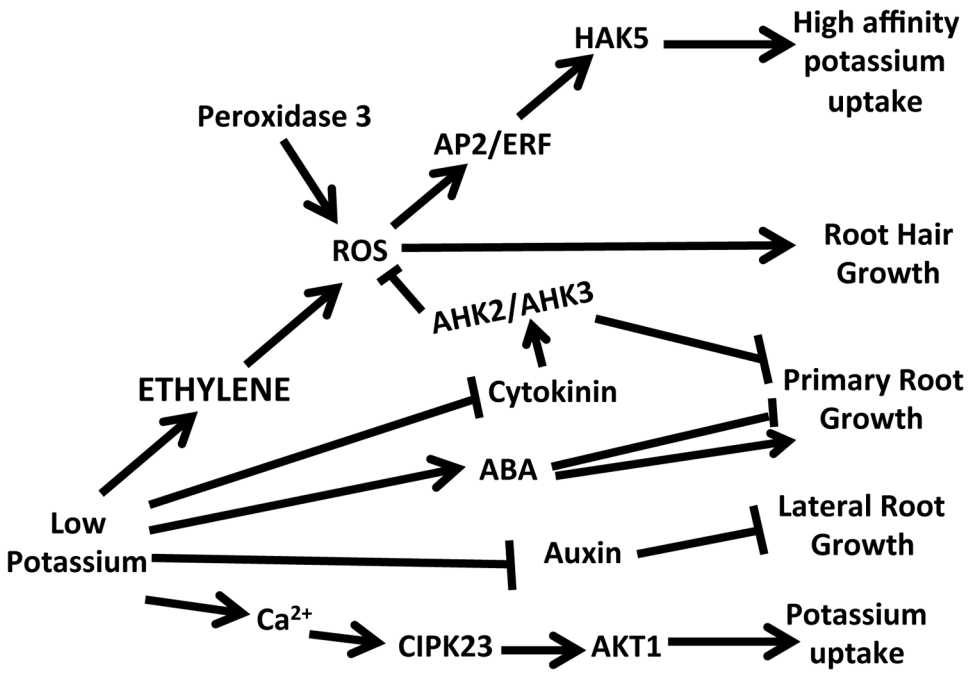

FIGURE 1 | A schematic of the hormone and calcium responses in *Arabidopsis* following potassium deprivation. Low potassium leads to increases in ethylene, ABA and calcium and decreases in cytokinin and auxin. Changes in these hormone levels are linked to changes in potassium uptake and different aspects of root growth.

signaling responses and to confirm the role of ethylene in the low K^+ signal transduction cascade, the expression was monitored of a high affinity K^+ transporter (*HAK5*), whose expression is triggered by low K^+ conditions. A promoter luciferase construct was used to study the effects of inhibitors and ethephon and real time PCR was used to study *HAK5* expression in several different ethylene mutants (Jung et al., 2009). The analysis of the promoter luciferase constructs showed that ethephon could induce expression of *HAK5* under full nutrient conditions and that inhibitors of ethylene reduced expression but did not totally eliminate expression under low K^+ conditions (**Figure 1**). This result supports the inference that there is also an ethylene-independent pathway in the low potassium signal transduction pathway. In the mutant of *ein2-1* that is insensitive to ethylene *HAK5* expression was reduced under low K^+ conditions whereas in two triple mutants (*etr1-6*, *etr2-3*, *ein4-4* and *etr2-3*, *ers2-3*, *ein4-4*) in which ethylene signaling is constitutively active, *HAK5* expression was elevated under full nutrient conditions.

In contrast to the inhibition of lateral root growth that is observed upon deprivation of potassium in *Arabidopsis* (Shin and Schachtman, 2004), root hair growth is increased (Pitts et al., 1998; Jung et al., 2009; **Figure 1**). In K^+ deprived *Arabidopsis* longer root hairs would be expected because more ethylene is produced in response to low K^+. In studies on *Arabidopsis* the ethylene insensitive mutants were tested as well as the inhibitors AVG and Ag^+ (Jung et al., 2009). Results showed that the ethylene insensitive mutants did not alter the elongation of root hairs under low K^+, but inhibitors even at very low concentrations did abolish the root hair elongation in response to low K^+. These results on root hair elongation correspond to what was found with the induction of root ROS, where inhibitors, but not the mutants tested completely abolished the increase in ROS. These results support the conclusion that ethylene is a positive regulator of root hair length under low K^+ conditions.

In addition to the phenotypes described above related to root growth and root hair elongation, the formation of root cortical aerenchyma (RCA) has been highlighted as an important mechanism involved in root adaptation to low concentrations of nutrients such as potassium, nitrogen, and phosphorus (Postma and Lynch, 2011). This single adaptive change in root structure has been shown to increase maize growth under low K^+ conditions by 72% (Postma and Lynch, 2011). Although there are no data demonstrating a direct role for ethylene under low K^+ conditions in triggering RCA formation, it has been shown that ethylene is involved in signaling RCA formation (Drew et al., 2000a,b). Together the increase in ethylene under low K^+ conditions and the strong adaptive advantage of RCA under low K^+ conditions suggest the two may be linked, and therefore warrant further experimentation to demonstrate this as a possible avenue for increasing plant growth under low K^+ conditions.

As part of the studies related to signal transduction processes involved in *Arabidopsis* root responses to low K^+ it was shown that the downregulation of *MYB77* (Shin et al., 2007) plays a role in the reduction of lateral root growth under low potassium conditions. Ethylene may also play a role in reducing lateral root growth in K^+ deprived roots as it has been shown to decrease lateral root growth in *Arabidopsis* (Lewis et al., 2011). In roots of plants grown in full nutrients the response to ethylene is mediated by high rates of auxin transport via *PIN3* and *PIN7,* which prevents the localization of auxin that is needed for lateral root formation (Lewis et al., 2011). Under low K^+ conditions there may be important interactions between ethylene and auxin that lead to altered lateral root growth.

Similar to what has been shown in *Arabidopsis*, several genes involved in ethylene biosynthesis and signaling were upregulated by K^+ deprivation in watermelon (*Citrullus lanatus* Thunb.; Fan et al., 2014). In that study two varieties of watermelon with contrasting K^+ efficiencies were used for transcriptional profiling to identify changes in gene expression and to reveal differences in the molecular basis of varietal differences. The roots of the watermelon varieties were profiled 6 and 120 h after K^+ deprivation. At the 120 h time point ACC was upregulated, as well as genes related to ethylene perception and signaling including: *ERF1*, *ERF-1,* and *EBF1* in the less efficient watermelon variety (8424; Fan et al., 2014). At the earlier stage, 6 h after deprivation, several ethylene-related genes were downregulated highlighting a slight difference between *Arabidopsis* and watermelon. In another study that compared barley lines that differed in response to low potassium the genes involved in ethylene biosynthesis or signaling were either not altered by low potassium or down regulated (Zeng et al., 2014). These gene expression differences across plant species could be due to different signaling pathways, or more likely differences in physiological processes involved in response time to low K^+.

Aside from studies on *Arabidopsis* and watermelon roots there is one other study that characterized the ethylene response of sunflower leaves in plants that were grown under potassium deficient conditions. In sunflower an interesting interaction was shown. Closure of stomata due to drought was partially short circuited by K^+ deprivation. Stomatal conductance of droughted and K^+ deprived plants was higher than plants that were only drought stressed (Benlloch-Gonzalez et al., 2008). In this system the leaves of the drought stressed and K^+ deprived sunflower plants were shown to be producing more ethylene, which was correlated with increased stomatal conductance (Benlloch-Gonzalez et al., 2010). To further strengthen the correlation between ethylene and stomatal conductance under low K^+ conditions, cobalt was used as an inhibitor of ethylene synthesis. When ethylene was inhibited by low levels of cobalt, plants showed a decrease in stomatal conductance under low K^+ conditions lower than wild type. Under low K^+ conditions in sunflower ethylene plays a role in modulating stomatal conductance under drought, which may be an adaptive response that increases the transport of K^+ from roots to leaves (Benlloch-Gonzalez et al., 2010).

Ethylene plays very important roles in the *Arabidopsis* signal transduction network for plant adaptation to low potassium conditions as well as deprivation of other nutrients including phosphorus, sulfur, iron and nitrogen (Garcia et al., 2015). In *Arabidopsis* ethylene is an early signaling molecule that leads to the induction of ROS and ultimately to the expression of a high affinity potassium transporter that is important in the uptake of K^+. Ethylene also plays a role in root hair elongation and in modulating stomatal conductance in response to drought and

low K^+. Although these findings suggest a key role for ethylene in plant response to low K^+ there are still many open questions. One major question that has not yet been explored is the cross talk among ethylene and the other hormones (**Figure 1**) that have been shown to be important under low K^+ conditions (Armengaud et al., 2004; Schachtman and Shin, 2007; Shin et al., 2007; Kim et al., 2009; Nam et al., 2012) as well as the interactions with other nutrient stresses (Iqbal et al., 2013).

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Links Between Ethylene and Sulfur Nutrition—A Regulatory Interplay or Just Metabolite Association?

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Multiple reports demonstrate associations between ethylene and sulfur metabolisms, however the details of these links have not yet been fully characterized; the links might be at the metabolic and the regulatory levels. First, sulfur-containing metabolite, methionine, is a precursor of ethylene and is a rate limiting metabolite for ethylene synthesis; the methionine cycle contributes to both sulfur and ethylene metabolism. On the other hand, ethylene is involved in the complex response networks to various stresses and it is known that S deficiency leads to photosynthesis and C metabolism disturbances that might be responsible for oxidative stress. In several plant species, ethylene increases during sulfur starvation and might serve signaling purposes to initiate the process of metabolism reprogramming during adjustment to sulfur deficit. An elevated level of ethylene might result from increased activity of enzymes involved in its synthesis. It has been demonstrated that the alleviation of cadmium stress in plants by application of S seems to be mediated by ethylene formation. On the other hand, the ethylene-insensitive *Nicotiana attenuata* plants are impaired in sulfur uptake, reduction and metabolism, and they invest their already limited S into methionine needed for synthesis of ethylene constitutively emitted in large amounts to the atmosphere. Regulatory links of EIN3 and SLIM1 (both from the same family of transcriptional factors) involved in the regulation of ethylene and sulfur pathway, respectively, is also quite probable as well as the reciprocal modulation of both pathways on the enzyme activity levels.

Keywords: abiotic stress, ethylene, sulfur nutrition, LSU, SLIM1, signaling

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INTRODUCTION

Sulfur (S) is an important macronutrient for all organisms. Plants can metabolize inorganic sulfur that is taken up from the soil in the oxidized form (sulfate) and then it is reduced and incorporated into a broad range of primary and secondary metabolites. Some of them serve as precursors of other important (but not S-containing) cellular compounds. A schematic overview of the S assimilation pathway, including most of the related metabolites, is shown in **Figure 1**. The crosstalk between sulfur assimilation and ethylene signaling in plants attracts more attention because of the growing number of data concerning the influence of S nutrition on ethylene signaling and production, as well as the impact of ethylene on the expression of S genes, activity of S enzymes and level of S metabolites (Iqbal et al., 2013). Here, we briefly summarize the most important facts and observations related to the links between ethylene and S nutrition and propose a working model of the complex signaling and regulatory interplay between these two factors.

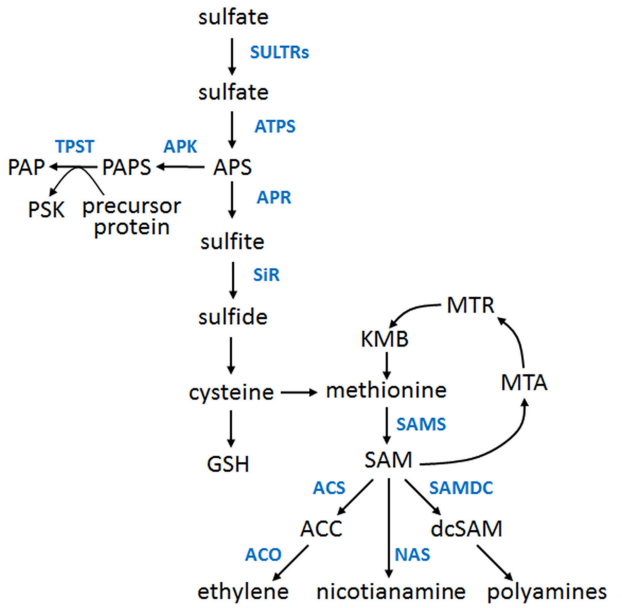

FIGURE 1 | An overview of the sulfur assimilation pathway and major sulfur metabolites. Only the selected metabolites (black fonts) and selected enzymes (blue fonts) of the pathway are presented. ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC oxidase; ACS, ACC synthase; APK, APS kinase; APR, APS reductase; APS, adenosine 5′-phosphosulfate; ATPS, ATP sulfurylase; dcSAM, decarboxylated SAM; GSH, reduced glutathione; KMB, α-keto-γ-methylthiobutyric acid; MTA, S-methyl-5′-thioadenosine; MTR, S-methyl-5-thio-D-ribose; NAS, nicotianamine synthase; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PSK, phytosulfokine; SAM, S-adenosylmethionine; SAMDC, SAM decarboxylase; SAMS, SAM synthase; SiR, sulfite reductase; SULTR, sulfate transporter; TPST, tyrosylprotein sulfotransferase.

SULFUR METABOLITES AS PRECURSORS IN ETHYLENE SYNTHESIS

Methionine (Met), a sulfur-containing amino acid is a substrate for S-adenosylmethionine synthase (SAMS) responsible for the synthesis of S-adenosylmethionine (SAM or AdoMet), an important metabolite in animals and plants (Fontecave et al., 2004; Roje, 2006). SAM serves as a donor of methyl, amino, ribosyl, and aminoalkyl groups. It is also a source of controlled 5′-deoxyadenosine radicals. In plants, SAM is a precursor of polyamines (PA), nicotianamine (NA) used to produce phytosiderophores, and ethylene. Production of ethylene is a two-step reaction with 1-aminocyclopropane-1-carboxylate (ACC), as a product of the first reaction, catalyzed by ACC synthase, and the substrate for the second reaction catalyzed by ACO (ACC oxidase; **Figure 1**). Met and SAM used for PA, NA and ethylene biosynthesis are recycled in the Met salvage cycle (known also as a Yang cycle). Noteworthy, soluble Met is apparently a rate-limiting metabolite of ethylene biosynthesis (Katz et al., 2006; Bürstenbinder et al., 2007), however for further details on the additional salvage cycles, regulatory circuits and complex relationships between the metabolites and enzymes, please see the reviews (Amir, 2010; Sauter et al., 2013). A new player, a plasma membrane receptor-like kinase, FERONIA, involved in the regulation of SAMS in *Arabidopsis thaliana,* has been recently reported (Mao et al., 2015).

Additional complexity is added by the fact that ACC seems to have more functions than just being the precursor of ethylene. It is a subject of short- and long-distance dedicated transport, can be conjugated to form three different derivatives. It also seems to be a signaling molecule by itself (Van De Poel and Van Der Straeten, 2014).

SULFUR NUTRITION AFFECTS ETHYLENE SYNTHESIS DURING VARIOUS STRESSES

Sulfur nutrition has been reported to modulate the stress response by increasing ethylene production in several stresses. The most intensively studied is cadmium (Cd)-induced stress. The results of experiments with mustard and wheat indicated that the reduced sensitivity of plants to ethylene due to Cd exposure is elevated with additional S supply. S application increased photosynthesis and dry mass, and resulted in the alleviation of oxidative stress by increasing the levels of antioxidant compounds, such as reduced glutathione (GSH; Masood et al., 2012; Asgher et al., 2014; Khan et al., 2015).

Drought stress has been shown to down-regulate S metabolism and ethylene enzymes in medicago roots and nodules (Larrainzar et al., 2014). Also in cassava grown during the dry season, the association of ethylene level with sulfur metabolism and GSH level in root cortex tissues was observed (Saithong et al., 2015). The regulatory aspect of various primary and secondary S metabolites in relation to drought response, including the role of 3′-phosphoadenosine 5′-phosphate (PAP) produced from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in retrograde signaling, were recently reviewed (Chan et al., 2013). Besides, the authors underline that various osmoprotectants (for example PA), accumulating during drought stress, require restoring the sulfur moiety in SAM through the Yang cycle.

Moreover, it has been shown that the effects of salt stress (inhibition of photosynthesis) in mustard can be reversed by excess S, and this reversal involved ethylene because the inhibition of ethylene biosynthesis counteracted the effects of S excess on salt stress alleviation (Nazar et al., 2014). The authors suggest that under salt stress, S was used for GSH synthesis instead of ethylene formation, while excess S resulted in increased ethylene, stimulating more efficient utilization of intracellular CO_2 for photosynthesis (Nazar et al., 2014).

Several clusters of genes upregulated by iron (Fe) deficiency in Arabidopsis were reported; one of them contains genes with a function predominantly linked to S assimilation and genes induced by S deficiency (Ivanov et al., 2012). However, the regulatory links between Fe and S metabolism are still unclear. There are contradicting reports on the influence of Fe nutrition on the expression of genes encoding sulfate transporters. On one hand, genes encoding two high affinity sulfate transporters were induced during Fe starvation in tomatos (Paolacci et al., 2014), while, on the other hand, Fe starvation reduced the expression

of *SULTR1;1*, encoding the high affinity sulfate transporter in the Arabidopsis roots (Forieri et al., 2013). Moreover, S deprivation limited Fe-deficiency responses in tomatos (Zuchi et al., 2009), however additional S nutrition ameliorated the damages in photosynthetic apparatus caused by Fe deficiency in oilseed rape (Muneer et al., 2014). The existence of co-regulation of S and Fe metabolism was recently discussed in terms of a possible role of several metabolic processes, including the involvement of [Fe–S] clusters in creating the important feedback signal leading to adjustment of the metabolism, for example, Fe and S uptake (Forieri et al., 2013). The role of ethylene in such co-regulation is unclear.

Transcriptomic analysis of grape berries treated with SO_2 revealed the reprogramming of transcriptome after treatment. Transcripts involved in auxin, ethylene and jasmonate signaling were strongly upregulated, including transcripts encoding auxin responsive proteins, ACC synthase, ACC oxidase, ethylene responsive proteins and lipoxygenase (Giraud et al., 2012). In addition to the S supply, S limitation also results in induction of the ethylene pathway. For example, a short-term S limitation (2 days) resulted in increased expression of some ethylene-related genes (Lewandowska et al., 2010) and elevated ethylene level (Moniuszko et al., 2013) in tobacco and a long-term S limitation (35 days) resulted in an increased amount of ACS in oilseed rape plants (D'hooghe et al., 2013). Interestingly, no increase of ethylene synthesis was observed when tomato plants were starved for S and Fe simultaneously (Zuchi et al., 2009).

VICE VERSA: ETHYLENE AND ETHYLENE SIGNALING AFFECTS SULFUR METABOLISM

Accumulation of APR activity as a result of the treatment of Arabidopsis with 0.2 mM ACC has been shown (Koprivova et al., 2008). Additionally, ethylene has been shown to increase ATP-sulfurylase activity and S accumulation in mustard (Iqbal et al., 2012). However, these few reports cannot be extrapolated into a universal hypothesis that ethylene stimulates S metabolism and accumulation. In fact, despite the above-mentioned increased production of ethylene during a response to S deficiency in *Nicotiana tabacum* (Moniuszko et al., 2013) and *Solanum lycopersicum* (Zuchi et al., 2009), the transcription of only a fraction of ethylene responsive genes was affected. Similar results could be extracted from microarray studies on Arabidopsis (Hirai et al., 2003; Nikiforova et al., 2003).

Consistent lack of correlation of the transcriptomics data with ethylene measurements suggests an association of S deficit with ethylene signaling machinery rather than with ethylene production. Moreover, recent reports put forward the possible occurrence of the cross talk between Sulfur LIMitation 1 transcription factor (SLIM1, described in the next chapter) and ethylene receptors. The re-analysis of the Arabidopsis microarray data showed that silver nitrate mimics the signal for perception of sulfur deficiency in plants at the transcriptome level (Moniuszko, 2015). The author identified 20 genes that were similarly regulated under S deficit and $AgNO_3$ treatment. Noteworthy, all 20 are considered S deficiency markers, and three of them (*LSU1, LSU2,* and *SULTR1;2*) are candidates for regulators of responses to S deficiency (Moniuszko et al., 2013; Zhang et al., 2014). Only two of them (APR2 and APR3) cannot be linked with SLIM1 during the plant's early response to S deficiency. The analysis also showed that the similarity between S deficit and $AgNO_3$ treatment is rather linked to the silver nitrate action on ethylene receptors than to other $AgNO_3$ effects (Moniuszko, 2015).

This mostly theory driven conclusion is supported by previously overlooked studies. It has been shown that *Eruca sativa* proteomic response to Ag+ ions is related to S metabolism (Vannini et al., 2013). The observed changes in S metabolites of *E. sativa* due two Ag+ exposures strongly suggest SLIM1 involvement. In addition, the heterologous expression of the Arabidopsis ethylene receptor gene, *etr1-1* (which encodes mutated ETR1 protein unable to relay ethylene signal after hormone binding), in *N. attenuata* resulted in impaired sulfate uptake and S metabolism (Meldau et al., 2013). Abnormal phenotypes of such seedlings under optimal sulfate supply (similar to plants grown under S deficit) suggest a defect in SLIM1 action as a result of changes in ethylene signaling at the receptor level. Apparently, the etr1-1 receptor, despite (and in addition to) its inability to properly function in a classic linear ethylene-signaling pathway, was mimicking the signal of S deficiency.

On the other hand, proper ethylene signaling was found to be necessary for increased GSH accumulation after ozone treatment. In the *ein2* Arabidopsis mutant plants, 6 h after ozone exposure, the increment of GSH level was much lower than in the control plants (Yoshida et al., 2009). Research involving the extrapolation of such regulation on different stresses falls way behind. Presently, the cross talk between GSH biosynthesis and ethylene signaling has been proposed only for Cd and drought (Masood et al., 2012; Saithong et al., 2015). Both cases have been discussed above regarding the S nutrition effect on ethylene production. However, we want to emphasize here that in the case of Cd treated mustard, the effects of additional S supply were reversed by the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG), and that similar effects were achieved by additional S supply and ethephon treatment (Masood et al., 2012). Thus, the authors suggested a prominent role of ethylene (possibly on GSH biosynthesis) in S-induced alleviation of Cd stress. However, this might be the reflection of a switch between the ethylene receptors' role in S status sensing and linear ethylene signaling, as discussed in a recently proposed model (Moniuszko, 2015). Nevertheless, further studies are needed to clarify the exact molecular mechanism behind the observed effects of ethylene and ethylene signaling on sulfur metabolism and its regulation.

POSSIBLE REGULATORY MECHANISMS RESPONSIBLE FOR COUPLING SULFUR AND ETHYLENE SIGNALING AND METABOLISM

The transcriptional control of gene expression very often serves to reprogram plant metabolism in order to cope with environmental challenges. So far the only described transcription factor exclusively assigned to affect gene expression during S

deficiency is SLIM1 from Arabidopsis (Maruyama-Nakashita et al., 2006). Certainly, attracting attention in the perspective of this review is the fact that SLIM1 belongs to the same plant protein family as EIN3, the main transcription factor controlling the expression of ethylene-responsive genes. It was initially identified as *ETHYLENE-INSENSITIVE-LIKE 3 (EIL3)* coding for a putative transcription factor of unknown function (Guo and Ecker, 2004). Analyses of the knockout mutants revealed that SLIM1 affects the expression of various genes facilitating the increased flux through the sulfate assimilation pathway and translocation of sulfate to the shoot, but it also controls the degradation of glucosinolates under sulfur deficient conditions (Maruyama-Nakashita et al., 2006). The functional complementation of the *slim1* mutant was only successful with SLIM1 and not any other protein member of EIL family, pointing out its specificity. Moreover, the treatment of plants with the precursor of ethylene, ACC, does not affect the transcription of any of SLIM1-dependent genes (Maruyama-Nakashita et al., 2006). It is tempting to speculate that the C-terminal part of the EIL proteins is responsible for that functional separation since all of them are highly homologous to one another, mainly in their N-terminal half of around 300 amino acid residues. All six members of the Arabidopsis EIL family share highly acidic N-terminal amino acids, five small clusters of basic amino acids scattered mostly in the first half of the protein and a proline-rich domain (Chao et al., 1997). SLIM1 served as a template to model the unique DNA-binding domain of the EIL family, consisting of five alpha helices, packed together into a globular shape as a whole (Yamasaki et al., 2005). The DNA-binding abilities of EIN3, EIL1, and EIL2 proteins have been demonstrated with ethylene response DNA elements, which are 28-nt imperfect palindromes, using an electro-mobility shift assay (Solano et al., 1998). The interaction of SLIM1 with those sequences is very unstable and is only detectable with surface plasmon resonance (Yamasaki et al., 2005), demonstrating the binding preferences between EIL family members. SLIM1 strongly binds to 20-nt consensus, called the UPE-box, which is only present in the promoters of eight genes that are strongly induced by S deficiency in Arabidopsis (Wawrzynska et al., 2010). Yet three of these genes encode proteins from the LSU family, homologs of tobacco UP9C protein (Sirko et al., 2014). Silencing of *UP9C* expression in tobacco led to disturbances of the ethylene signaling and synthesis pathways during conditions of S deficiency (Moniuszko et al., 2013).

In contrast to EIN3, not much is known about SLIM1 posttranslational modifications or its interaction with other proteins (Wawrzynska and Sirko, 2014). Its transcription level is not modulated by the changes of S conditions (Maruyama-Nakashita et al., 2006); however a strong elevation is observed in root tissue during Fe deficiency (Garcia et al., 2010). SLIM1 can bind with MYB72, which together with MYB10 induce the nicotianamine synthase gene *NAS4* governing proper homeostasis of Fe during its deficiency. However, this also triggers jasmonate/ethylene-dependent systemic resistance (Van

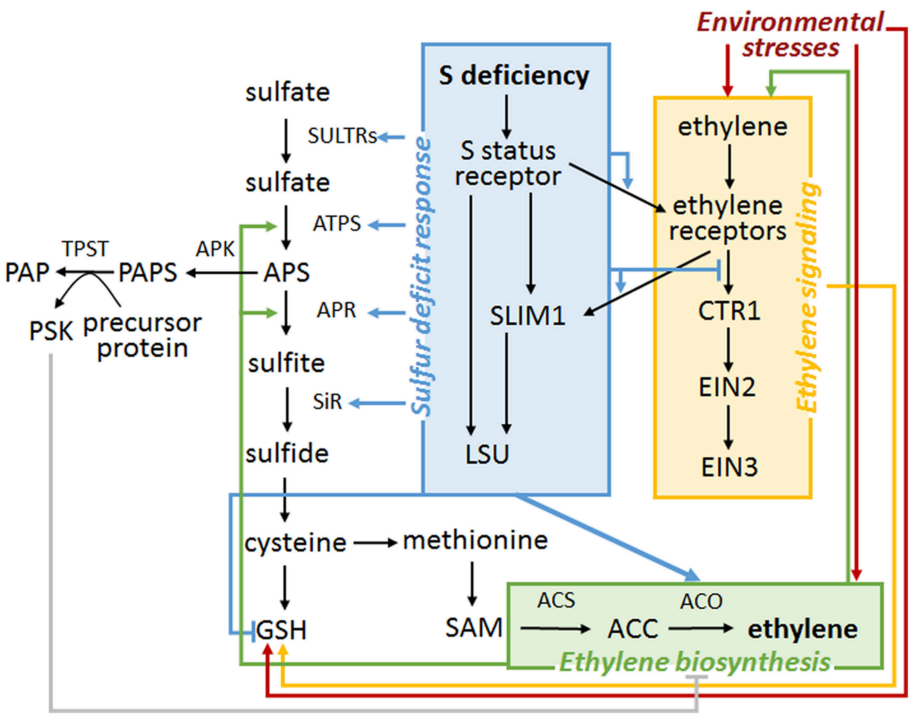

FIGURE 2 | A hypothetical model of regulatory links between S– and ethylene sensing and signaling. Only the selected metabolites, enzymes and other players are presented. The black arrow represents one-step or multiple-step signaling or metabolic pathway progress. Colored arrows (gray, red, blue, green, orange) represent regulatory mechanisms reported in the published studies. At the current stage, most of these mechanisms are obscurely documented and need further research. Additionally, the S status sensor is elusive.

Der Ent et al., 2008; Palmer et al., 2013). On the other hand, MYB72 is a direct target of FIT, a central regulator of Fe assimilation in roots (Sivitz et al., 2012). FIT abundance is controlled by interaction with EIN3, which reduces FIT proteasomal degradation leading to a higher level of expression of the genes involved in Fe acquisition (Lingam et al., 2011). Both SLIM1 and EIN3, therefore, seem to tune up Fe homeostasis when plants meet the conditions of deficiency.

Despite the possible cross talk between ethylene and S deficiency signals on the level of EIN3 and SLIM1 transcriptional factors, the regulation on the level of stability of enzymes involved in ethylene synthesis might be also envisaged. Such possibilities might be deduced from the reported interaction of the above-mentioned UP9C protein with ACO in tobacco (Moniuszko et al., 2013). Interestingly, many members of the LSU family are induced during S starvation and it is tempting to speculate that the interaction of these proteins with ACO serves some regulatory reason because of the lack of S-deficiency induced elevation of ethylene level in tobacco plants with lowered expression of *UP9C* (Moniuszko et al., 2013). Notably, the posttranslational regulation of ACS is a well-known phenomenon; however information about such regulation of ACO is thus far limited. Nevertheless, this possibility is supported by the transcriptomic-based kinetic model for ethylene synthesis in tomato fruits that indicates the existence of potential posttrancriptional regulation of ACO (Van De Poel et al., 2014).

Moreover, the small (five amino acids) peptide, phytosulfokine (PSK), a growth factor containing sulfated tyrosine might be an additional player in this complex signaling and regulatory network. PSK is produced from an 80-amino-acid-long precursor (there exist six *PSK* genes in Arabidopsis) via tyrosine sulfation and proteolytic processing (Matsubayashi, 2014; Sauter, 2015). Recent analysis of the Arabidopsis *tpst-2* mutant defective in tyrosylprotein sulfotransferase revealed that PSK suppresses ethylene production (Wu et al., 2015).

The hypothetical model explaining possible co-regulation of sulfur and ethylene signaling in plants is shown in **Figure 2**. According to the model, S availability modulates ethylene sensitivity due to a switch of ethylene receptor function. S deficiency might also affect ethylene production by stabilizing ACO level or activity. On the other hand, ethylene production is negatively affected by the sulfated phytohormone, PSK. The functional ethylene pathway is necessary for increased level of GSH in some stresses. Moreover, ethylene seems to stimulate the activity of several enzymes involved in S assimilation.

CONCLUSIONS

Ethylene production and sulfur assimilation pathways have close boundaries and share some metabolites. Thus, they might have also common regulatory elements. Although numerous observations suggest that these two pathways might indeed share some sensing or signaling elements, the molecular details are still obscure. Additional experiments are required to clarify and explain some contradicting and imprecise data. Answers to the following questions might help to elucidate the molecular basis of the postulated cross-talk of both signaling pathways: What is the S deficiency signal? What molecules function as the S status receptors? What factors are directly involved in linking these two pathways?

AUTHOR CONTRIBUTIONS

AS drafted the manuscript. All authors were involved in the writing process and preparing the final version.

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Ethylene Participates in the Regulation of Fe Deficiency Responses in Strategy I Plants and in Rice

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Iron (Fe) is very abundant in most soils but its availability for plants is low, especially in calcareous soils. Plants have been divided into Strategy I and Strategy II species to acquire Fe from soils. Strategy I species apply a reduction-based uptake system which includes all higher plants except the Poaceae. Strategy II species apply a chelation-based uptake system which includes the Poaceae. To cope with Fe deficiency both type of species activate several Fe deficiency responses, mainly in their roots. These responses need to be tightly regulated to avoid Fe toxicity and to conserve energy. Their regulation is not totally understood but some hormones and signaling substances have been implicated. Several years ago it was suggested that ethylene could participate in the regulation of Fe deficiency responses in Strategy I species. In Strategy II species, the role of hormones and signaling substances has been less studied. However, in rice, traditionally considered a Strategy II species but that possesses some characteristics of Strategy I species, it has been recently shown that ethylene can also play a role in the regulation of some of its Fe deficiency responses. Here, we will review and discuss the data supporting a role for ethylene in the regulation of Fe deficiency responses in both Strategy I species and rice. In addition, we will review the data about ethylene and Fe responses related to Strategy II species. We will also discuss the results supporting the action of ethylene through different transduction pathways and its interaction with other signals, such as certain Fe-related repressive signals occurring in the phloem sap. Finally, the possible implication of ethylene in the interactions among Fe deficiency responses and the responses to other nutrient deficiencies in the plant will be addressed.

Keywords: ethylene, Fe deficiency responses, iron, regulation, rice, Strategy I, Strategy II

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INTRODUCTION

Iron (Fe) is very abundant in most soils, mainly as Fe^{3+}, although its availability to plants is low, especially in high pH calcareous soils (Römheld and Marschner, 1986). On the other hand, excessive Fe accumulation by the plant may lead to toxic effects (Romera et al., 2014; Brumbarova et al., 2015). Therefore, Fe acquisition is highly regulated. Based on the mechanisms developed to facilitate mobilization and uptake of Fe, plants are classified into Strategy I species and Strategy II species. Strategy I species include all higher plants excluding the Poaceae and Strategy II species

include the Poaceae (Römheld and Marschner, 1986; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012).

The main characteristic of Strategy I species is the necessity for reduction of Fe^{3+} to Fe^{2+}, by means of a plasma membrane ferric reductase, prior to its root absorption through a Fe^{2+} transporter (**Figure 1**; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012). When grown under Fe deficiency, Strategy I species induce several physiological and morphological responses in their roots, that facilitate Fe mobilization to roots and uptake (see Section Role of Ethylene in the Regulation of Fe Deficiency Responses in Strategy I Species).

To obtain Fe from the soil, Strategy II species release PS (PhytoSiderophores) from their roots, which form stable Fe^{3+}-chelates. These Fe^{3+}-chelates (Fe^{3+}-PS) are then taken up by specific epidermal root cell plasma membrane transporters (**Figure 2**; Kobayashi and Nishizawa, 2012). Under Fe-deficient conditions, Strategy II species greatly increase the production and release of PS, the number of Fe^{3+}-PS transporters and develop other physiological and regulatory responses (Kobayashi and Nishizawa, 2012; see Section Role of Ethylene in the Regulation of Fe Deficiency Responses in Rice and Strategy II Species). Rice, traditionally considered a Strategy II species (Kobayashi and Nishizawa, 2012), presents some characteristics of Strategy I species, such as enhanced Fe^{2+} uptake through a Fe^{2+} transporter (**Figure 2**; Ishimaru et al., 2006, 2011; Kobayashi et al., 2014). For this reason, some authors consider it as a plant species that uses a combined strategy (Ricachenevsky and Sperotto, 2014).

Once adequate Fe has been absorbed, Fe deficiency responses need to be down regulated to avoid toxicity and to conserve energy. The regulation of these responses is not fully understood but several hormones and signaling substances have been proposed to participate in their activation, like auxin (Landsberg, 1984), ethylene (Romera and Alcántara, 1994), and NO (nitric oxide; Graziano and Lamattina, 2007), as well as in their suppression, like cytokinins (Séguéla et al., 2008), jasmonic acid (Maurer et al., 2011), and brassinosteroids (Wang et al., 2012). These hypotheses have been mainly focused on Strategy I species while the role of hormones and signaling substances on the regulation of Fe deficiency responses in Strategy II species has been less studied.

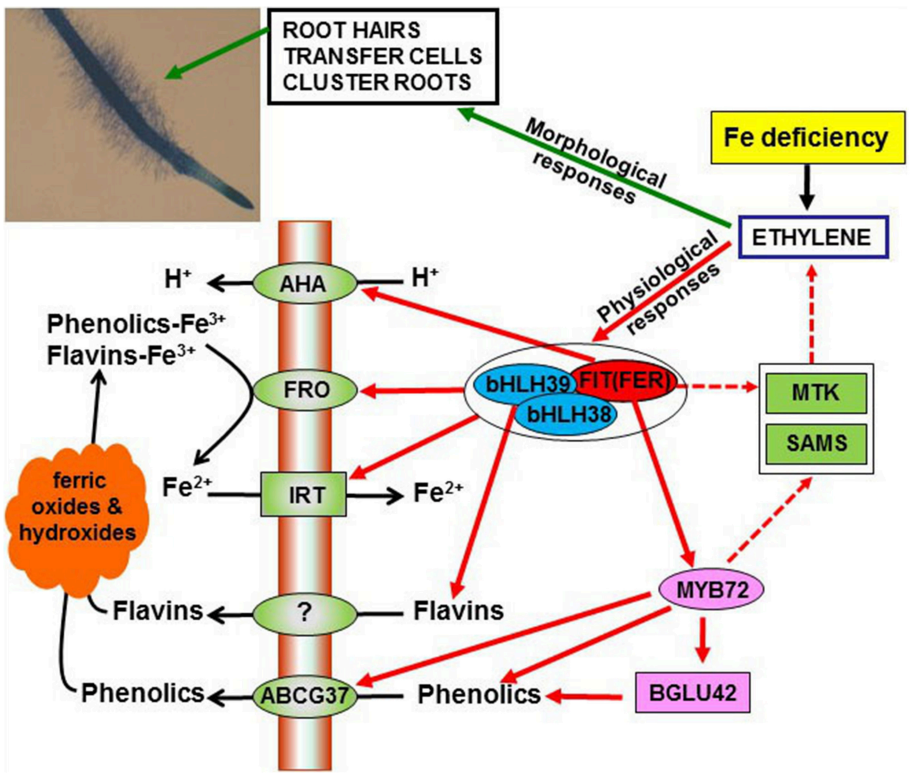

FIGURE 1 | Overview of the role of ethylene on the regulation of morphological and physiological responses to Fe deficiency in Strategy I species. Ethylene, through the activation of the transcription factors FIT (FER), bHLH38 and bHLH39, can up-regulate the expression of *FRO* (ferric reductase), *IRT* (iron transporter) and flavin synthesis genes, thus increasing ferric reductase activity, Fe^{2+} uptake and flavin synthesis. Similarly, ethylene, through FIT (FER), can up-regulate *AHA* (H^+-ATPase) genes, thus causing acidification, and activate the MYB72 transcription factor, which in turn up-regulates genes related to phenolics synthesis. Moreover, MYB72 activates the β-glucosidase BGLU42 and the phenolic efflux transporter ABCG37, both being implicated in the secretion of phenolic compounds. Ethylene has also been implicated in the development of different morphological responses, such as subapical root hairs, root epidermal transfer cells and cluster roots. For the development of these morphological responses, FIT (FER) could indirectly act by affecting ethylene synthesis, through the upregulation of *MTK* and *SAMS* (see **Figure 3**).

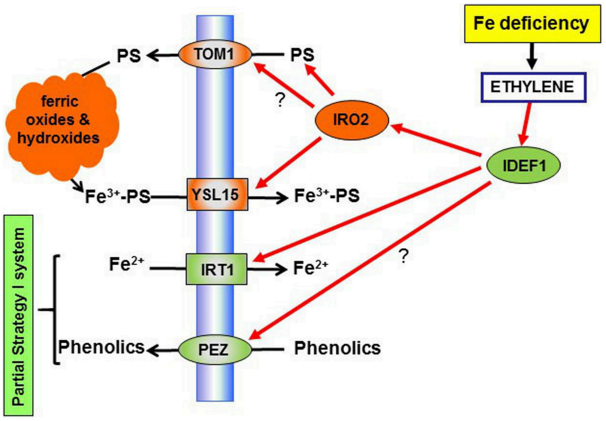

FIGURE 2 | Overview of the role of ethylene on the regulation of physiological responses to Fe deficiency in rice. Ethylene, through the subsequent activation of the transcription factors IDEF1 and IRO2, could activate the synthesis of PS (through up-regulation of *NAS* genes; see **Figure 3**), the expression of the PS efflux transporter TOM1 (not demonstrated yet) and of the PS-Fe^{3+} transporter, YSL15. Moreover, through the activation of the transcription factors IDEF1, ethylene could up-regulate the Fe^{2+} transporter IRT1, and the phenolic efflux transporter PEZ (not demonstrated yet). PS, physotiderophores.

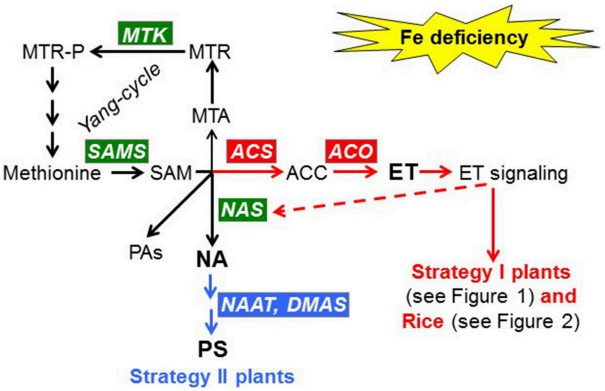

FIGURE 3 | Effect of Fe deficiency on the synthesis of NA (nicotianamine), PS (phytosiderophores), and ET (ethylene) in Strategy I and Strategy II species. Fe deficiency induces NA production in both Strategy I and Strategy II species by up-regulating the expression of genes implicated in the Yang-cycle, like *MTK* and *SAMS*, and by up-regulating *NAS* genes (in green). In Strategy I species and Rice, Fe deficiency induces ET production by up-regulating *ACS* and *ACO* genes (in red). ET can then up-regulate *NAS* genes and activate Fe responses in Strategy I plants and Rice. In Strategy II species, Fe deficiency induces PS production by up-regulating the expression of genes implicated in PS synthesis, such as *NAAT* and *DMAS* (in blue). MTK, methylthioribose kinase; SAMS, S-adenosyl metionine synthetase; ACS, ACC synthase; ACO, ACC oxidase; NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; DMAS, deoxymugineic acid synthase; PAs, polyamines.

In Strategy I species, accumulating evidence supports a role for auxin, ethylene and NO in the activation of Fe deficiency responses through the upregulation of Fe-related genes (Lucena et al., 2006; Graziano and Lamattina, 2007; Waters et al., 2007; Chen et al., 2010; García et al., 2010, 2011; Bacaicoa et al., 2011; Lingam et al., 2011; Meiser et al., 2011; Meng et al., 2012; Wu et al., 2012; Yang et al., 2013, 2014). The implication of all these substances is not unexpected, since auxin, ethylene and NO are closely interrelated (Romera et al., 2011, in press).

This review will focus on the role of ethylene in the regulation of Fe deficiency responses in Strategy I species, although some results related to rice and Strategy II species will also be presented and discussed. We will also review results that suggest interactions among Fe deficiency responses and the responses to other nutrient deficiencies. The possible implication of ethylene in these interactions will also be discussed.

ETHYLENE SYNTHESIS AND SIGNALING UNDER Fe DEFICIENCY

Ethylene is synthesized from methionine via a pathway that requires the enzymes SAMS (SAM synthetases), ACS (ACC synthases) and ACO (ACC oxidades; **Figure 3**; Sauter et al., 2013). Besides ethylene, SAM (S-adenosyl methionine) is also the precursor for the synthesis of NA (nicotianamine), PAs (polyamines) and PS (phytosiderophores; **Figure 3**; Kobayashi and Nishizawa, 2012; Sauter et al., 2013). The Yang-cycle allows the recycling of methionine, being MTK (Methyl Thioribose Kinase) one of the enzymes that participates in this cycle (**Figure 3**; Sauter et al., 2013). Although ethylene's mode of action is not fully understood, a linear signaling pathway has been proposed in Arabidopsis (Shakeel et al., 2013; Wang et al., 2013b):

$$\text{ET} \dashv \text{ETreceptors} \rightarrow \text{CTR1} \dashv \text{EIN2} \rightarrow \text{EIN3/EILs} \rightarrow \text{ERFs} \rightarrow \text{ETresponses}$$

In the absence of ET (ethylene), the kinase CTR1 phosphorylates EIN2 (which is localized to the ER membrane), preventing the cleavage and translocation of the EIN2 C-terminal fragment into the nucleus. In the presence of ethylene, CTR1 is inactivated, resulting in dephosphorylation of EIN2 and its cleavage. The EIN2 C-terminal fragment is then translocated into the nucleus, where it participates in stabilization of the transcription factor EIN3 and downstream gene activation (Shakeel et al., 2013; Wang et al., 2013b). EIN3 belongs to a small family of transcription factors that also includes various EIN3-like proteins: EIL1, EIL2, and EIL3 (Wang et al., 2013b). Mutants of CTR1 present constitutive activation of ethylene signaling, while mutants of EIN2 and EIN3 display reduced sensitivity to ethylene (Shakeel et al., 2013; Wang et al., 2013b). The ERF (Ethylene Response Factor) transcription factors act downstream of EIN3 to activate or repress ethylene-responsive genes although some ERFs can be activated by ethylene-independent transcription factors, not related to EIN3 (Wang et al., 2013b; Thirugnanasambantham et al., 2015).

Fe deficiency can influence both ethylene synthesis and signaling. Additionally, ethylene production can increase upon Fe excess (Yamauchi and Peng, 1995; Li et al., 2015).

Ethylene Synthesis and Signaling in Strategy I Species

Romera et al. (1999) showed that Fe-deficient roots of several Strategy I species produced more ethylene than the Fe-sufficient ones, even before the plants showed any other symptom of deficiency. This excludes that the higher ethylene production could be due to stimulation of wound ethylene in necrotic tissues (Lynch and Brown, 1997). After this report, the higher ethylene production by Fe-deficient roots of different Strategy I species has been confirmed by other authors (Waters and Blevins, 2000; Li and Li, 2004; Molassiotis et al., 2005; Zuchi et al., 2009; Wang et al., 2012; Li et al., 2014a).

The higher ethylene production described for Fe deficiency has been further supported by results showing upregulation of genes implicated in ethylene synthesis, such as *SAMS*, *ACS*, and *ACO* (**Figure 3**; García et al., 2010; Stein and Waters, 2012 and references therein; Li et al., 2014a; Moran Lauter et al., 2014; Romera et al., in press and references therein). At the proteomic level, several studies have also shown a significant increase in the SAMS protein (**Figure 3**) under Fe deficiency (reviewed by López-Millán et al., 2013). Additionally, MTK, an enzyme involved in methionine recycling for a sustained ethylene production (**Figure 3**), is also induced by Fe deficiency (García et al., 2010; Zamboni et al., 2012; Romera et al., in press and references therein).

Besides ethylene synthesis, Fe deficiency can also affect ethylene responsiveness by altering the expression of genes implicated in ethylene signaling. Several of these genes are upregulated in different Strategy I species under Fe deficiency, like *ETRs* and *ERSs* (coding for ethylene receptors), *EIN2*, *EIN3*, *EILs*, and *ERFs* (see above; O'Rourke et al., 2007; García et al., 2010, 2014; Wang et al., 2014a). Whether the expression of these genes enhances or decreases the sensitivity to ethylene is not known yet. It is possible that ethylene sensitivity increases at the earlier stages of Fe deficiency and then decreases as a dampening mechanism, slowing down the ethylene response once it has been initiated. In any case, this deserves further investigation.

Ethylene Synthesis and Signaling in Rice and Strategy II Species

There are few publications relating ethylene and Fe deficiency in Strategy II species. Morgan and Hall (1962) showed that Fe-deficient sorghum plants treated with 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin) produced more ethylene than the Fe-sufficient ones. However, they determined ethylene when plants were very chlorotic and, consequently, the higher ethylene production could be an indirect effect of the advanced stress. After this report, Romera et al. (1999) found that roots from several Fe-deficient Strategy II species (maize, wheat, barley) did not produce more ethylene than the Fe-sufficient ones. Recently, Wu et al. (2011) showed similar results with barley plants. However, Fe-deficient roots from rice, that presents a combined strategy (**Figure 2**; see Section Introduction), produced more ethylene than the Fe-sufficient ones (Wu et al., 2011). Moreover, several ethylene synthesis genes, like *OsACS*, *OsACO*, *OsSAMS,* and *OsMTK*, were up-regulated in rice under Fe deficiency (**Figure 3**; Kobayashi et al., 2005; Zheng et al., 2009; Kobayashi and Nishizawa, 2012; Itai et al., 2013). Zheng et al. (2009) also found upregulation of a gene coding for a transcription factor relating to ethylene signaling in rice (Os03g64260) under Fe deficiency.

Additionally to rice, *SAMS* and *MTK* genes are also up-regulated in Strategy II species, such as barley and maize, under Fe deficiency (**Figure 3**; Suzuki et al., 2006; Li et al., 2014b). This is in agreement with the participation of both genes in NA and PS synthesis, besides their participation in ethylene synthesis (**Figure 3**).

ROLE OF ETHYLENE IN THE REGULATION OF Fe DEFICIENCY RESPONSES IN STRATEGY I SPECIES

In response to Fe deficiency, Strategy I species induce several physiological and morphological responses in their roots, aimed to facilitate Fe acquisition. These responses are down regulated once Fe uptake is sufficient to meet plants needs, to avoid toxicity and to conserve energy. Romera and Alcántara (1994) showed for the first time that ethylene could be involved in the regulation of both physiological and morphological responses to Fe deficiency in Strategy I species. Both kind of responses work together to effectively increase Fe uptake (Lucena et al., 2006). Consequently, it is not surprising the coordination of their regulation through the participation of the same signal (ethylene) for both of them. Romera and Alcántara (1994), based on the use of ethylene inhibitors and precursors, proposed that Fe deficiency could cause an enhanced production of ethylene and that then ethylene would trigger the activation of both physiological and morphological responses. This hypothesis has been further confirmed by different results, some of them already considered in previous reviews (Romera and Alcántara, 2004; Romera et al., 2007). In addition to the higher ethylene production of Fe-deficient roots (see previous Section), other results also support a role for ethylene in the regulation of Fe deficiency responses in Strategy I species. These other results are based on a variety of experimental approaches, such as the use of ethylene inhibitors, like Co (cobalt), AOA (aminooxyacetic acid), AVG (aminoethoxyvinylglycine) or STS (silver thiosulfate), the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid), the ethylene-releasing substance ethephon, ethylene itself, ethylene mutants (ethylene insensitive, ethylene constitutive or ethylene overproducers), and molecular biology techniques, such as transgenic lines, transcriptomics, proteomics, bimolecular fluorescence complementation, and yeast two-hybrid (Romera and Alcántara, 1994, 2004; Schmidt et al., 2000b; Schmidt and Schikora, 2001; García et al., 2010; Lingam et al., 2011; Meiser et al., 2011; López-Millán et al., 2013; Yang et al., 2014). Ethylene, whose production increases under Fe deficiency, acts as activator of most Fe deficiency responses. Consequently, ethylene inhibitors block the responses while ethylene itself or ethylene precursors (ACC or ethephon) promote them (**Figure 4**; Romera and Alcántara, 1994, 2004; Molassiotis et al., 2005; Lingam et al., 2011).

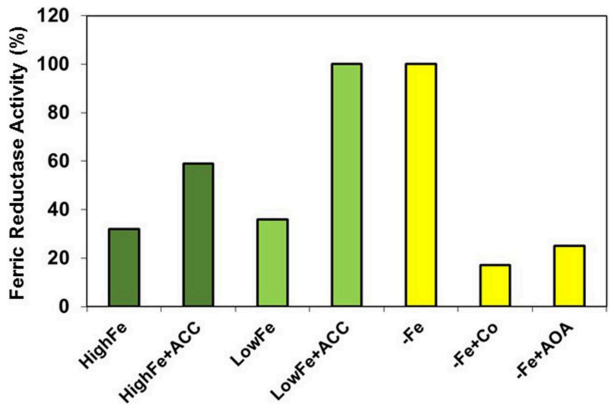

FIGURE 4 | Effect of ethylene precursors and inhibitors on the ferric reductase activity of Strategy I species. Ethylene (ACC) activates physiological responses, e.g., ferric reductase activity, to a lesser degree in plants grown with high levels of Fe than in plants grown with low levels. This suggests that ethylene acts in conjunction with some Fe-related repressive signals. On the other hand, ethylene inhibitors, like Co or AOA, block drastically the induction by Fe deficiency of this response, which suggests that ethylene is necessary for its activation. Data of ferric reductase activity expressed as percentage of –Fe plants (re-elaborated from Romera and Alcántara, 1994).

Here, we will review the more recent results supporting a role for ethylene in the regulation of both physiological and morphological responses to Fe deficiency in Strategy I species, most of them obtained with molecular biology techniques.

Role of Ethylene on Physiological Responses

As previously stated, Strategy I species need to reduce Fe^{3+}, the most abundant form in soils, to Fe^{2+}, prior to uptake. This reduction is mediated by a plasma membrane ferric reductase (encoded by *AtFRO2* in Arabidopsis; Robinson et al., 1999) and then Fe^{2+} is taken up by a Fe^{2+} transporter (encoded by *AtIRT1* in Arabidopsis; Eide et al., 1996). Ferric reductases and iron transporter genes have also been cloned from other plant species, like tomato (Eckhardt et al., 2001; Li et al., 2004), pea (Waters et al., 2002; Cohen et al., 2004), and cucumber (Waters et al., 2007). When grown under Fe deficiency, Strategy I species enhance both ferric reductase activity (due to increased expression of *AtFRO2*-like genes) and Fe^{2+} uptake capacity (due to increased expression of *AtIRT1*-like genes) (Walker and Connolly, 2008; Kobayashi and Nishizawa, 2012).

In addition to enhanced ferric reductase activity and Fe^{2+} uptake capacity, Strategy I species can develop other Fe deficiency responses aimed to facilitate Fe mobilization and uptake from the soil. This includes the capacity to acidify the rhizosphere medium (due to increased expression of plasma membrane proton-ATPase genes, such as *AtAHA2*, *AtAHA7*, *CsHA1,* and *MxHA7*), which contributes to Fe solubilisation (Santi et al., 2005; Waters et al., 2007; Zha et al., 2014); and the increased synthesis and release of Fe^{3+}-related compounds such as flavins and phenolics (Jin et al., 2007; Rodríguez-Celma et al., 2013; Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). The exact function of flavins and phenolics is not totally clear, but it has been proposed that they could act as "Iron Binding Compounds" contributing to Fe mobilization in the rhizosphere (Rodríguez-Celma and Schmidt, 2013). Alternatively, their main function could be related to Fe^{3+}reduction, acting as long distance electron shuttle (Rodríguez-Celma and Schmidt, 2013).

The regulation of the Fe-related genes described above is not totally understood but in the last years several transcription factors that participate in their activation have been found. The master regulator of most of the responses to Fe deficiency is the tomato SlFER, identified as a bHLH transcription factor (Ling et al., 2002), or its homologs AtFIT in *Arabidopsis* (bHLH29; Colangelo and Guerinot, 2004; Jakoby et al., 2004; Bauer et al., 2007) and MxFIT in *Malus xiaojinensis* (Yin et al., 2014). The tomato *fer* mutant (**Figure 5**) and the Arabidopsis *fit* mutant are very chlorotic and lack the ability to activate most Fe responses in roots (Brown et al., 1971; Ling et al., 2002; Colangelo and Guerinot, 2004; Jakoby et al., 2004). In Arabidopsis, the AtFIT regulatory network comprises other bHLH transcription factors, such as AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101. All of them have redundant functions and can interact with AtFIT to form heterodimers that activate the expression of the Fe acquisition genes *AtFRO2* and *AtIRT1* (**Figure 1**; Yuan et al., 2008; Wang et al., 2013a; Maurer et al., 2014; Brumbarova et al., 2015). FIT(FER) is induced in roots in response to Fe deficiency while the other bHLHs are induced in both roots and leaves in response to Fe deficiency (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Brumbarova and Bauer, 2005; Wang et al., 2007; Yuan et al., 2008; Brumbarova et al., 2015). In addition to the Arabidopsis AtFIT regulatory network, the AtPYE (AtPOPEYE) regulatory network has been described and implied in the regulation of a different subset of stele expressed Fe-related genes (Long et al., 2010; Ivanov et al., 2012; Brumbarova et al., 2015; Zhang et al., 2015). Other transcription factors related to Fe deficiency responses in Arabidopsis are AtMYB72 and AtMYB10 (Colangelo and Guerinot, 2004; Palmer et al., 2013; Zamioudis et al., 2014). They have redundant functions and have been implicated in the Fe deficiency induced up-regulation of *AtNAS4* (encoding NicotianAmine Synthase), playing an opposite role to AtPYE (Palmer et al., 2013).

The addition of ethylene inhibitors to Fe-deficient plants inhibits the induction of most of their physiological Fe responses, such as ferric reductase activity (**Figure 4**), Fe^{2+} uptake capacity, rhizosphere acidification, and flavin excretion (Romera and Alcántara, 1994, 2004; Lucena et al., 2006; Romera et al., 2007; Waters et al., 2007). The ferric reductase activity and other Fe responses are also drastically inhibited by ethylene inhibitors in mutants that show constitutive Fe responses and, as a consequence, accumulate toxic concentrations of Fe in their leaves, like the pea *brz* (*bronze*) and *dgl* mutants. Upon treatment with ethylene inhibitors, these mutants lower Fe uptake and, consequently, avoid Fe toxicity symptoms (**Figure 6**; Romera et al., 1996, 2014; see Supplementary Material). In contrast to ethylene inhibitors, the addition of ethylene, ACC or ethephon to Fe-sufficient plants induces some physiological Fe responses, such as enhanced ferric reductase activity (**Figure 4**), located in the subapical regions of the roots where formation of root

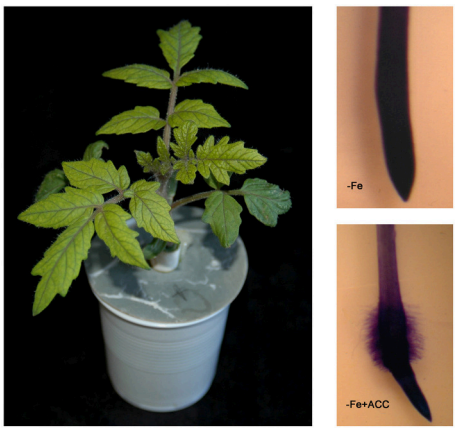

FIGURE 5 | Subapical root hairs in the tomato *fer* mutant. The tomato *fer* mutant (left) does not develop either subapical root hairs (right, top; Romera and Alcántara, 2004) or root transfer cells (Schmidt et al., 2000a) when grown under Fe deficiency. However, it develops both root hairs (right, bottom; Romera and Alcántara, 2004; see Supplementary Material) and transfer cells (Schmidt et al., 2000a) upon ACC treatment. This suggests that FER could influence these morphological responses through its possible indirect effect on ethylene synthesis (see **Figure 1**).

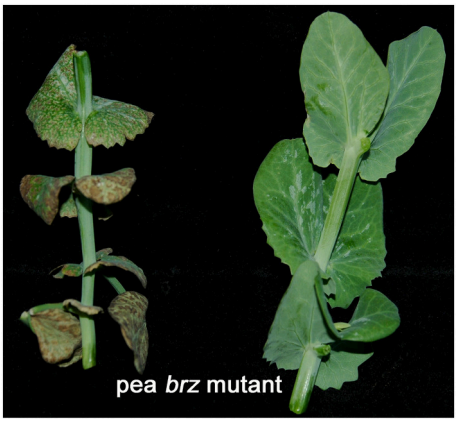

FIGURE 6 | The pea *brz* mutant can accumulate toxic levels of Fe in its leaves. Some mutants that present constitutive activation of Fe physiological responses, like the pea *brz* mutant (above; Romera et al., 1996) and the pea *dgl* mutant (Romera et al., 2014), can accumulate high levels of Fe and other metals in their leaves (Romera et al., 1996, 2014), which causes toxicity symptoms (left). Upon treatment with ethylene inhibitors, the Fe physiological responses are blocked and the concentration of Fe accumulated in the leaves diminish (Romera et al., 1996, 2014; see Supplementary Material). Consequently, the toxicity symptoms disappear (right). The *brz* plants (25-d-old) were transferred to nutrient solution with 20 μM FeEDDHA (left) or to nutrient solution with 20 μM FeEDDHA and the ethylene inhibitor AOA (at 20 μM; right) during 7 days.

hairs is also induced (**Figure 5**; Romera and Alcántara, 2004; Lucena et al., 2006; Waters et al., 2007; Romera et al., 2007; García et al., 2010, 2011). In supporting the role of ethylene, it should be mentioned that Fe-efficient cultivars of pea (Kabir et al., 2012) and *Medicago truncatula* (Li et al., 2014a) produce more ethylene than the Fe-inefficient ones. This suggests that the higher ethylene production would allow these cultivars to better activate their responses to Fe deficiency.

The participation of ethylene in the activation of physiological responses is further supported when analyzing its effects on the genes controlling these responses (Lucena et al., 2006; Waters et al., 2007; García et al., 2010; Lingam et al., 2011; Yang et al., 2014; Romera et al., in press). Ethylene up-regulates the expression of the key transcription factors AtFIT (or its tomato homolog SlFER), AtbHLH38, AtbHLH39, and AtMYB72 (**Figure 1**; Lucena et al., 2006; García et al., 2010, 2013, 2014; Lingam et al., 2011). The link between ethylene and AtFIT has been recently reinforced. It has been shown that AtEIN3 and AtEIL1, two transcription factors in the ethylene signaling pathway (see Section Ethylene Synthesis and Signaling under Fe Deficiency), interact with AtMED16 (Mediator) and AtMED25, to form a complex implicated in the transcription of *AtFIT* (**Figure 7**; Yang et al., 2014; Zhang et al., 2014; Brumbarova et al., 2015). Moreover, AtEIN3 and AtEIL1 can also influence the posttranscriptional stability of AtFIT (Lingam et al., 2011). AtbHLH38 and AtbHLH39 interact with AtFIT to form heterodimers that activate the expression of the Fe acquisition genes *AtFRO2* and *AtIRT1* (**Figures 1**, **7**; Yuan et al., 2008; Wang et al., 2013a). Consequently, the ferric reductase activity (depending on *AtFRO2*-like genes) and the Fe^{2+} uptake capacity (depending on *AtIRT1*-like genes) can be regulated by ethylene through AtFIT, AtbHLH38, and AtbHLH39. Similarly, the acidification capacity (depending on *AtAHA*-like genes, also activated by AtFIT-like transcription factors; Colangelo and Guerinot, 2004) can be regulated by ethylene too (**Figure 1**; Romera and Alcántara, 1994, 2004; Waters et al., 2007). Flavin excretion is another response inhibited by ethylene inhibitors (Romera and Alcántara, 2004). Since AtbHLH38 and AtbHLH39 have been implicated in the activation of flavin production (Vorwieger et al., 2007), and both transcription factors can be induced by ethylene (García et al., 2010), these results reinforce the hypothesis that flavin production is also controlled by ethylene (**Figure 1**). In relation to the excretion of phenolics, another response to Fe deficiency (Römheld and Marschner, 1986), some indirect studies suggest that the biosynthesis of some phenolics, like caffeic, coumaric and ferulic acids, could be stimulated by ethylene through an increase in the activity of the phenylalanine ammonia lyase enzyme (Rhodes and Wooltorton, 1973; Heredia and Cisneros-Zevallos, 2009; Liang et al., 2013). Very recently, Zamioudis et al. (2014) have found that the β-glucosidase AtBGLU42 is very important for the secretion of phenolic compounds under Fe deficiency. Furthermore, they have shown that the expression of *AtBGLU42*, as well as the expression of the phenolic efflux transporter *AtABCG37* (formerly named *AtPDR9*) and other genes involved in phenolic synthesis, are dependent on the transcription factor AtMYB72 (**Figure 1**; Zamioudis et al., 2014). Since *AtMYB72* expression is activated by ethylene (García et al., 2010), probably through AtFIT (AtMYB72 is one of its targets; Sivitz et al., 2012), this suggests that ethylene can also regulate phenolic secretion (**Figure 1**). In supporting this view, it should be noted that

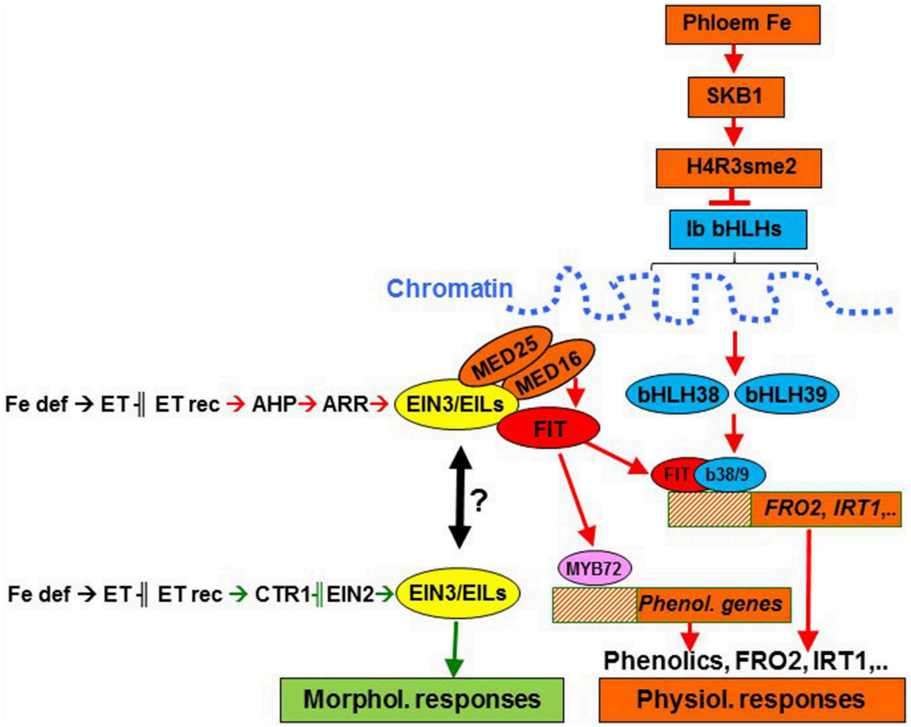

FIGURE 7 | Ethylene may regulate morphological and physiological responses to Fe deficiency through different signaling pathways in Strategy I species. Ethylene could regulate morphological responses through a pathway including CTR1 and EIN2 (in green) and physiological responses through a CTR1-EIN2-independent pathway (in red), instead using AHPs (phosphotransfer proteins) and ARRs (response regulators; Shakeel et al., 2013). Both pathways could converge through EIN3/EILs activity under certain circumstances, since EIN3 and EIL transciption factors have been involved in the regulation of both physiological (Lingam et al., 2011; Yang et al., 2014) and morphological responses, e.g., root hairs (Zhu et al., 2011) and transfer cells (Andriunas et al., 2011). For the regulation of physiological responses, ethylene may interact with some Fe-related repressive signals, probably moving through the phloem (García et al., 2013; Mendoza-Cózatl et al., 2014; Zhai et al., 2014). This phloem-Fe, through SKB1, could favor the chromatin package, where are located the bHLH38 and bHLH39 genes, thus inhibiting their transcription (Fan et al., 2014). Fe def, Fe deficiency; ET rec, ethylene receptors.

AtBGLU42 expression is also activated by ethylene (García et al., 2010).

Besides genes related to Fe acquisition from the growth medium, there are other genes that are induced under Fe deficiency in roots and/or leaves, like *AtFRD3* (Ferric Reductase Defective3; Rogers and Guerinot, 2002) and *AtNASs* (NicotianAmine Synthase; Klatte et al., 2009), that are very important for internal Fe mobilization and homeostasis. The FRD3 protein belongs to the multidrug and toxin efflux (MATE) family and has been implicated in the loading of citrate into the xylem, which is necessary for Fe translocation from roots to shoots (Rogers and Guerinot, 2002; Durrett et al., 2007; Roschzttardtz et al., 2011). The higher expression of *FRD3* under Fe deficiency is generally associated with increased synthesis of organic acids, like citrate and malate (Landsberg, 1981; Kabir et al., 2012; Li et al., 2014a). *In toto*, both responses cooperate for the internal translocation of Fe from roots to shoots. NAS (NA Synthase) proteins participate in the synthesis of NA (nicotianamine), which is a chelating agent implied in the long-distance transport of Fe (and other metals) and that facilitates the transport of Fe through the phloem to sink organs (Klatte et al., 2009; Schuler et al., 2012). NA is also the precursor for the biosynthesis of PS, that play a key role in Strategy II species (**Figure 3**; Kobayashi and Nishizawa, 2012). The mutants related to these genes (Arabidopsis *frd3* and *nas4x*, and the tomato *NAS* mutant *chloronerva*) show constitutive activation of Fe responses, even when grown under Fe-sufficient conditions (Rogers and Guerinot, 2002; Klatte et al., 2009; García et al., 2013). This suggests that the precise distribution of Fe throughout the plant has a decisive role in the control of Fe deficiency responses. Ethylene has been implicated in the activation of both *AtNAS* (*AtNAS1* and *AtNAS2*) and *AtFRD3* genes (García et al., 2010).

Role of Ethylene on Morphological Responses

In addition to physiological responses, Strategy I species can develop some morphological responses in their roots under Fe deficiency, like subapical root hairs (**Figure 1**), root epidermal transfer cells and cluster roots (also named proteoid roots; Kramer et al., 1980; Römheld and Marschner, 1986; Romera and Alcántara, 1994, 2004; Schmidt et al., 2000a; Waters and Blevins, 2000; Schmidt and Schikora, 2001; Schikora and Schmidt, 2002; Zaid et al., 2003; Romera et al., 2007; García et al., 2015). Transfer cells have increased surface area, due to invaginations of the plasma membrane (Kramer et al., 1980; Schmidt et al., 2000a; Schikora and Schmidt, 2002). Proteoid roots are clusters of

closely spaced short lateral rootlets formed in some plant species adapted to poor soils (Waters and Blevins, 2000; Zaid et al., 2003; Wang et al., 2014b). Most of these root modifications are also formed under P deficiency since both Fe and P may be poorly available in soils (Schmidt and Schikora, 2001; Schikora and Schmidt, 2002; Wang et al., 2014b). Root hairs, root epidermal transfer cells and cluster roots enhance nutrient uptake by increasing the surface of contact of roots with soil and by chemically modifying the soil environment (Wang et al., 2014b). Besides these specialized morphological responses, Fe deficiency, depending on the extent of the deficiency (mild, moderate, or severe), can also change root system architecture by altering the number, length, and diameter of roots. Generally, Fe deficient plants exhibit a shallower architecture that results from inhibition of primary root elongation (Kramer et al., 1980). Additionally, Fe deficiency can cause an increase in lateral root density (Kramer et al., 1980; Jin et al., 2008).

Ethylene and auxin, along with other hormones and signaling substances, have been implicated in all the morphological changes described in the above paragraph (Romera and Alcántara, 1994, 2004; Schmidt et al., 2000a; Waters and Blevins, 2000; Schmidt and Schikora, 2001; Schikora and Schmidt, 2002; Zaid et al., 2003; Romera et al., 2007; Muday et al., 2012; Wang et al., 2014b; García et al., 2015). Both hormones synergistically inhibit root elongation while play an antagonistic role on lateral root formation (Muday et al., 2012). Moreover, ethylene influences auxin distribution at the root tip, thus affecting the development of subapical root hairs (Muday et al., 2012; Lee and Cho, 2013).

The implication of ethylene on the regulation of morphological responses to Fe deficiency has been based on the use of ethylene inhibitors and precursors, and the use of ethylene mutants. The addition of ethylene inhibitors to Fe-deficient plants inhibited subapical root hairs, transfer cells and cluster roots, while the addition of ethylene or ethylene precursors (ACC or ethephon) to Fe-sufficient plants promoted them (Romera and Alcántara, 1994, 2004; Schmidt et al., 2000a; Schmidt and Schikora, 2001; Schikora and Schmidt, 2002; Zaid et al., 2003; Romera et al., 2007). In the same way, some ethylene insensitive mutants, like the *Arabidopsis etr1* and *ein2*, the soybean *etr1* and the *Medicago truncatula sickle*, did not develop subapical root hairs either under Fe-deficiency or upon ACC treatment, while the wild-types did (reviewed in Romera and Alcántara, 2004). By contrast, the *Arabidopsis* ethylene constitutive mutant *ctr1* developed subapical root hairs even under Fe-sufficient conditions (Romera and Alcántara, 2004).

The implication of ethylene on the development of transfer cells has also been reinforced by some studies with cotyledons. Ethylene functions as a key inductive signal for wall ingrowth and, consequently, transfer cell formation in epidermal cells of cotyledons (Zhou et al., 2010) while glucose functions as a negative regulator (Andriunas et al., 2011). Glucose modulates the amplitude of the ethylene-stimulated wall ingrowth induction by down-regulating the expression of ethylene synthesis and signaling genes, such as *EIN3/EILs* (Andriunas et al., 2011). These antagonistic effects of ethylene and glucose suggest that EIN3/EILs (see Section Ethylene Synthesis and Signaling under Fe Deficiency) act as integrators of glucose and ethylene regulation of transfer cell formation.

Are Physiological and Morphological Responses Regulated Similarly by Ethylene?

Since ethylene has been implicated in the regulation of most of the physiological and morphological responses to Fe deficiency in Strategy I species (**Figures 1**, **7**), a question emerged: Are all of them regulated by ethylene in the same way? The answer to this question, based on results with ethylene mutants, is that different responses can be regulated by ethylene through different signaling pathways. On the other hand, many results suggest that ethylene acts in conjunction with other hormones and signaling substances to regulate the responses. Some of these signals (auxin, nitric oxide) act positively on the regulation of Fe deficiency responses in Strategy I species while other ones (ABA, cytokinins, jasmonic acid, brassinosteroids) act negatively (Giehl et al., 2009; Hindt and Guerinot, 2012; Brumbarova et al., 2015; García et al., 2015; Romera et al., in press).

Results from ethylene insensitive mutants suggest that physiological and morphological responses can be regulated through different signaling pathways. For instance, the development of subapical root hairs is impaired in the Arabidopsis ethylene insensitive mutants *ein2* and *etr1*, either under Fe deficiency or upon ACC treatment (Schmidt and Schikora, 2001; Romera and Alcántara, 2004), while the enhanced ferric reductase activity and the expression of Fe acquisition genes is not impaired (Lucena et al., 2006; García et al., 2010). On the other hand, both the Arabidopsis ethylene constitutive mutant *ctr1* and the Arabidopsis ethylene overproducer mutant *eto* have constitutive subapical root hairs (a Fe deficiency response) in complete nutrient solution; however, neither of these mutants have full constitutive activation of Fe physiological responses (**Figure 8**; Schmidt et al., 2000b; Romera and Alcántara, 2004; García et al., 2007, 2014, 2015; see Supplementary Material). In the same way, root hairs, transfer cells and cluster roots are almost fully induced by ACC or ethephon in plants grown with high levels of Fe while physiological responses are activated to a lesser degree than when applied to plants grown with low levels, or in absence, of Fe (**Figure 4**; Romera and Alcántara, 1994; Schmidt et al., 2000a; Zaid et al., 2003; Lucena et al., 2006; García et al., 2013).

From the results above, several conclusions can be drawn. First, morphological and physiological responses can be differently regulated by ethylene. Second, for some morphological responses, like root hairs, ethylene acts through a signaling pathway including ETR1, CTR1, and EIN2 (**Figure 7**). Third, for some physiological responses, like ferric reductase activity, ethylene could act through a pathway where EIN2, and possibly CTR1, are not strictly required (**Figure 7**). Fourth, for the regulation of physiological responses, ethylene could act in conjunction with Fe-related repressive signals since the *ctr1* and *eto* mutants do not have full constitutive activation of these responses (see above).

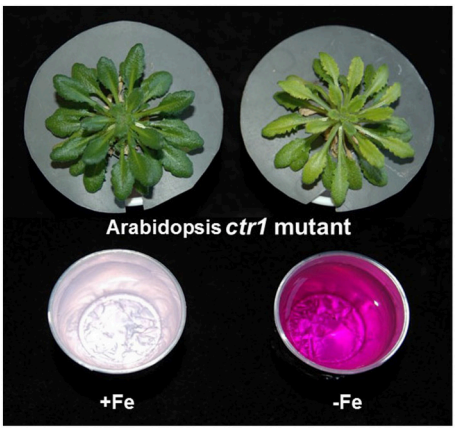

FIGURE 8 | Induction of ferric reductase activity in the Arabidopsis ethylene constitutive mutant *ctr1* under Fe deficiency. Neither the Arabidopsis ethylene constitutive mutant *ctr1* (above; García et al., 2014) nor the Arabidopsis ethylene overproducer mutant *eto* (Schmidt et al., 2000b) present constitutive activation of physiological responses, like ferric reductase activity, when grown under Fe-sufficient conditions (Schmidt et al., 2000b; García et al., 2014; see Supplementary Material). This suggests that ethylene can not activate these responses until some Fe-related repressive signals are eliminated (see **Figures 4**, **7**). Some *ctr1* plants (45-d-old) grown in nutrient solution with Fe were transferred to nutrient solution without Fe during the last 7 days. After that, ferricreductase activity was determined (García et al., 2014). The ferric reductase activity is enhanced under Fe deficiency and denoted by the purple color of the assay solution (right, bottom).

The existence of an alternate route for ethylene signaling, besides the conventional one including CTR1 and EIN2 (**Figure 7**; Shakeel et al., 2013), is further supported by results showing that the Arabidopsis *ctr1* and *ein2* mutants respond to both ACC (García et al., 2010, 2014) and ethylene inhibitors (García et al., 2007) for physiological responses. Does this mean that CTR1 and EIN2 do not participate at all in the regulation of these responses? Some experimental results suggest that, although not strictly required, CTR1 and EIN2 can participate in this regulation. As examples, hypoxia inhibited physiological responses in Fe-deficient wild type plants but not in *ctr1* mutant plants (García et al., 2014), which suggests that CTR1 can play a role in their regulation. A possible explanation for the participation of CTR1 and EIN2, without being strictly required, could be the possible interaction between the conventional route for ethylene signaling and the alternate one under certain circumstances. In such cases, both routes could converge downstream through EIN3/EILs (**Figure 7**). In supporting this view, it should be noted that EIN3/EILs transcription factors have been involved in the up-regulation of FIT (activator of most physiological responses; **Figure 7**; Yang et al., 2014; Brumbarova et al., 2015) but also in the development of morphological responses, such as root hairs (**Figure 7**; Zhu et al., 2011) and transfer cells (Andriunas et al., 2011).

In addition to physiological responses (**Figures 1**, **7**), FER (FIT homolog) has also been implicated in the activation of morphological responses. The tomato *fer* mutant does not develop either subapical root hairs or transfer cells under Fe deficiency (Schmidt et al., 2000a). However, it does develop both subapical root hairs (**Figure 5**; Romera and Alcántara, 2004; see Supplementary Material) and root transfer cells (Schmidt et al., 2000a) upon ACC treatment. This suggests that FER could indirectly activate morphological responses by influencing ethylene synthesis. This suggestion is further supported by two experimental results. First, the *AtMTK* gene, implicated in ethylene synthesis (**Figure 3**), is activated by ethylene through *AtFIT* (**Figure 1**; Colangelo and Guerinot, 2004; García et al., 2010). Second, one *AtSAMS* gene implicated in ethylene synthesis (**Figure 3**) is up-regulated by the AtMYB72 transcription factor (Zamioudis et al., 2014), that is a direct target of AtFIT (**Figure 1**; Sivitz et al., 2012).

In relation to the existence of Fe-related repressive signals, we can speculate that since physiological responses are not fully activated by ACC in "high Fe" plants (**Figure 4**), and are not fully activated in Fe-sufficient *ctr1* (**Figure 8**) and *eto* mutants, some Fe-related signals are acting negatively to block physiological responses. It has been proposed that these Fe-related repressive signals are not associated with the total Fe in roots but with some Fe compound(s) moving through the phloem, which could negatively interact with ethylene signaling to regulate Fe physiological responses (García et al., 2013; Mendoza-Cózatl et al., 2014; Zhai et al., 2014; Romera et al., in press). This does not preclude that these signals can also affect ethylene synthesis.

A possible step affected by the Fe-related repressive signals would be the Shk1 binding protein 1 (SKB1/AtPRMT5) in Arabidopsis (Fan et al., 2014). SKB1 associates with the chromatin of the Ib subgroup bHLH genes (AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101) and symmetrically dimethylates histone H4R3, thereby increasing H4R3sme2 and inhibiting transcription (**Figure 7**). The quantity of SKB1 that associates with the chromatin of these bHLH genes depends on the Fe status of the plants, in such a way that Fe sufficiency increases its association, thus inactivating the transcription of the Ib subgroup bHLH genes (**Figure 7**; Fan et al., 2014; Brumbarova et al., 2015). The possible effect of the Fe-related repressive signals through SKB1 would explain several experimental results. First, it would explain why the overexpression of *AtFIT* does not activate physiological responses in plants grown with high levels of Fe (Colangelo and Guerinot, 2004; Jakoby et al., 2004) while the overexpression of *AtFIT* with either *AtbHLH38* or *AtbHLH39* does (Yuan et al., 2008). Second, it would explain why the foliar application of Fe inhibits more drastically the expression of *AtbHLH38* and *AtbHLH39* than that of *AtFIT* (García et al., 2013). Third, it would explain why Fe physiological responses are not fully activated in Fe-sufficient *ctr1* (**Figure 8**) and *eto* mutants. Finally, it would explain why the application of ACC to Fe-sufficient plants does not fully activate physiological responses (**Figure 4**; Romera and Alcántara, 1994; Lucena et al., 2006; García et al., 2013). Taken together, the results described above indicate that ethylene can not activate Fe physiological responses until some Fe-related repressive signal is removed. This combinatorial control would provide Fe-specificity to the system, suggesting that both Fe deficiency and ethylene action are necessary for full transcriptional activation (Lucena et al., 2006).

ROLE OF ETHYLENE IN THE REGULATION OF Fe DEFICIENCY RESPONSES IN RICE AND STRATEGY II SPECIES

To our knowledge, specialized morphological responses to Fe deficiency have not been described in Strategy II species, although they can change their root system architecture. As example, Fe-deficient maize plants developed more lateral roots than Fe-sufficient ones (Li et al., 2014b). Therefore, here we will only describe the role of ethylene in the regulation of physiological responses to Fe deficiency in Strategy II species.

The Strategy II response relies on biosynthesis and secretion of PS (PhytoSiderophores), of the MA (Mugineic Acid) family, which are synthesized from SAM (*S*-Adenosyl Methionine; **Figure 3**). This pathway includes three sequential enzymatic reactions mediated by NAS (NA Synthase), NAAT (NA AminoTransferase), and DMAS (DeoxyMugineic Acid Synthase; **Figure 3**; Kobayashi and Nishizawa, 2012). Some years ago, Nozoye et al. (2011) identified the PS efflux transporter OsTOM1 (Transporter Of MAs 1) from rice and the PS efflux transporter HvTOM1 from barley. The Fe^{3+}-PS are taken up into root cells by Fe^{3+}-PS transporters, like ZmYS1 (YELLOW STRIPE 1) and YSL (YELLOW STRIPE 1–like) transporters (**Figure 2**; Curie et al., 2001; Inoue et al., 2009).

Rice, despite being traditionally considered a Strategy II species (Kobayashi and Nishizawa, 2012), possesses some characteristics of Strategy I species, such as a Fe^{2+} transporter, OsIRT1, which allows it to absorb Fe^{2+} from the soil, in addition to its Strategy II-based Fe^{3+}-PS uptake system, and its capacity to release phenolics (**Figure 2**; Ishimaru et al., 2006). Some authors consider rice as a plant species that uses a combined strategy (Ricachenevsky and Sperotto, 2014; see Section Introduction).

Under Fe deficiency, Strategy II species induce the expression of genes implicated in PS synthesis (*NAS*, *NAAT*, and *DMAS*) as well as genes implicated in PS efflux (*TOM1*) and Fe^{3+}-PS uptake (YS1, YSL15; **Figures 2**, **3**; Kobayashi and Nishizawa, 2012; Itai et al., 2013; Kobayashi et al., 2014). Additionally, Strategy II species, similarly to Strategy I species, increase the synthesis of organic acids under Fe deficiency (Landsberg, 1981). Rice also induces the expression of the *OsIRT1* gene (Ishimaru et al., 2006) and increases the production and release of phenolic compounds to the rhizosphere, through the OsPEZ1 phenolic efflux transporter (**Figure 2**; Ishimaru et al., 2011).

Similarly to Strategy I species, a bHLH transcription factor, OsIRO2, with homology to AtbHLH38-39, activates the expression of most of the genes related to PS production, secretion and uptake (*NAS1*, *NAS2*, *NAAT*, *DMAS*, *TOM1*, *YSL15*; **Figure 2**), and genes involved in the methionine cycle (Kobayashi and Nishizawa, 2012). Other Fe deficiency-induced bHLH genes in rice are *OsIRO3*, with similarity to *AtPYE*, and *OsbHLH133* (Zheng et al., 2010; Kobayashi and Nishizawa, 2012; Wang et al., 2013c).

The expression of the master regulator *OsIRO2* is strongly induced under Fe deficiency (Ogo et al., 2006) and is positively regulated by the IDEF1 transcription factor (**Figure 2**; Kobayashi et al., 2009). IDEF1 is an ABI3/VP1 transcription factor (Kobayashi et al., 2007) that can bind Fe^{2+} and Zn^{2+} (Kobayashi and Nishizawa, 2015), and is especially important for the early response to Fe deficiency (Kobayashi et al., 2009). IDEF2 is a NAC transcription factor (Ogo et al., 2008) that regulates OsYSL2 and other Fe deficiency-inducible genes, which may be involved in Fe translocation (Kobayashi et al., 2014). Both IDEF1 and IDEF2 are constitutively expressed in vegetative and reproductive tissues without induction by Fe deficiency (Kobayashi and Nishizawa, 2012).

The role of ethylene and other hormones in the regulation of Fe deficiency responses has been less studied in Strategy II species. In rice, as occurred in Strategy I species (see previous Section), several hormones (ethylene, auxin, jasmonic acid, ABA) have been implicated in the regulation of its Fe deficiency responses (reviewed by Kobayashi et al., 2014). Very recently, Shen et al. (2015) have shown that OsARF16, a transcription factor regulating auxin redistribution, is required for Fe deficiency response in rice.

In relation to ethylene, the few existing data do not support an important role for this hormone in the regulation of responses to Fe deficiency in Strategy II species. Firstly, roots from several Fe-deficient Strategy II species did not produce more ethylene than the Fe-sufficient ones, as observed in Strategy I species (Romera et al., 1999; Wu et al., 2011; see Section Ethylene Synthesis and Signaling in Rice and Strategy II Species). Secondly, the addition of ACC to barley plants did not increase either the production of PS (Welch et al., 1997) or the expression of Fe-related genes (Wu et al., 2011). In the case of rice, Zheng et al. (2009) and Wu et al. (2011) have found that ethylene production, as well as the expression of some ethylene synthesis genes, increase under Fe deficiency (see Section Ethylene Synthesis and Signaling in Rice and Strategy II Species). Wu et al. (2011) have also shown that ethylene is implicated in the activation of some Fe-related genes, such as *OsIDEF1*, *OsIRO2*, *OsITR1*, *OsYSL15*, *OsNAS1,* and *OsNAS2* (**Figures 2**, **3**). Some of these genes are generally associated to the Strategy I system, like *OsITR1*, while other ones are specifically associated to the Strategy II system, like *OsIDEF1*, *OsIRO2*, and *OsYSL15* (Kobayashi and Nishizawa, 2012; Kobayashi et al., 2014). Moreover, as in Strategy I species, the addition of ACC to Fe-sufficient plants upregulates the expression of the above genes to a lesser degree than when applied to Fe-deficient plants (Wu et al., 2011; see Section Are Physiological and Morphological Responses Regulated Similarly by Ethylene?).

Ethylene, NA and PS are synthesized from L-methionine, and some genes, e.g., *MTK*, *SAMS* and *NAS*, are induced by Fe deficiency in both Strategy I and Strategy II species (**Figure 3**; see Section Ethylene Synthesis and Signaling under Fe Deficiency). This implies that both strategies could share the first steps in the regulation of their responses to Fe deficiency while differ in the last steps. Probably, both kind of plants have diverged along the evolution in such a way that Strategy I species have devoted SAM to NA and ET synthesis while Strategy II species have devoted SAM to NA and PS synthesis (**Figure 3**).

CROSS TALKS BETWEEN Fe DEFICIENCY AND OTHER NUTRIENT DEFICIENCIES. ¿IS ETHYLENE A COMMON REGULATORY SIGNAL FOR DIFFERENT NUTRIENT DEFICIENCIES?

Although the responses to Fe deficiency are specifically induced under the deficiency of this metal, it is relatively frequent to find induction of some Fe responses under other nutrient deficiencies. On a reciprocal basis, frequently the induction of responses to other nutrient deficiencies occur under Fe deficiency (**Table 1**). These cross talks among nutrient deficiencies could be related to the common participation of similar regulatory signals, like ethylene, auxin and NO, in the induction of their responses (reviewed in García et al., 2015). In supporting this view, ethylene has also been implicated in the activation of responses to P deficiency (Lei et al., 2011; Nagarajan and Smith, 2012; Wang et al., 2014b), to K deficiency (Jung et al., 2009), to S deficiency (Moniuszko et al., 2013), and to other deficiencies (García et al., 2015 and references therein; See other reviews in this Issue).

Several Fe deficiency responses are up-regulated by P, S, K, or Cu deficiency in Strategy I species (**Table 1**; Wang et al., 2002, 2014b; Romera et al., 2003; Abel, 2011; Bernal et al., 2012). Under P deficiency, plants can induce changes like proliferation of root hairs, cluster roots, increased exudation of phenolics, citrate and protons, and an increased ferric reductase activity, strongly resembling the Fe deficiency response of Strategy I species (Wang et al., 2014b). Some Fe acquisition genes, such as *IRT1*-like, *FRO2*-like and *FIT*-like genes (**Figure 1**), were upregulated by P deficiency in Arabidopsis, tomato, and lupin plants (Wang et al., 2002; Abel, 2011 and references therein; Wang et al., 2014b). The up-regulation of Fe responses by P deficiency is further supported by the higher Fe accumulation in P-deficient plants (Ward et al., 2008). In oilseed rape, the Fe acquisition genes *BnIRT1* and *BnFRO1* were up-regulated by S deficiency during the earlier stages of the deficiency (Muneer et al., 2014). Similarly, *SlIRT1* was up-regulated by K deficiency in tomato plants (Wang et al., 2002). K deficiency also induces proliferation of subapical root hairs where ethylene has been involved (Jung et al., 2009). Ferric reductase activity was induced by Cu deficiency in soybean and other Strategy I species (Romera et al., 2003 and references therein). This response, as well as *AtIRT1* and *AtFRO2* expression, was also induced in Cu-deficient Arabidopsis plants in a SPL7 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7) dependent manner, being induced higher in Cu-deficient *spl7* mutant plants than in the wild type (Bernal et al., 2012). The data suggest that Cu deficiency leads to a lower translocation of Fe from roots to shoots in the *spl7* mutant. This results in greater Fe deficiency in the *spl7* shoots, which in turn triggers Fe deficiency responses (Bernal et al., 2012). As seen above, different nutrient deficiencies can induce Fe responses. On the other hand, Fe deficiency can up-regulate responses to other nutrient deficiencies (**Table 1**; Wang et al., 2002; García et al., 2010; Perea-García et al., 2013). As examples, the tomato P transporter, *SlPT1*, and the K channel, *SlKC1*, were up-regulated in roots of Fe-deficient tomato plants (Wang et al., 2002). Similarly, sulfate transporters, like *AtSULTR1;1* in Arabidopsis (García et al., 2010), and *SlST1.1*, *SlST1.2,* and *SlST2.1* in tomato (Paolacci et al., 2014), were induced under Fe deficiency. In the same way, the Cu transporter, *AtCOPT2*, was induced under Fe deficiency in Arabidopsis (Colangelo and Guerinot, 2004; García et al., 2010; Perea-García et al., 2013). These cross talks among

TABLE 1 | Cross talk between Fe deficiency and other nutrient deficiencies.

Name	Nutrient deficiency	References
Fe RESPONSES OR Fe-RELATED GENES INDUCED UNDER OTHER NUTRIENT DEFICIENCIES (Str. I)		
Ferric Reductase Activity	-P, -Cu	Romera et al., 2003; Bernal et al., 2012; Wang et al., 2014b
FRO (Ferric reductase)	-P, -S, -Cu	Abel, 2011; Bernal et al., 2012; Muneer et al., 2014; Wang et al., 2014b
IRT (Fe^{2+} transporter)	-P, -S, -K, -Cu	Wang et al., 2002; Abel, 2011; Bernal et al., 2012; Muneer et al., 2014; Wang et al., 2014b
Acidification	-P	Wang et al., 2014b
Phenolics	-P	Wang et al., 2014b
Organic acids	-P	Wang et al., 2014b
Root hairs	-P, -K	Jung et al., 2009; Wang et al., 2014b
NUTRIENT-RELATED GENES INDUCED UNDER Fe DEFICIENCY (Str. I)		
SlPT1 (P transporter)	-Fe	Wang et al., 2002
SlKCl (K channel)	-Fe	Wang et al., 2002
AtSULTR1; 1 (Sulfate transporter)	-Fe	García et al., 2010
SlST1.1 (Sulfate transporter)	-Fe	Paolacci et al., 2014
SlST1.2 (Sulfate transporter)	-Fe	Paolacci et al., 2014
SlST2.1 (Sulfate transporter)	-Fe	Paolacci et al., 2014
AtCOPT2 (Cu transporter)	-Fe	Colangelo and Guerinot, 2004; García et al., 2010; Perea-García et al., 2013
NUTRIENT-RELATED GENES INDUCED UNDER Fe DEFICIENCY (Str. II)		
S assimilatory pathway genes	-Fe	Ciaffi et al., 2013

Str. I, Strategy I; Str. II, Strategy II.

deficiencies also occur in Strategy II species: in wheat, several genes of the S assimilatory pathway induced by S deficiency were also significantly up-regulated by Fe deficiency (Ciaffi et al., 2013).

Besides these mutual and positive influences among nutrient deficiencies, some elements, either under deficiency or excess, can negatively affect the responses to Fe deficiency. As examples, S deficiency, depending on its severity and extent, can limit the development of Fe responses in Strategy I species, like tomato (Zuchi et al., 2009) and oilseed rape (Muneer et al., 2014). Similarly, S deficiency can also limit Fe responses in Strategy II species, like the release of PS (Astolfi et al., 2006). Interestingly, S, through methionine and its derivatives, participates in the synthesis of NA, PS, and ethylene (**Figure 3**; Sauter et al., 2013). Co excess can cause Fe deficiency by inhibiting Fe responses, which is logical since Co is a potent ethylene inhibitor (Romera and Alcántara, 1994, 2004 and references therein). Other heavy metals, like Ni, Cu and Cd, when applied at concentrations between about 5–20 μM in nutrient solution, can block the induction of some Fe physiological responses (Alcántara et al., 1994). Nonetheless, the relationship of these heavy metals with ethylene needs further research.

Besides the above interactions between Fe and other nutrients, there are intriguing cross talks between Fe responses and some defense responses, like the ISR (Induced Systemic Resistance). This latter cross talk suggests that responses to biotic and abiotic stresses can share some common regulatory components. ISR is a mechanism by which selected plant growth-promoting bacteria and fungi in the rhizosphere prime the whole plant body for defense against a broad range of pathogens and insect herbivores (Pieterse et al., 2014). Some Fe-related genes, like *AtMYB72*, *AtBGLU42*, *AtABCG37*, *AtFRO2*, *AtIRT1* and others (**Figure 1**), are upregulated in Arabidopsis roots colonized by ISR-inducing *Pseudomonas* strains (Pieterse et al., 2014; Zamioudis et al., 2014). Using the Arabidopsis ethylene mutant *eir1*, which is insensitive to ethylene in roots, it was shown that ethylene is required for the expression of ISR (Pieterse et al., 2014). The common implication of ethylene in the regulation of Fe deficiency responses and ISR could partially explain the cross talk between both processes.

CONCLUSIONS

The participation of ethylene in the activation of most of the morphological and physiological responses to Fe deficiency in Strategy I species implies that it acts as a general coordinator of their control. Nonetheless, morphological and physiological responses seem to be regulated by ethylene through different signaling pathways. Additionally, several results suggest that ethylene acts in conjunction with other positive signals, like auxin and NO, and with negative signals, e.g., probable Fe-related repressive signals moving through the phloem. Ethylene has also been implicated in the regulation of some Fe responses in rice, that possesses combined characteristics of both Strategy I and Strategy II species. In Strategy II species, the few existing data do not support an important role for ethylene in the regulation of their Fe deficiency responses. The common involvement of ethylene in the regulation of responses to other nutrient deficiencies and in the regulation of the ISR, could partially explain the cross talk between Fe deficiency responses and responses to other deficiencies, and Fe deficiency responses and the ISR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01056

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MPK3/MPK6 are involved in iron deficiency-induced ethylene production in Arabidopsis

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Iron (Fe) is an essential micronutrient that participates in various biological processes important for plant growth. Ethylene production induced by Fe deficiency plays important roles in plant tolerance to stress induced by Fe deficiency. However, the activation and regulatory mechanisms of 1-Aminocyclopropane-1-carboxylic acid synthase (ACS) genes in this response are not clear. In this study, we demonstrated that Fe deficiency increased the abundance of *ACS2*, *ACS6*, *ACS7*, and *ACS11* transcripts in both leaves and roots as well as the abundance of *ACS8* transcripts in leaves and *ACS9* transcripts in roots. Furthermore, we investigated the role of mitogen-activated protein kinase 3 and 6 (MPK3/MPK6)-regulated ACS2/6 activation in Fe deficiency-induced ethylene production. Our results showed that MPK3/MPK6 transcript abundance and MPK3/MPK6 phosphorylation are elevated under conditions of Fe deficiency. Furthermore, *mpk3* and *mpk6* mutants show a lesser induction of ethylene production under Fe deficiency and a greater sensitivity to Fe deficiency. Finally, in *mpk3, mpk6*, and *acs2* mutants under conditions of Fe deficiency, induction of transcript expression of the Fe-deficiency response genes *FRO2, IRT1,* and *FIT* is partially compromised. Taken together, our results suggest that the MPK3/MPK6 and ACS2 are part of the Fe starvation-induced ethylene production signaling pathway.

Keywords: *Arabidopsis*, ethylene, Fe deficiency, mitogen-activated protein kinase (MPK), 1-Aminocyclopropane-1-carboxylic acid synthase (ACS)

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INTRODUCTION

Iron (Fe) is an essential micronutrient that plays an important role in plant growth. It participates in several metabolic processes including respiration, photosynthesis, and chlorophyll biosynthesis (Kobayashi and Nishizawa, 2012). Since Fe is poorly soluble in neutral or basic soils, it is not readily available to plants in these conditions (Kim and Guerinot, 2007). To counteract Fe deficiency, plants have developed a set of responses to control the uptake, utilization, and storage of Fe. Most plants, with the exception of those in the Graminaceae family, use strategy I, which is also known as the reduction strategy. Strategy I plants, via H^+-ATPases, excrete protons into the rhizosphere to increase the solubility of Fe. Ferric-chelate reductase oxidases present on the root surface then reduce Fe^{3+} into Fe^{2+}, after which Fe^{2+} transporters take Fe^{2+} into the plant. Consistent with the roles of these proteins in strategy I plants, in Arabidopsis, expression of the

plasma membrane H^+ ATPase (*AHA2*), the major ferric-chelate reductase oxidase (*FRO2*), and the major Fe^{2+} transporter (*IRT1*) is strongly induced under Fe deficiency (Eide et al., 1996; Robinson et al., 1999; Santi and Schmidt, 2009). The Graminaceae family plants use strategy II, which is also known as the chelate strategy (Kim and Guerinot, 2007; Walker and Connolly, 2008). Strategy II plants release phytosiderophores, which can directly bind Fe^{3+} (Conte and Walker, 2011), into the rhizosphere. The chelated complexes are then transported into the roots through the YS/YSL family of transporters (Curie et al., 2001, 2009).

Recent studies have shown that in strategy I plants, phytohormones such as ethylene, auxin, cytokinins, and nitric oxide (NO) are involved in the regulation of Fe deficiency responses (Romera et al., 2011; Ivanov et al., 2012). In particular, Fe deficiency increases production of ethylene in roots of strategy I plants (Romera et al., 1999; Romera and Alcantara, 2004). It is thought that ethylene regulates *FRO2* and *IRT1* gene expression through the modulation of the major transcription factor FER or FER-like (Lucena et al., 2006). In support of this, in conditions of Fe deficiency, expression of Arabidopsis *FIT,* which is homolog of tomato *FER* decreases upon inhibition of ethylene synthesis or activity, and increases upon addition of ethylene precursor (García et al., 2010). And the expression of Fe-related genes and ferric reductase activity were also induced by ethylene level (Romera and Alcantara, 1994; Li and Li, 2004; Lucena et al., 2006; Waters et al., 2007; García et al., 2010). The genes involved in ethylene biosynthesis and signaling could also be up-regulated under Fe deficiency (García et al., 2010). Ethylene biosynthesis involves three enzymatic steps: (1) S-AdoMet synthetase converts methionine to S-adenosyl-L-methionine (S-AdoMet); (2) S-AdoMet is converted to ACC by ACC synthase (ACS); (3) ACC is oxidized by ACC oxidase (ACO) and is thereby converted to ethylene (Yang and Hoffman, 1984; Sato and Theologis, 1989; Zarembinski and Theologis, 1994; Wang et al., 2002; Chae and Kieber, 2005). Unlike ACO, ACS has very low basal activity and can be rapidly increased under conditions that promote ethylene production (Yang and Hoffman, 1984). Thus, ACS is considered to be the rate-limiting enzyme in ethylene biosynthesis.

Arabidopsis has nine genes encoding ACS isoforms that are classified into three types according to the phosphorylation sites in their C-termini. ACS1, ACS2, and ACS6 are the type I ACS isoforms and have phosphorylation sites for mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs; Liu and Zhang, 2004; Kamiyoshihara et al., 2010). ACS2 and ACS6 could be regulated by the MAPKs MPK3, and MPK6 at both the transcriptional and posttranslational levels (Liu and Zhang, 2004; Han et al., 2010; Li et al., 2012). Type II ACSs include ACS4, ACS5, ACS8, and ACS9, and have putative CDPK phosphorylation sites, but not MAPK phosphorylation sites, in their C-termini. ACS7 and ACS11 are classified into Type III ACS isoforms, which lack both types of phosphorylation sites. Previous studies have shown that expression of ACS isoforms is tissue-specific, and that different ACS isoforms respond differently to extracellular stimuli (Zarembinski and Theologis, 1994; Wang et al., 2002).

In plants, MAPK cascades, which consist of MAPKKK, MAPKK, and MAPK, play vital roles in development and in a number of stress responses, including those to wounding, pathogen infection, temperature, salinity, drought, osmolarity, ozone, UV irradiation, ROS, and nutrient deficiency (Group et al., 2002; Pedley and Martin, 2005; Zhang et al., 2007; Pitzschke et al., 2009; Rodriguez et al., 2010; Tena et al., 2011). MPK3 and MPK6 can be regulated by different MAPKKs under different stress conditions (Teige et al., 2004; Liu et al., 2007; Takahashi et al., 2007; Wang et al., 2007, 2008, 2010; Xu et al., 2008; Yoo et al., 2008; Zhou et al., 2009). In addition, recent work shows that the MKK9-MPK3/MPK6 cascade is involved in phosphate (Pi) acquisition (Lei et al., 2014). However, the role of MAPKs in regulation of plant responses to Fe deficiency has not been studied.

Using quantitative RT-PCR (qRT-PCR) analysis, we found that the expression of *ACS2*, *ACS6*, *ACS7*, and *ACS11* transcripts in both leaves and roots, *ACS8* transcripts in leaves and *ACS9* in roots were up-regulated by Fe deficiency. Further analysis showed that MPK3/MPK6 participates in Fe deficiency-induced ethylene production. Loss function in MPK3 and MPK6 suppressed the expression of ACS2, ACS6, and the Fe deficient responses. As a result, the *mpk3* and *mpk6* plants had a reduced soluble Fe content and severe chlorosis symptoms compared to the wild-type (WT) plants when grown under Fe deficient conditions.

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana Columbia (Col-0) ecotype was used as the WT control. T-DNA insertion mutant alleles of *MPK3* (At3g45640), *MPK6* (At2g43790), *ACS2* (At1g01480), *ACS6* (At4g11280) were described previously (Liu and Zhang, 2004; Wang et al., 2007; Han et al., 2010). The high-order *acs* mutants generated in Dr. Athanasios Theologis' laboratory (Tsuchisaka et al., 2009) were obtained from the Arabidopsis Biological Resource Center (ABRC). The stock numbers of the high-order ACS mutants *acs2/acs4/acs5//acs6/acs7/acs9* and *acs1/acs2/acs4/acs5/acs6/acs7/ acs9/acs11* are CS16649 and CS16651, respectively.

Growth Conditions and Treatments

For hydroponic experiments, seeds were vernalized at 4°C for 3 days in the distilled water. Then they were sown in 1.5 ml bottom-cut centrifuge tubes containing 400 μL of 0.6% agarose gel. The tubes were held in the holes of a thin polyurethane raft floating on nutrient solution (Lucena et al., 2006). This arrangement allowed the plants growing in the float to uptake the nutrient solution via the agarose gel. The nutrient solution (without Fe) had the following composition: 2000 μM $Ca(NO_3)_2$, 500 μM KH_2PO_4, 750 μM K_2SO_4, 650 μM MgSO4, 50 μM KCl, 1 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 10 μM H_3BO_3, and 0.05 μM $(NH_4)_6Mo_7O_{24}$. Fe-EDTA was added or not added to the nutrient solution depending on the experiments. The pH of the nutrient solution was adjusted to 6.0. The growth chamber of seedlings was set 22°C day/20°C night temperatures, relative

humidity 60%, and a 10 h photoperiod at a photosynthetic irradiance of 300 $\mu molm^{-2}s^{-1}$ (Lucena et al., 2006).

Experiments using 10 day old seedlings were performed at swimming medium culture as described (Li et al., 2012). After being vernalized at 4°C for 3 days, the surface sterilized seeds were sown in liquid half-strength (1/2) Murashige and Skoog (MS) medium and grown in a growth chamber at 22°C with continuous light(70 $\mu E/m^{-2}sec^{-1}$). Five-day-old seedlings were transferred to 20-ml gas chromatography (GC) vials with 6 ml of liquid 1/2 MS medium (10 seedlings per vial) and the growth conditions maintained the same as before. Seedlings were grown in 1/2 MS swimming medium for 10 days, and then transferred to 1/2 MS medium with or without Fe. Ethylene were measured at 4 day or the indicated day (in the time course analysis) after the treatment, while analysis of transcript abundance was performed at 7 days after treatment.

RNA Isolation and qRT-PCR

According to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), Triozol reagent were used for total RNA isolation. To remove the residual genomic DNA, five micrograms of RNA were then treated with RNase-free DNase I (Takara Bio, Tokyo, Japan). Using M-MLV reverse transcriptase, First-strand cDNA was synthesized (Promega, Madison, WI, USA). qRT-PCR was performed as described previously (Zheng et al., 2009). A LightCycler 480 machine was used for PCR amplification of cDNA (Roche Diagnostics, Basel, Switzerland) with SYBR Premix Ex Taq Kit (Takara Bio). Quantitative assays were performed in triplicate on each sample and the reference gene β-*tubulin* was used as an internal control. Transcript levels relative to β-*tubulin* were calculated using the formula $2^{-\Delta\Delta Ct}$. All primer sequences used for the PCR reactions are provided in Supplementary Table 1.

Protein Extraction and Immunoprecipitation Kinase Assay

Protein extraction was performed as described previously (Ren et al., 2002). Total protein was extracted from whole seedlings by grinding in extraction buffer containing 100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 g ml^{-1} leupeptin, 5 g ml^{-1} aprotinin, and 5% glycerol. Supernatants were transferred into 1.5 mL tubes after centrifugation at 18,000 × g for 40 min. Samples should be quickly frozen in liquid nitrogen and stored at −80°C until further analyses. The concentration of protein extracts was determined using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard (Ren et al., 2002). Twelve micrograms of protein was loaded into each lane and separated by SDS-PAGE.

Immunoprecipitation kinase assay was performed as described (Lee and Ellis, 2007; Tsuda et al., 2009). Anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody, which specifically recognizes the dually phosphorylated-pTXpY- motif in phospho-MPK3 and phospho-MPK6, was used to detecting the amount of phosphorylated MPK3 and MPK6, i.e., the activities of the MPK3 and MPK6. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. The protein membranes were visualized with an Enhanced Chemiluminescence Kit (Roche) and then it was exposed to X-ray film.

Ethylene Measurement

After treatment, the GC vials which contain Arabidopsis seedlings were flushed and capped immediately. Twenty-four hours before the measurement of the ethylene production, the GC vials were flushed with fresh air to remove the ethylene accumulated before the day. Ethylene accumulated in the headspace of the GC vials over a 24 h period were determined by gas chromatography and mass spectrometry at indicated times (Kim et al., 2003; Liu and Zhang, 2004). Then the seedlings were harvested and weighed. Samples were frozen in liquid nitrogen for future analysis.

Chlorophyll Content Analysis

The SPAD value (a measure of total chlorophyll content) of the fully expanded youngest leaves was measured by a portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, JP).

Measurement of Soluble Fe Concentration

To determine the concentration of soluble Fe in plants, approximately 0.5–1 g of new leaves of treated seedlings were ground in liquid nitrogen. Five volumes of deionized water were added to extract the soluble Fe at room temperature. After centrifugation, the supernatant was collected in new tubes (Zheng et al., 2009). Inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, Santa Clara, CA, USA) was used for Fe concentration measurement.

RESULT

Effect of Fe Deficiency on Ethylene Production

A previous study found that ethylene production in the roots of the strategy I plants pea, tomato, and cucumber increased under conditions of Fe deficiency (Romera et al., 1999). A time-course experiment was carried out with WT seedlings to investigate whether Fe deficiency induced ethylene production in Arabidopsis (**Figure 1**). To accurately measure the ethylene production at different treatment time, the GC vials were flushed with fresh air at 24 h prior to the measurement. Ethylene accumulated over a 24 h period was determined at the day indicated. Upon initiation of Fe deprivation, the induction of ethylene production began at the 2nd day and reached its maximum level at the 4th day. The maximum level of ethylene production in Fe deficient conditions was approximately three times higher than that in Fe sufficient conditions. After reaching its maximum level, ethylene levels decreased gradually and dropped to basal levels at 5 days after initiation of Fe deficient conditions.

Effect of Fe Deficiency on ACS Transcription

Using semi-quantitative PCR, a previous study demonstrated that several *ACS* genes, including *ACS4*, *ACS6*, and *ACS9*, are up-regulated under the stressful conditions of Fe deficiency (García et al., 2010). To identify all the ACS isoforms involved in ethylene production under Fe deficiency, we used qRT-PCR to measure the expression of all nine *ACS* genes in Arabidopsis grown under Fe-sufficient and Fe-deficient conditions. We were able to detect the expression of eight of the nine *ACS* genes (all but *ACS1*) in leaf, root, or both (**Figure 2**). The transcript abundance of *ACS2*, *ACS6*, *ACS7*, and *ACS11* increased significantly in both leaf and root after 7 days of Fe deprivation. *ACS8* transcripts were detected only in leaf, and were four-fold more abundant under Fe deficient conditions than under Fe sufficient conditions. While *ACS4* transcripts were also detected only in leaf, Fe deficiency did not lead to a significant change in *ACS4* mRNA. *ACS5* mRNA and *ACS9* mRNA were detected only in root. *ACS9* expression was over 30-fold higher in conditions of Fe deficiency compared to those of Fe sufficiency, whereas expression of *ACS5* was reduced. These results suggest that *ACS2*, *ACS6*, *ACS7*, *ACS8*, *ACS9*, and *ACS11* may contribute to ethylene induction under conditions of Fe deficiency.

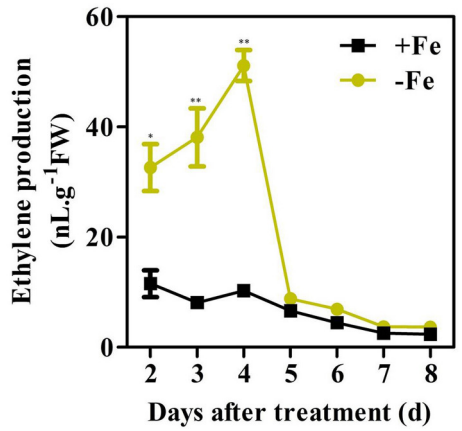

FIGURE 1 | Time-course analysis of ethylene production in WT plants under Fe-sufficient (+Fe) and -deficient (−Fe) conditions. Ten-day-old seedlings were transferred to Fe-sufficient (50 μM EDTA-Fe) or Fe-deficient (0 μM EDTA-Fe) nutrient solutions. For each time point shown, ethylene accumulated over a 24 h period from 10 seedlings in the headspace of GC vials was measured. Data are shown as the mean ±SEM ($n = 3$). Columns marked with "*" indicate a significant difference ($P < 0.05$), and "**" indicate a highly significantly difference ($P < 0.01$).

Mutation of *ACS2* Suppressed Ethylene Production Induced by Fe Deficiency

As the rate-limiting enzyme in ethylene biosynthesis, ACS is well positioned to influence ethylene production. To explore the involvement of ACS isoforms in Fe deficiency-induced ethylene production, *acs2*, *acs6*, and high-order *acs* mutants were used. In these mutants, expression of corresponding *ACS* genes was abolished (Supplementary Figure 1A). Under

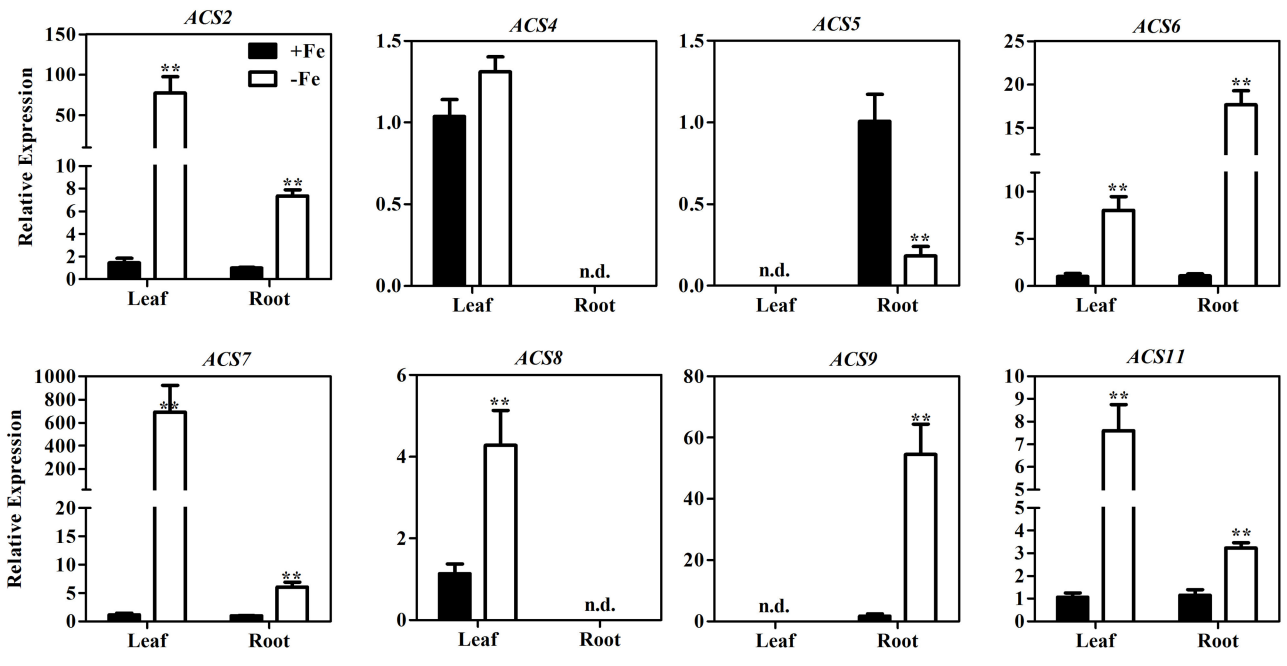

FIGURE 2 | Transcription levels of *ACS* genes in the leaves and roots of WT seedlings under Fe-sufficient (+Fe) and -deficient (−Fe) conditions. One-month-old seedlings were transferred to +Fe (50 μM EDTA-Fe) or −Fe (0 μM EDTA-Fe) nutrient solutions for 7 days. Leaves and roots were collected for RNA extraction. All values are expressed relative to the expression level under Fe-sufficient conditions (control—set to 1.0) as appropriate. Data are shown as the mean ±SEM ($n = 3$). Column marked with "**" indicate a highly significantly difference ($P < 0.01$). n.d. indicates "not detectable."

conditions of Fe deficiency, *acs2* seedlings produced ethylene at a level that was only 60% of that in WT seedlings (**Figure 3**). Surprisingly, *ACS6* gene mutation did not affect Fe-induced ethylene induction, as *acs6* seedlings produced the same amount of ethylene as the WT. Ethylene production in high-order *acs* mutants (*acs2/acs4/acs5/acs6/acs7/acs9* and *acs1/acs2/acs4/acs5/acs6/acs7/acs9/acs11*) was very low in both Fe sufficient and Fe deficient conditions.

Mutation of *ACS2* Suppressed the Upregulation of Fe Deficiency-responsive Genes

Ethylene regulates expression of *FER* (or *FER*-like) and thereby regulates gene expression of downstream Fe transporter, ferric reductase, and H^+-ATPase (Lucena et al., 2006). We did not observe obvious phenotypic differences between WT and *acs2* or *acs6* mutants in Fe sufficient or deficient conditions (Supplementary Figure 2A). However, in conditions of Fe deficiency, upregulation of Fe deficiency-responsive genes was attenuated in *acs2* mutants (**Figure 4**) and the high order ACS mutant (Supplementary Figure 3 Specifically, expression levels of *FIT*, *FRO2,* and *IRT1* genes in *acs2* mutants were reduced 40, 50, and 33% from levels in WT seedlings, respectively. In contrast, the induction of Fe deficiency-responsive gene expression did not change in the *acs6* mutants. To confirm the reduction of FRO2 expression indeed affected the Ferric-chelate reductase (FCR) activity, FCR assay was performed on *acs2, acs6,* and high order ACS mutants. Compared to the WT plants, the FCR activity in *acs2* was slightly reduced, but not significant (Supplementary Figure 2B). In contrast, FCR activity in the high order ACS mutants were significantly reduced. The FCR activity is in agreement with the *FRO2* transcript level.

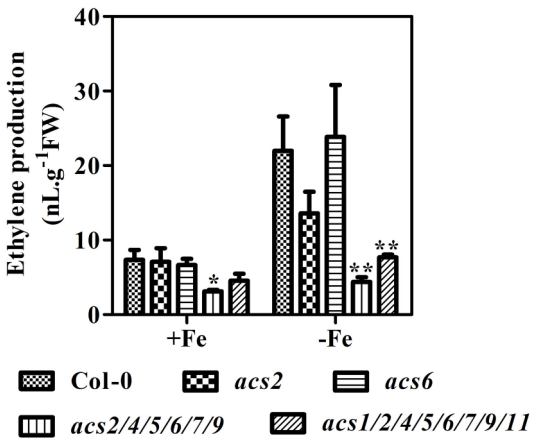

FIGURE 3 | Ethylene production of WT, *acs* mutant plants under Fe-sufficient (+Fe) and -deficient (−Fe) conditions. Ten-day-old seedlings were transferred to Fe-sufficient (50 μM EDTA-Fe) or -deficient (0 μM EDTA-Fe) nutrient solutions. Ethylene accumulated over a 24 h period from 10 seedlings in the headspace of GC vials was measured at 4 day after the treatment. Data are shown as the mean ±SEM ($n = 3$). Columns marked with "*" indicate a significant difference ($P < 0.05$), and "**" indicate a highly significantly difference ($P < 0.01$).

Fe Deficiency Activates MPK3/MPK6 at Both Transcript and Protein Levels

Previous research has shown that MPK3/MPK6-mediated phosphorylation of ACS2 and ACS6 proteins leads to their stabilization and accumulation (Liu and Zhang, 2004; Han et al., 2010; Li et al., 2012). qRT-PCR results confirmed that *ACS2* and *ACS6* transcript levels are increased under Fe deficiency conditions (**Figure 2**). To further understand the roles of MPK3 and MPK6 in Fe deficiency-induced ethylene production, both transcript and enzymatic activity levels of MPK3 and MPK6 were determined. Results showed that levels of *MPK3* and *MPK6* transcripts were significantly increased under Fe deficiency (**Figure 5A**). Specifically, expression of *MPK3* was induced eight-fold in leaf and 2.5-fold in root, whereas that of *MPK*6 was induced only 1.8-fold in leaf and 2.8-fold in root. Furthermore, immunoprecipitation kinase assay was performed using anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody to detect the amount of phosphorylated MPK3 and MPK6, (**Figure 5B**). While the activation of MPK6 persisted 4–5 days after treatment, the activation of MPK3 persisted only 1–2 days after treatment. Taken together, these results indicate that the

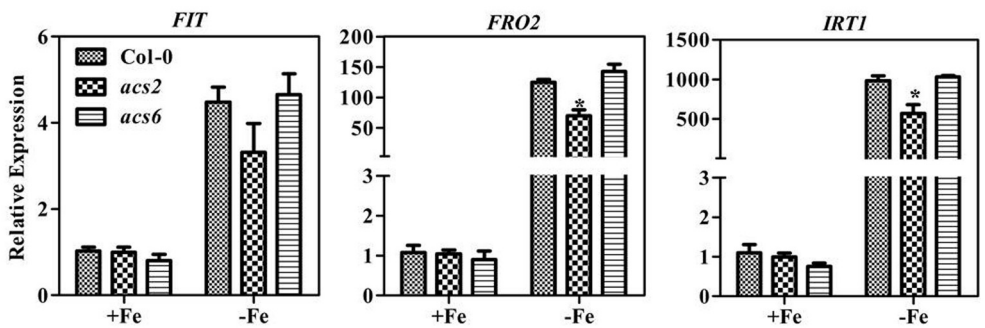

FIGURE 4 | Transcription levels of Fe-deficiency responsive genes in *acs2* and *acs6* mutant plants. Ten day old seedlings cultured in swimming medium were transferred to Fe-deficient medium for 7 days and sampled for RNA extraction. All values are expressed relative to the expression level under Fe-sufficient conditions (control—set to 1.0) as appropriate. Data are shown as the mean ±SEM ($n = 3$). Column marked with "*" indicate a significant difference ($P < 0.05$).

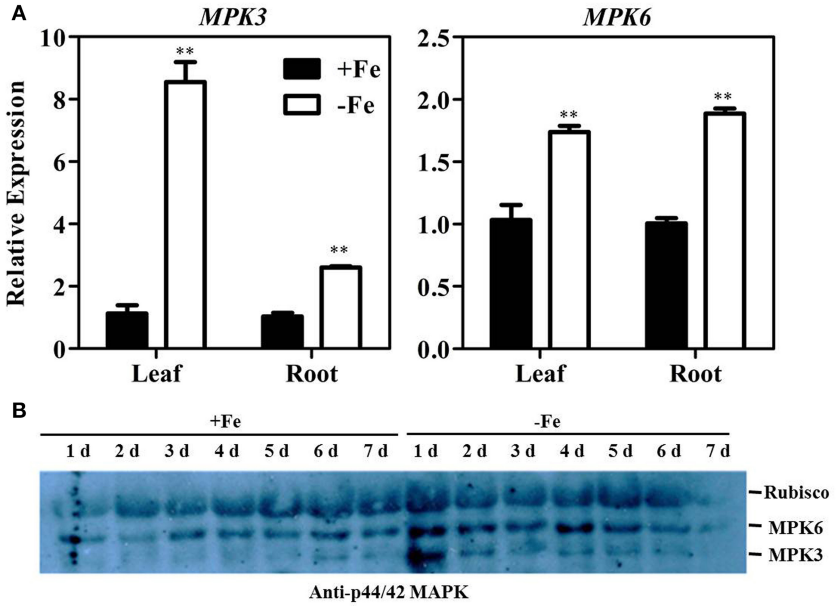

FIGURE 5 | Fe deficiency activates MPK3 and MPK6. (A) Transcription levels of MPK3 and MPK6 in the leaf and root of WT seedlings under Fe-sufficient (50 μM EDTA-Fe) and -deficient (0 μM EDTA-Fe) conditions. All values are expressed relative to the expression level under Fe-sufficient conditions (control—set to 1.0) as appropriate. Data are shown as the mean ±SEM ($n = 3$). **(B)** Immunoprecipitation kinase assay of MPK3 and MPK6 activity using Anti-p44/42 MAPK antibody. Ten-day-old seedlings were transferred to Fe-sufficient or -deficient nutrient solutions. Column marked with "**" indicate a highly significantly difference ($P < 0.01$).

transcript abundance and enzymatic activities of MPK3 and MPK6 were induced by Fe deficiency.

Loss of *MPK3* or *MPK6* Resulted in Reduction of Ethylene Production and the Expression Level of *ACS2* and *ACS6* Under Fe Deficiency

To further demonstrate that the MPK3/MPK6 cascade participates in Fe-induced ethylene production, two *mpk* mutants for each MPK were used in subsequent experiments: *mpk3-1*, *mpk3-2*, *mpk6-2,* and *mpk6-3*. Phosphorylation enzymatic activity assay showed that the mutants lost the function of the corresponding kinase activity (Supplementary Figure 1B). Fe deficiency-induced ethylene production in *mpk3-1* and *mpk3-2* was reduced by 15 and 28%, respectively, compared to that of WT seedlings (**Figure 6A**). In *mpk6* mutants, reduction of Fe deficiency-induced ethylene biosynthesis was much more severe. Compared to WT seedlings, both *mpk6* mutants maintained only 50% of ethylene production. These results provide further support for the involvement of MPK3 and MPK6 in ethylene production under Fe deficiency.

ACS2 and ACS6 are known to be regulated by the MPK3/MPK6 at both transcriptional level and posttranslational level (Li et al., 2012).To determine whether mutation in MPK3 and MPK6 would affect the expression ACS2 and ACS6, qRT-PCR was performed in the *mpk3* and *mpk6* plants grown under Fe sufficient or deficient conditions. While Fe deficiency induced the expression of *ACS2* and *ACS6,* the up-regulation was suppressed in the *mpk3* and *mpk6* mutants (**Figure 6B**).

The *MPK3* and *MPK6* Mutants Showed Severe Chlorosis Under Fe Deficiency

To further investigate the role of MPKs in Fe deficiency, the growth performance and the expression of the Fe deficiency-responsive genes were investigated in the *mpk* mutants. Under Fe sufficient conditions, growth performance of WT seedlings was not significantly different from that of mutants (**Figures 7A,B**). However, under Fe deficient conditions, the mutants, especially the *mpk3-1* and *mpk6-3* mutants, were smaller and showed more severe chlorosis than WT seedlings (**Figures 7A,B**). Consistent with the mutants' chlorosis phenotypes, chlorophyll content, measured as the leaf SPAD value, was lower in *mpk3-1, mpk6-2,* and *mpk6-3* mutants than in WT seedlings (**Figure 7C**).

To determine whether the severe chlorosis phenotype in the *mpk3* and *mpk6* mutants was due to a decrease in leaf Fe content, the total leaf Fe concentrations in the mutants were measured. Fe concentrations were at the same level in WT seedlings and mutants (Supplementary Figure 4). To further investigate the relationship between Fe content and SPAD value, soluble Fe concentrations in the leaves of mutants and WT seedlings were determined. Our results showed that soluble Fe in *mpk3-2* and both *mpk6* mutants was marginally lower than that in the WT (**Figure 7D**). Furthermore, Fe deficiency-responsive gene expression was suppressed in both the *mpk3-2* and *mpk6-2* mutants compared to WT seedlings (**Figure 8**). Specifically,

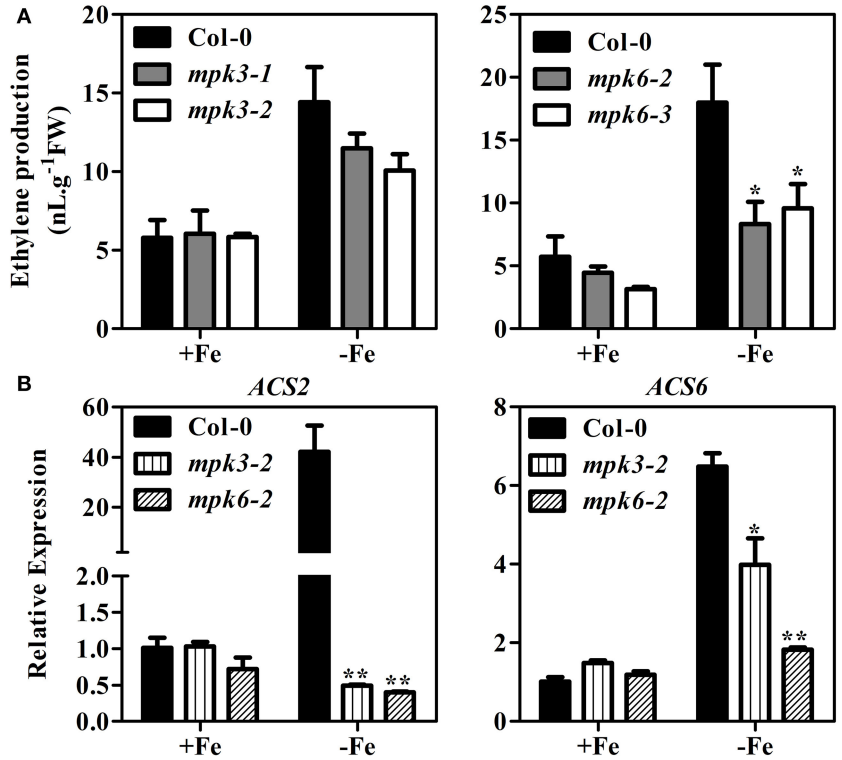

FIGURE 6 | Ethylene production and expression of *ACS2* and *ACS6* in *mpk3* and *mpk6* mutants. (A) Ethylene production in *mpk3* and *mpk6* mutants. **(B)** Transcript abundances of *ACS2* and *ACS6* genes in *mpk3* and *mpk6* mutants. Ten-day-old seedlings were transferred to Fe-sufficient (50 μM EDTA-Fe) or -deficient (0 μM EDTA-Fe) nutrient solutions. Ethylene accumulated over a 24 h period from 10 seedlings in the headspace of GC vials was measured at 4 days after the treatment. qRT-PCR was conducted using RNA extracted from whole seedlings at 7 days after –Fe treatment. Data are shown as the mean ±SEM ($n = 3$). Columns marked with "*" indicate a significant difference ($P < 0.05$), and "**" indicate a highly significantly difference ($P < 0.01$).

the induction of *FRO2* and *IRT1* in both mutants dropped to nearly 50% of WT levels. In the *mpk3-2* mutant, expression of *FIT* dropped to 30% of that in WT seedlings.

DISCUSSION

In strategy I plants, ethylene production is induced by Fe deficiency stress (Romera et al., 1999; Romera and Alcantara, 2004), which in return, functions as a positive regulator of Fe deficiency response (Romera and Alcantara, 1994; Li and Li, 2004; Lucena et al., 2006; Waters et al., 2007; García et al., 2010; Lingam et al., 2011; Romera et al., 2011). The aim of the study was to explore the regulatory mechanism of this physiological response. In the report, we demonstrated the involvement of MPK3 and MPK6 in the Fe-deficient induced ethylene production based on the following evidence. Firstly, the transcript abundance and enzymatic activities of MPK3 and MPK6 increased under Fe deprivation condition (**Figure 5**). ACS2 and ACS6 are known to be regulated by the MPK3/MPK6 at both transcriptional level mediated by a transcription factor and posttranslational level via the direct protein phosphorylation by MPK3/MPK6 (Li et al., 2012). The up-regulated expression of *ACS2, ACS6* in Fe-deficient plants at both root and leaf tissues (**Figure 6B**) is likely a consequence of up-regulation of MPK3 and MPK6. Secondly, the Fe-deficient induced ethylene production decreased in the *mpk3* and *mpk6* mutants (**Figure 6A**). Thirdly, the expression of Fe acquisition genes, *FIT, FRO2*, and *IRT1* in *mpk3* and *mpk6* mutants was less up-regulated by Fe deprivation than that in the WT (**Figure 8**). As a result, the *mpk3* and *mpk6* plants had a reduced soluble Fe content and severe chlorosis symptoms compared to the WT plants when grown under Fe deficient conditions (**Figure 7**).

The Fe-deficient induced ethylene production was not completely abolished in *mpk3, mpk6, acs2,* and *acs6* mutants (**Figure 3**, **Figure 6**). It suggests that other ACS isoforms are also involved in the process. Indeed, other than *ACS2* and *ACS6*, the expression of *ACS7, ACS9,* and *ACS11* genes in roots, and *ACS7, ACS8,* and *ACS11* in leaves were also up-regulated by Fe deficiency (**Figure 1**). Changes in the expression of the above ACS isoforms should also contribute to the Fe-deficient induced ethylene production. García et al. (2010) examined the expression of *ACS4, ACS6, ACS9,* and *ACS11* in Arabidopsis roots in response to Fe deficiency. They found that the expression of *ACS4, ACS6*, and *AC9* were up-regulated by Fe-deficiency. In contrast to that, the expression of *ACS4*, which was upregulated by Fe-deficiency in that research (García et al., 2010), was not detected in the study. In addition, the increased expression of

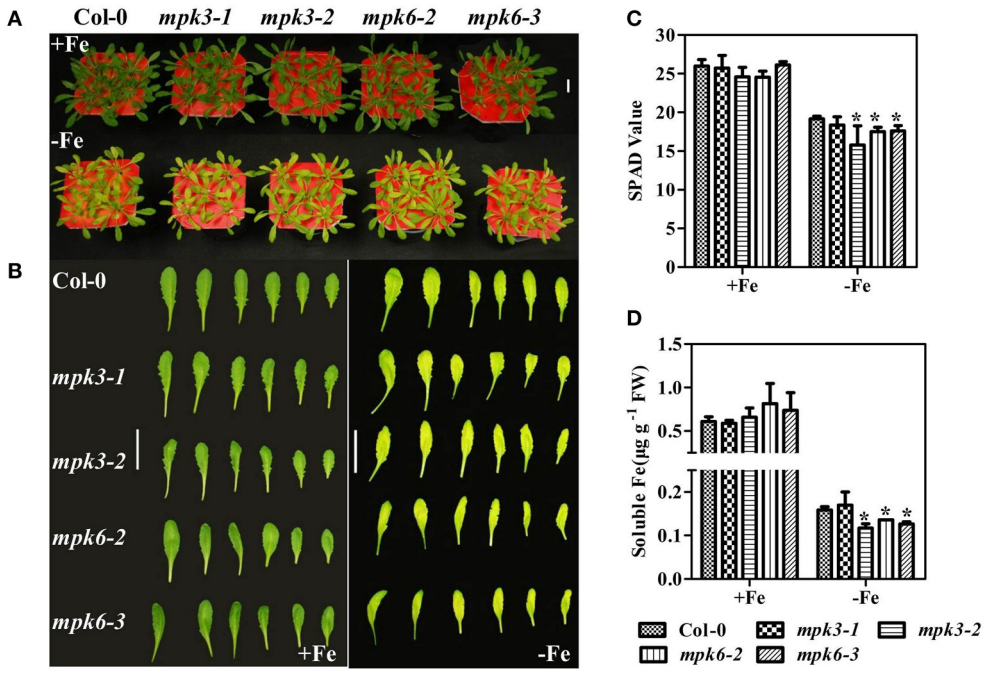

FIGURE 7 | Phenotypes of *mpk3* and *mpk6* mutants grown in Fe-sufficient or Fe-deficient conditions. (A) Growth performance of WT, *mpk3*, and *mpk6* mutant plants. 30 day-old seedlings were grown in Fe -sufficient (50 μM) or -deficient (0 μM) nutrient solution for 7 days. **(B)** Young leaves (from the third to the eighth) were detached to display their phenotypes. **(C)** SPAD values of WT, *mpk3*, and *mpk6* mutants. **(D)** Soluble Fe concentration of WT, *mpk3*, and *mpk6* mutants. Scale bar = 3 cm. Data are shown as the mean ±SEM ($n = 3$). Column marked with "*" indicate a significant difference ($P < 0.05$).

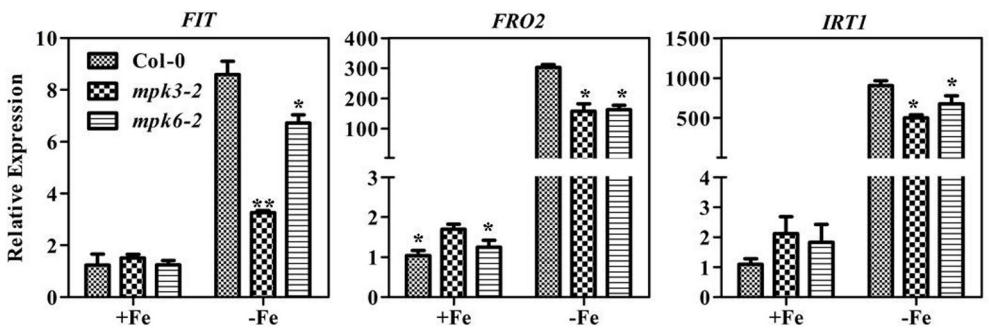

FIGURE 8 | Transcription levels of Fe-deficiency responsive genes in *mpk3* and *mpk6* mutants. Ten day old seedlings cultured in swimming medium were transferred to Fe-deficient medium for 7 days and sampled for RNA extraction. All values are expressed relative to the expression level under Fe-sufficient conditions (control—set to 1.0) as appropriate. Data are shown as the mean ±SEM ($n = 3$). Columns marked with "*" indicate a significant difference ($P < 0.05$), and "**" indicate a highly significantly difference ($P < 0.01$).

ACS11 in this study was not detected by García et al. (2010). The inconsistency between the two studies may be attributed to the different experimental conditions used. While García et al. (2010) examined the expression of *ACS* genes at 24 h after –Fe treatment, we did qRT-PCR on the plant tissues that had been treated with -Fe deficiency for 7 days. Seven days of Fe deprivation was chosen as that is when chlorotic symptoms are visible (Supplementary Figure 5). According to Vert et al. (2003), the level of *IRT1* and *FRO2* transcripts increases at 3 d and reaches to the maximum level at 5 d after –Fe treatment. In their paper, the *IRT1* and *FRO2* transcript abundance at day 7 is similar to day 3 of Fe deficiency (Vert et al., 2003). To verify that, we did a time-course qRT-PCR analysis using plant samples with different period of –Fe treatment, including 1, 3, 5, and 7 days of Fe-deprived treatment. Results showed that the up-regulation levels of the tested -Fe induced genes were stable from day 1 to day 7 of the treatment (Supplementary Figure 6). Thus, the expression of Fe acquisition related genes in response to 7 days of Fe deficiency is a good reflection of the transcript abundance for the genes measured. As to the expression of the *ACS* genes, after 7 days of –Fe treatment,

the up-regulation of rapid response *ACS* genes may have returned to a normal level. On the other hand, the long term Fe deficiency may turn on the general stress responsive genes, which is not directly related to Fe deficiency. Whether ACS4 and ACS11 are response to short or long term Fe-deficiency differently needs to be further investigated.

In summary, we demonstrated that the MPK3/MPK6 participates in Fe deficiency-induced ethylene production. Loss function in MPK3 and MPK6, or their downstream ACS2 isoform suppressed the Fe deficient responses. Roles of the other Fe-responsive ACS isoforms, such as ACS7, ACS 9, and ACS11 at leaves and roots, and ACS8 in leaves, in Fe deficiency-induced ethylene production remained to be explored. Additional studies, including studies of the regulation of upstream genes of MPK3/MPK6 in Fe deficiency-induced ethylene production and of other ACS isoforms would expand our understanding of the regulatory mechanisms of ethylene induction under Fe deficiency.

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SUPPLEMENTARY MATERIAL

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Root Responses to Boron Deficiency Mediated by Ethylene

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Low boron (B) supply alters the architecture of the root system in *Arabidopsis thaliana* seedlings, leading to a reduction in the primary root growth and an increase in the length and number of root hairs. At short-term (hours), B deficiency causes a decrease in the cell elongation of the primary root, resulting in a lower growth. Experimental approaches using ethylene insensitive *Arabidopsis* mutants, inhibitors of ethylene response, and GUS reporter lines suggest that ethylene is involved in these responses of the primary root to B deficiency. Furthermore, it has been shown that auxin participates in the inhibition of cell elongation under short-term B deprivation. These results support that an interaction between ethylene and auxin plays an important role in controlling the primary root elongation, in which a number of genes related to the synthesis, transport, and signaling of both phytohormones could modulate this effect. Evidence for a root cross-talk among both hormones and other possible intermediates (abscisic acid, calcium sensors, and reactive oxygen species) in response to B deficiency is provided and discussed.

Keywords: abscisic acid, auxin, boron deficiency, calcium signaling, ethylene, primary root, reactive oxygen species, root hairs

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ROLE OF BORON IN PLANT DEVELOPMENT

Boron (B)—an element with properties intermediate between metals and non-metals–is an essential nutrient for vascular plants, and its limited availability affects yields and quality of crops producing significant economic losses (Blevins and Lukaszewski, 1998; Tanaka and Fujiwara, 2008).

Three mechanisms to explain B uptake and transport in plants have been described: (i) passive diffusion through the plasma membrane, (ii) facilitated diffusion through channels (NIPs, nodulin 26-like intrinsic proteins), and (iii) active high-affinity transport mediated by BOR transporters and induced under low B availability (Brown et al., 2002; Takano et al., 2008; Miwa and Fujiwara, 2010; Reid, 2014).

Both boric acid and borate have the ability to react with compounds containing *cis*-diol groups resulting in stable borate ester complexes. Thus, the best-known role of B is its structural function in the cell wall, where borate acts to form a diester bond between apiose residues of two rhamnogalacturonan II monomers; this dimer—the first molecule linked by borate identified in the plant kingdom—contributes to the steadiness of the cell wall (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996). In addition, B has been related to other two main processes, namely, the maintenance of plasma membrane integrity through the formation of complexes with compounds containing *cis*-diol moieties (e.g., glycoproteins and glycolipids) and the support of metabolic activities, so that its deficiency affects numerous metabolic and physiological processes that take place during both reproductive and vegetative stages of a plant's life cycle (Blevins and Lukaszewski, 1998; Brown et al., 2002; Bolaños et al., 2004; Goldbach and Wimmer, 2007;

Camacho-Cristóbal et al., 2008a). To explain this apparently pleiotropic effect of B, it has been proposed that the main role of B is the stabilization of *cis*-hydroxyl-containing molecules, irrespectively of their function (Bolaños et al., 2004). Nonetheless, with the exception of the primary structural role of B in the cell wall, so far there is not a hypothesis which fully explains how so many plant processes are affected by short-term B deficiency.

ETHYLENE-AUXIN INTERACTION IN THE CONTROL OF ROOT DEVELOPMENT

Plant hormones regulate many aspects of growth and differentiation in plants, often through interaction between them. Thus, without exception, auxin, cytokinin, and ethylene are involved in regulation of root development (Ruzicka et al., 2007, 2009).

The roles of auxin and ethylene in controlling plant development have been thoroughly studied. It is well known that these two hormones act synergistically in regulating certain developmental processes, such as formation and elongation of root hairs, but also that they act antagonistically in other processes such as the development of lateral roots and hypocotyl elongation (Stepanova et al., 2007). The cross-talk between both hormones can be analyzed from the signaling pathways of ethylene and auxin (Muday et al., 2012). A first interaction occurs by activation of genes containing promoter regions that respond to ethylene and auxin, allowing both signaling pathways to directly regulate transcription. A second interaction occurs through the expression of genes that are auxin responsive, but whose activities regulate the synthesis, signaling or the response of ethylene, and vice versa (Muday et al., 2012). Therefore, ethylene and auxin can interact at three levels: reciprocally regulating their biosynthesis, influencing the response pathway, or acting on the same genes (Stepanova et al., 2007).

In some processes of plant growth and differentiation, auxin and ethylene can cause similar responses due to the capacity of auxin to promote ethylene synthesis by increasing ACC (1-aminocyclopropane-1-carboxylic acid) synthase activity. Exogenous application of IAA results in increased transcription of multiple genes responsible for ACC synthase (ACS), leading to an increase in ethylene production (Liang et al., 1992; Stepanova et al., 2007; Benková and Hejátko, 2009). Nevertheless, it has also been described that ethylene modulates the synthesis, transport, and auxin signaling in processes such as root growth and the formation of root hairs (Benková and Hejátko, 2009; Muday et al., 2012). Thus, while auxin can inhibit root growth in the absence of ethylene, ethylene inhibits root growth by increased auxin levels in certain areas of the root (Stepanova et al., 2007). For instance, ethylene enhances shootward auxin transport from the root apical to elongation zone by upregulating the transcription of AUX1 and PIN2, which mediate auxin delivery into cells of the elongation zone. Increased auxin levels elicit auxin responses in this zone that decrease cell elongation (Ruzicka et al., 2007).

Therefore, the maintenance of an appropriate ethylene-auxin balance is one of the most important mechanisms involved in root growth regulated by both hormones (Benková and Hejátko, 2009).

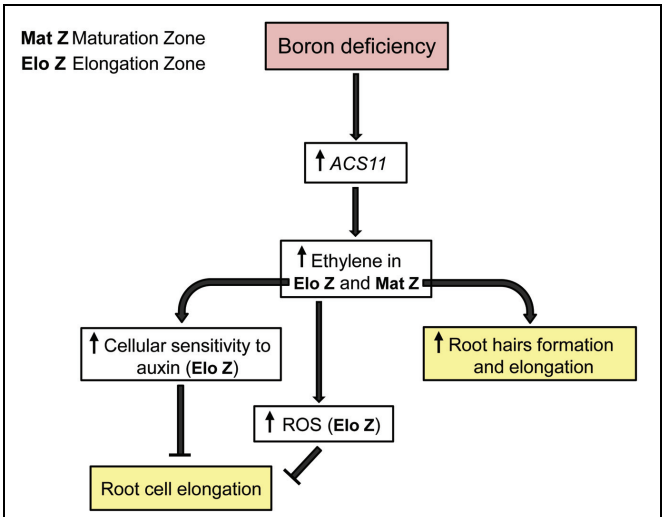

FIGURE 1 | Proposed model for short-term response of the primary root of *Arabidopsis thaliana* plants to B deficiency. For more details see the text.

BORON AVAILABILITY AFFECTS THE FORMATION OF ROOT HAIRS AND THE ROOT SYSTEM GROWTH *VIA* ETHYLENE

In vascular plants, the most rapid response to B deficiency is the growth inhibition of both primary and lateral roots (Dell and Huang, 1997). Furthermore, this mineral deficiency elicits root hair formation and elongation (Takano et al., 2006; Martín-Rejano et al., 2011) and a decrease in the cell elongation of the primary root (Dell and Huang, 1997; Camacho-Cristóbal et al., 2015). These changes in root architecture can seriously affect the ability of plants to take up water and nutrients.

Number and Length of Root Hairs

Interestingly, low B supply (0.4 μM) leads to an increase in the length and number of root hairs even after only 1 day of B deficiency (Martín-Rejano et al., 2011). This effect appears to be mediated by ethylene (**Figure 1**), which is supported by the following three facts: first, both the ethylene reporter EBS::GUS and the ACS11::GUS lines showed an increased expression in the maturation zone of primary root in response to low B supply (Martín-Rejano et al., 2011) and B deficiency (Camacho-Cristóbal et al., 2015), respectively, which suggests an accumulation of ethylene in this root zone; second, B limiting-induction of root hairs disappeared in an ethylene insensitive *(ein2-1) Arabidopsis thaliana* mutant (Martín-Rejano et al., 2011), which shows that the ethylene signal transduction to the nucleus through EIN2 protein is required to induce the formation and elongation of root hairs under conditions of limiting B; third, the effect of low B supply (0.4 μM, Martín-Rejano et al., 2013) or B deficiency (Camacho-Cristóbal et al., 2015) on root hair length was attenuated in the presence of Ag^{+}—an inhibitor

of ethylene response. In agreement with these results, ethylene has been reported to induce the formation and elongation of root hairs in *Arabidopsis* (Grierson et al., 2014 and references therein).

Additionally, it is well known that reactive oxygen species (ROS) are necessary for root hair growth in *Arabidopsis* (Foreman et al., 2003). In fact, *Arabidopsis rhd2* mutants, which lack respiratory burst oxidase homolog C (RBOHC, a plasma membrane NADPH oxidase), have markedly decreased levels of ROS and, consequently, form shorter root hairs; treatment of *rhd2* roots with ROS partly suppressed the mutant phenotype (Foreman et al., 2003). However, it has been reported that *Arabidopsis rhd2* plants grown on B-free solidified media formed root hairs, which were similar to those of control plants (Bassil et al., 2005). B lack in *Arabidopsis rhd2* mutants could induce the formation of root hairs by two non-exclusive hypotheses: (i) another mechanism in which a notable participation of ROS would not be essential, and/or (ii) the increase in the activity of other RBOs expressed in *Arabidopsis* roots. Consistent with this last hypothesis, NADPH oxidase activities increased in primary roots of *Arabidopsis* after short-term B deficiency (Camacho-Cristóbal et al., 2015).

Finally, it is noteworthy to mention that the higher number and length of root hairs under B limitation could be an efficient way to enhance the B uptake by NIP5;1 proteins localized to the plasma membrane of root hairs (Takano et al., 2006).

Primary Root Growth

Low B treatment (0.4 μM) for 1 to 4 days alters the architecture of the root system in *Arabidopsis* seedlings leading to a reduction in the primary root growth (Martín-Rejano et al., 2011). It is well known that ethylene plays critical roles in modulating root growth (Le et al., 2001; Swarup et al., 2007). The signals generated by ethylene converge in transcription factors, such as EIN3, which trigger a transcriptional cascade resulting in activation and repression of hundreds of genes (Stepanova and Alonso, 2009). Interestingly, EBS::GUS activity also increased in the elongation zone of the *Arabidopsis* primary roots treated with limiting B (Martín-Rejano et al., 2011). This suggests that the accumulation of ethylene mediates the inhibition of primary root growth under low B supply. In addition to ethylene, auxin seems to be involved in the inhibition of *Arabidopsis* primary root treated with limiting B supply (0.4 μ M). Two facts support this assumption: (i) the expression of the auxin reporter DR5::GUS increased in the elongation zone of primary roots and (ii) the growth of primary roots in the auxin resistant *aux1-22* mutant was less sensitive to low B treatment than in wild-type plants (Martín-Rejano et al., 2011).

Total primary root growth depends on two developmental processes: the division of cells in the meristematic region and the elongation of cells that leave the root meristem (Scheres et al., 2002). Several abiotic stresses such as B toxicity cause a decrease in meristem size because of a progressive reduction of cell division, which correlates with the inhibition of root growth (Aquea et al., 2012). However, other abiotic stresses, including B deficiency, affect mainly cell elongation in the growing tissues of plants (Dell and Huang, 1997; Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015). Thus, a short-term B deficiency has been shown to strongly inhibit the elongation of root cells as manifested by their short length, which results in a rapid inhibition of primary root growth (Camacho-Cristóbal et al., 2015). This inhibition in cell elongation can reasonably be attributed to the adverse effects of B deprivation on the physical stability of the cell wall, which is essential for the cell elongation process (De Cnodder et al., 2005). In fact, changes in cell wall polysaccharides and the structural proteins can moderate plant cell expansion during development (Cosgrove, 1997). Cell area is increased in an order of magnitude along the root elongation phase, and this requires a major restructuring of the cell wall and an increase in polysaccharide biosynthesis (Tsang et al., 2011). Hence, growing primary roots are sensitive to the cell wall damage. For instance, inhibition of cellulose biosynthesis or interference in the cell wall assembly rapidly reduced elongation (Tsang et al., 2011). Therefore, the structural damages in the cell wall caused by B deficiency, together with the downregulation of several cell wall-related genes (Camacho-Cristóbal et al., 2008b), could lead to disorders that affect cell elongation. Even though the processes that control the extent of root cell elongation under B deficiency are still not clearly defined, there is growing evidence supporting the mediation of ethylene (Camacho-Cristóbal et al., 2015). Thus, interestingly, *ACS11* gene, which encodes an isoform of ACC synthase, was rapidly overexpressed in the absence of B, a fact consistent with the increased expression of ACS11::GUS reporter line in the root elongation zone (Martín-Rejano et al., 2014; Camacho-Cristóbal et al., 2015). It has been shown that the expression of *ACS* genes increases when there are severe developmental problems (Tsuchisaka et al., 2009). Although *ACS11* gene expression was rapidly induced under B deficiency, this is not the case with other genes of the *ACS* family (Camacho-Cristóbal et al., 2015). These results agree with those obtained by Tsang et al. (2011) in which only the *ACS11* gene was induced in the root elongation zone upon treatment with the cell wall-damaging isoxaben. This rapid upregulation of the *ACS11* gene under B deficiency would suggest an enhancement of ACC and/or ethylene synthesis in *Arabidopsis* roots to inhibit expansion of cells leaving the root meristem (Le et al., 2001; Swarup et al., 2007). In addition, cell elongation of the ethylene-insensitive mutant *ein2-1* was less sensitive to B deficiency than that of the wild-type plants (Camacho-Cristóbal et al., 2015), which also supports the occurrence of this ethylene-dependent pathway to control the inhibition of cell elongation under B deficiency.

Ethylene, or ACC, causes an irreversible blockage in cell elongation from which cells cannot expand (Le et al., 2001; De Cnodder et al., 2005). Inhibition of root elongation is an evident effect of ethylene or ACC, which is a synergistic effect with auxin on this process (Rahman et al., 2001; Swarup et al., 2002). Analysis of inhibition of root growth by ethylene and auxin revealed that both reduce the rate of cell expansion in the central zone of elongation (Rahman et al., 2007; Swarup et al., 2007). Importantly, IAA exogenous application to *Arabidopsis*

seedlings grown under control conditions decreased root cell elongation in a similar way to that caused by B deprivation (Martín-Rejano et al., 2014). This would support that B deficiency induces a decrease in root cell elongation, in part, by increased levels of auxin. In addition, the higher expression of the auxin reporter IAA2::GUS in the elongation zone and the complete restoration of cell elongation by PEO-IAA—an auxin signaling inhibitor—in B-deficient roots indicate the requirement of auxin signaling in the response of cell elongation to B deficiency (Martín-Rejano et al., 2014; Camacho-Cristóbal et al., 2015). It has also been suggested that the shootward auxin transport *via* AUX1 and PIN2 proteins participates in this response, since the primary root growth in *aux1-22* and *pin2* mutants was less sensitive to B deprivation than in wild-type plants (Martín-Rejano et al., 2013; Camacho-Cristóbal et al., 2015).

Furthermore, an accumulation of ROS has been reported in the elongation zone of *Arabidopsis* roots when they were subjected to B deprivation, and that these early responses to B deficiency were mediated by ethylene probably acting upstream of ROS production (Oiwa et al., 2013; Camacho-Cristóbal et al., 2015). Interestingly, it has also been described that localized auxin accumulation increases ROS levels (Peer et al., 2013), which would also explain the observed accumulation of ROS in the elongation zone under B deficiency. In this regard, the shorter cell elongation in *Arabidopsis* roots under B deficiency has been related to oxidative damage by ROS (Camacho-Cristóbal et al., 2015), which can be produced by NADPH oxidases of plasma membrane (Suzuki et al., 2011). In fact, it has been proposed that the crosslinking between hydroxyproline-rich glycoproteins driven by ROS could be an important mechanism to inhibit root cell elongation (De Cnodder et al., 2005). Consistent with this, NADPH oxidase activity in *Arabidopsis* roots was significantly higher after short-term B deprivation and, in addition, diphenyleneiodonium, which inhibits ROS generation by NADPH oxidases, attenuated the effect of B deficiency on cell elongation, even in the presence of ACC (Camacho-Cristóbal et al., 2015). These results support the hypothesis of a relation between ethylene, auxin, ROS production and inhibition of root cell elongation under this mineral deficiency.

ROLE OF ETHYLENE AND AUXIN IN ROOT RESPONSE TO THE BORON DEFICIENCY: WHICH WORKS FIRST?

B deficiency causes a decrease in root growth that is mediated by ethylene and auxin, but which of the two phytohormones acts first triggering this response? When *Arabidopsis* seedlings grown with 10 μM B were treated with ACC, their primary root cell lengths decreased up to a similar size to those grown under B deficiency suggesting the involvement of ethylene in the B deficiency-induced response (Camacho-Cristóbal et al., 2015). Interestingly, when auxin signaling was inhibited by PEO-IAA in B-deficient seedlings, the length of their root cells increased up to the size of those treated with B sufficiency, even in the presence of ACC (Martín-Rejano et al., 2013, 2014; Camacho-Cristóbal et al., 2015). Furthermore, the blockage of ethylene signaling by Ag^{+} was able to abolish the effect of B deprivation on IAA2::GUS expression (Camacho-Cristóbal et al., 2015). These findings suggest that auxin signaling acts downstream of the ethylene signal in the root response to B deficiency, that is, ethylene would be acting previously to auxin. Consistent with these results, it has been described that the effect of ethylene on the root growth is largely mediated by an increase in the auxin response, which results in a lower elongation of root cells (Swarup et al., 2007).

According to this, a potential model is proposed to explain how seedling roots of *A. thaliana* respond in short-term to B deprivation, and how ethylene and auxin are associated with this response (**Figure 1**). B deficiency would trigger an increase in *ACS11* gene expression and, consequently, in the levels of ACC and ethylene. This rise would lead to alter auxin response in the primary root of *Arabidopsis* plants that, in turn, result in a decrease of the root cell length (Swarup et al., 2007; Muday et al., 2012). The increased auxin response in the elongation zone could explain the results observed with the auxin reporter line IAA2::GUS under B deficiency (Camacho-Cristóbal et al., 2015).

An intriguing fact is why B deficiency provokes a decrease in the cell elongation of the primary root while increases length of root hairs. It is well known that Ca^{2+} and ROS are necessary for root hair growth in *Arabidopsis* (Foreman et al., 2003; Takeda et al., 2008; Monshausen et al., 2009; Swanson et al., 2011). As B deprivation increases cytosolic levels of Ca^{2+} (Quiles-Pando et al., 2013; González-Fontes et al., 2014) and ROS (Oiwa et al., 2013; Camacho-Cristóbal et al., 2015) in the *Arabidopsis* roots, the higher levels of both could explain the enhanced length of root hairs under this mineral deficiency. However, under B deficiency, the auxin level in the elongation zone would exceed the threshold value of growth inhibition inducing local responses that inhibit the cell elongation.

Finally, and interestingly, it has recently been reported that abscisic acid (ABA) signaling activates two Ca^{2+}-dependent protein kinases—CPK4 and CPK11—that are capable of phosphorylating ACSs resulting in increased ethylene production, which inhibits primary root growth in *Arabidopsis* (Luo et al., 2014); as discussed by these authors, ABA would be acting not only upstream of ethylene, but also affecting auxin accumulation and/or auxin signaling *via* ROS production (Luo et al., 2014, and references therein). Moreover, it was shown that also a Ca^{2+}-dependent protein kinase is necessary for the phosphorylation of RBOHD protein associated with ROS generation (Dubiella et al., 2013). Curiously, it has been reported that B deprivation increases cytosolic Ca^{2+} concentration in both tobacco BY-2 cells (Koshiba et al., 2010) and *Arabidopsis* roots (Quiles-Pando et al., 2013; González-Fontes et al., 2014), and that two encoding genes of Ca^{2+}-dependent protein kinases *(CPK28* and *CPK29)* are also upregulated during short-term B deficiency (Quiles-Pando et al., 2013; González-Fontes et al., 2014). As B deprivation leads to increased

levels of ethylene and ROS (Camacho-Cristóbal et al., 2015), in light of all these data it would not be ruled out that there might be a cross-talk among ABA, Ca^{2+} signaling, ethylene, auxin, and ROS in responses to different plant stresses, including B deficiency.

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REVIEW
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The Role of Ethylene in Plants Under Salinity Stress

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Although the roles of ethylene in plant response to salinity and other stresses have been extensively studied, there are still some obscure points left to be clarified. Generally, in *Arabidopsis* and many other terrestrial plants, ethylene signaling is indispensable for plant rapid response and tolerance to salinity stress. However, a few studies showed that functional knock-out of some ACSs increased plant salinity-tolerance, while overexpression of them caused more sensitivity. This seems to be contradictory to the known opinion that ethylene plays positive roles in salinity response. Differently, ethylene in rice may play negative roles in regulating seedling tolerance to salinity. The main positive ethylene signaling components MHZ7/OsEIN2, MHZ6/OsEIL1, and OsEIL2 all negatively regulate the salinity-tolerance of rice seedlings. Recently, several different research groups all proposed a negative feedback mechanism of coordinating plant growth and ethylene response, in which several ethylene-inducible proteins (including NtTCTP, NEIP2 in tobacco, AtSAUR76/77/78, and AtARGOS) act as inhibitors of ethylene response but activators of plant growth. Therefore, in addition to a summary of the general roles of ethylene biosynthesis and signaling in salinity response, this review mainly focused on discussing (i) the discrepancies between ethylene biosynthesis and signaling in salinity response, (ii) the divergence between rice and *Arabidopsis* in regulation of salinity response by ethylene, and (iii) the possible negative feedback mechanism of coordinating plant growth and salinity response by ethylene.

Keywords: ethylene, salinity stress, MHZ, NtTCTP, negative feedback

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INTRODUCTION

Salinity has extensive negative effects on plant growth, including ion toxicity, osmotic stress, oxidative stress, and nutrient deficiency (Chinnusamy et al., 2006; Zhu, 2007). These hazards lead to growth inhibitory, crop yield reduction, and even death under prolonged high salinity condition. For improper irrigation and drainage, back flow of seawater, abuse of chemical fertilizer, and some other reasons, more and more arable land tends to be threatened by salinity. Salinity will usually lead to soil sodicity and alkalinity. Therefore, salinity stress has turned out to be one of the major factors limiting the sustainable development of agriculture. For a long time, people have focused on elucidating the physiological effects of salinity stress on plants, dissecting genetic components responsible for salinity stress response and resistance, and improving plant tolerance to salinity mainly through hybridization and gene transformation. Nowadays, studies at integrative levels become prevalent to uncover the intrinsic mechanism for plant rapid responses and self-modulation of vegetative and reproductive growth to adapt to salinity condition.

Throughout the life cycles of plants, phytohormones play vital roles in controlling the interaction between plants and environments, including plant responses to salinity stress. One of the phytohormones involved in salinity response is ethylene, which is also regarded as a stress-hormone besides its roles in regulation of plant growth and development (Abeles et al., 1992). Levels of ethylene and its direct precursor ACC could be obviously induced by salinity and other abiotic stresses in many plant species (Morgan and Drew, 1997). Compared to the glycophyte *Arabidopsis thaliana*, two halophytes *Cakile maritima* and *Thellungiella salsuginea* accumulated more ACC in both leaves and roots under high salinity (Ellouzi et al., 2014). In soybean, a research using 2-DE gel analysis found that several components of ethylene biosynthesis in the salt-tolerant genotype Lee 68 were more abundant than that in the salt-sensitive genotype Jackson under salinity stress (Ma et al., 2012). Application of ethylene or ACC could improve plant tolerance to high salinity (Cao et al., 2007), largely through enhancing the expression of reactive oxygen species (ROS) scavengers (Peng et al., 2014a). Further studies based on gene mutation and transformation analysis elucidated that the whole ethylene biosynthesis and signal transduction pathway are involved in plant response and adaptation to salinity. Generally, promotion of ethylene biosynthesis and signal transduction could enhance plant tolerance to salinity, while inhibition of it leads to increased plant sensitivity to salinity.

However, some other works showed that ACC may play a negative role in regulation of tomato seedling growth under salinity (Albacete et al., 2009). In tomato, ACC in leaves was increased prior to Na^+ accumulation, and was coincident with the onset of oxidative stress and leaf senescence under salinity stress (Ghanem et al., 2008). Although these results did not show the direct effects of ACC on plant response to salinity, and some other works revealed that ethylene is not the primary factor in salinity-induced plant growth inhibition (Shibli et al., 2007), it is still possible that ethylene may play a subtle negative role in plant response to salinity, at least at a certain growth stage. A further discussion is therefore necessary for us to understand the actual roles of ethylene in plant response to salinity.

In this review, we summarized and discussed roles of the whole ethylene biosynthesis and signaling pathway in plant response to salinity stress. The interactions between ethylene and ABA, jasmonic acid (JA) and some other signaling molecules, and the cross-talks between plant response to salinity and response to nutrient deficiencies were also compared. Some possible divergent roles of ethylene in different plant species were discussed as well.

ETHYLENE BIOSYNTHESIS AND SALINITY STRESS

Ethylene is a simple gaseous hormone which plays multiple roles in regulation of plant growth and development, and also serves as a key modulator between plant response to environmental stresses and normal growth (Abeles et al., 1992). Under salinity and some other stresses, ethylene production is quickly stimulated (Morgan and Drew, 1997). In fact, several key steps of ethylene biosynthesis could be affected by salinity and other stresses. Ethylene in plant is synthesized through three enzymatic reaction steps: methionine is converted to *S*-adenosyl-methionine (*S*-AdoMet) by *S*-AdoMet synthetase; then the direct precursor of ethylene ACC is synthesized from *S*-AdoMet by ACS (ACC synthase); and finally ethylene is produced through the oxidation of ACC by ACO (ACC oxidase) (reviewed in Lin et al., 2009). *S*-AdoMet is also the precursor for the synthesis of polyamines, which also plays a role in plant response to biotic and abiotic stresses. The by-product MTA (5′-methylthioadenosine) from the second step is recycled to methionine through the Yang Cycle to maintain a stable methionine pool even when ethylene is rapidly synthesized (Yang and Hoffman, 1984; Kende, 1993).

As a rate-limiting enzyme, ACS is the major target for regulation of ethylene production under stresses (Yang and Hoffman, 1984; Kende, 1993; Bleecker and Kende, 2000; Wang et al., 2002). There are eight functional *ACS* genes in *Arabidopsis*. They have redundant role in ACC biosynthesis (Tsuchisaka and Theologis, 2004; Tsuchisaka et al., 2009), while each with unique function in the regulation of plant growth and development. The regulation of *ACSs* under stress occurs at both transcriptional and post transcriptional levels. Under salinity, transcripts of *ACS2* and *ACS7* in *Arabidopsis* were increased dramatically (Achard et al., 2006). Through GUS staining, *Arabidopsis ACS5* and *ACS7* promoters were found to be induced by salinity (Wang et al., 2005). A more systematic research revealed that the MAPK cascade-induced by stress might activate WRKY33 which then promoted the expression of *ACS2*/*ACS6* genes in *Arabidopsis* (Li et al., 2012). In tobacco, the transcripts of *ACS1* were induced by salinity (Cao et al., 2006). In cotton, a series of *ACSs* were up-regulated under both short- and long-time salinity condition (Peng et al., 2014b). Recent work in *Arabidopsis* found that four *ACSs* (*ACS2*, *ACS6*, *ACS7*, and *ACS8*) were induced by high salinity, while a moderate low salinity pretreatment (known as salt acclimation) alleviated this induction (Shen et al., 2014). This result provides us a cue that promotion of ethylene production by a strong and sudden salinity stress might have multiple effects for plant response to salinity. It seems that no extra ethylene was needed for the process of salt acclimation, because there were no changes on the expression of genes related to ethylene biosynthesis (Shen et al., 2014). Four *ACSs* were up-regulated no matter under NASS (non-acclimated salt stress) or SASS (salt acclimated salt stress; Shen et al., 2014), indicating that promotion of ethylene production is still necessary for plant adaption to the stress condition. Nevertheless, transcripts of ACSs under SASS were less than those under NASS (Shen et al., 2014). These results suggest that a tight control of ethylene biosynthesis might be important for plant response and adaption to salinity stress.

Besides transcriptional regulation, some *ACSs* are also regulated post-transcriptionally under salinity, mainly through stress-activated MAPK (mitogen-activated protein kinase) cascades which phosphorylate ACSs protein and then prevent them from 26S proteasome-mediated degradation. Under biotic and abiotic stresses, *Arabidopsis* MPK6 was activated rapidly to phosphorylate ACS2 and ACS6 to elevate ethylene production (Liu and Zhang, 2004). In tomato, it was shown that calcium-dependent protein kinases (CDPKs)-mediated phosphorylation stabilized ACS2, leading to increased ACC content in stressed

tissues (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010). In *Arabidopsis*, loss-of-function of MPK6 diminished the effects of salt acclimation, leading to more sensitivity to high salinity (Shen et al., 2014).

Another key ethylene biosynthesis enzyme ACO is also regulated by salinity. Under high salinity, both ACC content and ACO activity were increased to enhance ethylene release in *Cicer arietinum* root (Kukreja et al., 2005). In cotton, several *ACOs* were up-regulated under both short- and long-time salt treatment (Peng et al., 2014b). From these results, we can propose that plants under salinity and other abiotic and biotic stresses tend to produce more ethylene mainly through enhancing ACSs and ACOs. Nevertheless, transcripts of *ACO1* in wheat were decreased under salinity and other abiotic stresses (Chen et al., 2014).

Although ACSs and ACOs are usually up-regulated under salinity, these key enzymes for ethylene biosynthesis may play negative roles in plant salinity-response. In *Arabidopsis*, loss-of-function of ACS7 conferred less ethylene emission, promoted vegetative growth and enhanced tolerance to salinity (Dong et al., 2011). Constitutive expression of wheat *ACO1* in *Arabidopsis* led to salinity-sensitivity, possibly through increasing the expression of *AtMYB15* while suppressing some stress-responsive genes like *AtRAB18*, *AtCBF1*, and *AtCBF3* (Chen et al., 2014). Expression of MKK9 in transgenic plants activated the endogenous MPK3 and MPK6 kinases, promoted the synthesis of ethylene and camalexin, and finally conferred increased sensitivity to salinity, whereas loss-of-function mutant *mkk9* showed enhanced tolerance to salinity (Xu et al., 2008). Recent work in rice found that a lectin receptor-like kinase SIT1 (salt intolerance 1) mediated salinity sensitivity through regulation of ethylene homeostasis (Li et al., 2014a). In presence of salinity stress, SIT1 was activated rapidly to phosphorylate MPK3/6 and then promote ethylene production (Li et al., 2014a). Overexpression of SIT1 in rice and *Arabidopsis* both conferred increased sensitivity to salinity, while loss-of-function of MPK3/6 alleviated this effect (Li et al., 2014a). Expression of SIT1 in rice and *Arabidopsis* also enhanced the ROS accumulation in an MPK3/6- and ethylene signaling-dependent manner (Li et al., 2014a). All indicate a SIT1-MPK3/6 cascade mediates salt sensitivity by regulating ethylene homeostasis. In addition, plant growth-promoting rhizobacteria (PGPR) produced AcdS (ACC deaminase), which facilitated the growth and stress tolerance of hosts via a reduction in levels of ethylene (Ali et al., 2014; Barnawal et al., 2014; Kim et al., 2014). Transgenic *Arabidopsis* expressing AcdS showed reduced sensitivity to exogenous ACC but increased tolerance to high salinity. In contrast, AcdS-silenced *Trichoderma* mutants were less effective in promoting plant tolerance to salinity, indicating that *Trichoderma* can also ameliorated plant growth under salinity stress by decreasing the ethylene biosynthesis in hosts (Brotman et al., 2013).

In contrast to the studies above, some other works showed that ethylene biosynthesis has positive effects on salinity-response. As stated above, MPK6, a key regulator of ethylene biosynthesis under stresses, was necessary for salt acclimation (Shen et al., 2014). That means plants may need MPK6 to stabilize ACSs and maintain a relative high ethylene level to accomplish the salt acclimation. The BTB ubiquitin ligases ETO1, EOL1, and EOL2 could interact with ACS5, promoted its ubiquitination and then accelerated its degradation (Christians et al., 2009). The expression of *ETOL1* in rice was induced under 200 mM NaCl treatment (Du et al., 2014). In *Arabidopsis*, loss-of-function of ETO1 increased ethylene production and improved seedling tolerance to soil-salinity. Lack of ETO1 reduced root Na^+ influx and so restricted root-to-shoot delivery of Na^+, and these effects were associated with increased RBOHF-dependent ROS accumulation in root stele. In addition, loss-of-function of ETO1 enhanced the tissue K^+ status through an RBOHF-independent manner associated with increased transcripts of the K^+-transporter *HAK5* (Jiang et al., 2013).

From these results, a definitive conclusion could not be drawn as to whether ethylene biosynthesis plays positive or negative roles in plant response to salinity. The discrepancy in the role of ethylene biosynthesis in salt response may be due to that, usually a single member of genes in the ethylene biosynthesis pathway, but not the whole status, was evaluated. And the optimal ethylene level for normal plant growth may be varied at different stages and in different plant species. From an evolutionary perspective, the induction of ethylene under stress condition should have some advantages for plant survival. In fact, pretreatment with ACC enhanced the salinity-tolerance of *Arabidopsis* seedlings (Peng et al., 2014a). We propose that ethylene indeed plays a positive role in the early stage of plant self-adjustment or salt acclimation for better survival under high salinity stress. After the stage of self-adjustment, excessive ethylene in plants will inhibit plant growth and development, which is disadvantageous for plants to survive under high salinity stress. Additionally, various ethylene receptors, whose functions are negatively regulated by ethylene, may also "neutralize" the ethylene role in salt stress. A homeostasis between ethylene and its receptors may facilitate plant survival under salinity stress.

There are multiple ACSs and ACOs members with functional redundancy on ethylene synthesis. Single mutation of one member only decreased ethylene emission in a limited level, and this may have little effect on salt acclimation. However, under high salinity, single mutation of ACS or ACO might decrease the induction of ethylene and alleviate excessive ethylene-induced growth inhibition, and hence keep higher growth potential for plants. Another possible reason is the competition for *S*-AdoMet as the precursor between ethylene and PA (polyamines) biosynthesis. Decreasing the ACS and ACO activity in tobacco could promote the biosynthesis of PA, and so enhance plant tolerance to abiotic stresses including high salinity through PA-mediated pathway (Wi and Park, 2002). Additionally, ACC itself might act as a signaling molecule in plants (reviewed in Yoon and Kieber, 2013), and so possibly mediates some unknown salinity response pathways. Further research based on analysis of multiple mutants of ACSs or treatments with different ethylene biosynthesis inhibitors will shine a light on the mechanisms involved.

ETHYLENE SIGNALING AND SALINITY STRESS

Based on the classical "triple response" (inhibited hypocotyl and root elongation, enhanced horizontal growth and exaggerated

apical hook of etiolated seedlings), a series of ethylene response mutants were characterized in *Arabidopsis*. The ethylene signal transduction model was then established through genetic and biochemical analysis of these mutants. Main components of this model include five ethylene receptors, a negative regulator CTR1, a key positive regulator EIN2, primary transcription factors EIN3/EILs and many downstream ethylene-response factors. In normal condition, ethylene receptors interact with and activate CTR1, which then phosphorylates EIN2 and prevents its translocation into nucleus, therefore inhibits ethylene signal transduction. When developmental or environmental signals induce ethylene production, ethylene binds with receptors to inhibit their interaction with CTR1, and CTR1 is inactivated, leading to the dephosphorylation and cleavage of EIN2. Then the C-terminus of truncated EIN2 is translocated into nucleus to stabilize downstream transcription factors EIN3/EILs (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). Finally, transcriptions of downstream ethylene response factors are activated by EIN3/EIL1 and lead to extensive ethylene responses. In this pathway, EIN2 is regulated by ETP1/ETP2 mediated protein turnover (Qiao et al., 2009) and EIN3 is regulated by EBF1/EBF2-dependent ubiquitination and degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007; An et al., 2010). Ethylene receptors are also regulated by proteasome-mediated protein degradation (Chen et al., 2007; Kevany et al., 2007; Shakeel et al., 2015; Tao et al., 2015). More recently, two groups reported new advances of ethylene signaling. Li et al. (2015a) found that ethylene induced EIN2 to associate with the 3′-UTR of *EBF1/2* mRNA and target *EBF1/2* mRNA to the cytoplasmic processing-body (P-body) through interaction with multiple P-body factors, leading to the stabilization of EIN3/EIL1 and activation of downstream events. Merchante et al. (2015) adopted genome-wide ribosomal footprinting and RNA-seq methods to identify translationally-regulated genes by ethylene, and they found that EIN2 and non-sense-mediated decay proteins UPFs are required for the translational regulation of *EBF1/2*.

As a stress hormone, the whole signaling pathway of ethylene is involved in plant response to salinity stress and possibly mediated the stress signal transduction. First, the expressions of many ethylene signaling genes are regulated by salinity and other stresses. In *Arabidopsis*, the expression of ETR1 was suppressed by osmotic stress including salinity (Zhao and Schaller, 2004). In tobacco, the mRNA quantity of ethylene receptor NTHK1 was dramatically increased under salinity stress (Zhang et al., 2001; Cao et al., 2006, 2007; Zhou et al., 2006). Further research discovered that NTHK1 was regulated by proteasome-mediated degradation, while its interaction with NtTCTP could stabilize itself (Tao et al., 2015). In cotton, several ethylene receptor genes (*ETR1*, *ETR2*, and *EIN4*), ethylene signaling genes (*CTR1*, *EIN3*, *ERF1*, and *ERF2*) and MAPK cascade genes (*MEKK1-MKK2-MPK4/6*) were all up-regulated under both short- and long-time salt treatments (Peng et al., 2014b). Besides, salt treatment promoted the degradation of EBF1/EBF2 and so enhanced the EIN3 protein accumulation in an EIN2-independent manner (Peng et al., 2014a). Additionally, salinity also promoted the transcriptional activity of EIN3 in an EIN2-dependent manner (Peng et al., 2014a).

Second, based on mutation and transgenic analysis, almost all ethylene signaling components were found to participate in plant response to salinity and other stresses. In *Arabidopsis*, *etr1* loss-of-function mutants showed enhanced tolerance to high salinity, whereas gain-of-function mutant *etr1-1* displayed increased sensitivity to salt stress (Zhou et al., 2006; Cao et al., 2007; Peng et al., 2014a). Overexpression of *NTHK1* in tobacco and *Arabidopsis* led to reduced sensitivity to ethylene but enhanced sensitivity to salinity (Cao et al., 2006). Addition of ACC to the treatment alleviated the salinity sensitivity caused by the overexpression of NTHK1 in *Arabidopsis*, but has no effects on the gain-of-function mutants of ethylene receptors (*etr1-1* and *ein4-1*, Cao et al., 2007). Further truncation and mutation analysis of NTHK1 showed that the transmembrane domain, the kinase domain and the kinase activity were indispensable for its roles in conferring reduced sensitivity to ethylene but enhanced sensitivity to salinity (Zhou et al., 2006; Chen et al., 2009). Besides redundant roles in ethylene perception, each ethylene receptor has some specific roles (reviewed in Shakeel et al., 2013). The specificity of ethylene receptors was reflected partly in their roles in salinity response. In *Arabidopsis*, ETR1 and ETR2 had contrasting roles in seed germination during salt stress, which seems to be mediated by affecting the ABA signaling but independent of ethylene signaling (Wilson et al., 2014). Tobacco subfamily II receptor NTHK1 played stronger roles than the subfamily I receptor ETR1 in regulation of seedling growth and salinity response (Chen et al., 2009). By yeast two-hybrid screening, an ankyrin domain-containing protein NEIP2 was identified to interact with NTHK1 and mediated plant response to salinity stress (Cao et al., 2015).

Besides the receptors, downstream ethylene signaling components also participated in salinity response. Loss-of-function of the key negative ethylene signaling factor CTR1 led to more tolerance to salinity stress (Achard et al., 2006; Peng et al., 2014a), possibly through modulation of shoot Na^+ and K^+ homeostasis which was dependent on ETR1-CTR1-regulated ethylene signaling (Jiang et al., 2013). The key positive ethylene signaling factor downstream of CTR1 is EIN2, which is found to confer salinity tolerance. *Arabidopsis* seedlings with loss-of-function of EIN2 became more sensitive to salinity, while overexpression of the C-terminus of EIN2 in *ein2-5* suppressed the salinity sensitivity (Cao et al., 2007; Lei et al., 2011a; Peng et al., 2014a). By yeast two-hybrid screening, a MA3 domain-containing protein ECIP1 was identified to interact with EIN2. Loss-of-function of ECIP1 led to minor enhanced ethylene response and salt tolerance during seedling growth but conferred salt sensitivity during seed germination process (Lei et al., 2011a). EIN3/EILs are the primary transcription factors for ethylene signal transduction from EIN2 to nuclear transcriptional regulation. In *Arabidopsis*, loss-of-function mutant *ein3-1* and the double mutant *ein3eil1* exhibited severe sensitivity to salinity, whereas overexpression of EIN3 enhanced seedling tolerance to salinity (Achard et al., 2006; Lei et al., 2011a; Peng et al., 2014a). Single mutant *ebf1-1* and double mutant *ebf1-1ebf2-1* also showed enhanced tolerance to salinity in an EIN3-dependent manner (Achard et al., 2006; Peng et al., 2014a).

Generally, in plant response to salinity, positive components of ethylene signaling are up-regulated by salinity and play positive roles, whereas negative factors are correspondingly down-regulated and play negative roles. In one word, ethylene signaling is necessary for plant response and adaption to salinity stress. However, ethylene response and signaling in rice, a semi-aquatic plant, seems to be somewhat different from *Arabidopsis* (Yang et al., 2015a). Based on the ethylene "double response" (promotes coleoptile growth but inhibits root elongation of dark-grown etiolated seedlings) in rice, a series of ethylene-response mutants *mhz* (*maohuzi*) were identified (Ma et al., 2010, 2013). These mutants were insensitive to ethylene on root elongation but showed differential responses on coleoptile growth. Unexpectedly, but interestingly, except for MHZ7 (a homolog of EIN2) and MHZ6 (a homolog of EIN3), other MHZs were either novel components with no homologies to ethylene signaling pathway proteins in *Arabidopsis* or cross-talk points interacting with other hormones (Ma et al., 2013), indicating a special feature for ethylene signaling pathway in rice. Both MHZ4 and MHZ5 were involved in ABA biosynthesis, and mediated ethylene-controlled root growth (Ma et al., 2014; Yin et al., 2015). MHZ6/OsEIL1 and OsEIL2 respectively regulated ethylene-controlled root and coleoptile growth of etiolated seedlings. Unlike the positive roles of EIN2 and EIN3 in salinity response in *Arabidopsis*, MHZ7/OsEIN2, MHZ6/OSEIL1, and OsEIL2 exhibited the opposite effects on the salinity tolerance of rice seedlings. Functional knock-out of MHZ7/OsEIN2, MHZ6/OSEIL1, or OsEIL2 led to enhanced salt tolerance, while overexpressing each of them increased seedling sensitivity to salinity (Yang et al., 2015b). Na^+ measurement and downstream gene analysis revealed that MHZ6/OSEIL1 and OsEIL2-regulated salinity responses were mediated through controlling the expression of *OsHKT2;1*, a Na^+ transporter gene, and the homeostasis of Na^+ in plants (Yang et al., 2015b). EMSA and luciferase assay showed that both MHZ6/OSEIL1 and OsEIL2 could bind to the promoter region of *OsHKT2;1* and promoted its transcription (Yang et al., 2015b). Particularly, there is no homolog of OsHKT2;1 in *Arabidopsis*, indicating that OsHKT2;1 may be the major reason of difference between rice and *Arabidopsis* on the role of ethylene in salinity response. It is likely that rice adopts the ethylene signaling pathway to activate *OsHKT2;1* expression and Na^+ uptake for cell ion homeostasis in water-saturated soil. However, this mechanism would lead to the excessive Na^+ uptake under high salinity condition and result in the salt sensitivity. Further studies on the roles of other rice ethylene signaling components in salinity response would generate more valuable data for elucidating the differences between terrestrial plants and aquatic/semi-aquatic plants on salinity response.

DOWNSTREAM EVENTS IN ETHYLENE MEDIATED SALINITY RESPONSE

The main ethylene signaling components downstream of EIN3 are ERFs (ethylene-responsive element binding factors), which are plant-specific transcription factors responsible for nuclear transcriptional regulation of a series of effectors related to ethylene response. In *Arabidopsis*, the three classes of ERFs, with either transcriptional-activation or -repression activities, were differentially regulated by ethylene and abiotic stresses (Fujimoto et al., 2000). EIN3/EILs could bind directly to the promoter of *ERF1* and activate its expression. Similar to *EIN3*-overexpression in transgenic plants, expression of *ERF1* also activated a variety of ethylene response genes and led to constitutive ethylene response (Solano et al., 1998). The expressions of *AtERF1*, *AtERF2* and *AtERF5* were all induced by ethylene treatment in an EIN2-dependent manner. The induction of *AtERF3* and *AtERF4* by high salinity stress was regulated by EIN2-mediated ethylene signaling (Fujimoto et al., 2000). Further analysis showed that *ERF1* was highly induced by salinity and drought stress in an ethylene and JA signaling-dependent manner. Overexpression of *ERF1* enhanced plant tolerance to salt, drought and heat stress (Cheng et al., 2013). Although overexpression of many *ERFs* genes could enhance salt tolerance, most of them seem to be independent of ethylene signaling. Based on microarray analysis, three *ERFs* genes in *Arabidopsis*, named as *ESE1* to *ESE3*, were found to be ethylene- and salt-inducible. Among them, *ESE1* was positively regulated by ethylene signaling at transcriptional level, and was downstream of EIN3/EIL1. Further analysis revealed that EIN3 could physically bind to the promoter of *ESE1* and activate its transcription. Then, ESE1 bound to the promoters of *RD29A*, *COR15A* and some other salinity-responsive genes to promote their transcription, and eventually enhanced plant tolerance to salinity (Zhang et al., 2011b).

Besides ERFs, some other factors were also found to be responsive to ethylene signaling and involved in salinity response. A NAC-type transcription factor gene *AtNAC2* was induced by ACC, ABA, NAA, and salinity treatments. The salt induction of *AtNAC2* was enhanced in ethylene-overproducing mutant *eto1-1*, but repressed in ethylene-insensitive mutants *etr1-1* and *ein2-1*, and auxin-insensitive mutant *tir1-1*. Overexpression of *AtNAC2* promoted lateral root development under both normal and salinity conditions (He et al., 2005). In *Arabidopsis*, NEK6, a NIMA-related kinases (NEKs), was induced by ACC and salinity. NEK6 positively regulated plant growth, seed yield and plant response to salinity and osmotic stresses, probably through suppression of ethylene biosynthesis and activation of cell division (Zhang et al., 2011a). In addition, many other stress-responsive factors, such as TINY (a GCC/DRE-binding protein-like transcription factor; Sun et al., 2008), AtMYB15 (a negative regulator of DREB1/CBF), RAB18 (an ABA responsive factor; Chen et al., 2014), and SIED1 (salt-induced and EIN3/EIL1-dependent 1; Peng et al., 2014a), were also found to be regulated by ethylene signaling and mediate ethylene-involved salinity responses.

Through these factors, ethylene-mediated salinity signaling was transferred into a series of nuclear transcriptional cascades which led to the expression changes of numerous stress-related effectors. Generally, these effectors could be classified into three major types according to their bio-functions in stress responses: (1) ROS scavengers such as SOD and POD (Kukreja et al., 2005; Peng et al., 2014a); (2) ion transporters such as HAK5 and HKTs (Moller et al., 2009; Jiang et al., 2013; Yang et al., 2015b); (3) osmolyte synthetic enzymes such as P5CS (Hsieh et al., 2013). Changes of

these effectors usually led to physiological modification (such as homeostasis of ROS and Na^+/K^+) of plants for better adaptation to salinity condition.

INTERACTIONS BETWEEN ETHYLENE AND OTHER SIGNALS UNDER SALINITY

In addition to directly regulating salinity-related effectors, ethylene also coordinates with some other phytohormones and stress signaling molecules to modulate plant response to salinity and normal growth. As an essential stress hormone, ABA participates in plant response to a series of biotic and abiotic stresses. Interactions between ABA and ethylene signaling in seed germination are extensively investigated. Ethylene ETR1-CTR1-EIN2 signaling suppressed ABA signaling in seeds, thus alleviated ABA-mediated inhibition of seed germination (Beaudoin et al., 2000). And NO was proposed to be an interactor between ethylene and ABA in seed (reviewed in Arc et al., 2013). The relationship between ethylene and ABA in salinity response is not as clear as in seed germination. Nevertheless, from ABA-associated expression pattern and mutant phenotypes of ethylene-related factors, we proposed that the whole ethylene biosynthesis and signal transduction pathway interacted with ABA in regulating salinity response. The expressions of *AtACS5*, *AtACS7*, *TaACO1*, *OsERF3*, *GmERF3*, *GhERF1*, and some other ethylene-related genes (Wang et al., 2005; Qiao et al., 2008; Zhang et al., 2009, 2013; Chen et al., 2014) were all regulated by ABA and salinity. Mutation of *ACS7* enhanced plant tolerance to salt, osmotic and heat stresses, possibly through elevating the expression of stress-responsive genes involved in ABA signaling under salinity (Dong et al., 2011). In *Arabidopsis*, *etr1* loss-of-function mutants showed reduced sensitivity to ABA and accelerated seed germination, while *etr2* loss-of-function mutants became more sensitive to ABA and germinated slower than the wild type, indicating contrasting roles of ETR1 and ETR2 in seed germination during salt stress. But this seemed to be mediated by affecting the ABA signaling but independent of ethylene signaling (Wilson et al., 2014). Disruption of EIN2 increased ABA level but substantially reduced the induction of *RD29B* under high salinity (Wang et al., 2007). Actually, the central ethylene signaling component EIN2 plays an important role in mediating the interactions between ethylene and several other hormones, including ABA.

In rice, MHZ4 (homologous to *Arabidopsis* ABA4) and MHZ5 (carotenoid isomerase), identified from *mhz* ethylene-response mutants, are involved in ABA biosynthesis (Ma et al., 2014; Yin et al., 2015). Mutation of either *MHZ4* or *MHZ5* reduced the ABA level but promoted ethylene production in etiolated seedlings. Ethylene treatment induced the expressions of *MHZ4* and *MHZ5*, thus increased the accumulation of ABA in roots. Loss-of-function mutants of *MHZ4* and *MHZ5* showed alleviated ethylene-inhibition of root growth, and this could be largely rescued by ABA treatment. Genetic analysis revealed that both MHZ4 and MHZ5-dependent ABA pathways acted downstream of ethylene receptors to positively regulate root response to ethylene (Ma et al., 2014; Yin et al., 2015). These findings first uncovered a different mechanism of controlling root growth in rice by ethylene-ABA interaction, while in *Arabidopsis* ABA is not necessary for ethylene to inhibit root growth. Given that ABA production and signaling are necessary for plant responses to salinity and other stresses, MHZ4 and MHZ5 are anticipated to have some roles in plant responses to salinity and other stresses. Thus, besides regulating seedling growth, MHZ4 and MHZ5 may also mediate the interaction between ethylene and ABA on controlling stress responses.

In addition, some other stress-related phytohormones are also related to ethylene in salinity response, including JA, SA (salicylic acid), BR (brassinosteroid), and so on. It was reported that the cross-effects between ethylene, JA, SA, and BR signaling pathways played important roles in plant defense response (Divi et al., 2010; Yang et al., 2013). They may function synergistically or antagonistically to precisely regulate defense responses. Loss-of-function of EIN2 eliminated plant response to JA, and expression of the EIN2-CEND was sufficient to recover this responsiveness, indicating that EIN2 is a molecular link between ethylene and JA signaling pathway (Alonso et al., 1999). It was shown that EIN3/EIL1-ERF1 might act as a node to integrate JA and ethylene signaling and regulate plant development as well as stress defense (Lorenzo et al., 2003; Zhu et al., 2011). In *Arabidopsis*, ERF1 played positive roles in plant tolerance to salinity, drought and heat stress by regulation of stress-related genes, which integrated the ethylene, JA and ABA signals (Cheng et al., 2013). In rice, the ethylene, JA and SA pathways are all involved in the induction of OsPR10 by stress, and OsERF1 may function in both ethylene and JA pathway to regulate this induction (Takeuchi et al., 2011). Ethylene insensitive mutant *ein2* exhibited hypersensitivity to salinity on seed germination. This hypersensitivity could be rescued by treatment with 24-epibrassinolide (EBR; Divi et al., 2010). Besides these stress-hormones, ethylene also interacts with auxin extensively on regulation of plant growth and development (reviewed in Vandenbussche et al., 2012). Recently, several auxin-related small proteins (SAURs and ARGOS) from *Arabidopsis* were found to act as brakes of ethylene signaling and as accelerators of cell proliferation and/or cell expansion to coordinate seedling growth and ethylene response (Li et al., 2015b; Rai et al., 2015). We propose that these proteins may also act as modulators in regulation of plant growth under salinity and other stresses.

Moreover, on regulation of salinity response, ethylene also interacts with many stress signaling molecules, including ROS and cGMP. In rice, salinity triggered MAPK cascades to stabilize ACSs, led to enhanced ethylene production and ethylene signaling, which then promoted ROS accumulation and growth inhibition (Li et al., 2014a; reviewed in Steffens, 2014). In *Arabidopsis*, salinity-induced EIN3/EIL1 conferred enhanced tolerance to salinity by promoting the ROS scavenging in an EIN2-independent manner (Peng et al., 2014a). Both ACC and cGMP treatments could promote the ethylene production and alleviate salinity-induced injury by homeostasis of Na^+/K^+. Further analysis based on the ethylene-insensitive mutant *etr1-3* and treatments with ethylene biosynthesis inhibitor and guanylate cyclase inhibitor revealed that cGMP modulated ethylene-mediated salinity response pathway by regulation of ethylene biosynthesis and perception (Li et al., 2014b).

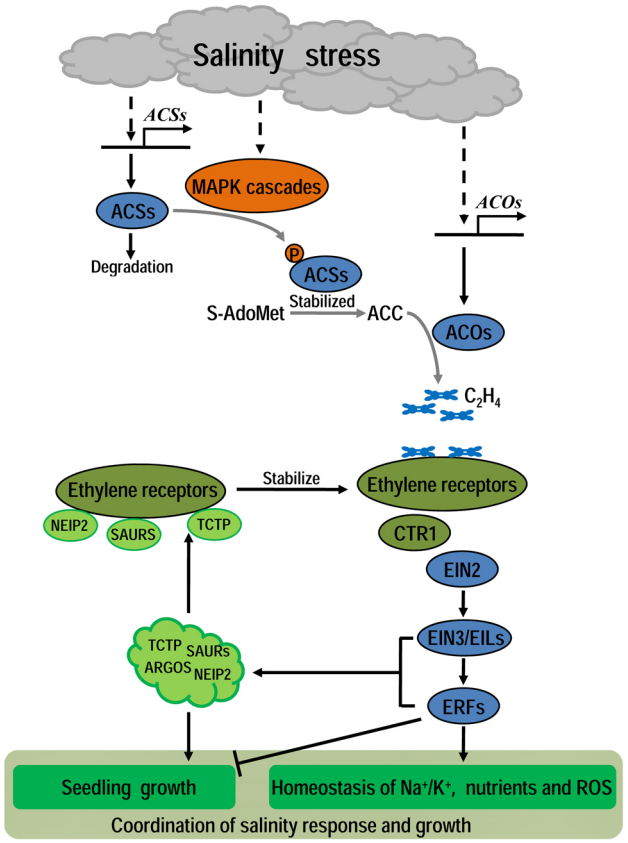

FIGURE 1 | A diagram of the ethylene's role in plant under salinity. Under salinity, stress signals could activate the MAPK phosphorylation cascades which then stabilize some ACSs to promote ethylene production. The transcripts level of *ACSs* and *ACOs* are also up-regulated under salinity. Then, salinity-induced ethylene signal is transduced mainly through the classical receptors-CTR1-EIN2-EIN3 pathway to regulate many effectors involved in plant growth and salinity response. Under proper concentration, ethylene promotes the homeostasis of Na^+/K^+, nutrients and ROS to enhance plant tolerance to salinity, with no irreversible inhibition of normal growth, while excessive ethylene will lead to harmful hyper-inhibition of plant growth. To avoid damage from extreme ethylene response, plants have evolved some negative feed-back mechanisms to alleviate ethylene response and promote seedling growth. In these mechanisms, some small proteins such as NtTCTP, NEIP2, AtSAURs, and AtARGOS are supposed to act as key modulators to coordinate plant growth and ethylene/salinity responses.

NOVEL NEGATIVE FEEDBACK MECHANISMS IN ETHYLENE SIGNALING

Generally speaking, ethylene is supposed to be a coordinator between stress response and growth. Intrinsic ethylene production and signaling is indispensable for plant rapid response to salinity and self-modification for better survival. But excessive ethylene production under continuous stress tends to largely inhibit plant growth and development, even leads to death. Therefore, tight control of ethylene homeostasis is critical for plants to survive under salinity and recover growth later. Indeed, there are multiple positive and negative feedback mechanisms for regulation of ethylene biosynthesis and signaling (reviewed in Vandenbussche et al., 2012). In addition, some ethylene and salinity-responsive small proteins were identified as restrictors in alleviating the ethylene-inhibited growth through suppression of ethylene response and promotion of cell proliferation and/or expansion. The most typical restrictors are NEIP2 and NtTCTP, two ethylene receptor NTHK1-interacting proteins in tobacco (Cao et al., 2015; Tao et al., 2015). Both NEIP2 and NtTCTP proteins were induced by ethylene treatment. Overexpressing either of them led to reduced sensitivity to ethylene and improved vegetative growth. Further investigation revealed that NtTCTP could stabilize the ethylene receptor NTHK1 and promote cell proliferation to reduce ethylene sensitivity and alleviate ethylene-inhibition of vegetative growth (Tao et al., 2015). NTHK1 phosphorylates NEIP2 *in vitro* and NEIP2 can be phosphorylated *in planta* in response to ethylene and salt treatment. Overexpression of NEIP2 improved plant tolerance to salt and oxidative stresses (Cao et al., 2015). Recently, two studies found that *ARGOS* (*auxin regulated gene involved in organ size*) genes in *Arabidopsis* were induced by ethylene dose-dependently, but this induction was suppressed in ethylene-insensitive mutants. Increasing the expression of ARGOS family members reduced seedling sensitivity to ethylene (Rai et al., 2015; Shi et al., 2015). More recently, we found that AtSAUR76/77/78 could associate with subfamily II ethylene receptor ETR2 and EIN4 to reduce ethylene response and promote plant growth (Li et al., 2015b). These findings reveal novel negative feedback mechanisms of precisely desensitizing excessive ethylene response but promoting growth recovery from ethylene-inhibited growth. All these small proteins are supposed to act as brakes of ethylene signaling and accelerators of cell proliferation/expansion to coordinate normal growth and ethylene response (**Figure 1**). Further investigation of the roles of these factors in ethylene-mediated response to salinity and other stresses would be valuable for elucidating the overall roles of ethylene in modulation of plant growth under environmental stresses.

Considering the key role of NEIP2-type factors in constraint of ethylene signaling, gene manipulation of these factors would be an effective way to enhance plant tolerance to salinity stress. This might be achieved by using salinity-responsive promoters. A previous study showed that distinctive expression of HKT1;1 in the root stele of *Arabidopsis* reduced root-to-shoot Na^+ delivery, thus promoting seedling tolerance to salinity, while constitutive expressing HKT1;1 increased Na^+ accumulation in shoot and led to more sensitive to salinity (Moller et al., 2009). In addition, it was shown that ethylene promoted salinity tolerance largely through improving the homeostasis of Na^+/K^+ (Jiang et al., 2013). These findings suggest another way to enhance salinity tolerance of plants by cell type-specific engineering of ethylene signaling.

CROSS-TALKS BETWEEN RESPONSES TO SALINITY AND RESPONSES TO NUTRIENT DEFICIENCIES

Besides ion toxicity, osmotic and oxidative stresses, high salinity also led to nutrient deficiencies (Chinnusamy et al., 2006). The most direct salinity-related nutrient is potassium (K), which has critical roles in maintaining enzyme activities, ion homeostasis,

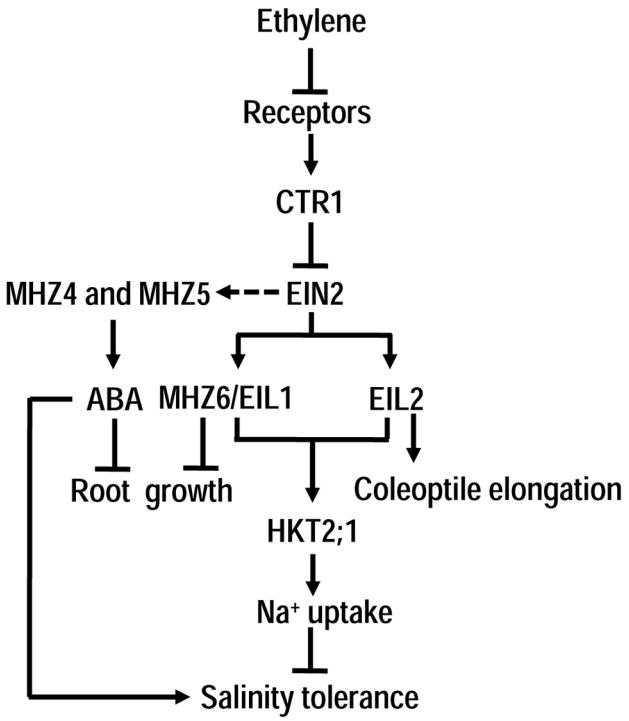

FIGURE 2 | The role of ethylene signaling in rice under salinity. Unlike in *Arabidopsis*, the classical CTR1-EIN2-EILs ethylene signaling in rice plays negative role in regulating plant tolerance to salinity mainly through up-regulation of OsHKT2;1, a high affinity Na^+ transporter with no homolog in *Arabidopsis*. Interestingly, EIL1 and EIL2 in rice respectively regulates ethylene-controlled roots and coleoptiles growth of etiolated seedlings. Besides, two novel ethylene signaling components MHZ4 and MHZ5 in rice may mediate the interaction between ethylene and ABA in regulation of root growth and salinity response.

internal pH, and etc. For similar chemical feature, excessive sodium ions inhibit potassium uptake and lead to potassium deficiency. Usually, high concentration of sodium and low concentration of potassium status (high sodium/potassium ratio) is most harmful for plant cells. Similar to the vital roles of ethylene in high salinity response, ethylene also played important roles in response to potassium deficiency and acted upstream of ROS (Jung et al., 2009). One piece of evidence was that the low K^+-inducible *HAK5* expression was dependent on ethylene signaling (Jung et al., 2009). In addition, changes of ethylene biosynthesis and signaling often lead to alteration of sodium/potassium contents under salinity. In tobacco, ethylene receptor *NTHK1*-overexpressing tobacco seedlings showed higher sodium/potassium ratio than the WT (Cao et al., 2006). In fact, Na^+/K^+ homeostasis is the key point for ethylene regulation of plant salinity response (Jiang et al., 2013).

In addition to potassium deficiency, ethylene also regulates plant responses to many other nutrient (N, P, Ca, and Fe) deficiencies through different pathways (reviewed in García et al., 2015). Among them, the most frequently studied is iron (Fe) deficiency. Previous works showed that ethylene participated in up-regulation of many important Fe-regulated genes in Strategy I plants, including ferric reductase, H^+-ATPase gene and several Fe acquisition genes *FIT*, *FRO2*, and *IRT1* (Lucena et al., 2006; Waters et al., 2007; García et al., 2010). Simultaneously, Fe deficiency promoted the expressions of many genes related to ethylene biosynthesis and signaling in the roots (García et al., 2010). Moreover, recent work showed that hypoxia and bicarbonate, two main factors causing Fe chlorosis in Strategy I plants, negatively regulated the expressions of Fe acquisition genes, probably by affecting ethylene synthesis and/or signaling (García et al., 2014). Meanwhile, salinity stress usually reduced the Fe uptake and led to Fe deficiency (Rabhi et al., 2007; Yousfi et al., 2007). Considering the positive roles of ethylene in both salinity tolerance and Fe acquisition, we can deduce that ethylene mediates the cross-talk between salinity and Fe deficiency responses, possibly through regulation of many genes involved in Fe homeostasis under salinity condition.

Another focused research field is the cross-talk between phosphate (P) deficiency and ethylene, and a simple model has been supposed (Lei et al., 2011b). First, P starvation might induce ethylene production and enhance plant sensitivity to ethylene (He et al., 1992; Borch et al., 1999). Then enhanced ethylene production and ethylene responses would promote the development of root hair and the expression of *PSI* (*P starvation-induced*) genes (Lei et al., 2011b). These changes could directly affect P uptake, remobilization and redistribution, which facilitate plants to maintain P homeostasis under P-deficient condition. Interestingly, ethylene alone seemed to be not enough to promote the *PSI* gene expression to the degree induced by P starvation, suggesting a cross-talk between ethylene and P-deficiency-induced signals in controlling the *PSI* gene expression under low P (Lei et al., 2011b). Moreover, it was found that inhibition of ethylene biosynthesis or signaling could respectively rescued the increased primary root elongation and root hair formation caused by overexpression of the P transporter gene *Pht1;5*, providing another evidence of cross-talk between ethylene and P signaling (Nagarajan et al., 2011). Indeed, ethylene plays an integrative role in regulating both local P-deficiency responses and systemic P signaling pathways (reviewed in Nagarajan and Smith, 2012). Usually, high salinity led to reduced P uptake (Rai and Sharma, 2006), and phosphate-accumulating mutants *siz1* and *pho2* showed reduced uptake and reduced accumulation of Na^+, hence enhanced plant tolerance to salinity stress (Miura et al., 2011). All these studies indicate that ethylene may mediate the cross-talk between salinity and P deficiency stresses.

Recently, the cross-talk between nitrate (N) deficiency and ethylene was investigated. Low nitrate treatment rapidly induced ethylene production and up-regulated the expression of *EIN3/EIL1* and *NRT2.1*, while enhanced ethylene production and signaling down-regulated the expression of *NRT2.1* and thus decreased the high-affinity nitrate uptake, indicating a negative feedback regulation of nitrate acquisition by ethylene under nitrate deficiency (Zheng et al., 2013). More recently, it was reported that cadmium and sodium stresses-induced ethylene and JA signaling converged at EIN3/EIL1 to up-regulate *NRT1.8* expression but down-regulate *NRT1.5* expression, thus mediated the stress-initiated nitrate allocation to roots (SINAR), which decoupled nitrate assimilation and photosynthesis, and finally

decreased plant growth but promoted plant tolerance to stress in a nitrate reductase-dependent manner (Zhang et al., 2014).

All these findings suggest that ethylene mediates the cross-talks between plant response to salinity and responses to nutrient deficiencies. Ethylene may be a linking point in regulation of nutrient homeostasis under salinity stress to coordinate stress response and normal growth.

CONCLUSION AND PERSPECTIVES

From above studies, a general conclusion could be made that inherent ethylene production is necessary for the establishment of salt acclimation, and ethylene signaling is indispensable for plant self-adjustment in rapid response to salinity stress and better adaptation to the stress condition (summarized in **Figure 1**).

Different from *Arabidopsis*, ethylene signaling in rice seems to be more complex, so does the ethylene's role in salinity response (**Figure 2**). Ethylene treatment of rice seedlings increased salinity sensitivity, while 1-MCP (a blocker of ethylene perception) treatment led to enhanced tolerance to salinity. Rice MHZ7/OsEIN2, MHZ6/OsEIL1 and OsEIL2 conferred reduced tolerance to salinity (Yang et al., 2015b). MHZ6/OsEIL1 and OsEIL2 could bind to the promoter region of *OsHKT2;1* (encoding a Na^+ transporter) and activated its expression for Na^+ uptake. Ethylene-induced *OsHKT2;1* expression and Na^+ uptake may represent a mechanism for maintaining ion homeostasis in water environment (Yang et al., 2015b). This mechanism could lead to sensitive response especially under high salinity. Different mechanisms in *Arabidopsis* and rice in response to salt stress may arise from the evolutionary divergence under different growing conditions or due to different plant species.

Although much progress has been made in terms of ethylene roles in salt stress responses, there are still some uncertain points left to be clarified. First, whether the functions of ACSs and ACOs on salinity response must be executed through downstream ethylene signaling? This could be solved directly through construction and analysis of double loss-of-function mutants between ACS and EIN2 or EIN3. Second, a few studies showed that enhanced ethylene production led to salinity sensitivity (Xu et al., 2008; Li et al., 2014a), but whether this sensitivity arises from inherent weakened salinity resistance or just from ethylene-inhibited growth and promoted senescence is unclear. This could be clarified through alleviating the effects of ethylene on plant growth and development by genetic method. Third, the appropriate ethylene quantity and signaling intensity for plant response to salinity may be varied during different growing stages. Thus, precise control of ethylene production and signaling may be critical for promotion of plant salinity tolerance. This could be achieved by gene manipulation of key ethylene biosynthesis and signaling factors using specific promoters. Last, further investigation of the differences between rice and *Arabidopsis* on the role of ethylene in salinity response is valuable for elucidating the evolutionary divergence of ethylene in different plant species. Additionally, identification of more ethylene-response mutants and evaluation of their salt response may reveal novel components linking ethylene and salt stress. Analysis of sequence variations of known components among different cultivars may identify better alleles for salt tolerance. Uncovering these problems will largely broaden our horizon and enrich our knowledge on how plant self-adjust to coordinate external stresses and internal growing motivation for better survival. This will be helpful for precisely controlling ethylene production and signaling to enhance salinity tolerance and improve agronomic traits of crops.

AUTHOR CONTRIBUTIONS

JJT, HWC, SYC, and JSZ conceived the topic. JJT wrote the manuscript. All authors revised the manuscript.

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REVIEW
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Ethylene and Metal Stress: Small Molecule, Big Impact

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The phytohormone ethylene is known to mediate a diverse array of signaling processes during abiotic stress in plants. Whereas many reports have demonstrated enhanced ethylene production in metal-exposed plants, the underlying molecular mechanisms are only recently investigated. Increasing evidence supports a role for ethylene in the regulation of plant metal stress responses. Moreover, crosstalk appears to exist between ethylene and the cellular redox balance, nutrients and other phytohormones. This review highlights our current understanding of the key role ethylene plays during responses to metal exposure. Moreover, particular attention is paid to the integration of ethylene within the broad network of plant responses to metal stress.

Keywords: ethylene, metals, oxidative stress, signal transduction, crosstalk

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SETTING THE SCENE

With the global population exceeding nine billion by 2050, it is of increasing importance to optimize plant growth and ensure food and feed supply. However, plant yield is severely affected by environmental stress factors such as drought, nutrient deficiency, salinity and metal pollution (Mittler, 2006; Dolferus, 2014). Toxic metals and metalloids accumulate in the environment because of industrial applications. Contamination peaks occurred throughout history (e.g. the Roman Empire and Industrial Revolution) and current production rates are still high. In addition, the contribution of metal-contaminated fertilizers, pesticides and sewage sludge to overall metal pollution should not be ignored (Alloway, 2012). Metals such as cadmium (Cd), mercury (Hg) or lead (Pb) are not essential for plants. Therefore, even low concentrations interfere with plant growth and development and cause significant yield losses worldwide. On the other hand, excess levels of essential micronutrients such as copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn) are phytotoxic as well (Cuypers et al., 2009; Hänsch and Mendel, 2009).

Plants are primary producers and therefore constitute an important bridge between the soil elemental composition and the food chain. Non-essential trace elements such as As and Cd opportunistically enter plant tissues via the same transport systems used to take up essential nutrients (Verbruggen et al., 2009; Seth et al., 2012). Excessive accumulation of toxic metals in food and feed crops represents a severe threat to human health (Järup, 2003), indicating the need to remediate metal-contaminated soils. However, recent efforts regarding the use of plants to clean-up soils via phytoextraction are often hampered by metal phytotoxicity (Vangronsveld et al., 2009). Therefore, it is crucial to enhance our current understanding of metal-induced stress responses in plants and provide scientific clues to ameliorate phytoextraction strategies.

A recurring cellular response in metal-exposed plants, independent of the species and exposure time, is an increased generation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$; Schützendübel and Polle, 2002; Sharma and Dietz, 2009). Under optimal physiological conditions, ROS are constantly produced as by-products of aerobic metabolism in chloroplasts, mitochondria and peroxisomes. However, their

production is tightly controlled and maintained at a low level by the antioxidative defense network of plant cells. This system consists of enzymes neutralizing $O_2^{\bullet-}$ and H_2O_2 such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POD) and peroxiredoxins (Prx), complemented by metabolites such as ascorbate (AsA) and glutathione (GSH). All subcellular compartments are equipped with specific antioxidative enzymes and metabolites maintaining the cellular redox balance within certain limits (Mittler et al., 2004). However, under abiotic stress conditions such as metal exposure, the equilibrium between ROS production and detoxification is disturbed in favor of the former. While redox-active metals such as Cu and Fe are able to directly generate ROS via Fenton and Haber-Weiss reactions, metals without redox properties (e.g. Cd or Hg) only indirectly contribute to ROS production (Schützendübel and Polle, 2002; Verbruggen et al., 2009).

Whereas ROS are closely linked to hormonal signaling networks in a developmental context (Overmyer et al., 2003; Diaz-Vivancos et al., 2013), it is now widely accepted that they also constitute an ambiguous role during stress responses (Dat et al., 2000). Being toxic molecules, ROS are able to oxidatively injure cells (Møller et al., 2007), but they also regulate defense pathways leading to cellular protection and acclimation (Mittler et al., 2004; Petrov and Van Breusegem, 2012). In addition, recent research also suggests a major role for plant hormones interacting with redox signaling to control adaptive responses to environmental stresses (Mittler et al., 2011; Bartoli et al., 2013; Baxter et al., 2014). More specifically, ethylene has been put forward as an important stress hormone under abiotic stress conditions (Dietz et al., 2010). Therefore, the aim of this review is to highlight our current understanding of the role ethylene plays during metal stress in plants. Experimental evidence for the relationship between ethylene and metal exposure is discussed at the level of ethylene biosynthesis as well as signaling, in which different reports support a link between ethylene and metal tolerance or sensitivity. Finally, special attention is paid to the growing body of evidence suggesting a clear integration between ethylene and the broad network of signaling responses activated in metal-exposed plants.

WEIGHING THE EVIDENCE FOR A RELATION BETWEEN ETHYLENE AND METAL STRESS

In the following sections, results of different studies are discussed and point toward a role for ethylene during metal stress responses in plants (**Table 1**). However, when interpreting these results, it is important to take various aspects related to the experimental design into account. First of all, metal-specific properties should be considered. As discussed before, both essential and non-essential metals cause phytotoxic responses, albeit at different exposure levels. Furthermore, experiments can be conducted using massive or environmentally realistic metal concentrations. Under severe stress conditions, ethylene production might be simply increased by tissue damage and necrosis (Lynch and Brown, 1997). Stress severity will affect the activation of specific signal transduction pathways, for example those related to ethylene (Kacperska, 2004). Although Kacperska (2004) proposed that increased ethylene synthesis is a characteristic feature of the alarm situation during severe stress, it was also observed during exposure to mild and environmentally realistic Cd concentrations (Schellingen et al., 2014). Nonetheless, the extent and consequences of augmented ethylene production should always be interpreted with the applied exposure concentrations in mind (Thao et al., 2015).

It is important to discriminate between primary and secondary metal stress-induced events in plants. For example, metal toxicity often leads to nutrient deficiency (Lynch and Brown, 1997; Cuypers et al., 2009), which in its turn is related to alterations in ethylene biosynthesis and signaling (Iqbal et al., 2013a). Furthermore, one of the primary responses of plants to metal stress is the generation of ROS and induction of an oxidative challenge. Redox-active and non-redox-active metals affect the cellular redox state in a different way, which might also influence plant responses related to ethylene as discussed in the section "Interaction between Ethylene and ROS Signaling." Although some kinetic studies have been conducted (Montero-Palmero et al., 2014a; Schellingen et al., 2014, 2015a,b), more in-depth research is required to decipher the exact order of both primary and secondary events affecting ethylene production under metal stress.

Chelation followed by vacuolar sequestration is a common strategy exploited by plants to maintain low concentrations of free metal(loid)s in the cytosol. Important chelators either contain thiol groups [e.g. metallothioneins, glutathione and phytochelatins (PCs)] or not (e.g. histidine, nicotianamine and organic acids; Seth et al., 2012; Anjum et al., 2015). Especially for GSH, evidence is pointing toward a relationship with ethylene biosynthesis and signaling under metal stress (see section "Crosstalk between Ethylene and GSH"). However, it should be noted that not every metal(loid) is equally connected to this chelating compound (Anjum et al., 2015). Therefore, it is important to consider metal-specific properties when discussing the link between ethylene and GSH.

Finally, different experimental strategies are used to unravel the functional role of ethylene during metal stress. On the one hand, ethylene biosynthesis or signaling can be pharmacologically inhibited. On the other hand, different results can be obtained when studying mutants defective in one or both processes. Furthermore, not all mutations will lead to complete inhibition of ethylene biosynthesis or signaling due to functional redundancy (e.g. different ethylene receptors). Some studies use transformants that overexpress ethylene-related genes, often derived from other plants or even organisms, to study the functional role of ethylene in metal tolerance. Correct data interpretation is therefore only possible when the setup is taken into account (cfr. *infra*; Thao et al., 2015). The studies summarized in this review clearly point toward an intimate relationship between ethylene and metal stress in plants. However, much work remains to be done to finally determine the mechanistic processes underlying this link and apply this knowledge in field conditions, e.g. during phytoremediation.

TABLE 1 | Metal exposure differentially affects ethylene biosynthesis and signaling in plants.

Metal	Concentration	Exposure time	Tissue type	Species	Observations	References
Al	10 or 50 μM $AlCl_3$	24 h	Root apices	*L. japonicus*	↑ ACO activity ↑ ethylene (max after 30 min) Al and cobalt/AVG: ↓ ethylene ↓ inhibition of root elongation	Sun et al., 2007
	10 μM $AlCl_3$	2 and 24 h	Root apices	*M. truncatula*	↑ *ACS* and *ACO* expression	Sun et al., 2007
	50 μM $AlCl_3$	24 h	Root apices Roots	*A. thaliana*	↑ ethylene (max after 30 min) Al and cobalt/AVG/$AgNO_3$: ↓ inhibition of root elongation	Sun et al., 2010
	50 μM $AlCl_3$	0.5, 2, and 12 h	Roots	*A. thaliana*	↑ *ACS* and *ACO* expression	Sun et al., 2010
As	100 and 200 μM As(V)	1.5 to 3 h	Roots	*A. thaliana*	↑ expression of ethylene-related genes in tolerant Col-0 ecotype *ERF* = As tolerance-associated	Fu et al., 2014
Cd	0.5 mM $CdCl_2$	14 h	Leaf discs	*T. aestivum*	↑ ethylene	Groppa et al., 2003
	14, 28 or 42 mg kg^{-1}	10 days	Chloroplast membranes	*H. vulgare*	↑ ethylene (14 and 28 mg kg^{-1}) ↓ ethylene (42 mg kg^{-1})	Vassilev et al., 2004
	5 or 50 μM $CdSO_4$	2, 6, and 30 h	Shoots and roots	*A. thaliana*	↑ *ACS* and *ACO* expression (30 h, 50 μM Cd) ↑ *ERF* expression (all conditions)	Herbette et al., 2006
	50 μM $CdCl_2$	15 days	Roots	*P. sativum*	↑ ethylene	Rodríguez-Serrano et al., 2006
	10 or 50 μM Cd	2 h	Roots	*A. thaliana*	↑ *ACS* (50 μM) and *ERF* (10 and 50 μM) expression	Weber et al., 2006
	400 μM $CdSO_4$	24 h	Different plant parts	*A. thaliana*	↑ ethylene	Arteca and Arteca, 2007
	0.1 mM $CdSO_4$	75 h	Suspension cells	*L. esculentum*	↑ ethylene during the first 24 h Cd and AVG/STS: ↓ cell death	Iakimova et al., 2008
	50 μM $CdCl_2$	14 days	Leaves	*P. sativum*	↑ ethylene	Rodríguez-Serrano et al., 2009
	200 mg kg^{-1} $CdCl_2$	30 days	Leaves	*B. juncea*	↑ ACS activity ↑ ethylene	Masood et al., 2012
	10 or 25 mg l^{-1} $CdCl_2$	3, 6, and 24 h	Root tips (RNA) Whole plants (ethylene)	*G. max*	↑ *ACS* expression (3 and 6 h) ↑ ethylene	Chmielowska-Bąk et al., 2013
	50 μM $CdCl_2$	30 days	Leaves	*B. juncea*	↑ ACS activity ↑ ethylene	Asgher et al., 2014
	5 μM $CdCl_2$	15 days	Leaves	*H. vulgare*	↑ ethylene Cd-tolerant genotype: ↑ *ACO* expression Cd-sensitive genotype: ↓ ethylene responsive genes	Cao et al., 2014
	5, 10, 25 or 100 μM $CdSO_4$	24 and 72 h	Shoots and roots (RNA/ACC) Whole plants (ethylene)	*A. thaliana*	↑ *ACS* and *ACO* expression ↑ ACC (free and conjugated) ↑ ethylene ↑ ethylene responsive genes	Schellingen et al., 2014
	50 μM $CdCl_2$	3 h	Roots	*O. sativa*	↑ *ACO* expression	Trinh et al., 2014
	5 μM $CdCl_2$	16 days	Whole plants	*A. thaliana*	↓ ethylene	Carrió-Seguí et al., 2015
	200 mg kg^{-1} $CdCl_2$	30 days	Leaves	*T. aestivum*	↑ ACS activity ↑ ethylene	Khan et al., 2015
Cr	200 μM K_2CrO_4[Cr(VI)]	1 to 3 h	Roots	*O. sativa*	↑ *ACS*, *ACO* and *EIN3;4* expression	Trinh et al., 2014
Cu	10 mM $CuSO_4$	48 h	Leaves	*N. glutinosa*	↑ *ACO* expression	Kim et al., 1998
	25, 100 or 500 μM $CuSO_4$	7 h	Whole plants	*A. thaliana*	↑ ethylene Cu and AVG: ↓ ethylene	Mertens et al., 1999
	0.5 mM $CuCl_2$	14 h	Leaf discs	*H. annuus* *T. aestivum*	↑ ethylene	Groppa et al., 2003

(Continued)

TABLE 1 | Continued

Metal	Concentration	Exposure time	Tissue type	Species	Observations	References
Cu	10 μM Cu	2 h	Roots	*A. thaliana*	↑ *ACS* and *ERF* expression	Weber et al., 2006
	400 μM $CuSO_4$	24 h	Different plant parts	*A. thaliana*	↑ ethylene	Arteca and Arteca, 2007
	2.5 mM $CuCl_2$	0.5 to 6 h	Whole plants	*B. oleracea*	↑ *ACS* and *ACO* expression	Jakubowicz et al., 2010
	25 or 50 μM $CuSO_4$	9 days	Whole plants	*A. thaliana*	= ethylene	Lequeux et al., 2010
Fe	200 mg l^{-1} $FeSO_4$	24 h	Leaves	*O. sativa*	↑ ethylene	Yamauchi and Peng, 1995
	300 mg l^{-1} $FeSO_4$	10 days	Shoots and roots		= ethylene	
	300 mg l^{-1} $FeSO_4$	24 h	Leaves of derooted plants		↑ ethylene	
Hg	500 or 1000 μM $HgCl_2$	15 days	Roots	*H. vulgare*	↑ expression of ethylene responsive genes	Lopes et al., 2013
	10 μM $HgCl_2$	6, 12, 24, and 48 h	Whole plants	*M. truncatula*	Altered expression of ethylene responsive genes	Zhou et al., 2013
	25 μM Hg	1 to 3 h (short)	Root apices	*O. sativa*	↑ expression of *ACS*, *ACO* and ethylene responsive gene	Chen et al., 2014
		24 h (long)			↑ *ACO* expression	
	3 μM $HgCl_2$	3, 6, and 24 h	Roots	*M. sativa*	↑ expression of *ACS*, *ACO* and ethylene responsive genes Hg + 1-MCP: ↓ induction of ethylene-related genes	Montero-Palmero et al., 2014a
Li	0.1, 1, 10 or 50 mM LiCl	2 h	Whole plants	*A. thaliana*	↑ *ACS* expression	Liang et al., 1996
	30 mM LiCl	6 days	Leaves	*N. tabacum*	↑ ethylene Li and AVG: ↓ ethylene no necrotic spots	Naranjo et al., 2003
Ni	50, 100, 200, 400 and 800 μM $NiSO_4$	24 h	Inflorescence stalks and leaves	*A. thaliana*	= ethylene	Arteca and Arteca, 2007
	200 mg kg^{-1} $NiSO_4$	30 days	Leaves	*B. juncea*	↑ ACS activity ↑ ethylene	Khan and Khan, 2014
Pb	500 mg l^{-1} $Pb(NO_3)_2$	12 days	Shoots and roots	*S. drummondii*	↑ expression of a putative *ACS/ACO* gene (shoots)	Srivastava et al., 2007
	0.5 mM $Pb(NO_3)_2$	14 days	Whole plants	*A. thaliana*	↑ *EIN2* expression	Cao et al., 2009
Zn	25, 100 or 500 μM $ZnSO_4$	7 h	Whole plants	*A. thaliana*	↑ ethylene	Mertens et al., 1999
	50, 100, 200, 400 and 800 μM $ZnSO_4$	24 h	Inflorescence stalks and leaves	*A. thaliana*	= ethylene	Arteca and Arteca, 2007
	200 mg kg^{-1} $ZnSO_4$	30 days	Leaves	*B. juncea*	↑ ACS activity ↑ ethylene	Khan and Khan, 2014

For each study, the experimental setup (metal concentration, exposure time, tissue type and plant species) is shown to facilitate the interpretation of metal-induced responses related to ethylene biosynthesis and the induction of the ethylene signaling cascade. In some studies, the functional role of ethylene during metal stress is studied by inhibiting ethylene biosynthesis using aminoethoxyvinylglycine (AVG) or cobalt, as well as by inhibiting ethylene signaling using 1-methylcyclopropene (1-MCP), silver nitrate ($AgNO_3$) or silver thiosulfate (STS).

METAL STRESS AFFECTS ETHYLENE BIOSYNTHESIS AND SIGNALING AT MULTIPLE LEVELS

In 1901, the Russian plant physiologist Neljubov reported that etiolated pea plants grew horizontally in the laboratory and upright in outside air (Neljubov, 1901). He attributed this abnormal growth response to ethylene in illuminating gas and is therefore credited with its discovery as biologically active compound (Bleecker and Kende, 2000). It took 33 more years to provide chemical proof that plants indeed synthesize this volatile molecule themselves (Gane, 1934), providing an important indication to investigate the function of ethylene as endogenous signaling molecule. Currently, this simple two-carbon atom

molecule (C_2H_4) is "all around" and known to be involved in almost all developmental and physiological processes in plants (De Martinis et al., 2015). It triggers senescence, influences growth, leads to various morphogenetic effects and—important within the scope of this review—acts as "stress hormone" in diverse biotic and abiotic stress conditions (Bleecker and Kende, 2000; Lin et al., 2009; Vandenbussche et al., 2012; Van de Poel et al., 2015).

Ethylene Biosynthesis is Altered under Metal Stress

More than 30 years ago, Yang and co-workers elucidated the ethylene biosynthesis pathway, which involves the consecutive action of three enzymes (**Figure 1**; Yang and Hoffman, 1984). First, the amino acid methionine is converted to S-adenosyl-methionine (SAM) by SAM synthetase. Using SAM as a substrate, 1-aminocyclopropane-1-carboxylic acid (ACC) is produced by ACC synthase (ACS). This is the rate-limiting step in the ethylene biosynthesis pathway and releases 5′-methylthioadenosine (MTA), which is recycled back to methionine via the so-called "Yang cycle." In the presence of O_2, ACC is degraded by ACC oxidase (ACO) to produce ethylene, CO_2 and cyanide (HCN; **Figure 1**). The latter is detoxified by β-cyanoalanine synthase to prevent toxicity of accumulating HCN at high ethylene biosynthesis rates (Bleecker and Kende, 2000; De Paepe and Van Der Straeten, 2005; Lin et al., 2009).

Of the 12 members of the ACS multigene family in *Arabidopsis thaliana*, eight encode functional ACS enzymes (isoforms 2, 4–9 and 11). While *ACS1* is inactive and *ACS3* encodes a pseudogene, isoforms 10 and 12 encode aminotransferases (Yamagami et al., 2003; Van de Poel and Van Der Straeten, 2014). The complexity of the ACS family is further enhanced at the structural and functional level by the formation of heterodimers. Although individual members of the gene family display specific developmental and physiological roles, significant combinatorial interplay exists between different isoforms. Various internal as well as external stimuli [developmental cues (e.g. senescence and ripening), light, hormones (e.g. auxin, cytokinin and ethylene), biotic (e.g. pathogens), and abiotic (e.g. heat) stress factors] regulate the production of ethylene at the level of *ACS* gene expression (Tsuchisaka et al., 2009; Van de Poel and Van Der Straeten, 2014). For example, *ACS8* transcript levels are controlled by light and shade as well as the circadian clock (Vandenbussche et al., 2003; Thain et al., 2004). Expression of *ACS2* and *ACS6* often appears to be regulated by different stresses

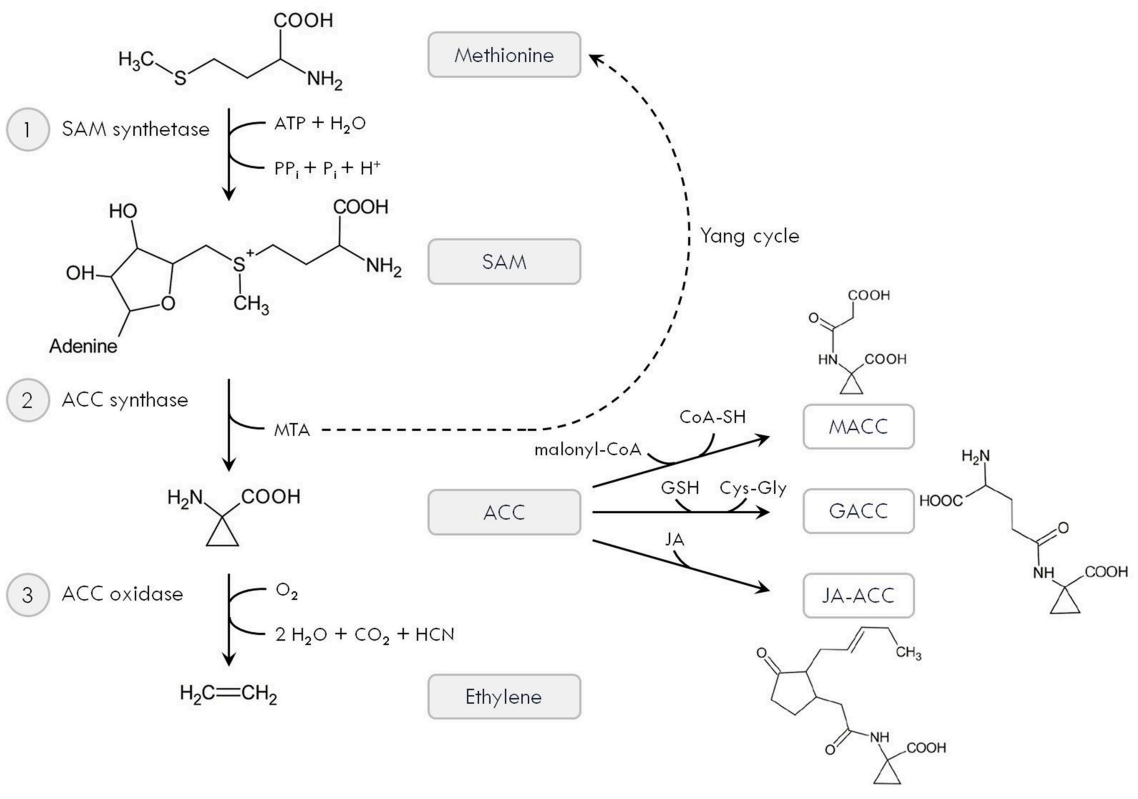

FIGURE 1 | Ethylene biosynthesis pathway. The amino acid methionine is converted to S-adenosyl-methionine (SAM) by SAM synthetase (1), which requires ATP. Using SAM as a substrate, 1-aminocyclopropane-1-carboxylic acid (ACC) is produced by ACC synthase (ACS) (2). This also releases 5′-methylthioadenosine (MTA), which is recycled back to methionine via the so-called "Yang cycle." Finally, ACC is oxidized by ACC oxidase (ACO) (3) to produce ethylene, CO_2 and cyanide (HCN). In addition, ACC can be converted to its major conjugate 1-malonyl-ACC (MACC) using malonyl-CoA. It can also react with GSH to form γ-glutamyl-ACC (GACC) or with JA to produce jasmonyl-ACC (JA-ACC).

such as ozone, salinity and hypoxia (Vahala et al., 1998; Arteca and Arteca, 1999; Peng et al., 2005). In addition, ACS enzymes have a highly variable carboxylic end that serves as a regulatory domain responsible for post-transcriptional regulation. This is due to the presence of mitogen-activated protein kinase (MAPK) and/or calcium-dependent protein kinase (CDPK) target sites, with phosphorylation playing an important role in ACS protein stability (Chae and Kieber, 2005; Yoon and Kieber, 2013).

In the final biosynthetic reaction, ACC is converted to ethylene by ACO. When ethylene production rates are high, for example during post-climacteric ripening of tomato fruit (Van de Poel et al., 2012), ACO can also act rate-limiting in ethylene biosynthesis. It is a ferrous-dependent non-heme oxygenase and uses a single electron from AsA to open the ACC ring (Murphy et al., 2014). In *A. thaliana*, five different *ACO* genes appear to be expressed in all tissues. However, differential accumulation of specific *ACO* transcripts is observed during various physiological processes and environmental conditions (De Paepe and Van Der Straeten, 2005; Argueso et al., 2007; Lin et al., 2009; Ruduś et al., 2012). Several *ACO* genes were shown to be auto-regulated by ethylene (De Paepe et al., 2004) and recently, evidence is suggesting post-transcriptional/translational regulation mechanisms for ACO as well (Dilley et al., 2013; Van de Poel et al., 2014; Van de Poel and Van Der Straeten, 2014).

Instead of being degraded by ACO, ACC can also be converted to its major conjugate 1-malonyl-ACC (MACC) using malonyl-coenzyme-A. Secondly, ACC can react with GSH to form γ-glutamyl-ACC (GACC). Finally, jasmonic acid also forms a conjugate with ACC, producing jasmonyl-ACC (JA-ACC; **Figure 1**). These conjugates could regulate the pool of available ACC and potentially affect ethylene production. However, the exact molecular and biochemical function of ACC conjugates deserves further investigation, as recent studies report ACC to function as a signal itself (Yoon and Kieber, 2013; Van de Poel and Van Der Straeten, 2014). Increased levels of conjugated ACC were observed in both roots and leaves of Cd-exposed *A. thaliana* plants (Schellingen et al., 2014; **Table 1**), supporting a role for ACC conjugation during metal stress. Future research should be conducted to reveal the molecular nature of these conjugates. In particular, GACC might be involved as GSH is known to play a central role in defense to metal stress via its chelating, antioxidant and signaling properties (Jozefczak et al., 2012; Hernández et al., 2015).

Several reports have shown that the effects of metal stress on ethylene production in plants are both metal- and concentration-specific (Abeles et al., 1992; Thao et al., 2015; **Table 1**). It has been suggested that Cd could be the most phytotoxic inorganic ion able to stimulate ethylene production by plants (Abeles et al., 1992; Arteca and Arteca, 2007). Cadmium-induced increases in ethylene production were observed in *Hordeum vulgare* (Vassilev et al., 2004), *Lycopersicon esculentum* (Iakimova et al., 2008), *Pisum sativum* (Rodríguez-Serrano et al., 2006, 2009), *Brassica juncea* (Masood et al., 2012; Asgher et al., 2014), *Glycine max* (Chmielowska-Bąk et al., 2013), *A. thaliana* (Schellingen et al., 2014) and *Triticum aestivum* plants (Khan et al., 2015). On the other hand, long-term (16 days) Cd exposure decreased ethylene release in *A. thaliana* (Carrió-Seguí et al., 2015). Interestingly, a Cd-tolerant *H. vulgare* genotype showed a larger increase in ethylene emission after 15 days of Cd exposure as compared to a Cd-sensitive genotype (Cao et al., 2014). Up to 6 h after exposure to excess Cu or Zn (25–500 μM), seven-days-old *A. thaliana* seedlings grown on hydroponics produced more ethylene than unexposed seedlings (Mertens et al., 1999). In contrast, no significant changes in ethylene emission were detected for *A. thaliana* seedlings *in vitro* grown in the presence of 25 or 50 μM Cu during 9 days (Lequeux et al., 2010), suggesting an effect of exposure time and/or plant age. Excess Cu (500 μM) did induce increased ethylene production in *Helianthus annuus* and *T. aestivum* leaf discs. On the other hand, exposure to 500 μM Cd only enhanced its emission in *T. aestivum* leaves (Groppa et al., 2003), pointing toward species-specific responses to metal stress. Moreover, different *A. thaliana* plant parts showed a various induction of ethylene release after exposure to excess Cu or Cd, with the highest production rate observed in inflorescences. This response declined with increasing age of the different plant parts and did not occur in plants exposed to Ni or Zn (Arteca and Arteca, 2007). Nonetheless, Ni and Zn exposure led to higher ethylene release from *B. juncea* leaves (Khan and Khan, 2014) and aluminum (Al) induced a rapid evolution of ethylene from *Lotus japonicus* (Sun et al., 2007) and *A. thaliana* root apices (Sun et al., 2010). Also Fe (Yamauchi and Peng, 1995) and lithium (Li) toxicity (Naranjo et al., 2003) were reported to be linked to stress-induced ethylene production (**Table 1**).

Although most studies only investigated the effects of metal exposure on ethylene release by plants, the mechanistic basis is becoming increasingly clear (**Table 1**). For example, Cu induced an increased expression of *ACO1* and *ACO3* genes in *Nicotiana glutinosa* (Kim et al., 1998). It has been suggested that upregulation of *ACO* genes serves as a good ethylene production indicator (Ruduś et al., 2012). Nevertheless, ACC production by ACS covers the rate-limiting step in the ethylene biosynthesis pathway. Sun et al. (2007) have attributed the induction of ethylene evolution from roots of Al-exposed *L. japonicus* plants to increased ACO activity, but also observed upregulated *ACS* and *ACO* gene expression in *Medicago truncatula* after Al exposure. While Li had a variable effect on *ACS* expression (Liang et al., 1996), Cu highly increased *ACS* transcript levels in *A. thaliana* plants (Weber et al., 2006). Activity of ACS increased in *B. juncea* plants exposed to Cd (Asgher et al., 2014), Ni or Zn (Khan and Khan, 2014), as well as in Cd-exposed *T. aestivum* plants (Khan et al., 2015). Transcript levels of *ACS* and *ACO* genes were rapidly enhanced in Cu-exposed *B. oleracea* (Jakubowicz et al., 2010), Al-exposed *A. thaliana* (Sun et al., 2010), chromium (Cr)-exposed *Oryza sativa* (Trinh et al., 2014), and Hg-treated *O. sativa* (Chen et al., 2014) and *M. sativa* plants (Montero-Palmero et al., 2014a). In addition, Cd was shown to enhance *ACS* gene expression in *G. max* (Chmielowska-Bąk et al., 2013) and *ACS* and/or *ACO* transcription in *H. vulgare* (Cao et al., 2014), *O. sativa* (Trinh et al., 2014) and *A. thaliana* plants (Herbette et al., 2006; Weber et al., 2006; Schellingen et al., 2014; **Table 1**). In the latter study, the Cd-induced increase

in ACC and ethylene biosynthesis was mainly attributed to upregulated *ACS2* and *ACS6* expression, as mutants lacking both isoforms did not show enhanced ethylene release when exposed to Cd (Schellingen et al., 2014). These enzymes are both phosphorylated by the MAPKs MPK3 and MPK6, increasing their half-life (Liu and Zhang, 2004; Joo et al., 2008; Lin et al., 2009; Han et al., 2010; Skottke et al., 2011). Furthermore, MPK3 and MPK6 are able to induce *ACS2* and *ACS6* transcription via the transcription factor WRKY33 (Li et al., 2012). As MAPKs are clearly implicated in metal-induced signaling responses in plants (Opdenakker et al., 2012), they might affect ethylene biosynthesis during metal stress. Finally, whereas most studies focused on *ACS* or *ACO* gene expression levels, Dorling et al. (2011) have pointed out the importance of also examining the effects of metal stress on enzyme abundance, activity and post-translational modifications.

Ethylene Signaling is Affected in Metal-Exposed Plants

The ethylene signaling cascade starts with its perception by a family of membrane-bound receptors that are predominantly localized at the endoplasmic reticulum (ER). Because of its volatile nature, ethylene can freely diffuse throughout the cell from the site of production to the ER. In *A. thaliana*, five genes encode a high affinity receptor for ethylene: *ETHYLENE RESISTANT 1* and *2* (*ETR1/2*), *ETHYLENE RESPONSE SENSOR 1* and *2* (*ERS1/2*), and *ETHYLENE INSENSITIVE 4* (*EIN4*). Although some functional specificity exists among the different isoforms, they are largely redundant in controlling the ethylene response in plants (Merchante et al., 2013). In the absence of ethylene, its receptors actively suppress the downstream response (Hua and Meyerowitz, 1998). All receptors possess an N-terminal transmembrane domain to bind ethylene, a domain involved in protein-protein interactions between different receptor types and a C-terminal domain to interact with downstream components of the signaling cascade. A functional receptor unit consists of a homo- or heterodimer able to bind ethylene, although associations of higher order can give rise to receptor clusters in the ER membrane (Merchante et al., 2013). REVERSION TO ETHYLENE SENSITIVITY 1 (RTE1) negatively regulates ethylene responses by specifically activating ETR1 (Resnick et al., 2006, 2008). Furthermore, Cu is required for ethylene binding as well as receptor functionality and is delivered to the receptors by the intracellular RESPONSIVE TO ANTAGONIST 1 (RAN1) Cu transporter (Hirayama et al., 1999). Although the role of Cu in ethylene perception is well established, recent results point toward its involvement in ethylene biosynthesis as well. Indeed, *A. thaliana* plants grown under Cu deficient conditions release less ethylene (Carrió-Seguí et al., 2015).

In the absence of ethylene, the receptors activate a Raf-like protein kinase called CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), which is a negative regulator of the downstream ethylene signaling cascade (Kieber et al., 1993; Ju et al., 2012). Because of its physical interaction with the ethylene receptors, CTR1 also resides at the ER membrane (Gao et al., 2003). Without ethylene binding to its receptors, CTR1 forms a homodimer and functions as serine/threonine protein kinase to phosphorylate—and thereby inactivate—the downstream molecule ETHYLENE INSENSITIVE 2 (EIN2) (**Figure 2**; Ju et al., 2012). The EIN2 protein is an essential positive regulator of ethylene signaling. Furthermore, it is the only gene of the ethylene pathway where a loss-of-function mutation leads to complete ethylene insensitivity (Alonso et al., 1999). Similar to CTR1, EIN2 interacts with the ethylene receptors and is therefore localized at the ER membrane (Bisson et al., 2009; Bisson and Groth, 2010).

Upon ethylene binding to its receptors, CTR1 is inactivated (Ju et al., 2012; Shakeel et al., 2015). As a result, EIN2 is released from its inhibition by CTR1 and transduces the signal via its C-terminal end that physically moves from the ER membrane to the nucleus to activate the downstream components ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1). These short-lived transcription factors act as positive regulators of the ethylene signaling pathway and activate target genes such as *ETHYLENE RESPONSIVE FACTOR 1* (*ERF1*) that in turn affect the expression of secondary response genes in the ethylene-dependent transcription cascade (**Figure 2**; Yoo et al., 2009; Merchante et al., 2013). The above-described linear signaling pathway is subject to feedback regulation and turnover of different signaling components at the mRNA and protein level as described elsewhere (Guo and Ecker, 2003; Qiao et al., 2009; Zhao and Guo, 2011; Merchante et al., 2013). As it has not been described yet if and how metal stress affects these regulatory mechanisms, this paves the way for future research in this area.

Several studies support a role for ethylene signaling in response to different metals, mostly related to *ERF* expression (**Table 1**). The ERF proteins belong to the APETALA2/ethylene response element binding protein (AP2/EREBP) transcription factor family, which is known to mediate and integrate hormonal and redox signaling pathways during abiotic stress (Dietz et al., 2010). Roots of *A. thaliana* plants exposed to 50 μM Cd for 2 h showed increased expression levels of *ERF1, ERF2* and *ERF5*, while only *ERF1* expression was induced when Cu (10 μM) was applied (Weber et al., 2006). In addition, exposure to 5 or 10 μM Cd induced expression of *ERF1*, *ETR2* and *ACO2* in roots and leaves of *A. thaliana* plants after 24 and 72 h (Schellingen et al., 2014). Expression of *ERF2* and *ERF5* was increased in *A. thaliana* roots and shoots after 2, 6 and 30 h exposure to 5 or 50 μM Cd (Herbette et al., 2006). Recently, a whole-genome transcriptional profile from *M. sativa* seedlings exposed to 3 μM Hg for 3, 6 and 24 h demonstrated significant upregulation of several ethylene-responsive genes such as *ERF1*, mostly during the earliest hours of exposure (Montero-Palmero et al., 2014a). Similarly, Hg exposure affected genes related to ethylene signaling in *M. truncatula* (Zhou et al., 2013), *O. sativa* (Chen et al., 2014), *H. vulgare* plants (Lopes et al., 2013). Furthermore, roots of *O. sativa* plants exposed to 200 μM Cr for up to 3 h showed an increased expression of the *EIN3;4* gene (Trinh et al., 2014), while the *EIN2* gene was induced in Pb-exposed *A. thaliana* plants (Cao et al., 2009; **Table 1**). It is clear from these studies that ethylene signaling is involved in the response of plants to toxic metals (Montero-Palmero et al., 2014b).

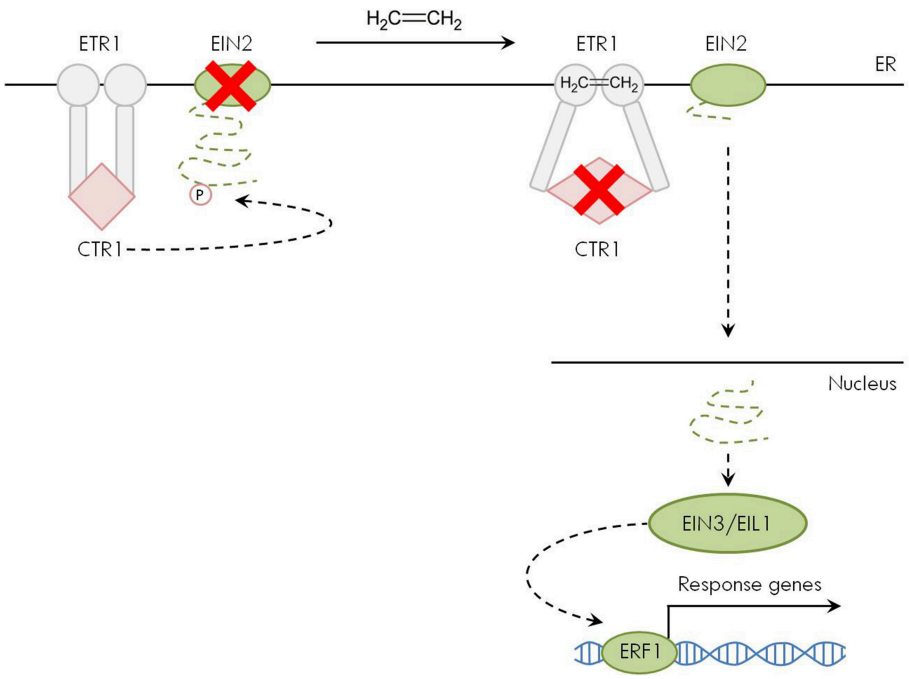

FIGURE 2 | Ethylene signal transduction pathway. In the absence of ethylene (left part), the ER-membrane embedded receptors such as ETHYLENE RESISTANT 1 (ETR1) activate CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). This Raf-like protein kinase phosphorylates ETHYLENE INSENSITIVE 2 (EIN2) at the C-terminal domain, which is thereby inactivated. When ethylene is present (right part), its binding to the receptors inactivates CTR1. The C-terminal domain of EIN2 translocates to the nucleus and activates the downstream signaling cascade via ETHYLENE INSENSITIVE 3 (EIN3)/EIN3-LIKE 1 (EIL1) and ETHYLENE RESPONSIVE FACTOR 1 (ERF1), finally affecting the transcription of ethylene responsive genes.

ETHYLENE IS A KEY REGULATOR OF PLANT RESPONSES TO METAL STRESS

Various reports discuss the potential implication of ethylene in plant adaptation or tolerance to toxic metals, and plant genotypes emitting more ethylene were suggested to be more metal resistant than those that release less (Lu and Kirkham, 1991). Moreover, the Pb-hyperaccumulator *Sesbania drummondii* showed increasing mRNA levels of a putative *ACS/ACO* gene upon exposure to Pb (Srivastava et al., 2007). Recently, Fu et al. (2014) conducted a transcriptome profiling of genes and pathways associated with As tolerance and toxicity in two *A. thaliana* ecotypes. In the more tolerant Columbia ecotype, genes encoding components of the ethylene signaling pathway were significantly enriched after short-term As exposure as compared to the sensitive Wassilewskija ecotype (Fu et al., 2014). Similarly, Cao et al. (2014) suggested that Cd tolerance in *H. vulgare* is related to the induction of ethylene signaling. Transgenic *N. tabacum* plants overexpressing an *ERF* gene from *Lycium chinense* displayed greater tolerance to Cd stress than non-transformed plants (Guan et al., 2015). On the other hand, ethylene insensitive *etr1-1* and *ein3-3 A. thaliana* mutants were shown to be less sensitive to Li than WT plants (Bueso et al., 2007). These apparent conflicting results can be attributed to metal-specific properties, but are definitely related to the chosen experimental setup as discussed before (metal concentration, exposure time, plant species; **Table 1**, see section "Weighing the Evidence for a Relation between Ethylene and Metal Stress"). Nevertheless, still little is known about the underlying mechanisms of ethylene regulating plant responses to metal stress and potentially affecting sensitivity vs. tolerance (Asgher et al., 2015).

Mutants defective in ethylene biosynthesis and signaling, together with pharmacological compounds to induce or inhibit these processes, have provided an elegant framework to further unravel the involvement of ethylene in plant metal stress responses. In this way, it was shown that ethylene signaling plays an important role during Cd-induced cell death in cultured tomato cells. Exposure to $CdSO_4$ induced rapid cell death and a transiently increased ethylene production within 24 h. Addition of aminoethoxyvinylglycine (AVG) to inhibit ethylene biosynthesis or silver thiosulfate (STS) to block the ethylene receptor led to a marked decrease in Cd-induced cell death (Iakimova et al., 2008). A similar inhibitory effect of AVG was observed during Al-induced cell death in tomato suspension cells (Yakimova et al., 2007).

Using the ethylene-insensitive *Never ripe (Nr)* tomato mutant, ethylene was demonstrated to be involved in Cd-induced lipid peroxidation in roots, leaves and fruits (Gratão et al., 2012). Mutant *A. thaliana* plants without functional *ACS2* and *ACS6* enzymes did not show an increased ethylene release upon

short-term (24 to 72 h) exposure to 5 or 10 μM Cd as compared to wild-type (WT) plants. Moreover, Cd-induced decreases in leaf fresh weight were less pronounced in mutants than in WT plants, pointing to a lower Cd sensitivity in the absence of ACS2/6 (Schellingen et al., 2014, 2015a). After prolonged exposure to the same Cd concentrations however, WT and *acs2-1/6-1* knockout mutants were equally sensitive, suggesting an early and transient role for ethylene in Cd-induced stress responses (Schellingen et al., 2015a).

Ethylene insensitive *ein2-1* mutants are more sensitive to Pb (Cao et al., 2009). This was attributed to an increased uptake of Pb and a diminished GSH content (Cao et al., 2009), revealing crosstalk between ethylene and the biosynthesis of this antioxidant and metal chelating compound. Also other studies link ethylene to the metal-induced oxidative stress response (Sun and Guo, 2013; Zhang et al., 2014; Montero-Palmero et al., 2014a; Schellingen et al., 2015a,b), as is discussed in the next section. These results clearly point toward the potential benefit of altering ethylene biosynthesis and/or signaling in future phytoremediation strategies (Montero-Palmero et al., 2014a). This is also supported by the fact that bacteria producing ACC deaminase (ACD) and thereby diminishing ethylene levels in their host plant, have been successfully used in laboratory and field conditions to protect plants from growth inhibition by elements such as As, Cd, Cu, Ni, Pb, and Zn (reviewed by Glick et al., 2007). This enzyme converts the ethylene precursor ACC into α-ketobutyrate and ammonia, which is subsequently used as nitrogen source by the bacteria. This reduces deleterious ethylene levels *in planta* and alleviates the associated stress symptoms (Arshad et al., 2007; DalCorso et al., 2013; Glick, 2014). However, it must be emphasized that the beneficial effects of plant-associated bacteria are also related to the increased availability of nutrients such as P and Fe, the production of phytohormones such as auxins and cytokinins and their competition with phytopathogens that could negatively affect plant health and growth (Weyens et al., 2009). Nonetheless, diminishing ethylene levels seems a promising path to explore, as transgenic plants expressing a bacterial ACD gene display a more resistant phenotype than non-transformed plants when exposed to different metals (Arshad et al., 2007; Glick et al., 2007). It has even been shown that plants possess ACD activity themselves (McDonnell et al., 2009), an intriguing asset which could also be exploited in phytoremediation of metal-polluted soils. However, ethylene production and signaling might also be a beneficial part of metal stress responses in plants (Cao et al., 2014; Fu et al., 2014; Thao et al., 2015). Indeed, ethylene can promote as well as inhibit plant growth (Pierik et al., 2006). Therefore, much work remains to be done prior to altering the ethylene response and improving phytoremediation of metal-contaminated soils.

With regard to plant growth in metal-polluted areas, the root architecture is of great importance. Interestingly, ethylene modulates local and systemic responses to low phosphate (Pi), thereby contributing to the remodeling of the root system architecture to increase Pi uptake (Nagarajan and Smith, 2011). As the root system of plants exposed to toxic metals is also drastically changed (Remans et al., 2012), this opens the window to study the potential involvement of ethylene in this response specifically (De Smet et al., 2015). For example, WT *A. thaliana* plants exposed to increasing Cd concentrations showed a higher lateral root density, which was abolished at higher exposure concentrations. In contrast, the Cd-induced increase in lateral root density was maintained at these higher exposure levels in ethylene insensitive *ein3-1* mutants. Ethylene might therefore modulate lateral root outgrowth during high Cd exposure (Remans et al., 2012). Furthermore, ethylene is implicated in the development of root hairs in Cd-exposed *B. napus* seedlings, as the use of the ethylene biosynthesis inhibitors cobalt chloride ($CoCl_2$) and aminooxyacetic acid (AOA) attenuated the Cd-mediated increase in root hair density (Sun and Guo, 2013). Ethylene may also inhibit primary root growth during the early response to Hg, as roots of *M. sativa* seedlings exposed to the ethylene receptor inhibitor 1-methylcyclopropene (1-MCP) as well as roots of ethylene insensitive *ein2-5 A. thaliana* mutants grew more in the presence of moderate Hg concentrations as compared to their untreated or WT counterparts (Montero-Palmero et al., 2014a). In addition, ethylene insensitive *etr1-3* and *ein2-1 A. thaliana* plants were more tolerant to Al stress, as root elongation of both mutants was less inhibited than in WT plants (Sun et al., 2010). For *ein2-1*, root and leaf growth was also less compromised as compared to the WT after 14 days of Al exposure (Zhang et al., 2014). Similarly, root elongation of Al-exposed *A. thaliana* plants was less affected in the presence of antagonists of ethylene biosynthesis (AVG and $CoCl_2$) and perception [silver nitrate ($AgNO_3$); Sun et al., 2010]. Recently, it was shown that ethylene negatively regulates Al-induced efflux of malate anions in wheat. As malate forms extracellular complexes with Al, this explains the increased Al tolerance observed in ethylene insensitive genotypes (Tian et al., 2014). Again, the potential benefits of ethylene able to reduce root and plant growth during metal stress should not be ignored when interpreting the above-mentioned results.

CROSSTALK BETWEEN ETHYLENE AND OTHER PLAYERS IN THE METAL STRESS NETWORK

Research over the past years points toward an intimate interaction between ethylene and other signaling components implicated in the response of plants to metal stress (**Figure 3**). In the following sections, the experimental evidence is summarized. Nonetheless, it must be emphasized that our knowledge is still scarce, revealing the need for future research to obtain an integrated picture and potentially apply this information in strategies to cope with phytotoxic metals (Thao et al., 2015).

Interaction between Ethylene and ROS Signaling

It is widely accepted that ROS act as signaling molecules in abiotic stress responses, interacting with other signaling pathways in a spatiotemporal manner (Bartoli et al., 2013; Baxter et al., 2014). Oxidative stress characterized by an imbalance between ROS and antioxidants in favor of the former is a recurrent response of metal-exposed plants (Sharma and Dietz, 2009),

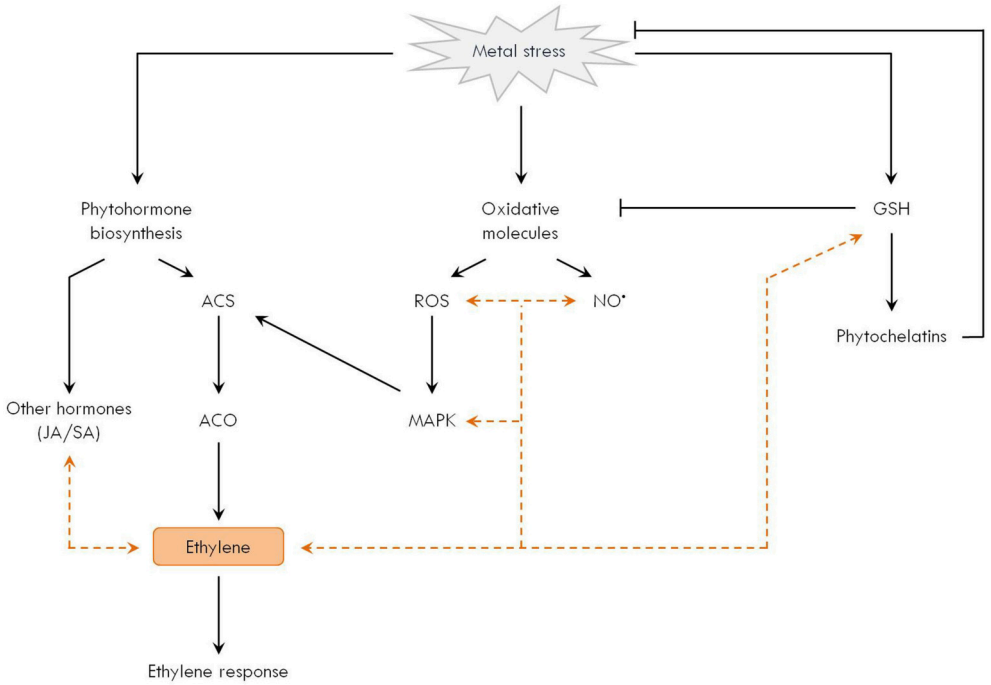

FIGURE 3 | Ethylene participates in the network of metal-induced signaling responses in plants. Different signaling pathways are affected by metal exposure in plants. (1) Phytohormones such as ethylene, jasmonic acid (JA) and salicylic acid (SA) are influenced by metal stress. In particular, ethylene biosynthesis is generally activated at the level of ACC synthase (ACS) and oxidase (ACO), thereby stimulating the ethylene signaling cascade. (2) Increased generation of reactive oxygen species (ROS, e.g. H_2O_2) and reactive nitrogen species (e.g. $NO^{\bullet}$) sets oxidative signaling pathways in motion, for example those mediated by mitogen-activated protein kinases (MAPK). (3) Glutathione (GSH) is a central player in the metal-induced stress network, not only because of its antioxidant function, but also as a precursor for metal-chelating phytochelatins. It is increasingly clear that these individual players integrate and interact within a broad signaling network in metal-exposed plants. Direct interaction between oxidative stress and ethylene biosynthesis is demonstrated by the MAPK-mediated activation of ACS. In addition, ethylene is shown to affect other players such as JA, SA, ROS, $NO^{\bullet}$, MAPK, and the GSH metabolism as well (indicated by orange dashed arrows).

which triggers downstream responses potentially leading to acclimation. Furthermore, it is increasingly clear that signals related to an increased ROS generation are linked to hormonal signaling pathways (Fujita et al., 2006; Baxter et al., 2014). Different studies demonstrated the involvement of ethylene in the stress-induced oxidative burst, as reviewed by Steffens (2014) during salinity, flooding and metal stress responses in *O. sativa*. When ethylene production or perception was inhibited by AVG or STS, respectively, camptothecin-induced H_2O_2 production was blocked in *L. esculentum* suspension cells (de Jong et al., 2002). As compared to WT plants, ethylene insensitive *ein2-1* *A. thaliana* plants produced less H_2O_2 and showed a lower $O_2^{\bullet-}$ production rate when exposed to paraquat. Consequently, mutant seedlings had a lower increase in malondialdehyde (MDA) content, which suggests less oxidative damage compared to the WT (Cao et al., 2006). Similarly, H_2O_2 production and MDA content were lower in *ein2-1* as compared to WT plants after Al exposure (Zhang et al., 2014). Furthermore, application of the ethylene receptor blocker STS significantly reduced the H_2O_2 content in roots of Cd-exposed *Phaseolus coccineus* plants after 1 and 2 h (Maksymiec, 2011). Cadmium-induced production of $O_2^{\bullet-}$ at the growing root hair tips of *B. napus* was blocked by the ethylene biosynthesis inhibitor AOA, suggesting that ethylene signaling acts upstream of $O_2^{\bullet-}$ (Sun and Guo, 2013). Finally, *A. thaliana cat2-1* mutants that accumulate more H_2O_2 under normal growth conditions were more tolerant to Li, although they took up more Li as compared to WT plants. Lithium-exposed *cat2-1* mutants produced less ethylene and showed less induction of ethylene responsive genes than the WT. Therefore, the authors attributed the increased Li tolerance of *cat2-1* mutants to a reduced ethylene production and sensitivity (Bueso et al., 2007).

These results suggest an interaction between ethylene and the ROS network of plants, with ethylene able to affect ROS producing as well as scavenging enzymes and metabolites. The ROS producing NADPH oxidases [also known as respiratory burst oxidase homologs (RBOH)] are put forward as critical signaling hubs in the response of plants to environmental stimuli (Suzuki et al., 2011) such as metal exposure (Remans et al., 2010). Ethylene is an important upstream regulator of $O_2^{\bullet-}$-producing NADPH oxidases (Chae and Lee, 2001), with a regulatory interaction between the ethylene biosynthesis gene *ACS1* and *RBOHD/F* transcription in *B. oleracea* seedlings (Jakubowicz et al., 2010). In *Ipomoea batatas*, the NADPH oxidase inhibitor diphenyleneiodonium decreased ROS production induced by ethephon, an ethylene releasing compound (Chen et al., 2013).

Furthermore, ethylene seems to stimulate the apoplastic release of H_2O_2 by activating NADPH oxidase isoform D (RBOHD) during biotic stress, as flagellin (flg22)-induced ROS generation diminished in ethylene insensitive *etr1-1* and *ein2-1 A. thaliana* mutants as compared to the WT (Mersmann et al., 2010). Recent reports also indicate a relationship between ethylene and NADPH oxidase during metal stress. For example, inhibition of the ethylene receptors by 1-MCP reduced or even abolished the increase in extracellular H_2O_2 production and NADPH oxidase activity observed during the first 6 h of Hg exposure in *M. sativa* root segments (Montero-Palmero et al., 2014a). In addition, Hg-exposed ethylene insensitive *ein2-5* mutants produced less H_2O_2 as compared to their WT counterparts (Montero-Palmero et al., 2014a). Upon Cd exposure, expression of *RBOHC* did not increase to WT levels in leaves of *acs2-1/6-1* knockout, *ein2-1*, and *ein2-5* mutant *A. thaliana* plants (Keunen et al., 2015), again supporting a link between ethylene and ROS production by NADPH oxidases during metal stress. Furthermore, ethylene signaling was also related to the transcriptional induction of ALTERNATIVE OXIDASE 1a/d (AOX1a/d), which was lower in leaves of Cd-exposed *ein2-1* and *ein2-5* mutants as compared to WT plants. This enzyme regulates ROS levels and is suggested to modulate the Cd-induced oxidative challenge in *A. thaliana*, requiring ethylene—either directly or indirectly via RBOHC—to be fully induced at the transcript level (Keunen et al., 2015). In line with this, AOX was demonstrated to be involved in ethylene-induced plant cell death as well (Lei et al., 2003).

On the other hand, ethylene might affect the plant's antioxidative defense network as shown by Cao et al. (2006). They demonstrated a constitutively higher transcription of *Cu/Zn SOD 2* (*CSD2*) and *CAT3* genes, leading to enhanced SOD and CAT enzyme activities in *ein2-1* mutants as compared to WT plants. This was also shown in Al-exposed *ein2-1* mutants, which showed differential responses at the level of SOD and CAT activities compared to WT plants (Zhang et al., 2014). The interaction between ethylene and antioxidative defense is further underlined by the fact that *ein2-1*, *ein3-1* and *ein4* mutant *A. thaliana* plants have a higher AsA content in leaves (Gergoff et al., 2010), which was also observed in ethylene insensitive *Nr* tomato fruits (Alba et al., 2005). Conversely, the *ctr1-1* mutant with a loss-of-function of the negative regulator CTR1 displayed lower leaf AsA levels (Gergoff et al., 2010). Concurrently, ethylene signaling was reported to suppress AsA synthesis and accumulation in tomato leaves (Mazorra Morales et al., 2014). In this regard, it is noteworthy to mention that ethylene biosynthesis requires AsA in the final step (cfr. *supra*), further supporting crosstalk between both compounds. Finally, mutants defective in ethylene perception (*etr1-1*) as well as those overproducing ethylene (*eto1-1*) showed up to five-fold higher α-tocopherol levels during leaf aging. Furthermore, ethylene insensitive *ein3-1* mutants showed a delayed increase in α-tocopherol during water stress (Cela et al., 2009). This antioxidant compound was shown to be essential for the tolerance of *A. thaliana* plants to metal-induced oxidative stress (Collin et al., 2008). Therefore, the interaction between ethylene and the antioxidative defense network mounted during metal exposure can ultimately affect responses leading to sensitivity or tolerance and deserves further investigation. In particular, a relation between ethylene and GSH is suggested and is discussed in a separate section.

Besides ROS, plant cells also produce reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$) during abiotic stress (Corpas et al., 2011) such as Cd exposure (Arasimowicz-Jelonek et al., 2011; Chmielowska-Bąk et al., 2014). However, the functional role of $NO^{\bullet}$ in metal-challenged plants is not yet fully understood. Different results point toward an interaction between $NO^{\bullet}$ and ethylene, as recently reviewed by Mur et al. (2013). During salt stress, $NO^{\bullet}$ and ethylene were shown to cooperate in the modulation of ion homeostasis. Salt stress-induced $NO^{\bullet}$ production greatly enhanced ethylene emission in *A. thaliana* callus. In its turn, ethylene stimulated the expression of plasma membrane H^+-ATPase genes, which has been suggested to facilitate Na^+ efflux into the apoplast and attenuate Na^+ toxicity under saline conditions (Wang et al., 2009). Such regulatory interactions between $NO^{\bullet}$ and ethylene might also be involved in the response of plants to metal stress. Interestingly, both ethylene and $NO^{\bullet}$ are involved in the upregulation of key genes related to Fe acquisition and homeostasis in *A. thaliana* (García et al., 2010). Iron deficiency is a well-known consequence of metal toxicity [e.g. Cd (Xu et al., 2015)] and seems to increase ethylene sensitivity (García et al., 2010), which potentially affects metal-induced responses related to ethylene as well. It has been shown that nutrient stress—either a reduced or increased availability—affects ethylene biosynthesis and perception in plants via the induction of an oxidative burst (Iqbal et al., 2013a), again highlighting the link between ethylene and ROS signaling.

Crosstalk between Ethylene and GSH

Tolerance to toxic metals is highly dependent on the metabolism of GSH, a widely distributed biothiol tripeptide (γ-glutamyl-cysteinyl-glycine) in plant cells. As metal chelator, antioxidant and signaling compound, GSH is a key player in metal-induced oxidative stress defenses (Seth et al., 2012). This multifunctional role is related to the sulfhydryl group in cysteine, which has a high affinity toward metals such as Cd and Hg. Furthermore, GSH is the precursor molecule for the synthesis of phytochelatins (PCs), which consist of 2 to 11 GSH molecules and limit the cellular concentration of free metal ions (Jozefczak et al., 2012; Hernández et al., 2015). Using ethephon, it was shown that ethylene induces the activity of ATP sulfurylase (ATPS), leading to an accumulation of sulfur (S) in *B. juncea* (Iqbal et al., 2012). Recently, Iqbal et al. (2013b) reviewed the crosstalk between S assimilation and ethylene signaling in plants. Since GSH synthesis is affected by S availability to produce the amino acid cysteine, ethylene might modulate this process in order to meet the increasing demands for GSH during metal stress. In this regard, it is important to mention that ethylene synthesis itself also requires cysteine to ultimately produce SAM (**Figure 4**).

Crosstalk between ethylene and GSH is suggested by the observed upregulation of different genes encoding ERF transcription factors in the severe GSH deficient *root meristemless 1-1* (*rml1-1*) *A. thaliana* mutants as compared to WT plants (Schnaubelt et al., 2015). On the other hand, *ERF2* expression was significantly repressed in *cadmium sensitive 2-1* (*cad2-1*)

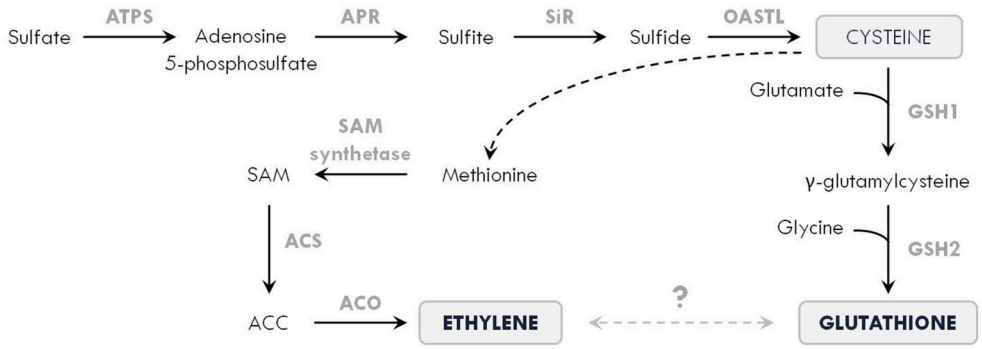

FIGURE 4 | Simplified scheme of the interaction between sulfur assimilation, ethylene and glutathione biosynthesis in plants. Sulfur is taken up from the soil as sulfate, which is converted into adenosine 5-phosphosulfate by the enzyme ATP sulfurylase (ATPS). This is further reduced by adenosine 5-phosphosulfate reductase (APR) into sulfite, which is subsequently reduced into sulfide by sulfite reductase (SiR). The enzyme O-acetylserine (thiol) lyase (OASTL) produces cysteine, which is one of the three building blocks that make up glutathione. During glutathione biosynthesis, cysteine is coupled to glutamate by γ-glutamylcysteine synthetase (GSH1) to form γ-glutamylcysteine. In the next step, glycine is added by glutathione synthetase (GSH2) to finally produce glutathione. In addition, cysteine is also required for ethylene formation, as methionine is derived from cysteine via different reactions (depicted by the dashed arrow). In the ethylene biosynthetic pathway, methionine is converted to S-adenosyl-methionine (SAM) by SAM synthetase. In the next steps, 1-aminocyclopropane-1-carboxylic acid (ACC) is produced by ACC synthase (ACS) and subsequently oxidized by ACC oxidase (ACO) to form ethylene (see **Figure 1**). As ethylene and glutathione fulfill important functions in metal-exposed plants, a trade-off between both might lie at the heart of their interaction regulating plant responses to metal stress.

A. thaliana mutants that also have lower GSH levels than WT plants, but not as low as those of *rml1-1* mutants (Han et al., 2013). Transgenic *N. tabacum* plants with an enhanced GSH content showed induced ethylene biosynthesis (*ACO*) and signaling (*ERF*) genes (Ghanta et al., 2014). The potential interplay between GSH and ethylene is further underlined by the results of Chen et al. (2013), who demonstrated that exogenous GSH mitigated the ethephon-induced increase in ROS production in sweet potato. Furthermore, ethylene was suggested to increase *de novo* biosynthesis of GSH in ozone-exposed *A. thaliana* plants, thereby protecting against leaf injury (Yoshida et al., 2009).

Increasing evidence points toward a close relationship between ethylene and GSH metabolism during metal stress. For example, ethephon treatment increased GSH levels in Cd-exposed *B. juncea* (Masood et al., 2012). Similarly, GSH levels in Ni- and Zn-exposed *B. juncea* plants were higher after ethephon application, which alleviated metal toxicity (Khan and Khan, 2014). While ACS activity and ethylene production decreased, GSH levels increased in Cd-exposed *T. aestivum* supplied with S (Khan et al., 2015). Transcript levels of genes encoding GSH biosynthesis enzymes were significantly less upregulated, concomitantly with lower GSH levels in leaves of Cd-exposed *acs2-1/6-1* knockout vs. WT *A. thaliana* plants. Therefore, increased ethylene biosynthesis upon Cd exposure seems crucial to mount effective defense responses related to GSH (Schellingen et al., 2015a). In addition, ethylene signaling is implicated in the accumulation of GSH in Al-exposed *A. thaliana* (Zhang et al., 2014), Cd-exposed *L. chinense* (Guan et al., 2015), and *A. thaliana* plants (Schellingen et al., 2015b). The increased Cd tolerance of transgenic tobacco plants overexpressing an *ERF* gene from *L. chinense* was related to an enhanced expression level of GSH biosynthesis genes (Guan et al., 2015). Similar results were obtained in Pb-exposed *A. thaliana* plants, where EIN2 is indispensable to confer metal resistance partially by increasing GSH levels (Cao et al., 2009). These results confirm the suggested interplay between ethylene and GSH in determining metal tolerance vs. sensitivity and open the window to future experiments exploiting this relationship in phytoremediation strategies (Hernández et al., 2015). As discussed before, it should be kept in mind that not all metal(loid)s are equally strong inducers of PC synthesis (Anjum et al., 2015) and will therefore differentially affect GSH levels in plants.

Interaction between Ethylene and MAPK Signaling Pathways

A signaling pathway linked to ethylene and worthwhile to discuss in the light of metal stress is the ROS-induced MAPK cascade. These kinases are activated at transcript and activity level in different plant species exposed to metals. Furthermore, they interfere with hormone biosynthesis and signaling to activate downstream responses (Opdenakker et al., 2012). As mentioned before, the stress-responsive MPK3 and MPK6 isoforms increase ethylene production by affecting *ACS2* and *ACS6* transcription as well as protein stability (Liu and Zhang, 2004; Li et al., 2012). In addition, MAPK kinase 9 (MKK9) was shown to activate the MPK3/MPK6 cascade and stimulate ethylene biosynthesis in *A. thaliana* (Xu et al., 2008). Other studies have indicated that MAPKs could be involved in ethylene signaling as well (Ouaked et al., 2003; Hahn and Harter, 2009). This might come as no surprise since the negative regulator of ethylene signaling, CTR1, shows sequence similarities with Raf protein kinases and has been presumed to function as a MAPK kinase kinase (MAPKKK). However, no conclusive CTR1-targeted kinases have been identified yet (Zhao and Guo, 2011; Ju and Chang, 2012; Merchante et al., 2013). Nonetheless, nuclear EIN3 was shown to be regulated not only by CTR1 but also by

a novel MAPK cascade mediated by MKK9 and MPK3/6 in *A. thaliana*. This cascade functions downstream of CTR1, is activated when ethylene binds to its receptors and stabilizes EIN3 by phosphorylation (Yoo et al., 2008). Recently, Schellingen et al. (2015b) proposed a model where MPK3/6 link ROS production and ethylene signaling during Cd stress in *A. thaliana* leaves. In this model, Cd exposure activates NADPH oxidases, which produce ROS that are sensed by the oxidative signal-inducible kinase1 (OXI1). This kinase then activates MPK3/6, both affecting ACS2/6 enzymes at multiple levels (Schellingen et al., 2015b). Furthermore, Liu et al. (2010) have shown that pretreatment with GSH reduced the activation of MPK3 and MPK6 under Cd stress in *A. thaliana*. This suggests that ROS are involved in Cd-induced MAPK signaling, with a relation to ethylene as both MPK3/6 are able to affect ethylene biosynthesis enzymes (Thao et al., 2015). Therefore, the potential implication of MAPK signaling and its relation with ethylene biosynthesis and/or signaling during metal stress should be explored in more detail.

Phytohormone Crosstalk During Metal Stress

Interactions between various phytohormones are required to integrate environmental signals and stress tolerance responses (De Paepe and Van Der Straeten, 2005; Kohli et al., 2013). In addition to ethylene, jasmonic acid (JA) and salicylic acid (SA) are mostly implicated in plant stress responses (Van de Poel et al., 2015) such as those mounted during metal exposure (De Smet et al., 2015). Different genes involved in ethylene and JA biosynthesis as well as genes responsive to these hormones were differentially expressed after Hg exposure in *M. sativa*, *M. truncatula* and *H. vulgare* plants (Montero-Palmero et al., 2014b). Gene expression profiling in Cr-stressed *O. sativa* roots indicated activation of ethylene and JA signaling pathways (Trinh et al., 2014). Furthermore, JA levels rapidly increased in *A. thaliana* and *P. coccineus* plants exposed to Cd or Cu (Maksymiec et al., 2005). From a signaling point of view, JA was shown to trigger ROS production in short-term Cd- and Cu-exposed *A. thaliana* plants, as inhibiting JA biosynthesis by propyl gallate decreased $O_2^{\bullet-}$ and H_2O_2 levels after metal exposure (Maksymiec and Krupa, 2006).

A mutual relationship exists between ethylene and JA signaling (Song et al., 2014), which might ultimately affect metal stress responses as well. For example, it has been shown that the JA receptor CORONATINE INSENSITIVE 1 (COI1) is implicated in the inhibition of *Arabidopsis* root growth mediated by ethylene in the light (Adams and Turner, 2010). Furthermore, ethylene insensitive *ein2-1* mutants become ethylene responsive by reducing JA levels via a genetic or chemical approach (Kim et al., 2013). Crosstalk between ethylene and SA during metal stress was supported by the results of Zhang et al. (2014). They showed that *Arabidopsis* mutants insensitive to ethylene (*ein2-1*) or SA [*nonexpressor of pathogenesis-related proteins 1-1* (*npr1-1*)] were more tolerant to Al exposure as compared to WT plants. However, *ein2-1*/*npr1-1* double mutants were less tolerant than WT plants, indicating that the tolerant phenotype of *ein2-1* and *npr1-1* single mutants depended on remaining NPR or EIN function, respectively (Zhang et al., 2014). These results further support studying the complex interaction between various hormonal signaling pathways mediating metal stress responses in plants.

As for ethylene, different studies support a functional link between GSH and JA as well. Mutant *A. thaliana* plants without functional GR1 displayed a constitutive increase in oxidized glutathione disulfide (GSSG), which affected the expression of genes involved in JA metabolism dependent on day length conditions (Mhamdi et al., 2010). Expression levels of two JA signaling marker genes [plant defensin 1.2 (*PDF1.2*) and vegetative storage protein 2 (*VSP2*)] were significantly lower in GSH deficient *cad2-1* or *regulator of APX2 1-1* (*rax1-1*) mutants. Similar results were obtained when GSH biosynthesis was chemically inhibited by buthionine sulfoximine (BSO) in WT plants. In addition, microarray analysis revealed a multitude of genes involved in JA synthesis, activation and signaling to be differentially expressed in *cad2-1* mutants, indicating that the basal expression of JA-associated genes is affected by the content of GSH (Han et al., 2013). In the conditional oxidative stress signaling mutant *cat2* with H_2O_2-induced accumulation of GSSG under ambient air and moderate light conditions, the JA pathway was upregulated. However, this response was attenuated in a *cat2 cad2* double mutant, showing that GSH also regulates oxidative stress-induced JA-related gene expression in *A. thaliana* (Han et al., 2013). In addition to JA, SA was also shown to interact with GSH, as transgenic tobacco plants with an enhanced GSH content showed induction of SA-related genes such as *PATHOGENESIS-RELATED PROTEIN 1a* (*PR1a*; Ghanta et al., 2014). As ethylene and GSH are clearly intertwined during metal stress responses, it might be worthwhile to investigate the involvement of JA and SA in this interaction. In addition, JA signaling also involves the MAPK cascade MKK3/MPK6 (Takahashi et al., 2007) and $NO^{\bullet}$ does not only affect ethylene but also JA and SA signaling cascades (Mur et al., 2013). Therefore, it is recommended to further dissect the hormonal crosstalk affecting plant responses to metal stress (also reviewed by Thao et al., 2015) and address their interaction with oxidative stress and particularly GSH, the MAPK cascade and $NO^{\bullet}$ in future experiments.

CONCLUDING REMARKS

Ethylene is involved in many processes throughout the entire life cycle of plants, including responses to environmental stimuli such as metal exposure. Our current knowledge on the role of ethylene in metal-induced stress responses, as well as its integration within a broad network of signaling compounds, is gradually expanding. Recent evidence points toward an intimate link between ethylene, the cellular redox balance with GSH as important antioxidant and other phytohormones such as JA and SA, finally affecting plant metal sensitivity vs. tolerance. Nevertheless, much work remains to be done before this information can be applied in practice.

AUTHOR CONTRIBUTIONS

EK, KS, JV and AC participated in the conception of the topic. EK and AC wrote the manuscript. All authors read and approved the final manuscript after critically revising it for important intellectual content.

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