

Role of Ethylene and Its Cross Talk with Other Signaling Molecules in Plant Responses to Heavy Metal Stress¹

Nguyen Phuong Thao², M. Iqbal R. Khan², Nguyen Binh Anh Thu, Xuan Lan Thi Hoang, Mohd Asgher, Nafees A. Khan, and Lam-Son Phan Tran*

School of Biotechnology, International University, Vietnam National University, Ho Chi Minh 70000, Vietnam (N.P.T., N.B.A.T., X.L.T.H.); Plant Physiology and Biochemistry Section, Department of Botany, Aligarh Muslim University, Aligarh 202002, India (M.I.R.K., M.A., N.A.K.); and Signaling Pathway Research Unit, RIKEN Center for Sustainable Resource Science, Tsurumi, Yokohama 2300045, Japan (L.-S.P.T.)

Excessive heavy metals (HMs) in agricultural lands cause toxicities to plants, resulting in declines in crop productivity. Recent advances in ethylene biology research have established that ethylene is not only responsible for many important physiological activities in plants but also plays a pivotal role in HM stress tolerance. The manipulation of ethylene in plants to cope with HM stress through various approaches targeting either ethylene biosynthesis or the ethylene signaling pathway has brought promising outcomes. This review covers ethylene production and signal transduction in plant responses to HM stress, cross talk between ethylene and other signaling molecules under adverse HM stress conditions, and approaches to modify ethylene action to improve HM tolerance. From our current understanding about ethylene and its regulatory activities, it is believed that the optimization of endogenous ethylene levels in plants under HM stress would pave the way for developing transgenic crops with improved HM tolerance.

In addition to common abiotic stresses seen in agricultural production, such as drought, submerging, and extreme temperatures (Thao and Tran, 2012; Xia et al., 2015), heavy metal (HM) stress has arisen as a new pervasive threat (Srivastava et al., 2014; Ahmad et al., 2015). This is mainly due to the unrestricted industrialization and urbanization carried out during the past few decades, which have led to the increase of HMs in soils. Plants naturally require more than 15 different types of HM as nutrients serving for biological activities in cells (Sharma and Chakraverty, 2013). However, when the nutritional/nonnutritional HMs are present in excess, plants have to either suffer or take these up from the soil in an unwilling manner (Nies, 1999; Sharma and Chakraverty, 2013). Upon HM stress exposure, plants induce oxidative stress due to the excessive production of reactive oxygen species (ROS) and methylglyoxal (Sharma and Chakraverty, 2013). High levels of these compounds have been shown to negatively affect cellular structure maintenance (e.g. induction of lipid peroxidation in the membrane, biological macromolecule deterioration, ion leakage, and DNA strand cleavage; Gill and Tuteja, 2010; Nagajyoti et al., 2010) as well as many other biochemical and physiological processes (Dugardeyn and Van Der Straeten, 2008). As

a result, plant growth is retarded and, ultimately, economic yield is decreased (Yadav, 2010; Anjum et al., 2012; Hossain et al., 2012; Asgher et al., 2015). Moreover, the accumulation of metal residues in the major food chain has been shown to cause serious ecological and health problems (Malik, 2004; Verstraeten et al., 2008).

Plants employ different strategies to detoxify the unwanted HMs. Among the common responses of plants to HM stress are increases in ethylene production due to the enhanced expression of ethylene-related biosynthetic genes (Asgher et al., 2014; Khan and Khan, 2014; Khan et al., 2015b) and/or changes in the expression of ethylene-responsive genes (Maksymiec, 2007). Conventionally, this hormone has been established to modulate a number of important plant physiological activities, including seed germination, root hair and root nodule formation, and maturation (fruit ripening in particular; Dugardeyn and Van Der Straeten, 2008). On the other hand, although ethylene has also been suggested to be a stress-related hormone responding to a number of biotic and abiotic triggers, little is known about the exact role of elevated HM stress-related ethylene in plants (Zapata et al., 2003). Enhanced production of ethylene in plants subjected to toxic levels of cadmium (Cd), copper (Cu), iron (Fe), nickel (Ni), and zinc (Zn) has been shown (Maksymiec, 2007). As an example, Cd- and Cu-mediated stimulation of ethylene synthesis has been reported as a result of the increase of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) activity, one of the enzymes involved in the ethylene synthesis pathway (Schlagnhauser and Arteca, 1997; Khan et al., 2015b).

Plants tend to adjust or induce adaptation or tolerance mechanisms to overcome stress conditions. To develop

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² These authors contributed equally to the article.

* Address correspondence to son.tran@riken.jp.

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stress tolerance, plants trigger a network of hormonal cross talk and signaling, among which ethylene production and signaling are prominently involved in stress-induced symptoms in acclimation processes (Gazzarrini and McCourt, 2003). Therefore, the necessity of controlling ethylene homeostasis and signal transduction using biochemical and molecular tools remains open to combat stress situations. Stress-induced ethylene acts to trigger stress-related effects on plants because of the autocatalytic ethylene synthesis. Autocatalytic stress-related ethylene production is controlled by mitogen-activated protein kinase (MAPK) phosphorylation cascades (Takahashi et al., 2007) and through stabilizing ACS2/6 (Li et al., 2012). Strong lines of evidence have shown the multiple facets of ethylene in plant responses to different abiotic stresses, including excessive HM, depending upon endogenous ethylene concentration and ethylene sensitivities that differ in developmental stage, plant species, and culture systems (Pierik et al., 2006; Kim et al., 2008; Khan and Khan, 2014). Under HM stress conditions, plants show a rapid increase in ethylene production and reduced plant growth and development, suggesting a negative regulatory role of ethylene in plant responses to HM stress (Schellingen et al., 2014; Khan et al., 2015b). On the other hand, a potential involvement of ETHYLENE INSENSITIVE2 (EIN2), a central component of the ethylene signaling pathway, as a positive regulator in lead (Pb) resistance in *Arabidopsis thaliana* has also been demonstrated (Cao et al., 2009). More recently, Khan and Khan (2014) showed that ethylene-regulated antioxidant metabolism maintained a higher level of reduced glutathione (GSH) and alleviated photosynthetic inhibition in mustard (*Brassica juncea*) plants exposed to Ni, Zn, or Cd through the optimization of ethylene homeostasis (Masood et al., 2012). Taken together, the purpose of this review is to update the research community with our current understanding of the roles of ethylene and its signaling in plant responses to HM stress. Moreover, the cross talk of ethylene with other phytohormones and signaling molecules upon HM stress will also be discussed.

ETHYLENE AND PLANT RESPONSES TO HM STRESS

The role of ethylene in plant responses to HMs has been a concern of many plant molecular biologists, biochemists, and physiologists, but in-depth and convincing research on how ethylene regulates different HM tolerance mechanisms is still a matter of task. Under unstressed conditions, ethylene is synthesized from an activated form of Met in plants (Xu and Zhang, 2015). ACS converts S-adenosyl-methionine (SAM) to ACC, and the oxidization of ACC is then executed by ACC oxidase (ACO) to form ethylene (Fig. 1). ACS and ACO, the two major enzymes in ethylene biosynthesis, are encoded by multigene families, which are also the primary regulation points in the ethylene biosynthetic pathway (Xu and Zhang, 2015). HM stress increases the activity of these two enzymes, resulting in increased

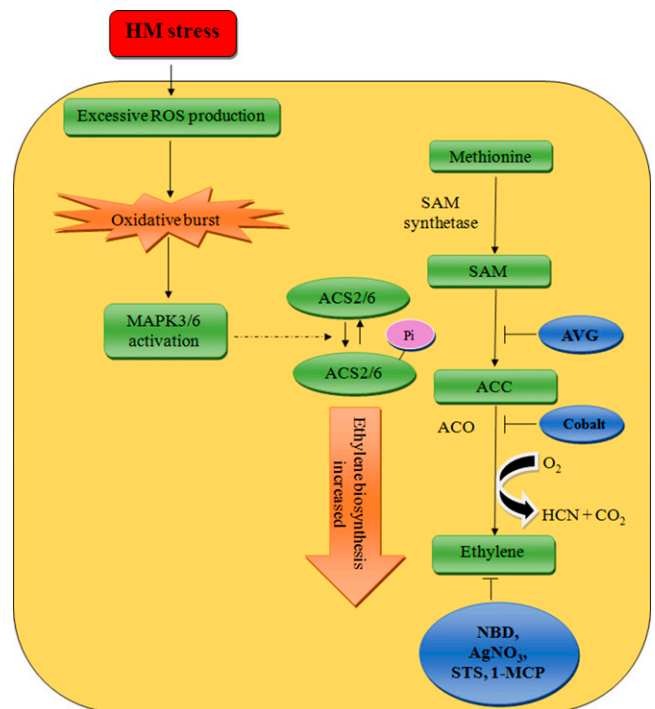


Figure 1. Ethylene biosynthesis under normal conditions and HM stress. Ethylene biosynthesis under normal conditions starts from the conversion of Met into SAM catalyzed by SAM synthetase. Furthermore, SAM is catalyzed by ACS to form ACC, an immediate precursor of ethylene. At the last step, ACC is oxidized by ACO to form ethylene. At this step, CO₂ and cyanide (HCN) are produced as by-products. Under HM stress, ethylene biosynthesis rapidly increased due to the excessive ROS production, resulting in oxidative burst of the cell and activation of the MAPK3 and MAPK6 cascade. The activated MAPK cascade phosphorylates ACS2 and ACS6 enzymes. Both native and phosphorylated ACS enzymes are functional; however, phosphorylated ACS is more stable and active compared with native ACS. Phosphorylated ACS induces stress ethylene. However, HM-induced stress ethylene can be controlled either by the manipulation of ethylene biosynthetic genes using biotechnological tools or by pharmacological tools, such as the ethylene biosynthesis inhibitors aminoethoxyvinylglycine (AVG) and cobalt (Co) that inhibit ACS and ACO activities, respectively. Additionally, stress ethylene action can be blocked by using ethylene receptor inhibitor norbornadiene (NBD), silver nitrate (AgNO₃), 1-methylcyclopropene (1-MCP), or silver thiosulfate (STS). The dashed line indicates possible regulation under HM stress. Arrows and T-bars represent positive and negative regulation, respectively, upon HM stress. Pi, Inorganic phosphate.

ethylene production (Schellingen et al., 2014; Khan et al., 2015b). The Cu-inducible expression of the ACS genes in potato (*Solanum tuberosum*) and the accumulation of the ACS transcripts in different varieties of tobacco (*Nicotiana tabacum*) have been reported (Schlaginhauser et al., 1997). Recently, transcriptome analysis of chromium-treated rice (*Oryza sativa*) roots also indicated enhanced expression of four ethylene biosynthesis-related genes (ACS1, ACS2, ACO4, and ACO5), suggesting the participation of ethylene in chromium signaling in rice (Steffens, 2014; Trinh et al., 2014). These findings together demonstrated that ethylene is enhanced in response to various

excessive metals in a wide range of plant species (Maksymiec, 2007; Peñarrubia et al., 2015).

A classic example illustrating the involvement of ethylene in plant responses to HM stress was the study of Sandmann and Böger (1980), which demonstrated that the synthesis of ethylene and the inhibition of photosynthetic electron transport in isolated spinach (*Spinacia oleracea*) chloroplasts were induced by Cu stress. It is possible that the high content of ethylene led to the inhibition of the photosystems, which might also trigger senescence processes at the late phase of growth or after a longer exposure to the excessive Cu in runner bean (*Phaseolus coccineus*; Maksymiec and Baszyński, 1996). Moreover, Arteca and Arteca (2007) showed that the application of Cu or Cd induced various levels of ethylene production in different plant parts, among which the highest amount was recorded in inflorescences. This group affirmed that Cu and Cd induced similar levels of ethylene production in both inflorescence stalks and leaves. This observation was different from earlier results that demonstrated that Cd promoted a greater increase in ethylene production in bean leaves than Cu or other HMs tested (Rodecap et al., 1981; Fuhrer, 1982). Interestingly, it was reported that ethylene biosynthesis was diminished in the *Arabidopsis copper transporter5 (cpt5)* mutant, which is defective in Cu transport, resulting in the hypersensitivity of *cpt5* to Cd stress (Carrió-Seguí et al., 2015). This finding suggests that an optimal endogenous Cu level might help plants better tolerate HM stress. Another independent study noticed that Ni and Zn did not stimulate ethylene production in *Arabidopsis* (Arteca and Arteca, 2007). However, these two HMs increased ethylene levels in mustard plants by enhancing ACS activity (Khan and Khan, 2014). In other recent studies, Jakubowicz et al. (2010) reported that 2.5 mM Cu induced ethylene biosynthesis in broccoli (*Brassica oleracea*) seedlings, and Franchin et al. (2007) noted significantly enhanced ethylene production with Cu concentration within a range of 5 to 500 μM , causing leaf toxicity and impairing root formation in poplar (*Populus alba*). In contrast, Cu at 25 and 50 μM did not significantly induce ethylene production in *Arabidopsis* seedlings (Lequeux et al., 2010). Collectively, these data might suggest that the HM-induced ethylene production is plant specific and/or dose dependent.

Ethylene was shown to be involved in the regulation of *P. coccineus* responses to Cd stress (Maksymiec, 2011). The Cd-induced ROS decreased in roots, and Cd-induced inhibition of leaf growth was completely ameliorated by the ethylene action inhibitor STS (Maksymiec, 2011). More recently, Schellingen et al. (2014) reported that the expression of ethylene-responsive genes, such as *ACO2*, *ETHYLENE RESPONSE2 (ETR2)*, and *ETHYLENE RESPONSE FACTOR1 (ERF1)*, was up-regulated by Cd treatment, while ethylene elevation during stress resulted in negative effects on leaf biomass in *Arabidopsis* plants. Together, these data suggest that the induction of ethylene by HMs may cause unbeneficial symptoms in plants that were exposed to HMs. However,

although it was also reported that HM stress-induced ethylene had negative effects on mustard plants, an optimized level of ethylene, which was lower than the HM stress-induced ethylene level but still higher than the ethylene level of control plants under unstressed conditions, could lead to beneficial plant responses, such as increased photosynthesis under Cd stress (Masood et al., 2012). These findings together suggest the complex and biphasic regulatory function of ethylene under stressful environments, which depends on its endogenous level.

EFFECTS OF ETHYLENE MODULATORS ON ETHYLENE BIOSYNTHESIS UNDER HM STRESS

It has been evident that the ethylene biosynthesis pathway is well regulated under HM stress in plants. The increase of endogenous ethylene levels under HM stress caused negative effects on plant growth and developmental processes (Maksymiec, 2011; Schellingen et al., 2014). By contrast, reducing HM-induced ethylene production to keep ethylene at an optimized level shows the positive regulatory role of ethylene in plant responses to various HMs (Maksymiec, 2011). Understanding these important issues, scientists have been able to control plant growth and development under HM stress conditions, including Cd, Ni, and Zn stresses, using ethylene action or ethylene biosynthetic inhibitors at low concentrations (Maksymiec and Krupa, 2007; Khan et al., 2015b). More interestingly, the inhibitors of ethylene production do not protect the commodity from exogenous ethylene (Zhang and Wen, 2010; Iqbal et al., 2012). They disrupt the ethylene biosynthesis pathway by targeting either ACS or ACO, whereas ethylene action inhibitors occupy ethylene receptors and block ethylene action (Serek et al., 2006).

Co, a beneficial metal for plant development at moderate levels, is known as an inhibitor of ethylene production (Palit et al., 1994; Yıldız et al., 2009; Chmielowska-Bąk et al., 2014). Although many studies showed that Cd, Cu, Fe, and Zn induce ethylene production in plants (Wise and Naylor, 1988; Maksymiec, 2007), excessive Co treatment of HM-stressed plants does not lead to enhanced ethylene levels, since Co inhibits the ACO enzymatic activity in the ethylene synthetic pathway. Thus, Co has been widely used as an ethylene biosynthesis inhibitor to study the effects of ethylene on plant responses to HM stress (Sun et al., 2010; Chmielowska-Bąk et al., 2014). However, in soybean (*Glycine max*) seedlings, coapplication of Co and Cd negatively affected cell viability as well as the expression of Cd-induced genes encoding MAPK KINASE2, DNA BINDING WITH ONE FINGER1 (DOF1), and BASIC LEUCINE ZIPPER2 (bZIP2) transcription factors, suggesting that Co increased Cd toxicity to soybean plants and that this happened independently from ethylene action (Chmielowska-Bąk et al., 2014). Moreover, excessive Co also increased oxidative stress and photosynthesis inhibition as well

as caused alterations in germination, sex ratio, photoperiodism, and uptake of other elements (Yıldız et al., 2009; Hasan et al., 2011). Therefore, the use of Co as an ethylene biosynthesis inhibitor in research should be interpreted with caution.

AVG, another inhibitor of ethylene synthesis, has been shown to decrease ethylene production by inhibiting ACS activity (Masood et al., 2012). Iakimova et al. (2008) reported that the combination of ethylene and Cd treatments to tomato (*Solanum lycopersicum*) suspension cells resulted in increased cell death, which could be rescued by adding AVG (Fig. 1). Besides the application of ethylene biosynthesis inhibitors, ethephon, an exogenous ethylene-releasing compound, has also been widely used to control endogenous ethylene production and function under Cd stress (Masood et al., 2012) and Ni or Zn stress (Khan and Khan, 2014). Although under nonstressed conditions, ethephon treatment has been shown to increase the level of endogenous ethylene in plants (Cooke and Randall, 1968; Khan, 2004), interestingly, the level of HM-induced ethylene was shown to be decreased by ethephon treatment, which led to the induction of an antioxidant system and increased photosynthesis. As a result, ethephon-treated plants were found to be more tolerant to HM stress (Masood et al., 2012; Khan and Khan, 2014). More investigations should be carried out to better clarify the role of ethephon in the regulation of ethylene homeostasis and sensitivity under HM stress.

ETHYLENE SIGNALING AND PLANT RESPONSES TO HM STRESS

Ethylene receptors are similar to bacterial two-component receiver domains. Ethylene in Arabidopsis is perceived by a five-member family of ethylene receptors, including products encoded by the *ETR1* and *ETR2*, *ETHYLENE RESPONSE SENSOR1* (*ERS1*) and *ERS2*, and *EIN4* genes (Clark et al., 1998; Yoo et al., 2009). In Arabidopsis, in the absence of ethylene, *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), a Raf-like MAPK KINASE KINASE, interacts with the ethylene receptors to suppress the downstream component *EIN2* by directly phosphorylating its cytosolic C-terminal domain, leading to the inactivation of *EIN3* and *ETHYLENE-INSENSITIVE3-LIKE1* (*EIL1*; Guo and Ecker, 2004; Ju et al., 2012; Shan et al., 2012). Upon the binding of ethylene to the receptors with the help of the Cu ions delivered by the Cu transporter *RESPONSIVE TO ANTAGONIST1* (*RAN1*), *CTR1* becomes inactivated, consequently resulting in the cleavage of *CARBOXYL END OF EIN2* from the endoplasmic reticulum-located *EIN2*. As a result, the moving of *EIN2* to the nucleus is facilitated, which leads to the stabilization of *EIN3* protein that initiates the signaling cascade (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). The MAPK cascade has been shown to be involved in ethylene signaling and/or ethylene biosynthetic pathways by targeting at least *ACS2* and *ACS6* (Liu and

Zhang, 2004; Hahn and Harter, 2009; Yoo et al., 2009; Opdenakker et al., 2012). Under HM stress, such as Cd stress, ethylene production has also been found to be induced mainly through the accumulation of *ACS2* and *ACS6* transcripts (Schellingen et al., 2014). The Arabidopsis *acs2-1 acs6-1* double knockout mutant exposed to Cd showed a decreased ethylene level, leading to a positive effect on leaf biomass (Schellingen et al., 2014), suggesting the negative regulation of HM stress-induced ethylene in plant development. As the number of studies on ethylene signaling under HM stress has been limited, more effort should be taken in this important research area.

Since blockers of the ethylene receptor protect the tissues from both endogenous and exogenous ethylenes, ethylene action inhibitors are considered very potent for agricultural use (Sisler and Serek, 1997; Feng et al., 2000). They are more specific than ethylene biosynthetic inhibitors because they bind to a specific receptor (Sisler and Serek, 1997; Hua and Meyerowitz, 1998; Klee, 2004). The use of 1-MCP, a blocker of ethylene action in plants, has been reviewed extensively (Sisler and Serek, 1997; Blankenship and Dole, 2003), and numerous applications of 1-MCP in the amelioration of stress responses in plants have been reported (Grimmig et al., 2003; Huang and Lin, 2003; Yokotani et al., 2004). Recently, Montero-Palmero et al. (2014b) reported the involvement of ethylene as a negative regulator of mercury (Hg)-induced responses in alfalfa (*Medicago sativa*) using 1-MCP. Similarly, the application of STS, an inhibitor of ethylene reception, is another efficient means of controlling ethylene action and thus is being used for both agronomic and research purposes (Ichimura and Niki, 2014; Pacifici et al., 2014). Silver is thought to occupy the Cu-binding site of ethylene receptors and to interact with ethylene to inhibit the ethylene response (Rodríguez et al., 1999; Zhao et al., 2002; Binder et al., 2007). NBD, the third ethylene action inhibitor compound, is also a very common tool used to reduce ethylene-induced stress effects under Ni and Zn treatment (Sisler and Serek, 1997; Khan and Khan, 2014). Using NBD, which was expected to inhibit ethylene action by blocking receptors, Khan and Khan (2014) have verified the involvement of ethylene in the reversal of photosynthetic inhibition by Ni and Zn stress, which was caused by changes in PSII activity, and the enhancement of photosynthetic nitrogen use efficiency and antioxidant capacity. These findings together suggest that appropriate control of ethylene action using ethylene action inhibitors could lead to the positive regulation role of this hormone in plant responses to HM stress.

ETHYLENE AND ITS CROSS TALK WITH OTHER HORMONES AND SIGNALING MOLECULES IN THE REGULATION OF PLANT TOLERANCE TO HM STRESS

The molecular mechanism of how plants can cope with different HM stresses varies from plant to plant, but in general, ethylene and its cross talk with other

phytohormones or with signaling molecules are important for plant adaptation to HM-induced oxidative stress (Thapa et al., 2012; Montero-Palmero et al., 2014a, 2014b). It has been found that not only the production of ethylene but other phytohormones are also affected by excessive HM. Upon exposure to the stress, the levels of jasmonic acid (JA), salicylic acid (SA), abscisic acid, and ethylene increase, while the contents of GA₃ and auxin decrease in plants (Metwally et al., 2003; Cánovas et al., 2004; Atici et al., 2005; Maksymiec et al., 2005).

Taking a case study of aluminum (Al) application in *Arabidopsis* as an example, it was observed that Al treatment led to the increased expression of ethylene biosynthesis-related genes, including both *AtACS* (*AtACS2*, *AtACS6*, and *AtACS8*) and *AtACO* (*AtACO1* and *AtACO2*) genes (Sun et al., 2010). Moreover, in wild-type plants, this Al treatment also increased the transcript of *AUXIN RESISTANT1* (*AtAUX1*) and *PINFORMED2* (*AtPIN2*), yet the ethylene synthesis inhibitors Co and AVG, and the ethylene perception inhibitor silver, abolished this Al-induced expression of *AtAUX1* and *AtPIN2*. In the auxin-insensitive single mutants *aux1-7* and *pin2*, the Al-induced inhibition of root elongation was lower than that in the wild type. These data suggested that Al-induced ethylene production may lead to auxin redistribution by affecting auxin polar transport systems through AUX1 and PIN2 (Sun et al., 2010), which is an indicator of possible cross talk between ethylene and auxin in plant responses to HM stress. Interestingly, it was not PIN2 or AUX1 but PIN1 that was reported to be required for Cu-induced auxin redistribution in *Arabidopsis* (Yuan et al., 2013). Furthermore, the study of Yuan et al. (2013) also showed that both *ein2-1* and wild-type plants exhibited similar effects on the inhibition of primary root elongation under Cu stress, indicating that ethylene-mediated signaling is not required for the Cu-inhibited primary root elongation. Together, these findings suggested that genes involved in the control of auxin redistribution might be specific, and they act dependently or independently of ethylene/ethylene signaling, depending on the type of HMs to which the plants are exposed.

Recently, the ethylene and JA signaling pathways have been shown to converge at two ethylene-stabilized transcription factors, EIN3 and EIL1, and to function synergistically in the regulation of gene expression in *Arabidopsis* (Zhu et al., 2011). Moreover, other studies further indicated that the posttranslational regulation of ERFs by ethylene and JA was independent of EIN3/EIL1 (Bethke et al., 2009; Van der Does et al., 2013). When *Arabidopsis* plants were exposed to excessive Cd, these two hormone signaling pathways were activated, leading to the up-regulation of *NITRATE TRANSPORTER1.8* (*NRT1.8*) and the down-regulation of *NRT1.5*, which mediated the stress-initiated nitrate allocation to roots to enhance the tolerance to Cd stress (Zhang et al., 2014).

By studying the *gibberellin insensitive ethylene overproducing2-1* double mutant, a functional GA₃ signaling

pathway was shown to be required for the increased ethylene biosynthesis in *Arabidopsis*, suggesting a possible link between ethylene and GA₃ (De Grauwe et al., 2008). More recently, Masood and Khan (2013) suggested that treatment with GA₃ and/or sulfur (S) at sufficient levels reduced undesirable stress ethylene induction, resulting in the alleviation of photosynthetic inhibition caused by Cd stress. It is well established that S assimilation leads to Cys biosynthesis, which is required for both ethylene and GSH biosyntheses under normal conditions (De Grauwe et al., 2008; Iqbal et al., 2013). On the other hand, under HM stress, application of S to Cd-treated plants was reported to adjust stress-induced ethylene content to an optimized level, which subsequently led to a maximal GSH content, thereby providing effective protection against oxidative stress and, thus, alleviating unbeneficial Cd-induced symptoms in plants (Asgher et al., 2014). Furthermore, both ethylene and S assimilation pathways were also affected by Cd stress and were shown to regulate GSH biosynthesis under Cd stress (Masood et al., 2012). This further suggested the role of the GSH pathway in the mitigation of HM stress through ethylene and ethylene signaling that might also involve the S pathway (i.e. the GSH pathway might be the check point of the cross talk between S and ethylene in plant responses to HM stress). The role of GSH in HM detoxification might be explained by numerous physiological, biochemical, and genetic studies that have confirmed that GSH is the substrate for phytochelatin (PC) biosynthesis (Cobbett, 2000). In *Arabidopsis* and fission yeast (*Schizosaccharomyces pombe*), PCs were shown to play an important role in Cd and arsenic detoxification by using PC synthase-deficient mutants (Ha et al., 1999). Down-regulation of *GSH1* and a decrease in GSH content were observed in the *Arabidopsis ein2-1* mutant, which led to the impaired GSH-dependent Pb tolerance (Cao et al., 2009), indicating that ethylene signaling positively regulates HM responses through the GSH pathway. On the other hand, there was also evidence that the *EIN2* gene mediates Pb resistance through a GSH-independent *PLEIOTROPIC DRUG RESISTANCE TRANSPORTER12* (*AtPDR12*)-mediated mechanism (Cao et al., 2009). *PDR12*, which is a member of the ATP-binding cassette transporter G family and is induced by auxin, abscisic acid, ethylene, JA, and SA, was reported to be up-regulated in *Arabidopsis* plants treated with AuCl⁻⁴ (Shukla et al., 2014).

ROS itself was also reported to have an interaction with ethylene in plant responses to HMs. Ethylene and hydrogen peroxide were believed to act in a synergistic manner in tomato, and hydrogen peroxide plays an important role in ethylene-related Cd-induced cell death (Liu et al., 2008). Several studies have shown that HMs, such as Cd, Cu, Fe, Zn, Hg, manganese, and Al, can induce ROS production and alter the activities of antioxidant enzymes, including catalase, superoxide dismutase (SOD), peroxidase, ascorbate peroxidase (APX), and glutathione reductase (GR), in plants (Sun

et al., 2010; Yuan et al., 2013; Montero-Palmero et al., 2014a; Khan et al., 2015b; Mostofa et al., 2015b). It was found that the application of ethephon or NBD could somehow adjust the stress-induced ethylene, thereby alleviating photosynthetic inhibition and decreasing oxidative stress, perhaps by the enhancement of SOD, APX, and GR metabolism, in mustard plants treated with Ni and Zn (Khan and Khan, 2014). More recently, Liu et al. (2010) reported that pretreatment of Cd-stressed *Arabidopsis* plants with GSH, a ROS scavenger, inhibited the activation of MAPK3 and MAPK6, which had been activated by Cd-induced ROS accumulation. MAPK3 and MAPK6 have been demonstrated to be involved in the regulation of ethylene biosynthesis and potentially in the ethylene signaling pathway, although this last possibility remains controversial (Ecker, 2004; Hahn and Harter, 2009), providing a hint about the potential interaction between ROS and ethylene through these MAPKs in the regulation of plant responses to HM stress.

In response to HMs, not only ethylene but other hormones, including brassinosteroids, auxin, SA, GA₃, and cytokinin, were shown to stimulate the antioxidant responses in order to scavenge different ROS when plants were grown under Cd, Cu, or Pb stress (Hayat et al., 2007; Noriega et al., 2012; Piotrowska-Niczyporuk et al., 2012). SA treatment increased the GSH content and resulted in an induction of antioxidant and metal detoxification systems, which led to Cd stress tolerance in wheat (*Triticum aestivum*) and pea (*Pisum sativum*) as well as amelioration of the negative effects of Cu stress in *Brassica napus* (Srivastava and Dwivedi, 1998; Khademi et al., 2014; Kovács et al., 2014). In contrast, JA was found to increase metal bio-sorption and ROS generation in the green microalga *Chlorella vulgaris* (Chlorophyceae) exposed to excessive Cd, Cu, or Pb (Piotrowska-Niczyporuk et al., 2012). Moreover, ROS production was triggered by JA in *Arabidopsis* treated with Cu or Cd (Maksymiec and Krupa, 2006). However, it has also been reported that JA-induced ROS is mediated by the oxidative status of GSH and that JA induced the expression of GSH metabolic genes (Xiang and Oliver, 1998; Mhamdi et al., 2010). Thus, the mechanism of how JA is involved in HM-induced oxidative stress and plant tolerance still requires further experiments. It would be interesting to see the changes in the levels of all other hormones, ROS, and antioxidant systems in ethylene-deficient or -overproducing plants under normal and HM stress conditions to learn more about the cross talk between ethylene and other hormones in plant responses to HM stress.

Nitric oxide (NO), another signaling molecule, is well known to have a regulatory role in various plant responses, including ethylene emission (Leshem and Haramaty, 1996), biotic and abiotic responses (Leshem and Haramaty, 1996; Clark et al., 1998; Durner et al., 1998; Delledonne et al., 2001; Mostofa et al., 2015a), cell proliferation and plant development (Ribeiro et al., 1999), senescence (Corpas et al., 2004), programmed cell

death (Magalhaes et al., 1999; Clarke et al., 2000; Pedroso et al., 2000), and stomatal closure (García-Mata and Lamattina, 2002; Neill et al., 2002). However, similar to ethylene, NO plays a controversial role in HM tolerance. Exogenous NO was shown to contribute to the enhancement of plant tolerance to excessive Cd, Ni, and Al (Laspina et al., 2005; Wang and Yang, 2005; Singh et al., 2008; Kazemi et al., 2010), whereas endogenous NO was reported to be involved in Cd toxicity in plants (Groppa et al., 2008; Besson-Bard et al., 2009; Ma et al., 2010). Recently, it was reported that the Cd-induced activation of MAPK6 is mediated by NO (Hahn and Harter, 2009; Ye et al., 2013), which might suggest a link between NO and ethylene through MAPK6 in plant responses to HM stress. NO could act as an antioxidant to scavenge ROS and, directly or indirectly, increase the activity of antioxidant enzymes in leaves of plants treated with Ni or Cd (Kazemi et al., 2010; Ye et al., 2013). The accumulation of ethylene and ROS, and the diminution of NO, led to Cd-induced senescence processes in pea (Rodríguez-Serrano et al., 2006). Moreover, ethylene, polyamines, NO, MAPKs, and several transcription factors, including MYB22, bZIP62, and DOF1, were proposed to integrate the responses to short-term Cd stress in young soybean seedlings (Chmielowska-Bak et al., 2014). Together, these findings further suggest a possible role of NO in the HM-induced ethylene pathway. On the other hand, under Ni stress, application of both NO and SA significantly reduced Pro accumulation, lipid peroxidation, and ROS level in *Brassica napus* leaves as well as improved the chlorophyll content, thus reducing the toxic effects of Ni on this crop plant (Kazemi et al., 2010). These findings collectively indicate a complex mechanism of how phytohormones, including ethylene, and signaling molecules interact in response to HMs (Fig. 2).

IMPROVEMENT OF PLANT TOLERANCE TO HM: AN APPROACH OF MODIFYING ETHYLENE ACTION

HM stress has become a significant concern because of its severe impact on human health and plant productivity (Thapa et al., 2012). Understanding the changes in ethylene biosynthesis and signaling triggered by HMs at the molecular level may help identify gene(s) responsible for the expression of an HM-tolerant genotype, thus providing biotechnological approaches to improve plant fitness in HM-polluted areas.

Manipulation of ethylene response/signaling and/or ethylene endogenous production plays an important role in the improvement of plant HM tolerance (Asgher et al., 2014; Khan and Khan, 2014; Khan et al., 2015b; Table I). Several studies have proved that the application of ethylene biosynthesis modulators adjusted endogenous stress-induced ethylene content to an optimized level and, consequently, resulted in beneficial effects in plants treated with Ni and Zn (Iqbal et al.,

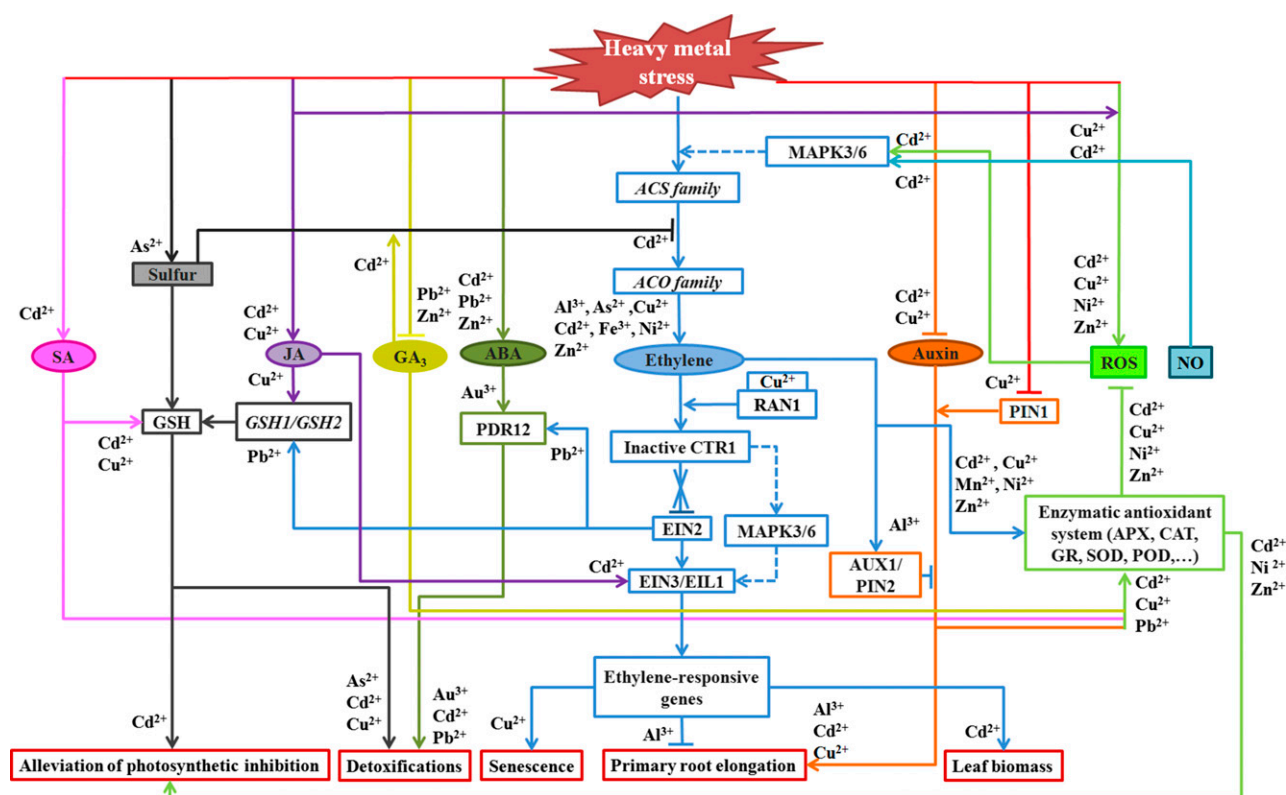


Figure 2. Generalized model of ethylene biosynthesis and signaling pathways under HM stress in cross talk with other phytohormones and signaling molecules. Different colors show different networks of ethylene, auxin, SA, JA, GA₃, abscisic acid (ABA), ROS, NO, and S assimilation in plants under HM stress. Arrows and T-bars indicate positive and negative regulatory interaction, respectively. Dashed lines indicate possible regulation under HM stress. The cross represents release from inhibition. Au, Gold; CAT, catalase; Mn, manganese.

2012; Khan and Khan, 2014), Cd (Iakimova et al., 2008; Sun et al., 2010; Chmielowska-Bak et al., 2014), or Al (Sun et al., 2010). Additionally, S application has proved to be effective in the alleviation of Cd stress, which was related to the reduction of undesirable stress-induced ethylene production in mustard, suggesting that S might be used to optimize the ethylene level for developing HM stress-tolerant cultivars as well (Asgher et al., 2014; Khan et al., 2015a). Furthermore, a combined treatment of mustard plants with GA₃ and/or S decreased Cd-induced stress ethylene production and promoted a photosynthetic response to Cd stress (Masood and Khan, 2013). As supportive evidence for the approach of reducing stress ethylene levels to improve HM tolerance, Schellingen et al. (2014) reported that the ethylene-deficient *acs2-1 acs6-1* double mutant showed alleviated growth inhibition of leaves in Cd-exposed Arabidopsis plants, as discussed earlier. These findings together suggest that the alteration of endogenous levels of ethylene can be used to mitigate the HM toxicity of plants, and the manipulation of endogenous ethylene levels, therefore, can be considered as a potential biotechnological approach for the development of crop cultivars with improved HM tolerance.

However, in many floral plants, targeting the ethylene signal transduction pathway is a preferred strategy (Ma et al., 2014). The ethylene-insensitive *Nr* mutant of tomato avoided or withstood Cd-induced stress by increasing antioxidant enzymes and affecting the intercellular spaces and the size of the mesophyll (Gratao et al., 2009; Monteiro et al., 2011). A single amino acid change in the sensor domain of *Nr* (LeETR3), which shows high homology to the Arabidopsis ethylene receptor ETR1, resulted in the loss of its capacity to respond to either endogenously generated or exogenously applied ethylene (Lanahan et al., 1994; Wilkinson et al., 1995). This observation in the *Nr* mutant has suggested that not only the manipulation of ethylene production but also of ethylene perception can be used to control plant responses to HM stress. Other studies also suggested that an appropriate control of ethylene signaling could be used as a biotechnological approach to improve HM stress tolerance. In Arabidopsis, *EIN2* gene function was found to be required for plant Al and Hg sensitivities, as root growth inhibition under HM stress was alleviated in all the Arabidopsis *ein2-1*, *ein2-5*, and *etr1-3* single mutants (Sun et al., 2010; Montero-Palmero et al., 2014a). By contrast, the *EIN2* gene was reported to be important for Pb resistance in

Table 1. Summary of the experimental manipulation of ethylene levels and the ethylene signaling pathway in plant responses to HM stress. The ↓ and ↑ arrows indicate decrease and increase, respectively. *Nr*, Never ripe.

Stress	Species	Genetic Approaches	Physiological Traits	References
Al	Arabidopsis	<i>etr1-3</i> mutant	↓ Inhibition of root elongation	Sun et al. (2010)
Al	Arabidopsis	<i>ein2-1</i> mutant	↓ Inhibition of root elongation	Sun et al. (2010)
Cd	Arabidopsis	<i>acs2-1 acs6-1</i> double mutants	↓ Inhibition of leaf biomass	Schellingen et al. (2014)
Cd	Tomato	<i>Nr (LeETR3)</i> mutant	↓ Root diameter	Gratao et al. (2009)
Cd	Tomato	<i>Nr (LeETR3)</i> mutant	Maintenance of pigment content; ↓ leaf senescence	Monteiro et al. (2011)
Cd + S	<i>B. juncea</i>	None	Optimization of ethylene level; ↓ undesirable Cd-induced symptoms	Asgher et al. (2014)
Cd + GA ₃ + S	<i>B. juncea</i>	None	Optimization of ethylene level; ↓ undesirable Cd-induced symptoms	Masood and Khan (2013)
Cd + ethephon + S	<i>B. juncea</i>	None	↑ Ethylene sensitivity; ↑ photosynthesis	Masood et al. (2012)
Cd + STS	<i>P. coccineus</i>	None	↓ Inhibition of leaf growth	Maksymiec (2011)
Cu	Arabidopsis	<i>ein2-1</i> mutant	Similar inhibition of root elongation relative to the wild type	Yuan et al. (2013)
Hg	Arabidopsis	<i>ein2-5</i> mutant	↓ Inhibition of root growth	Montero-Palmero et al. (2014a)
Ni + Zn + ethephon	<i>B. juncea</i>	None	Optimization of ethylene level; ↓ photosynthetic inhibition	Khan and Khan (2014)
Pb	Arabidopsis	<i>ein2-1</i> mutant	Inhibition of root length; ↑ Pb content; ↓ GSH content	Cao et al. (2009)

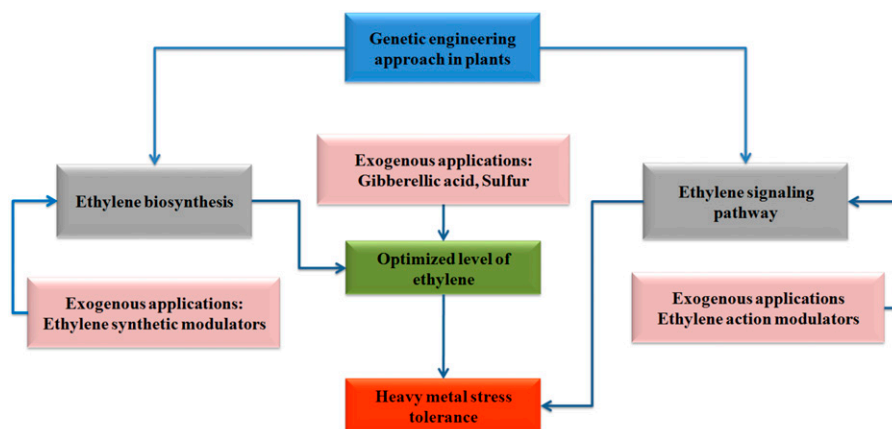
Arabidopsis plants (Cao et al., 2009), suggesting that the role of ethylene in plant responses to HM stress is complex and, perhaps, depends on the types of HMs to which the plants are exposed.

It is noteworthy that the manipulation of ethylene signaling-related genes encoding upper components in the ethylene pathway, between the receptor and EIN2, such as knocking out *OsETR2* or *OsCTR2*, normally causes a pleiotropic phenotype (Wuriyanghan et al., 2009; Wang et al., 2013). The tissue-specific or stress-inducible promoter should be considered for use to alleviate these side effects (Ma et al., 2014). Additionally, ERF transcription factors were reported to play an important role in regulating the expression of specific stress-related genes under

Cd stress (DalCorso et al., 2010). Because each form of ERFs is likely to be involved in a specific response mechanism pathway to cope with stress, *ERF* genes are highly considered as ideal targets for a genetic engineering approach on ethylene action in order to improve plant tolerance while conferring minimal pleiotropic effects (Ma et al., 2014).

In addition, the use of ethylene action inhibitors to alleviate stress symptoms in plants exposed to various HM stresses, including Al (Sun et al., 2010), Hg (Montero-Palmero et al., 2014b), Cd (Maksymiec, 2011), and Ni or Zn (Khan and Khan, 2014), has been discussed previously in this review. An integrated approach for the improvement of plant tolerance to HM stress is presented in Figure 3.

Figure 3. Potential targets for biotechnological applications to improve crop tolerance to HM stress.



CONCLUSION AND FUTURE PERSPECTIVES

HM contamination and its toxicity have been recognized as a substantial threat to sustainable agriculture worldwide. Current research has shown a significant contribution of ethylene in the regulation of physiological processes and the mediation of HM tolerance in plants. However, a clear model of ethylene under HM stress is not easy to be drawn, since its regulatory role in plant responses to HM stress may lead to positive or negative effects on plant growth and reproduction. Since most up-to-date studies about the roles of ethylene and its signaling under HM stress have involved mostly physiological aspects, a molecular approach using mutants should take the lead in future studies in order to gain an in-depth understanding of the regulatory functions of ethylene in plant responses to HM stress at the molecular level. This will enable us to appropriately control the homeostasis of ethylene for the improvement of plant adaptation to HM stress as well as to open potential opportunities to select appropriate ethylene-related genes and promoters as promising candidates for genetic engineering aimed at developing HM stress-tolerant crop varieties.

In addition, as the conventional plant breeding methods for improving plant tolerance to HM stress are time consuming and costly, the use of ethylene modulators for optimizing ethylene can be a wise strategy to enhance HM tolerance with minimal side effects. To effectively apply this strategy, knowledge of the relationship (antagonism/synergism) between ethylene and ethylene-responsive genes, or between ethylene and other factors (other phytohormones/other signaling molecules) for HM stress tolerance, is equally valuable. Therefore, more efforts should be made to gain a better understanding of ethylene biology, ethylene cross talk with other signaling molecules, and HM stress tolerance in the whole context, which will surely bring more benefits for both basic and applied research in the future.

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