Delivering copper within plant cells

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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.

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Abbreviations

ATX1	ANTIOXIDANT1
BCB	*BLUE-COPPER-BINDING PROTEIN*
CCC2	*Ca^{2+}-SENSITIVE CROSS-COMPLEMENTER2*
CCH	COPPER CHAPERONE
EIN2	*ETHYLENE-INSENSITIVE2*
ETR1	ETHYLENE RECEPTOR1
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••].

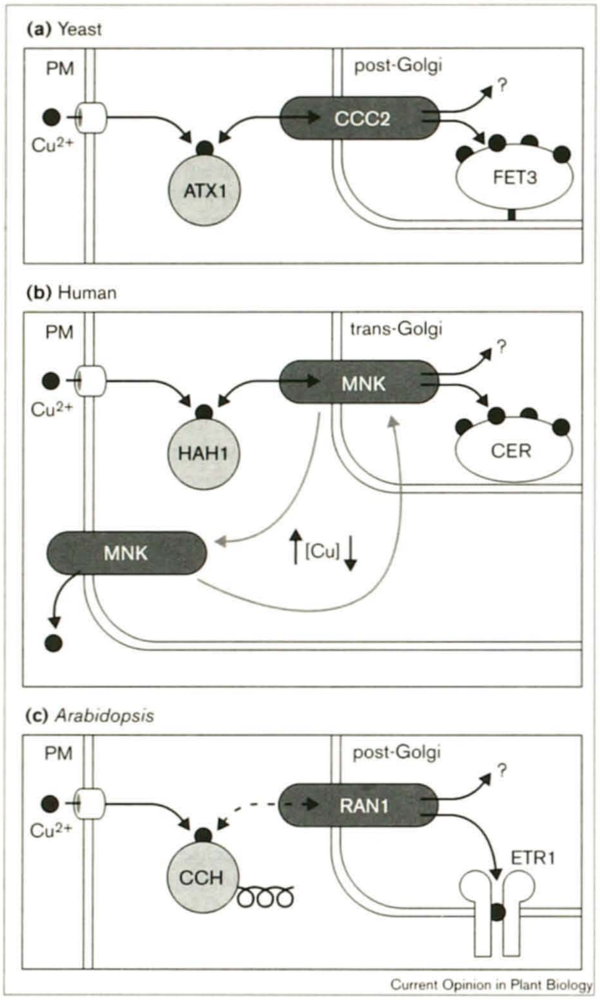
The study of intracellular copper trafficking began with the identification of a rare human metabolic disorder. The first report of what would become known as 'Menkes' kinky hair syndrome' detailed the symptoms of an untreatable, X-linked disease that was caused by a defective recessive gene and therefore 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, *Ca^{2+}-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

Figure 1



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 [16]. MNK cycles back to the trans-Golgi when copper levels decrease. **(c)** A possible copper-trafficking pathway in plants. Copper in the cytoplasm is bound by the ATX1/HAH1 homolog, CCH. Unlike ATX1 and HAH1, CCH has a carboxy-terminal helix domain. CCH may interact with RAN1 to transport copper into a lumen of some vesicle, possibly a post-Golgi vesicle. Although the destination of copper within this vesicle is unknown ('?'), ethylene receptors, such as ETR1, may depend on RAN1 for copper delivery [3••,4••]. PM, plasma membrane.

are mutated, have been identified from *Arabidopsis thaliana* [2••,3••] (Figure 1c). *Arabidopsis CCH* was initially identified by a search for genes that are upregulated during leaf senescence [2••]. CCH is similar to ATX1 at the amino-acid level, particularly in the location and content of its metal-binding domains. One feature that is unique to CCH is a 47-amino-acid carboxy-terminal extension that is absent from ATX1 as well as from its human and mouse homologs [2••]. This region is believed to form a helix with one positively charged face and one negatively charged face. The function of this helix is unknown, but the possibilities that it contributes to targeting or protein–protein interactions are under investigation.

RAN1 was identified by a screen for mutations that alter sensitivity to the hormone ethylene (the involvement of RAN1 in ethylene perception is discussed below). The sequence of *RAN1* revealed that it is the *Arabidopsis CCC2/MNK* homolog, and it has been shown that *RAN1* can rescue *ccc2* mutant yeast [3••]. The metal-binding, membrane-spanning and ATPase domains of RAN1 are similar to those of CCC2/MNK, yet both RAN1 and CCC2 do not appear to have an important targeting feature that is present in MNK [3••]. MNK contains two leucine repeats that form a targeting signal for retention in the trans-Golgi membrane [15•]. Interestingly, MNK appears to remain in the post-Golgi except under conditions of elevated cytoplasmic copper in which 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 [3••] 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

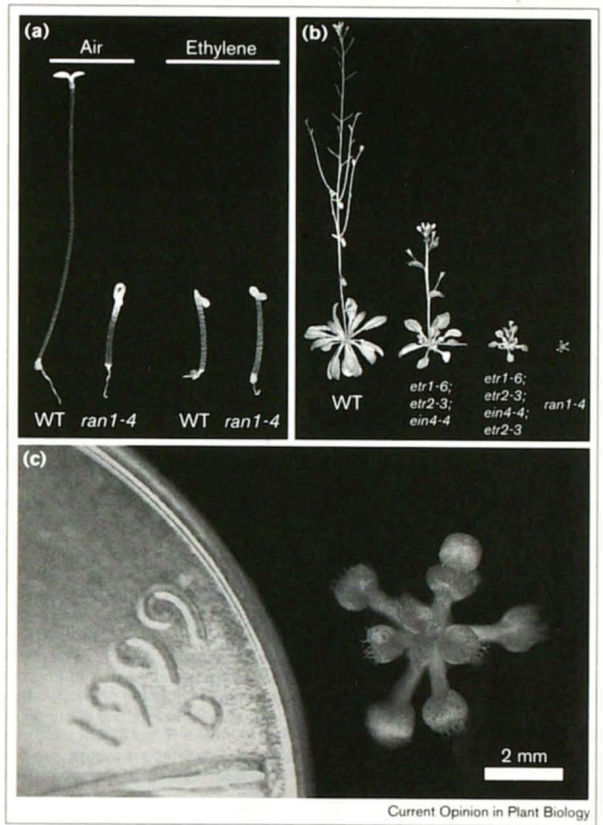
The *ran1* mutant is altered in ethylene perception [3••,4••]. The ethylene receptor, ETR1, forms a homodimer and is probably present in the plasma membrane. The ETR1 homodimer surrounds a single copper atom that is required for high-affinity ethylene binding [17•]. In *Arabidopsis*, there are five ethylene 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.

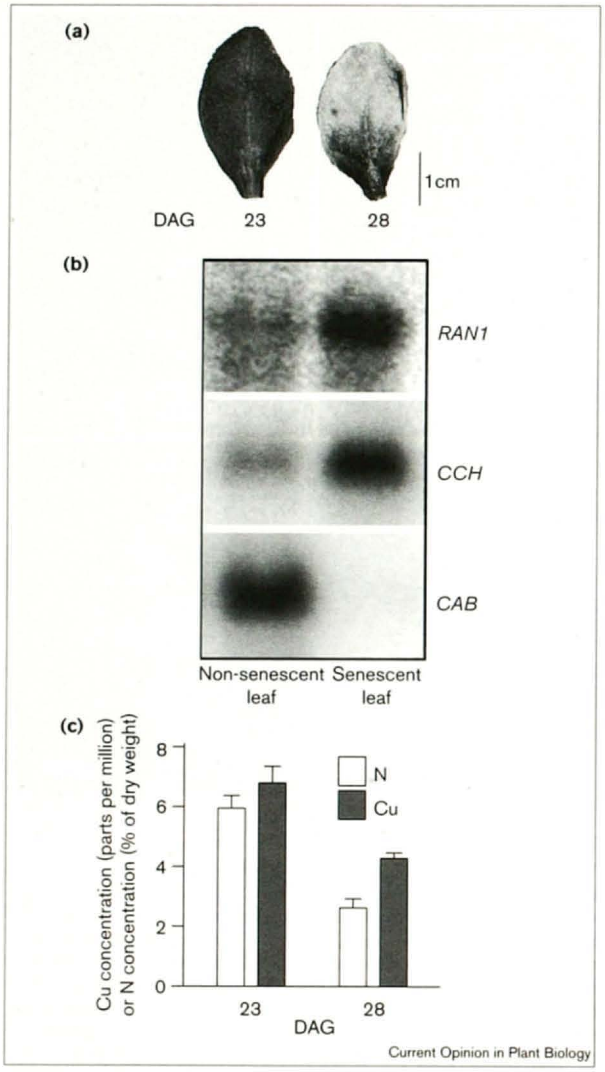
Gene expression studies of β-*chitinase*, a gene known to be upregulated in response to ethylene [23] have revealed that the *ran1* phenotype is not entirely a product of the ethylene response. *ran1* mutants express β-*chitinase* throughout development, supporting the notion that *ran1* produces a constitutive 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



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 either 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.

Figure 3



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).

be altered in many copper-related processes. The analysis of *ran1* phenotypes that are independent of ethylene perception will be an interesting area of future research.

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 *METALLOTHIONEIN 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 [2••,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 metallothionein, *LSC54*, that is also upregulated during leaf senescence has been identified in *Brassica napus* [32]. Interestingly, *MT2*, another *Arabidopsis* metallothionein gene is expressed in leaves prior to senescence but is not a *SAG* [31]. *COPPER-BINDING PROTEIN* (*BCB*) is also upreg during senescence yet its contribution to the senescen 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-binding proteins are upregulated during leaf senescence indicate that copper sequestration is an important activity, 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 delivery system to be identified within plant cells. In yeast and humans, other trafficking pathways deliver copper to superoxide 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 aluminum shown to inhibit the growth of wild-type roots. Nevertheless, when challenged with levels of copper sufficient 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

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nces and recommended reading

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

ecial interest
•• of outstanding interest

1. Valentine JS, Gralla EB: **Delivering copper inside yeast and human cells.** *Science* 1997, **278**:817-818.

2. Himelblau E, Mira H, Lin SJ, Culotta VC, Penarrubia L, Amasino RM:
•• **Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from *Arabidopsis*.** *Plant Physiol* 1998, **117**:1227-1234.

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, Kogan M, Guzman P,
•• Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR: ***RESPONSIVE TO ANTAGONIST1*, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*.** *Cell* 1999, **97**:383-393.

The *RESPONSIVE TO ANTAGONIST 1* (*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 *CCC2*, a gene involved in copper trafficking. The authors propose that RAN1 delivers copper to ethylene receptors in post-Golgi vesicles. They also observe that the *ran1* mutation produces a constitutive ethylene response.

4. Woste KE, Kieber JJ: **A strong loss-of-function mutation in *RAN1*
•• results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype.** *Plant Cell* 2000, **12**:443-455.

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 background, the authors show that the severe inhibition of cell expansion seen in the *ran1* mutant is not due to a constitutive ethylene response. They conclude that the *ran1* phenotype results from a combination of ethylene-dependent and ethylene-independent factors.

5. Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH: **A sex-linked recessive disorder with retardation of growth, peculiar hair and focal cerebral and cerebellar degeneration.** *Pediatrics* 1962, **29**:764-779.

6. Gillespie JM: **The isolation and properties of some soluble proteins from wool.** *Aust J Biol Sci* 1964, **17**:282.

7. Danks DM, Campbell PE, Stevens BJ, Mayne V, Cartwright E: **Menkes's kinky hair syndrome. An inherited defect in copper absorption with widespread effects.** *Pediatrics* 1972, **50**:188-201.

8. Mercer JF, Livingston J, Hall B, Paynter JA, Begy C, Chandrasekharappa S, Lockhart P, Grimes A, Bhave M, Siemieniak D: **Isolation of a partial candidate gene for Menkes disease by positional cloning.** *Nat Genet* 1993, **3**:20-25.

9. Chelly J, Tumer Z, Tonnesen T, Petterson A, Ishikawa-Brush Y, Tommerup N, Horn N, Monaco AP: **Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein.** *Nat Genet* 1993, **3**:14-19.

10. Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J: **Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase.** *Nat Genet* 1993, **3**:7-13. [Published erratum appears in *Nat Genet* 1993, **3**:273.]

11. Yamaguchi Y, Heiny ME, Suzuki M, Gitlin JD: **Biochemical characterization and intracellular localization of the Menkes disease protein.** *Proc Natl Acad Sci USA* 1996, **93**:14030-14035.

12. Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD: **The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake.** *Proc Natl Acad Sci USA* 1995, **92**:2632-2636.

13. Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV: **Metal ion chaperone function of the soluble Cu(I) receptor Atx1.** *Science* 1997, **278**:853-856.

14. Klomp LW, Lin SJ, Yuan DS, Klausner RD, Culotta VC, Gitlin JD: **Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis.** *J Biol Chem* 1997, **272**:9221-9226.

15. Petris MJ, Camakaris J, Greenough M, LaFontaine S, Mercer JFB:
• **A C-terminal di-leucine is required for localization of the Menkes protein in the trans-Golgi network.** *Hum Mol Genet* 1998, **7**:2063-2071.

The Menkes disease protein (MNK) 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. Mutagenized protein lacking this sequence, is no longer targeted to the TGN when expressed in cultured cells.

16. Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J: **Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking.** *EMBO J* 1996, **15**:6084-6095.

17. Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB:
• **A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*.** *Science* 1999, **283**:996-998.

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.

18. Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM: ***EIN4* and *ERS2* are members of the putative ethylene receptor gene family in *Arabidopsis*.** *Plant Cell* 1998, **10**:1321-1332.

19. Guzman P, Ecker JR: **Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants.** *Plant Cell* 1990, **2**:513-523.

20. Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR: ***CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases.** *Cell* 1993, **72**:427-441.

21. Hua J, Meyerowitz EM: **Ethylene responses are negatively**
• **regulated by a receptor gene family in *Arabidopsis thaliana*.** *Cell* 1998, **94**:261-271.

The authors identified loss-of-function alleles of four of the five ethylene receptors. By generating plants in which two, three or four of these receptor genes are disrupted, the authors demonstrate that the ethylene receptors negatively regulate the ethylene response. In particular, the quadruple receptor mutant has a strong, constitutive ethylene response.

22. Chang C, Kwok SF, Bleecker AB, Meyerowitz EM: ***Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators.** *Science* 1993, **262**:539-544.

23. Samac DA, Hironaka CM, Yallaly PE, Shah DM: **Isolation and characterization of genes encoding basic and acidic chitinase in *Arabidopsis thaliana*.** *Plant Physiol* 1990, **93**:907-914.

24. Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR: **EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*.** *Science* 1999, **284**:2148-2152.

25. Bleecker AB: **The evolutionary basis of leaf senescence: method to the madness?** *Curr Opin Plant Biol* 1998, **1**:73-78.

26. Mauk CS, Nooden LD: **Regulation of mineral redistribution in pod-bearing soybean explants.** *J Exp Botany* 1992, **43**:1429-1440.

27. Drossopoulos JB, Bouranis DL, Bairaktari BD: **Patterns of mineral nutrient fluctuations in soybean leaves in relation to their position.** *J Plant Nutr* 1994, **17**:1017-1035.

28. Hocking PJ: **Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat.** *J Plant Nutr* 1994, **17**:1289-1308.

29. Weaver LM, Himelblau E, Amasino RM: **Leaf senescence: gene expression and regulation.** In *Genetic Engineering*, vol 19. Edited by Setlow JK. New York: Plenum Press; 1997:215-234.

30. Weaver LM, Gan S, Quirino B, Amasino RM: **A comparison of the**
• **expression patterns of several senescence-associated genes in response to stress and hormone treatment.** *Plant Mol Biol* 1998, **37**:455-469.

The authors examine the expression of many senescence-associated genes (*SAGs*). Plants are subjected to a variety of treatments, some which induce senescence and some which inhibit senescence. *SAG* expression is examined in the leaves of the treated plants. The *SAG* expression patterns indicate that *SAG* regulation is complex as each *SAG* is induced or repressed by a slightly different set of conditions. This paper also provides a useful catalog of some of the known *SAGs*.

31. Zhou J, Goldsbrough PB: **Functional homologs of fungal metallothionein genes from *Arabidopsis*.** *Plant Cell* 1994, **6**:875-884.

32. Buchanan-Wollaston V: **Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus*. Identification of a gene encoding a senescence-specific metallothionein-like protein.** *Plant Physiol* 1994, **105**:839-846.

33. Van Gysel A, Van Montagu M, Inze D: **A negatively light-regulated gene from *Arabidopsis thaliana* encodes a protein showing high similarity to blue copper-binding proteins.** *Gene* 1993, **136**:79-85.

34. Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD: **The copper chaperone for superoxide dismutase.** *J Biol Chem* 1997, **272**:23469-23472.

35. Glerum DM, Shtanko A, Tzagoloff A: **Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase.** *J Biol Chem* 1996, **271**:14504-14509.

36. Lin SJ, Pufahl RA, Dancis A, O'Halloran TV, Culotta VC: **A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport.** *J Biol Chem* 1997, **272**:9215-9220.

37. Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM, Kaplan J: **The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake.** *Cell* 1994, **76**:403-410.

38. Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A: **A permease–oxidase complex involved in high-affinity iron uptake in yeast.** *Science* 1996, **271**:1552-1557.

39. Lohman K, Gan S, John M, Amasino RM: **Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*.** *Physiol Plant* 1994, **92**:322-328.

40. Ezaki B, Gardner RC, Ezaki Y, Matsumoto H: **Expression of aluminum-induced genes in transgenic *Arabidopsis* plants can ameliorate aluminum stress and/or oxidative stress.** *Plant Physiol* 2000, **122**:657-665.