Delivering copper within plant cells
Edward Himelblau* and Richard M Amasino†

Two genes recently identified in Arabidopsis thaliana may be involved in sequestering free copper ions in the cytoplasm and delivering copper to post-Golgi vesicles. The genes COPPER CHAPERONE and RESPONSIVE TO ANTAGONIST1 are homologous to copper-trafficking genes from yeast and humans. This plant copper-delivery pathway is required to create functional ethylene receptors. The pathway may also facilitate the transport of copper from senescing leaf tissue. In addition, several other genes have been identified recently that may have a role in copper salvage during senescence.

Addresses
Department of Biochemistry, 433 Babcock Drive, University of Wisconsin, Madison, WI 53706, USA
* e-mail: ethimelb@students.wisc.edu
† e-mail: amasino@biochem.wisc.edu

Abbreviations
ATX1 ANTIOXIDANT1
BCB BLUE-COPPER-BINDING PROTEIN
CCC2 Ca2+-SENSITIVE CROSS-COMPLEMENTER2
CCH COPPER CHAPERONE
ETR1 ETHYLENE-SENSITIVE2
HAH1 HUMAN ATX HOMOLOG1
MNK MENKES
MT METALLOTHIONEIN
RAN1 RESPONSIVE TO ANTAGONIST1
SAG SENESCENCE-ASSOCIATED GENE

Introduction
Copper is an excellent catalyst for redox reactions. Thus, it is not surprising that copper is an essential component of many of the electron carriers involved in oxidative phosphorylation and photosynthesis. In addition, copper participates in the detoxification of oxygen radicals generated by metabolism. Nevertheless, the reactivity of copper that makes it so useful in redox reactions also makes it toxic. For example, free copper will readily oxidize the thiol bonds within proteins causing a disruption of their secondary structure. Thus, cells must accumulate copper and distribute it to the cellular components that require it while preventing its toxic effects.

Work in yeast, mice and humans, has resulted in the emergence of a picture of specific, intracellular copper trafficking pathways [1]. The first components of these pathways are a variety of cytoplasmic copper chaperones. These chaperones sequester copper in a nonreactive form and interact with other transport proteins to deliver copper to where it is needed within cells. Recently, two genes have been identified from Arabidopsis thaliana that encode the first components of the intracellular copper-delivery system to be identified in plants. The products of these genes, COPPER CHAPERONE (CCH) and RESPONSE TO ANTAGONIST1 (RAN1) may interact to move copper from the cytoplasm into post-Golgi vesicles. Little is known of the contribution of this pathway to copper homeostasis and metabolism in plants. Initial characterization of the copper-trafficking pathway components suggests that they may be involved in the delivery of copper to ethylene receptors and in the transport of copper from senescing leaves [2-4].

The study of intracellular copper trafficking began with the identification of a rare human metabolic disorder. The first report of what would become known as ‘Menkes’ kinky hair syndrome’ detailed the symptoms of an untreatable, X-linked disease that was caused by a defective recessive gene and therefore primarily affected males [5]. Menkes’ disease patients suffered from retarded growth and severe cerebral degeneration that caused their death within their first three years. Associated with these symptoms was the growth of wiry, brittle hair. Investigators later realized that this brittle hair is similar to the wool of sheep grazed on copper-poor forage [6]. It was eventually established that Menkes’ disease resulted from a defect in copper transport that caused ‘copper starvation’ symptoms in some tissues. Despite adequate dietary copper, patients had defective intestinal absorption of copper [7].

The defective gene in Menkes’ disease (MNK) has been cloned and shown to encode a metal-binding ATPase that is localized to the trans-Golgi [8-11]. It is therefore suspected that in Menkes’ disease patients, the absence of this ATPase prevents some form of copper transport. Researchers studying the MNK homolog from yeast, Ca2+-SENSITIVE CROSS-COMPLEMENTER2 (CCC2), found the product of this gene in the membranes of post-Golgi vesicles [12], and found that it forms part of a specific copper-delivery pathway [13] (Figure 1a). CCC2 was found to interact with a cytoplasmic copper chaperone, ANTIOXIDANT1 (ATX1) [13]. Through this interaction, copper is transferred from ATX1 to CCC2 and then into the lumen of the post-Golgi vesicle. Once inside the post-Golgi vesicle, the copper can be inserted into copper-requiring proteins as they make their way to the plasma membrane, the endomembrane system or to be secreted. A human homolog of ATX1, HUMAN ATX HOMOLOG (HAH1) (Figure 1b), has been found, indicating that this pathway is conserved in eukaryotes [14]. In this review we describe recent advances in the study of an intracellular copper trafficking pathway in plants. This pathway may supply copper to ethylene receptors and transport copper during leaf senescence.

Identification of CCH and RAN1
Functional homologs of both ATX1/HAH1 and CCC2/MNK, which have the ability to rescue yeast in which these genes...
Comparison of copper-trafficking pathways in yeast, human and Arabidopsis. (a) In yeast, when copper (Cu^{2+}, shown as a black dot) enters the cytoplasm it is bound by ATX1 [36]. ATX1 interacts with a membrane-bound ATPase, CCC2, in the membrane of post-Golgi vesicles [13]. As a result of this interaction, the copper is transferred into the lumen of the vesicle. Once inside, copper may have many destinations (represented by '?'), but the best characterized is FERROUS TRANSPORT3 (FET3), a copper-dependent iron oxidase [12,37,38]. (b) The human homologous copper-trafficking pathway. Copper is bound in the cytoplasm by HAH1, the human ATX1 homolog. HAH1 interacts with the CCC2 homolog, MNK, to deliver the copper into the lumen of the trans-Golgi. Although copper may then become incorporated into other proteins ('?') a known destination is the human FET3 homolog, ceruloplasmin (CER) [37]. When cytoplasmic copper concentrations are elevated, MNK travels to the plasma membrane where it functions in copper efflux from the cell [16]. Once copper levels are reduced, MNK returns to the trans-Golgi [16]. Thus, ligand-mediated targeting allows MNK to function in both copper trafficking and in defense against copper accumulation. Although RAN1 lacks the leucine repeats that act as signals for ligand-mediated targeting [39] it is unclear whether RAN1 contains other plant-specific Golgi retention signals —indeed, it has not been determined whether RAN1 is localized to the plant post-Golgi at all. It will be interesting to determine whether RAN1 can shuttle between the plasma membrane and some internal membrane, and if so, whether cytoplasmic copper levels influence this movement.

**Copper delivery and ethylene perception**

The ran1 mutant is altered in ethylene perception [3**,4**]. The ethylene receptor, ETR1, forms a homodimer and is probably present in the plasma membrane. The ETR1 homodimer surrounds a single copper atom that is required for high-affinity ethylene binding [17**]. In Arabidopsis, there are five ethylene receptors all of which contain a conserved cysteine residue that has been shown to be critical for copper binding in ETR1 [17**,18]. Therefore, it is probable that, like ETR1, all five ethylene receptors are copper-dependent. It has been suggested that the ethylene receptors dimerize and bind copper in the post-Golgi system as they move toward the membrane in which they act. If this is true, then RAN1 is a strong candidate for the delivery of copper to the receptors. This hypothesis is supported by the phenotype of ran1 mutants.
The ethylene response includes the ‘triple response’ in seedlings (i.e. hypocotyl elongation is inhibited, the hypocotyl exhibits radial swelling and the hypocotyl hook is exaggerated), the upregulation of ethylene-induced genes and the inhibition of cell expansion (see Figure 2) [19,20]. Ethylene receptors that have not bound ethylene negatively regulate the ethylene response through a cytoplasmic signaling domain [18,21*]. Ethylene binding probably induces a conformational change in the receptor that inactivates the signaling domain and thereby allows the ethylene response to occur [22]. Mutations that eliminate ethylene binding create dominant insensitivity to ethylene because the negative regulatory signaling domain is never inactivated [22]. Loss-of-function mutations that eliminate the receptors or disrupt the signaling domains show a constitutive ethylene response [21*].

*ran1* mutants have two possible ethylene-related phenotypes. First, the absence of copper from the ethylene-binding site could prevent ethylene binding and cause ethylene insensitivity. Second, and more likely, the absence of copper could prevent the functioning of the signaling domain possibly by inducing a conformational change in the receptors that target them for degradation. Either way, the loss of signaling function would cause a constitutive ethylene response. Indeed, plants in which *RAN1* expression is undetectable because of co-suppression and *ran1* loss-of-function mutants appear to have a constitutive ethylene response ([3**,4**]; E Himelblau, RM Amasino, unpublished data) (Figure 2a). Loss-of-function mutants have been identified for four of the five ethylene receptors [21*]. Interestingly, genetic experiments in which double, triple and quadruple receptor mutants were constructed reveal that the strength of the constitutive ethylene-response phenotype increases with the number of receptors mutated [21*] (Figure 2b). The *ran1* mutant appears to have a stronger ethylene response than even the quadruple mutant (Figure 2b,c) ([3**,4**]; E Himelblau, RM Amasino, unpublished data). It is possible, therefore, that the *ran1* mutation biochemically creates the ‘quintuple mutant’ in which all of the ethylene receptors are inactive. In addition, the loss of activity of copper-requiring proteins may contribute to the *ran1* phenotype.

Gene expression studies of β-chitinase, a gene known to be upregulated in response to ethylene [23] have revealed that the *ran1* phenotype is not entirely a product of the ethylene response. *ran1* mutants express β-chitinase throughout development, supporting the notion that *ran1* produces a constitutive ethylene response [4**]. Double mutants have been constructed that contain mutations in both *RAN1* and *ETHYLENE-INSENSITIVE2* (*EIN2*) [4**]. The *EIN2* gene product acts downstream of the ethylene receptors and is essential for the ethylene response. Therefore, loss-of-function *ein2* mutations cause ethylene insensitivity [24]. Because *ein2* eliminates the ethylene response, the *ran1; ein2* double mutant should theoretically display only *ran1* phenotypes that are independent of the ethylene response. As expected the *ran1; ein2* double mutant appears to be ethylene insensitive as a seedling and does not exhibit β-chitinase induction at any point in its development, indicating that ethylene responses are absent in this background [4**]. Interestingly, the *ran1; ein2* adult is indistinguishable from the *ran1* mutant having severe inhibition in cell expansion [4**]. Thus, it appears that the dwarfed phenotype of the *ran1* mutant is independent of the ethylene response, and that the *ran1* mutant is likely to

![Figure 2](current-opinion-in-plant-biology)

The Arabidopsis *ran1* mutant has altered ethylene sensitivity. (a) Response of dark-grown seedlings to ethylene. Wild-type (WT) seedlings grown in air have an etiolated phenotype (i.e. a long, thin hypocotyl). In the presence of ethylene WT seedlings show the ‘triple response’ (i.e. hypocotyl elongation is inhibited, the hypocotyl exhibits radial swelling and the hypocotyl hook is exaggerated) [19]. A *ran1-4* mutant shows a constitutive triple response when grown in air or ethylene. The *ran1-4* allele (E Himelblau, RM Amasino, unpublished data) is caused by a transfer-DNA insertion in the coding region of *RAN1*. (b) The *ran1-4* mutant is phenotypically similar to plants with loss of ethylene receptor function. Lines were created in which increasing numbers of ethylene receptors are disrupted [21*]. The triple mutant has disruptions in the ethylene receptors ETR1, ETR2 and EIN4. The quadruple mutant has disruptions in the ethylene receptors ETR1, ETR2, EIN4 and ERS2. The degree of inhibition of cell expansion is proportional to the number of ethylene receptors disrupted. The *ran1* mutant is severely inhibited in cell expansion ([3**,4**]; E Himelblau, RM Amasino, unpublished data). (c) The *ran1* mutant shown next to a US penny.
Changes in gene expression, copper concentrations and nitrogen concentrations during Arabidopsis leaf senescence. (a) Leaves of Arabidopsis thaliana at 23 and 28 days after germination (DAG). At 23 DAG, the leaf is fully expanded but does not show the leaf-yellowing that is indicative of senescence. At 28 DAG, the leaf has lost approximately one-half of its chlorophyll and is midway through senescence. (b) An RNA blot analysis of copper-trafficking genes during senescence. The steady-state levels of COPPER CHAPERONE (CCH) and RESPONSIVE TO ANTAGONIST1 (RAN1) are increased in the senescent leaf. The mRNA levels of CHLOROPHYLL A/B BINDING PROTEIN (CAB) were also determined. CAB is known to be downregulated in senescing leaves [39]. (c) Concentrations of nitrogen and copper in the leaves at 23 and 28 DAG. The fall in nitrogen and copper concentrations indicate the extent of nutrient salvage from senescent leaves ([2*]; E Himelblau, RM Amasino, unpublished data).

Copper transport during leaf senescence

Both CCH and RAN1 are upregulated during leaf senescence suggesting that they have a role in that process ([2*]; E Himelblau, RM Amasino, unpublished data) (Figure 3). During leaf senescence, nitrogen, phosphorus and certain metal ions contained in leaves are mobilized and transported to seeds, fruits, storage organs or other growing parts of the plants. This mobilization provides the plant with a means of 'recycling' important nutrients from old, shaded or damaged leaves that no longer contribute photosynthates to the plant [25]. Copper is among the nutrients transported from senescent leaves in many species including Arabidopsis [2*,26–28] (Figure 3).

Several possible roles have been suggested for CCH and RAN1 in the process of copper recycling during senescence. One role could be to sequester copper as it is released by the degradation of copper-containing proteins in the chloroplast. Such sequestration would prevent free copper from poisoning the cell and preventing the salvage of nutrients. Potentially, CCH could bind newly freed copper in the cytoplasm and then interact with RAN1 to further sequester the copper in a storage vesicle. A second possible role for CCH and RAN1 could be to deliver copper to a system that exports it from the leaf. In this scenario, cytoplasmic copper could be bound by CCH, passed to RAN1 and then into a post-Golgi vesicle. Within the vesicle, copper carriers bind the copper prior to fusion of the vesicle with the plasma membrane. In a third possible scenario, RAN1 could pump copper out of the cell directly if, like MNK, RAN1 is localized to the plasma membrane during periods when copper is accumulating in the cytoplasm [16]. The unique carboxy-terminal helix of CCH may also have a senescence-specific role either in targeting or protein–protein interactions or in targeting CCH to a distinct intracellular location, but this remains to be determined. It will be important to determine whether a mutation in CCH or RAN1 can prevent the export of copper from senescing leaves.

Studies of leaf senescence have focused on identifying genes that are upregulated during senescence [29]. These 'senescence-associated genes' (SAGs) are thought to carry out the processes that constitute leaf senescence. Several of the SAGs identified thus far appear to be involved in maintaining copper homeostasis. As discussed above, because copper is so reactive, sequestration of newly freed copper would seem to be necessary to prevent its toxic effects that would kill the cell before the senescence process was complete. One metallothionein encoding gene METALLOTHIONEINE 1 (MT1), is upregulated during leaf senescence in Arabidopsis [30]. MT1 expression in leaves is typically low but this gene is induced in leaves that have higher than normal copper concentrations, suggesting that MT1 has a role in toxicity defense [29–31]. Indeed, the Arabidopsis MT1 can defend transgenic yeast from toxic concentrations of copper in the growth medium [31]. These observations are consistent with a role for MT1 in thwarting copper toxicity during leaf senescence when
Copper is freed from the chloroplasts. A similar metallo­

dionein, LSC54, that is also upregulated during leaf senescence

has been identified in Brasica napus [32]. Interestingly, MT2,

another Arabidopsis metallo­
dionein gene is expressed in

leaves prior to senescence but is not a SAG [31]. COPPER-BINDING PROTEIN (BCB) is also uppre-
during senescence yet its contribution to the senescent drome is less well defined [30]. BCB, a membrane-­

protein that is related to plastocyanin, is probably cap

electron transport [33]. As chloroplast membranes

down, the disruption of normal electron flow through the

light-harvesting complexes could result in oxidative damage.

BCB may sequester copper freed as the photosystems are

broken down (i.e. form an early step in the salvage of copper

from the senescing leaf cell).

The findings that several genes encoding copper-bind­
ing proteins are upregulated during leaf senescence indicate that copper sequestration is an important activ­

ity, even in a cell undergoing the final stage of development. Indeed, all of the genes discussed above are

expressed in other tissues at other times during development in addition to being upregulated during leaf senescence. These genes therefore have important housekeeping functions that may be required to a greater extent during senescence when catabolic processes are releasing copper into the cytoplasm.

Conclusions

CCH and RAN1 are components of the first copper deliv­

ery system to be identified within plant cells. In yeast and humans, other trafficking pathways deliver copper to super­
oxide dismutase [34] and to the mitochondria [35]. Given the high degree to which the CCH/RAN1 pathway is con­served among yeast, plants and animals, it is reasonable to assume that plants also contain homologs of the superoxide dismutase and mitochondrial delivery pathways. It will be of particular interest to determine how copper is delivered to the chloroplast as this may represent a novel form of copper trafficking. Ultimately, research in plants, yeast and animals will develop a complete picture of the ways in which potentially toxic copper atoms are delivered within cells to the organelles in which they are needed.

Update

Recent work suggests a role for Arabidopsis BCB in defense against aluminum toxicity. This work involved the generation of transgenic Arabidopsis plants expressing BCB under the control of a strong, constitutive promoter.

The roots of these plants are resistant to levels of alu­

minum shown to inhibit the growth of wild-type roots.

Nevertheless, when challenged with levels of copper suf­

ficient to inhibit root growth, the transgenic plants expressing BCB were inhibited to the same extent as wild-type plants. This finding indicates that ectopic expression of BCB is not sufficient to confer resistance to copper toxicity in roots [40].

Acknowledgements

We are grateful to Fernando Rodriguez, Tony Bleecker and Joe Kieber for insightful discussions and communication of recent results. We thank Elliott Meyerson for providing photographs of ethylene-sensitive mutants. Our work on senescence is funded by the Consortium for Plant Biotechnology Research (HR89-70227) and by the National Science Foundation (DBI-9602222).

References and recommended reading

particular interest, published within the annual period of review, highlighted as:

**of outstanding interest


The authors describe the cloning and characterization of COPPER CHAPERONE (CCH), the Arabidopsis homolog of the yeast copper chaperone, ANTIOXIDANT1 (ATX1), and the first intracellular metal chaperone described in plants. CCH can rescue yeast mutants that lack ATX1. In these experiments, rescue by CCH is copper-dependent. CCH is upregulated in leaves undergoing senescence and in leaves treated with ozone.

3. Hirayama T, Kieber JJ, Hirayama N, Kogen M, Guzman P:

** Noutroundel S, Alonso JM, Dailey WP, Daniels A, Ecker JR:

RESPONSIVE TO ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 1999, 97:589-599.

The RESPONSIVE TO ANTAGONIST1 (RAN1) gene from Arabidopsis is cloned. ran1 mutants were identified in a screen for plants that responded to the ethylene antagonist, trans-cyclooctene. Cloning of RAN1 shows that this gene is the Arabidopsis homolog of the yeast CCH2, a gene involved in copper trafficking. The authors propose that RAN1 delivers copper to eth­

ylene receptors in post-Golgi vesicles. They also observe that the ran1 mutation produces a constitutive ethylene response.


The authors describe a loss-of-function allele of RAN1. The mutant appears to have a constitutive ethylene response as a seedling and as an adult both in terms of morphology and gene expression. Nevertheless, in a series of genetic experiments in which ran1 is placed in an ethylene-insensitive back­
ground, the authors show that the severe inhibition of cell expansion seen in the ran1 mutant is not due to a constitutive ethylene response. They con­
clude that the ran1 phenotype results from a combination of ethylene-depen­
dent and ethylene-independent factors.


The authors identified loss-of-function alleles of four of the five ethylene receptor mutant genes. Copper-binding domain of ETR1 eliminates ethylene binding even in the presence of exogenous copper. Site-directed mutagenesis of several amino-acid residues in the putative cytosolic region of ETR1 show a significant increase in ethylene binding in the presence of exogenous copper. The Menkes disease protein (MNK1) usually resides in the trans-Golgi network (TGN). When cytoplasmic copper levels are elevated, however, MNK travels to the plasma membrane and pumps copper out of the cell. The authors identify a sequence in MNK that targets it to the TGN when expressed in cultured cells.

The authors present evidence that copper is required for high-affinity ethylene binding by the ethylene receptor, ETR1. Yeast expressing ETR1 show a significant increase in ethylene binding in the presence of exogenous copper whereas other metals have little effect on ethylene binding. Site-directed mutagenesis of several amino-acid residues in the putative copper-binding domain of ETR1 eliminate ethylene binding even in the presence of copper.

The authors present evidence that copper is required for high-affinity ethylene binding by the ethylene receptor, ETR1. Yeast expressing ETR1 show a significant increase in ethylene binding in the presence of exogenous copper whereas other metals have little effect on ethylene binding. Site-directed mutagenesis of several amino-acid residues in the putative copper-binding domain of ETR1 eliminate ethylene binding even in the presence of copper.

The Menkes disease protein (MNK1) usually resides in the trans-Golgi network (TGN). When cytoplasmic copper levels are elevated, however, MNK travels to the plasma membrane and pumps copper out of the cell. The authors identify a sequence in MNK that targets it to the TGN when expressed in cultured cells.

The authors present evidence that copper is required for high-affinity ethylene binding by the ethylene receptor, ETR1. Yeast expressing ETR1 show a significant increase in ethylene binding in the presence of exogenous copper whereas other metals have little effect on ethylene binding. Site-directed mutagenesis of several amino-acid residues in the putative copper-binding domain of ETR1 eliminate ethylene binding even in the presence of copper.

The authors present evidence that copper is required for high-affinity ethylene binding by the ethylene receptor, ETR1. Yeast expressing ETR1 show a significant increase in ethylene binding in the presence of exogenous copper whereas other metals have little effect on ethylene binding. Site-directed mutagenesis of several amino-acid residues in the putative copper-binding domain of ETR1 eliminate ethylene binding even in the presence of copper.

The authors present evidence that copper is required for high-affinity ethylene binding by the ethylene receptor, ETR1. Yeast expressing ETR1 show a significant increase in ethylene binding in the presence of exogenous copper whereas other metals have little effect on ethylene binding. Site-directed mutagenesis of several amino-acid residues in the putative copper-binding domain of ETR1 eliminate ethylene binding even in the presence of copper.