Ethylene Captures a Metal! Metal Ions Are Involved in Ethylene Perception and Signal Transduction

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More than three decades ago, transition metal such as copper or zinc were postulated to be required for the ethylene perception. However, there was no direct evidence for this metal requirement until very recently. Two studies using Arabidopsis thaliana, one genetic and the other biochemical, have provided complementary evidence for the role of copper in ethylene perception, closing this argument. Additional evidence for the importance of the metal in the ethylene-signaling pathway came with the recent discovery that EIN2, a central signal transducer in the ethylene-signaling pathway, has significant homology to the Nramp divalent cation transporters. These studies suggest that metal metabolism may have a critical role not only in ethylene perception but also in ethylene signaling.

Key words: Arabidopsis — Ccc2 protein — Ethylene — Menkes/Wilson disease protein — Nramp — Signal transduction.

A gaseous hormone ethylene is involved in diverse developmental and physiological processes of plants (Smalle and Van der Straeten 1997, Johnson and Ecker 1998). Treatment of etiolated seedlings with ethylene evokes dramatic morphological changes referred to as the “triple response” that includes exaggerated apical hook, radial swelling of hypocotyl and inhibition of hypocotyl and root elongation in Arabidopsis. These morphological changes are highly specific for ethylene. A genetic approach that relies on the triple response phenotype as a morphological marker has allowed the identification of several classes of mutants with impaired responses to ethylene. In Arabidopsis, these mutants can be classified into three groups; ethylene insensitive mutants [etr1 (Bleecker et al. 1988), etr2 (Sakai et al. 1998), ein2 (Guzman and Ecker 1990), ein3, ein4, ein6 (Roman et al. 1995) and ein5/ain1 (Van der Straeten et al. 1993, Roman et al. 1995)], constitutive ethylene response mutants [eto1, 2, 3 and ctrl (Guzman and Ecker 1990, Kieber et al. 1993)] and tissue-specific ethylene response mutants [hls1 (Lehman et al. 1996) and eir1 (Roman et al. 1995)]. Based on the results from extensive genetic studies with these mutants, a model has been drawn for the ethylene-signaling pathway, in which identified components act in a linear pathway (Ecker 1995, Roman et al. 1995). Isolation of the corresponding genes and molecular analysis of encoded proteins have greatly facilitated our understanding of the ethylene signaling pathway at the molecular level (Johnson and Ecker 1998, Solano and Ecker 1998, Chang and Shockey 1999).

According to the current view, the ethylene molecule is sensed by ethylene receptors, ETR1 and its related proteins, localized in the plasma membrane. A downstream component CTR1 encodes a Raf-type protein kinase, suggesting that a MAP-kinase cascade functions in the ethylene-signaling pathway (Kieber et al. 1993). EIN2, a membrane anchored protein, is thought to act downstream of CTR1 (Roman et al. 1995, Alonso et al. 1999). Further downstream, a new class of DNA binding protein, which includes EIN3 and its related proteins (EIL1, 2, 3), is responsible to the ethylene-mediated transcriptional activation of ethylene-inducible genes (Chao et al. 1997, Solano et al. 1998).

Recently, the linkage between metal ion homeostasis and ethylene-signaling system has been revealed. For more than three decades, it has been postulated that the recognition of ethylene, the smallest of the olefins, by the plant cells would require a transition metal such as copper or zinc (Burg and Burg 1967). Although this idea has been generally accepted, direct evidence for the requirement of metals in ethylene sensing has been lacking, even after the molecular cloning of receptor genes. Two recent studies, one biochemical and the other genetic, have provided strong evidence for the copper requirement in ethylene perception by the receptor proteins (Hirayama et al. 1999, Rodriguez et al. 1999). Metals may also participate in the ethylene signaling at the EIN2 level. Because of the complete ethylene insensitivity of ein2 mutant, EIN2 is believed to be a pivotal signal transducer in ethylene signaling. Interestingly, cloning of the EIN2 gene and functional analysis of

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Abbriviation: TCO, trans-cyclooctene.

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EIN2 protein using transgenic plants surprisingly suggest that EIN2 functions as a sensor for a divalent cation(s), offering another linkage between metal metabolism and ethylene signaling. In this brief review, the involvement of metal ions in the ethylene perception will be summarized. Along the way, the possible physiological roles of the metal ion metabolism in the ethylene signal transduction pathway will be discussed.

**Copper requirement in ethylene perception**

**Historical overview**—Small gaseous molecules act as signal transmitters in various organisms. In most cases, these signal molecules coordinate with their targets through transition metals. For example, NO in animal cells binds to GMP cyclase via an iron containing heme group and activates it. Burg and Burg studied the structural requirements of olefins for triggering an ethylene response and found a correlation between the ability of olefins to exert ethylene-like biological activity and ability to make a complex with silver ion. Based on this study, they proposed that ethylene receptors should contain metal ions. At that time, they speculated that zinc could be the best candidate since zinc-deficient tomato plants could not respond to ethylene (Burg and Burg 1967). In the 80’s, the characterization of copper-ethylene complex by spectroscopic and X-ray diffraction analyses revealed that copper-ethylene coordination chemistry is consistent with the idea of copper requirement in ethylene binding (Thompson et al. 1983). In the mid 90’s, the ethylene receptor genes, ETR1 and its related genes, were cloned (Chang et al. 1993). The predicted encoded proteins had membrane spanning domains and a cytoplasmic histidine kinase domain, characteristics consistent with the idea of ethylene receptors. The ethylene binding activity of the recombinant ETR1 protein was demonstrated in yeast cells, providing strong evidence for its function as an ethylene receptor (Schaller and Bleeker 1995). However, ETR1 and ETR1-like ethylene receptors lacked any known metal binding motifs. No direct evidence for the requirement of metals in ethylene perception has been presented until very recently.

**A genetic approach; RESPONSIVE-TO-ANTAGONIST1 mutants of Arabidopsis**—Two responsive to antagonist1 (ran1) mutants of Arabidopsis (ran1-1 and ran1-2) were isolated as mutants that showed an alternate ligand specificity in the ethylene perception (Hirayama et al. 1999). The ran1 mutants display the ethylene response phenotype in the presence of trans-cyclooctene (TCO), a potent ethylene competitive binding inhibitor. Activation of the ethylene-signaling pathway by TCO in ran1 mutants indicates that ran1 mutation changes the ligand specificity of ethylene receptors. The results from genetic analysis indicated that RAN1 acted at or upstream of ETR1, further indicating the involvement of RAN1 in the ethylene recognition. Interestingly, map-based positional cloning of the RANI gene and sequencing analysis revealed that the predicted RAN1 protein has a significant similarity to P-type copper transporting ATPases, such as human Menkes/Wilson disease proteins, and yeast Ccc2p (for review (Eide 1998, Camakaris et al. 1999, Nelson 1999)). According to recent studies these copper transporters are located in the membrane of the post-Golgi compartments and function in copper ion homeostasis. The copper ions are received by these copper transporters and delivered to specific proteins on the secretory pathway. Complementation analysis using yeast ccc2-disrupted mutants proved that RANI protein had a copper-transporting activity. Since mutations in both alleles of ran1 mutants were localized in the functional domains of copper transporter, it is likely that a reduction in copper transport activity of RANI protein causes a change in the ligand specificity of ethylene receptor. This idea is supported by the fact that when CuSO₄ is added to the medium, the TCO-induced triple response phenotype of ran1 mutants is suppressed (see later). These results strongly suggested that copper is an important cofactor for ethylene perception. The involvement of RANI in ethylene signaling is further demonstrated by the observation that co-suppression of the RANI gene by a 35S::RANI transgene results in the constitutive ethylene response phenotype.

Copper is an essential metal for aerobic organisms. It serves as a co-factor for enzymes such as cytochrome c oxidase, copper-zinc superoxide dismutase, lysyl oxidase, and dopamine β-hydroxylase, which are required for cell respiration, scavenging active oxygen, the maturation of connective tissues and neurotransmitter synthesis in animals, respectively (for review (Uauy et al. 1998)). Since copper is highly toxic, the intracellular concentration of this metal has to be tightly regulated, and extra copper ions are sequestered in non-reactive forms. Detailed analysis of copper metabolism in budding yeast cells has uncovered the sophisticated mechanisms for the regulation of intracellular copper ion concentrations. Yeast Ctr1p is a plasma membrane-localized copper transporter that imports copper ions into the cytoplasm. Imported copper ions are immediately bound by copper trafficking proteins. There are several distinct copper-trafficking proteins known, including Atx1p, Lys7p and Coo17p, which differ in their intracellular targets. Atx1p delivers copper ions to Ccc2p in the membrane of post-Golgi compartments, where Ccc2p, in turn, imports copper ions into the secretory pathway (Yuan et al. 1995). Lys7p delivers copper ions to Sod1p, a Cu-Zn superoxide dismutase, and Coo17p transports copper to cytochrome c oxidase in the mitochondria.

Mutations in copper-trafficking genes cause physiological disorders in many eukaryotes, supporting the idea that the proper regulation of copper metabolism is crucial. In fact, the basic mechanisms of copper transport and metabolism seem to be highly conserved in evolution.
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(Askwith and Kaplan 1998). In addition to the Menkes/Wilson disease genes (yeast CCC2/Arabidopsis RANI), human homologues of the yeast cytosolic copper metabolism genes have been uncovered, including human 
Cr1 gene [a homologue of yeast CTR1, (Zhou and Gitschier 1997)], human 
Hahl gene [a homologue of yeast ATX1, (Klomp et al. 1997)], human 
Cesl gene [a homologue of 
LYS7, (Culotta et al. 1997)] and human 
Cox17 gene [a homologue of yeast 
COX17, (Amaravadi et al. 1997)]. In 
Arabidopsis, several expressed sequence tags with significant similarity to the functional equivalent of yeast Atxlp have been identified [for example, 
CCH, (Himelblau et al. 1998)]. It is likely that other steps in the intracellular trafficking copper ions may also be conserved in the plant kingdom.

Based on the results from RANI study and by analogy with the function of yeast Ccc2p and human Menkes/Wilson disease proteins, we postulated that RANI is localized in the membrane of a post-Golgi compartment, transporting copper ions from Atx1-like proteins into the lumen of the post-Golgi compartment (Fig. 1). In the lumen, accumulated copper ions are incorporated into membrane-targeted ethylene receptor apoproteins. Once translocated to plasma membrane, the ethylene receptors are able to sense ethylene. In wild-type plants, ethylene inactivates the receptors upon binding (Hua and Meyerowitz 1998), presumably by causing a reduction in histidine kinase activity. This, in turn, results in the derepression of the downstream components in the signaling pathway and activation of these hormone response phenotypes. A severe

Fig. 1 A model for the function of RAN1 in the ethylene signaling pathway in Arabidopsis. RAN1 is presumed to be localized in the membrane of a post-Golgi compartment. Copper ions received from CCH, a putative copper chaperon, is transported by RAN1 into a post-Golgi compartment, delivering the metal to membrane-targeted ethylene receptor apoproteins that become able to coordinate ethylene after the incorporation of copper ions. In the absence of the hormone, the receptors are active and negatively regulate downstream signaling components, preventing hormone response phenotypes. Ethylene is expected to inactivate the receptors upon binding, presumably by causing a reduction in histidine kinase/phosphatase activity. This, in turn, results in derepression of downstream signaling components (EIN2, EIN3) and activation of hormone response phenotypes. The metal-deficient ethylene receptors are nonfunctional, resulting in a constitutively activated signaling pathway. Red and blue shapes indicate the active and inactive states of ethylene-signaling pathway components, respectively.
reduction of copper supply in RAN1 co-suppressed transgenic plants leads to inactivation of ethylene receptors, in turn activating ethylene signaling. In the partial loss-of-function RAN1 mutants (ranl-1 and ranl-2), reduced delivery of copper ions to the ethylene receptors may produce a state of suboptimal copper:apoprotein stoichiometry. Altered protein conformation of ethylene receptor may result in reduced ligand specificity, thereby allowing TCO to act as an agonist. Since ranl-1 and ranl-2 mutants show a normal response to ethylene, the structural change in the recognition cavity is presumed to be small. A severe reduction of copper supply in RAN1 co-suppressed transgenic plants leads to the activation of ethylene signaling.

Why do RAN1 co-suppressed transgenic plants (ranl-1 plants) show a constitutive ethylene-response phenotype? If copper is required for ethylene perception, ranl-1 plants should show the ethylene-insensitive phenotype, like etr1-1. The defect of ETR1-1 mutant protein in coordination with a copper molecule causes loss of ethylene binding activity (see below). One possible explanation is that the etr1-1 mutation (conversion Cys65 to Tyr) not only abolishes the copper-binding activity but also causes a conformational change that locks the protein in an active state. In contrast, in the complete copper-depleted post-Golgi vesicles of the ranl-1 plants, the wild type receptors will adopt a non-functional conformation. As indicated before, in the absence of functional receptors the downstream components in the signaling pathway will become active and therefore the plants will show the constitutive ethylene phenotype observed in the ranl-1 plants. In order to test this hypothesis, a functional assay for the kinase activity of the ethylene receptors will be required.

A biochemical approach; heterologous system using budding yeast—The ethylene binding activity of ETR1 was first demonstrated with the recombinant ETR protein expressed in yeast cells (Schaller and Bleecker 1995). The biochemical characteristics of ETR1 have been studied also using this heterologous system. The three N-terminal transmembrane domains of ETR1 are sufficient for ethylene binding. A cysteine residue located in the second membrane spanning domain in ETR1 is essential for ethylene binding. In yeast, copper transporter Ccc2p and copper chaperon Atx1p are required for the high affinity iron uptake. The expression of genes for those proteins are regulated by iron-sensing transcriptional activator Aft1p (Yamaguchi-Iwai et al. 1996, Lin et al. 1997), suggesting a feed-back effect in which the levels of iron control copper delivery in specific cell compartments regulating in this way the iron uptake. By analogy to this system, it is possible that plant cells control ethylene receptor activity by modulating copper supply. Upon onset of senescence, the copper level drops in Arabidopsis leaves (Himelblau et al. 1998). Since ethylene is known to promote leaf senescence, an intriguing possibility exists that senescing leaves are more sensitive to ethylene due to depletion in the copper supply. On the other hand, ranl-1 mutants that are presumed to have reduced copper supply to ethylene receptors do not show enhanced ethylene sensitivity compared to the wild-type plants (Hirayama et al. 1999). More studies need to be performed to finally address this issue.

EIN2; a sensor for a divalent cation?—The EIN2 function is required for the transduction of the ethylene signal from the Raf kinase CTR1 to the transcription factor EIN3 (Fig. 1). The role of EIN2 in this process is essential since the loss of function of the EIN2 protein (Rodriguez et al. 1999). These results suggest that ETR1 binds copper ion(s) and copper-receptor coordination are required for ethylene binding. Interestingly, addition of silver ion instead of copper also increases ethylene-binding activity of membrane preparation. This is quite surprising since silver ion is known to act as an ethylene response inhibitor in plant tissues. They speculated that although silver may be incorporated in ethylene receptors and facilitate ethylene binding, this binding may not induce appropriate conformational change in the receptor. ETR1 protein expressed in yeast ccc2-disrupted mutant cells could not bind to the ethylene molecule, supporting the idea that the ethylene-binding activity of ETR1 requires a copper delivery system in which Ccc2p/RAN1 functions in the secretory pathway (F.I. Rodriguez and A.B. Bleecker, personal communications). It is not yet known how ethylene receptors coordinate with copper ion(s) and how ethylene molecule(s) is recognized by the copper-receptor complex. To find the answers to these questions, the three-dimensional structure of the copper-ethylene receptor complex needs to be determined.

What is the physiological significance of the linkage between copper metabolism and ethylene perception?—As mentioned above, when the copper supply to the ethylene receptors is blocked, the receptors are shut down and become inactive. This implies that a change in the copper supply may result in a change in the ethylene sensitivity. Then a question arises; does RANI protein also regulate their sensitivity to ethylene controlling copper supply besides simply delivering copper ions to ethylene receptors? In yeast, copper transporter Ccc2p and copper chaperon Atx1p are required for the high affinity iron uptake. The expression of genes for those proteins are regulated by iron-sensing transcriptional activator Aft1p (Yamaguchi-Iwai et al. 1996, Lin et al. 1997), suggesting a feed-back effect in which the levels of iron control copper delivery in specific cell compartments regulating in this way the iron uptake. By analogy to this system, it is possible that plant cells control ethylene receptor activity by modulating copper supply. Upon onset of senescence, the copper level drops in Arabidopsis leaves (Himelblau et al. 1998). Since ethylene is known to promote leaf senescence, an intriguing possibility exists that senescing leaves are more sensitive to ethylene due to depletion in the copper supply. On the other hand, ranl-1 mutants that are presumed to have reduced copper supply to ethylene receptors do not show enhanced ethylene sensitivity compared to the wild-type plants (Hirayama et al. 1999). More studies need to be performed to finally address this issue.

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completely blocks the ethylene response. The \textit{EIN2} gene has been cloned by map-based positional cloning and it was found to encode a unique membrane-anchored protein (Alonso et al. 1999). The \textit{EIN2} protein consists of nearly 1300 amino acid residues. Computer analysis predicts twelve membrane-spanning domains in the N-terminal half. The amino acid sequence of this N-terminal half has a significant similarity to that of Nramp-family proteins, including yeast Smf1p, the \textit{malvolio} protein of \textit{Drosophila}, and mammalian Nram1 and Nram2 (DCT1). The long hydrophilic C-terminal domain does not have obvious similarity to any proteins in the data base. Functional information has been obtained for several of the members of the Nramp protein family. For example, in budding yeast Smf1p transports a variety of divalent cations such as Mn$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ (Supek et al. 1996); a mutation in the \textit{malvolio} (\textit{mlv}) gene affects taste behavior in \textit{Drosophila} (Rodrigues et al. 1995) and this phenotype can be reverted by feeding the flies with 10 mM MnCl$_2$ or FeCl$_2$ (Orgad et al. 1998); the mouse Nram2 (DCT1) is capable of transporting iron ions (Gunshin et al. 1997). Unlike \textit{EIN2}, those Nramp family proteins do not have an extended C-terminal domain. The metal ion transporting activity of \textit{EIN2} protein has been analyzed extensively in various heterologous systems, but no transporter activity has been detected. In addition, overexpression of the N-terminal half did not confer any ethylene related phenotypes to the wild type \textit{Arabidopsis} plants, neither did it suppress the ethylene insensitive phenotype of \textit{ein2} mutants, suggesting that the N-terminal half alone is not sufficient for \textit{EIN2} function. In contrast, the ectopic expression of the C-terminal half in \textit{ein2} mutant resulted in a constitutive ethylene response.

![Diagram of glucose transporters and sensors](image-url)

**Fig. 2** Does \textit{EIN2} function as a sensor for a divalent cation? Left panel; the relationships between glucose transporters and glucose sensors of budding yeast. Glucose sensors (Smf3p and Rgt2p) have twelve membrane spanning domains with similarity to glucose transporters and a long cytoplasmic domain. The membrane-anchored domain is presumed to bind the glucose molecule and the cytoplasmic domain transduces the signal to downstream components (such as Std1p). Right panel; \textit{EIN2} has twelve membrane-spanning domains similar to Nramp family proteins and a long cytoplasmic domain. Overexpression of C-terminal half of \textit{EIN2} in \textit{Arabidopsis} confers the constitutive ethylene response phenotype, implying that this domain functions as a signal transducer. Analogy to the glucose transporter and sensor couple, it might be expected that \textit{EIN2} function as a sensor for a divalent cation, although direct evidence is lacking.
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phenotype. Therefore, it has been postulated that the C-terminal end of EIN2 functions as a signal transmitter. Since the amino acid sequence of the C-terminal half is novel, the mechanism by which EIN2 transmits the ethylene signal is completely unknown (see below). What is the function of the membrane anchored domain in the N-terminal half? Probably this domain regulates the C-terminal's function, because overexpression of full length EIN2 could not confer the constitutive ethylene response phenotype.

The relationship between Nramp and EIN2 is reminiscent of that between yeast glucose transporters and glucose sensors. Budding yeast has two glucose sensors, Snf3p and Rgt2p. The common feature of these proteins is apparent in their long cytoplasmic C-terminal domain in addition to the twelve membrane spanning domains that have a significant similarity to glucose transporters (Hxtps). Originally, these two proteins were thought to be a member of glucose transporters because of its high similarity at the sequence level with transporters. However,

![Diagram of ethylene signaling pathway](https://academic.oup.com/plants/abstract/41/5/548/1830483)
recent studies have revealed that these proteins function as a glucose signal transduction in budding yeast (reviewed in (Kruckerberg et al. 1998), Fig. 2, Left). Overexpression of the Snf3p C-terminal domain activates glucose signaling (Coons et al. 1997). When the Snf3p C-terminal domain is fused to a glucose transporter the fusion protein gains the ability to sense glucose. (Ozcan et al. 1998). Furthermore, two hybrid screening using Std1p (a regulator for glucose responsive genes) as a bait identified Snf3p and Rgt2p as Std1p-interacting proteins (Schmidt et al. 1999), supporting the idea that their C-terminal domain functions as a signal transmitter. The transporter-like sensors are not restricted to the glucose-response pathway. An amino acid permease-like protein, Ssy1p, is involved in the serine-inducible expression of transporter and thought to sense serine in the media (Didion et al. 1998).

Two parallels between Snf3p, Rgt2p-Hxtps and EIN2-Nramp can be drawn. First, the similarity at the sequence level between the sensors and transporters is lower than the similarity among transporters. Second, overexpression of the long cytoplasmic C-terminal domain confers an ability to activate the signaling system. These analogies led to the proposal that EIN2 might function as a sensor for an upstream signal, presumably divalent cations (Fig. 2, Right). In yeast glucose sensors, a region of conserved amino acids that is presumed to interact with the downstream factors is required for signal transmission (Ozcan et al. 1998). EIN2 does not have such a sequence nor any known motifs in its C-terminal domains. The prediction of secondary structure of the C-terminal domain revealed a coiled-coil domain adjacent to the last membrane-spanning domain. Coiled-coil domains have been found in the regions where protein-protein interactions occur. It is likely that EIN2 C-terminal domain transduces the signal by interacting with other signal transducers through this coiled-coil domain. The identification and characterization of EIN2 interacting protein will shed some light on the way the ethylene signaling is transmitted from EIN2 to EIN3. Several EIN2 interacting proteins have been identified using a two-hybrid system and are currently being characterized (J.M. Alonso & J.R. Ecker, unpublished results).

There is no direct evidence for the requirement of any divalent cations in the ethylene signal transduction. Thus, if EIN2 is a divalent cation sensor, what is the EIN2 function in ethylene signaling? Several mutants with altered response to other phytohormones or physiological processes have turned out to be allelic to ein2. For example, recent study on the jasmonic acid response of Arabidopsis has revealed that EIN2 is required for the jasmonic acid dependent expression of the PDF1.2 gene that encode defensin (Penninckx et al. 1996), although other ethylene insensitive mutants did not show such a phenotype. This implies that ethylene signal is required for triggering other physiological responses. Alternatively, EIN2 itself may integrate information from several stimuli and trigger the appropriate response. There is an intriguing possibility that EIN2 may sense a divalent cation for the purpose of monitoring the physiological state of the cell or tissue and integrate this information with ethylene stimuli. The subcellular localization of EIN2 has yet to be determined, the answer to this question will help to clarify the role of EIN2 in ethylene signaling.

Future perspective—Ethylene is involved in a variety of growth and developmental processes, suggesting that the ethylene response should be controlled at multiple levels by many different signals including other phytohormones, developmental or environmental signals. The emerging picture of the ethylene signal transduction pathway that consists of at least five ethylene receptor molecules, a predicted MAP kinase cascade, and the multi-step gene activation supports this idea. As discussed above, the requirement of copper for ethylene receptors and the possibility of EIN2 functioning as a divalent cation sensor add a new level of complexity to this signaling pathway. Plants still cover the secret of how they sense ethylene molecule. The role of the copper delivery system in the control of ethylene sensitivity is obscure, and the means by which EIN2 transmits the ethylene signal is unknown. However we believe that the findings described here will encourage and enhance the research on the metal homeostasis in plants. Future researches should uncover more sophisticated and elaborate mechanisms for ethylene system and other signaling processes in plants.

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