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The promoters of *Arabidopsis thaliana* genes *AtCOX17-1* and *-2*, encoding a copper chaperone involved in cytochrome *c* oxidase biogenesis, are preferentially active in roots and anthers and induced by biotic and abiotic stress

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AtCOX17 genes encode *Arabidopsis thaliana* homologs of the yeast metallochaperone Cox17p, involved in the delivery of copper for cytochrome *c* oxidase (COX) assembly. Two different *AtCOX17* genes, located in chromosomes 1 and 3, are present in the *Arabidopsis* genome. Sequences available in data banks indicate that the presence of two genes is a common feature in monocots, but not in dicots, suggesting that *Arabidopsis* genes may be the result of a recent duplication. Sequences upstream from the translation start sites of *AtCOX17* genes, which include an intron located in the 5′ leader region, were introduced into plants in front of the *gus* gene. For both genes, expression was localized preferentially in young roots and anthers, but almost 10-fold higher β-glucuronidase activity levels were observed in plants transformed with *AtCOX17-1* upstream regions. Both promoters were induced to different extents by wounding, treatment of leaves with the bacterial pathogen *Pseudomonas syringae* and incubation with agents that produce oxidative stress and metals. *AtCOX17-2* showed similar responses to these factors, while *AtCOX17-1* was more strongly induced by relatively low (10–100 μ*M*) copper. The results indicate that both *AtCOX17* genes have similar, though not identical, expression characteristics and suggest the existence in their promoters of elements involved in tissue-specific expression and in responses to factors that may produce mitochondrial or cell damage. It can be speculated that *Arabidopsis* COX17 accumulates under stress conditions to actively replace damaged or inactive cytochrome *c* oxidase to sustain cyanide-sensitive respiration in plant cells.

Introduction

Mitochondrial respiratory complexes are composed of several different subunits, encoded either by the nuclear or the organellar genome. In the case of cytochrome *c* oxidase (COX) or Complex IV, for example, three subunits synthesized within mitochondria in most organisms are assembled with 7–10 polypeptides imported from the cytoplasm to conform an active enzyme (Barrientos et al. 2002). In addition to the genes that encode enzyme subunits, mutational studies in yeast have unveiled the

Abbreviations – 3-AT, 3-amino-1,2,4-triazole; cDNA, complementary DNA; COX, cytochrome *c* oxidase; EC, epidermal cells; GUS, β-glucuronidase; LP, leaf primordia; MUG, 4-methylumbelliferyl β-D-glucuronide; NSR, nascent secondary root; OR and YR, older and younger root, respectively; S, septum; SA, salicylic acid; SNP, sodium nitroprusside; VC, vascular cylinder; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

existence of about 20 additional nuclear genes whose products are specifically required to obtain an active COX (Barrientos et al. 2002, Herrmann and Funes 2005, McEwen et al. 1986, Tzagoloff and Dieckmann 1990). These proteins, generally termed COX assembly factors, are involved in different processes, like transcription, processing and translation of mitochondrial messenger RNAs, insertion of subunits into the membrane, heme A biosynthesis and copper delivery to the enzyme (Barrientos et al. 2002, Herrmann and Funes 2005). Homologs of some of these proteins are present in a wide range of eukaryotic organisms while others are also found in prokaryotes.

Plant COX is composed of about 10 polypeptides, as deduced from Blue-native gels of *Arabidopsis* mitochondrial extracts (Millar et al. 2004). Besides the three subunits encoded in the organelle (COX1 to COX3), three additional subunits (COX5b, 6a and 6b) can be recognized by homology search in the *Arabidopsis* nuclear genome, while others, including several plant-specific subunits, have been identified by direct protein sequencing (Millar et al. 2004). Homology search also allows the identification of several *Arabidopsis* genes encoding at least ten different COX assembly factors. Among these genes, two encode homologs of the yeast metallochaperone Cox17p, involved in the delivery of copper to mitochondria for its insertion into COX subunits 1 and 2. Briefly, it has been postulated that Cox17p participates in the transport of copper from an unknown cytoplasmic location to the mitochondrial intermembrane space, where two additional inner membrane proteins, Cox11p and Sco1p, act as intermediates for copper insertion into Cox1p and Cox2p, respectively (Beers et al. 1997, Glerum et al. 1996, Herrmann and Funes 2005, Horng et al. 2004). A role of Cox17p in copper delivery to mitochondria is supported by the fact that the protein can be found both in the cytoplasm and within the mitochondrial intermembrane space (Beers et al. 1997, Glerum et al. 1996). The existence of a Cox17p-independent non-proteinaceous copper pool within the mitochondrial matrix (Cobine et al. 2004) and the fact that Cox17p is active in COX assembly when strictly localized within the intermembrane space (Maxfield et al. 2004) do not agree, however, with a role in copper transport to the organelle. In view of these results, the function of Cox17p in the cytoplasm, if any, remains unclear.

The presence of a COX17 homolog in *Arabidopsis* (AtCOX17) has been previously reported by Balandin and Castresana (2002), who identified a complementary DNA (cDNA) clone corresponding to a gene located in chromosome 3. This cDNA was able to complement a yeast *cox17* null mutant, and hybridized to transcripts that were induced by effectors that produce an increase in reactive oxygen species. Careful examination of the *Arabidopsis* genome indicates that there are indeed two genes encoding similar, though not identical, COX17 homologs, located in chromosomes 1 and 3 (Wintz and Vulpe 2002). In the present study, we describe the structure of both *AtCOX17* genes and the patterns of expression conferred by sequences located upstream from their respective translation start codons. We have observed that both upstream sequences produce similar tissue-specific expression patterns, with high activity in young roots and anthers, and confer responsiveness to biotic and abiotic stress factors.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). Plants were grown in a growth chamber at 22–24°C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approximately 200 $\mu E\ m^{-2}\ s^{-1}$. Plants used for the different treatments were grown in Petri dishes containing Murashige and Skoog medium and 0.8% agar for 2 weeks and then transferred to soil for 2 additional weeks. Treatments were performed by vacuum infiltration (15 min in a desiccator) of excised rosette leaves suspended in solutions containing the different compounds at the concentrations stated in the figure legends. Effectors were dissolved in water (used as control of infiltration). During treatments, leaves were kept under illumination and gentle agitation. *Pseudomonas syringae* pv. tomato DC3000 (with or without the *avrRpm1* gene) was grown as described (Alvarez et al. 1998). After washing and resuspension in a solution containing 1 m*M* $MgCl_2$, plants were subjected to vacuum infiltration as described above. A 1 m*M* $MgCl_2$ solution was used for infiltration of control leaves.

Reporter gene constructs and plant transformation

A 1.7-kbp *Sal*I/*Bgl*II fragment comprising *AtCOX17-1* sequences located upstream from the translation start site was amplified from *Arabidopsis* genomic DNA using oligonucleotides 5′-GGCGTCGACTGAATGTCCCAAG-CAAGC-3′ and 5′-GGCAGATCTATCCTGTAGACAAC-GAAAAA-3′. A 1.9-kbp *Sal*I/*Bam*HI *AtCOX17-2* fragment was obtained in a similar way using oligonucleotides 5′-GGCGTCGACTTAAGTTTTGGTTTCAGATAC-3′ and 5′-GGGGGATCCCTCCGACCTATCAT-3′. Both fragments were cloned in front of the *gus* coding region into plasmid pBI101.3 digested with *Sal*I and *Bam*HI. The constructs

were introduced into *Agrobacterium tumefaciens* strain LBA4404, and transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the floral dip procedure (Clough and Bent 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR carried out on genomic DNA with primers specific for *AtCOX17-1* or *-2* and the *gus*-specific primer 5′-TTGGGGTTTCTACAGGAC-3′. Ten independent lines were further reproduced and homozygous T3 and T4 plants were used to analyze *gus* expression. Plants transformed with pBI101.3 were obtained in a similar way and used as negative controls of expression.

β-Glucuronidase assays

β-Glucuronidase (GUS) activity of transgenic plants was analyzed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as described by Hull and Devic (1995). Whole plants or separated organs were immersed in a 1 m*M* X-gluc solution in 100 m*M* sodium phosphate, pH 7.0, and 0.1% Triton X-100 and, after applying vacuum for 15 min, they were incubated at 37°C until satisfactory staining was observed. Tissues were cleared by immersing them in 70% ethanol.

Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) essentially as described by Jefferson et al. (1987). Total protein extracts were prepared by grinding the tissues in extraction buffer (50 m*M* sodium phosphate, pH 7.0, 10 m*M* ethylenediaminetetraacetic acid, 10 m*M* β-mercaptoethanol) containing 0.1% (w/v) sodium dodecyl sulfate and 1% Triton X-100, followed by centrifugation at 13 000 *g* for 10 min. GUS activity in supernatants was measured in extraction buffer containing 1 m*M* MUG and 20% methanol. Reactions were stopped with 0.2 *M* Na_2CO_3 and the amount of 4-methylumbelliferone was calculated by relating relative fluorescence units with those of a standard of known concentration. The protein concentration of extracts was determined as described by Sedmak and Grossberg (1977).

Results

Structure of *AtCOX17* genes

The *Arabidopsis* genome contains two genes encoding homologs of the yeast metallochaperone Cox17p, with arabidopsis genome initiative (AGI) numbers At1g53030 and At3g15352. cDNAs for these two genes have been annotated in data banks with accession numbers AF349684 (*COX17-1*) and AF349685 (*COX17-2*) for the genes located in chromosomes 3 and 1, respectively (Wintz and Vulpe 2002). Both genes contain a single intron, present within the 5′ untranslated region, one or two nucleotides upstream from the start codon for *AtCOX17-1* or *-2*, respectively. Introns are relatively large (709 and 929 bp) compared with the size of the mature transcripts, of about 480 bp. The encoded proteins differ markedly (25% identical amino acids) in their N-terminal third and are highly conserved (more than 90% identity) in the remaining portion (Fig. 1A). The conserved C-terminal portion contains the six cysteines putatively involved in copper binding and translocation into mitochondria via the Mia40 import pathway (Abajian et al. 2004, Mesecke et al. 2005).

A search in data banks revealed the existence of cDNAs encoding COX17 homologs in several plant species, apart from its presence in other eukaryotes. Notably, two different cDNAs could be observed for the monocots rice, maize and barley, among others, but not for dicots, with the exception of *Arabidopsis* (Fig. 1). This would lead to postulate the occurrence of two duplication events in *COX17* genes, an early one in monocots and a very recent one in *Arabidopsis*. The construction of a phylogenetic tree with COX17 protein sequences indicates, however, that a group of the monocot COX17 proteins (which we have arbitrarily named COX17-2) clusters apart from the branch containing the remaining monocot COX17 and dicot COX17 (Fig. 1B). This would indicate that duplication took place before the separation of monocots and dicots and that this was followed by an early loss of a copy in dicots. Alternatively, one of the monocot *COX17* genes may have undergone rapid changes leading it to cluster apart from the rest of plant *COX17* genes. Although the presence of duplicated *COX17* genes in dicots is evident only in *Arabidopsis*, it cannot be ruled out that a second gene with reduced expression levels is present in other dicot plants because the number of available expressed sequence tags (ESTs) is low compared with *Arabidopsis*. Whatever the case, the event that led to the presence of two genes in *Arabidopsis* seems to have occurred after the separation of monocot and dicot plants.

Tissue-specific expression patterns conferred by the upstream regions of *AtCOX17* genes

Gene duplication may lead to specialization of gene function by generating protein isoforms with either different properties or different expression patterns. Regarding AtCOX17, the strong conservation of protein regions known to be involved in function suggests that changes in expression characteristics may have occurred after duplication took place. To analyze this, we have constructed fusions of the respective upstream regions from *AtCOX17-1* and *AtCOX17-2* to the *Escherichia coli*

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Ce17   ----------MP----AEPQK-------STEAGSVAP-------------EK-KLKACCACPETKRVRDACIIENGE--EKCGKLIEAHKACMRAAGFNI----
Dm17   MGNSASQGVAAPSVSAAHPLTTASAATASTTTASAATA----------S-GEKPKCKACCACPETKRARDACIVENGE--ENCLALIEAHKKCMRDAGFNI----
Xl17   ------MSSLAA---------------ASCESLSPSAE----------S-QEKKPLKPCCACPETKKARDACIIENGE--EKCQHLIEAHKECMRSLGFKV----
Hs17   ----------MP---------------GLVDSNPAPPE----------S-QEKKPLKPCCACPETKKARDACIIEKGE--EHCGHLIEAHKECMRALGFKI----
Os17-2 ----------MGNTASGATEA-----SDSTEK-TEQAP----------PADTKPKKKICCACPDTKKLRDECIVQHGE--DACGKWIEAHRQCLRAEGFNV----
Hv17-2 ----------MGNSASPTAVA-----PVAEEK-TGQAP----------APDTKPKKKICCACPDTKRLRDECIVQNGE--DACGKWIEAHRQCLRAEGFNV----
Zm17-2 ----------MGNTTSVQAEA-----SDSAQKPATTSP----------APGSKPKK-ICCACPDTKKLRDDCIVQNGE--DACGKFIEAHLKCLRAEGFSV----
Os17-1 ----------MGSTEHPIPAQ-----SP--ACPTVSEGGS---AAPAPATDSKPKKKICCACPDTKRLRDECIVEHGE--SACTKWIEAHKRCLRAEGFNV----
Hv17-1 ----------MGSTESPAPVQ-----TPAAACSIVNQ-------APAPATDSKPKKKICCACPDTKRLRDECIVEHGE--SACTKWIEAHKQCLRAEGFKV----
Zm17-1 ----------MSCAELPLPVT-----VTLPETPAVNGEGSSAATAAAPGAGSKPKKKICCACPDTKRLRDECVVEHGE--PACTKWIEAHKRCLRAEGFNV----
Bn17   ----------MSGLQAQES-------ACSLSQPSKDVA----------ATETKPKKRICCACPDTKKLRDECIVENGE--SACTKWIEAHLMCLRSEGFKV----
At17-2 ----------MSGLQAQDS-------ACSLDKPSKDVV----------ATETKPKKRICCACPDTKKLRDECIVEHGE--SACTKWIEAHILCLRSEGFKV----
Ha17   ----------MSGLQTQDFPS-----ALRLTKAQKDQG--------STESETKPKKKICCACPDTKKLRDECIVEHGE--SACEKWIEAHRLCLRAEGFNV----
Gm17   ----------MSGAQLRSASP-----ALIIQGSQKNEGSV----AVATAAESKPKKKICCACPDTKRLRDECIVEHGE--SACTKWIEAHRLCLRAEGFNV----
At17-1 ----------MTDQPAQNGLI-----PPPTSEPSKAAA----------SAETKPKKRICCACPDTKKLRDECIVEHGE--SACTKWIEAHKICLRAEGFNV----
Dd17   ------------------------MSIAETNTT------------TEVAAPKKKMCCACPETKKVRDECIVANGE--EKCAALIELHKVCLRKEGFDV----
Cr17   ----------MGASGSKPEGA-----GPGPALPTPSPAPPG----VPIGPDGKPKK-ICCSCPDTKKLRDTCIAERGEEHAYCQALIEAHKACLRVEGFKV----
Sp17   ----------MSSSTEPSTAT-------KVSEPAPIAS------------EEKPKP--CCACPETKQARDACMLQSSNGPIECAKLIEAHKKCMAQYGYEV----
Sc17   ------------------------MTETDKKQEQEN----------HAECEDKPKPCCVCKPEKEERDTCILFNGQDSEKCKEFIEKYKECMKGYGFEVPSAN
                                                        ** *   *. ** *:   .:    *   ** :  *:   *:.:
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B

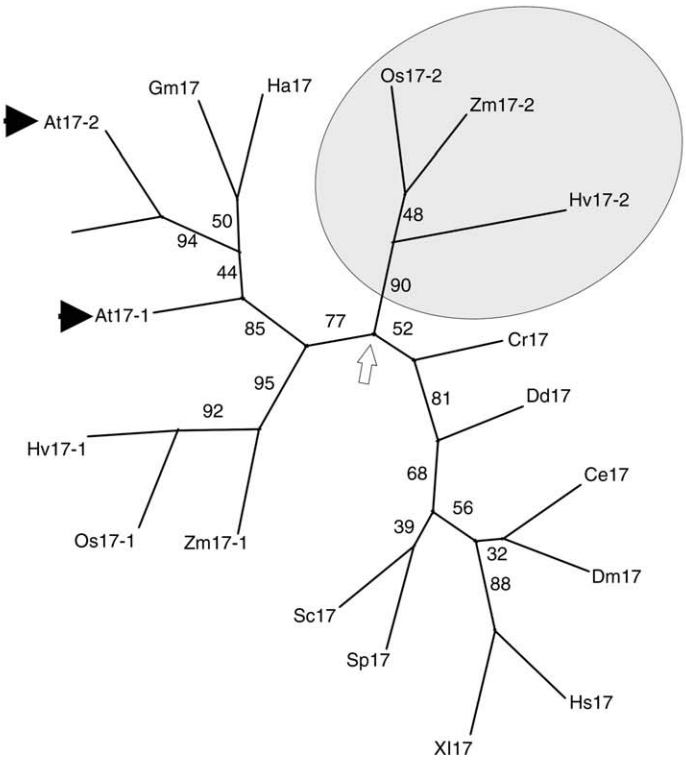

Fig. 1. Comparison of COX17 sequences from different eukaryotic organisms. (A) Alignment of COX17 protein sequences. Nucleotide sequences encoding full-length COX17 polypeptides were identified in data banks by similarity search. The corresponding protein sequences were aligned using CLUSTALW (Thompson et al. 1994). Sequences are ordered according to the results produced by this program, based on their similarity. Positions occupied by identical amino acids are marked with an asterisk; those occupied by related amino acids are marked with double dots. Positions with several conserved amino acids are marked with a single dot. Conserved cysteines are shadowed. (B) Phylogenetic tree derived from COX17 protein sequences, obtained using the PHYLIP group of programs (Felsenstein 1989). The tree is a neighbor-joining consensus one generated by CONSENSUS after bootstrap analysis of 100 trees performed with PROTDIST (with Dayhoff's percent accepted mutations (PAM) matrix) followed by NEIGHBOR. Numbers indicate bootstrap values for each of the groups. Black arrowheads indicate the position of AtCOX17 proteins. The white arrow shows a branch point leading to a clade containing only monocot COX17 protein sequences (shadowed) and another one containing monocot and dicot sequences. The sequences used for the analysis are from *Caenorhabditis elegans* (Ce17, accession no. NM_064774), *Drosophila melanogaster* (Dm17, accession no. NM_132770), *Xenopus laevis* (Xl17, accession no. BC084847), *Homo sapiens* (Hs17, accession no. NM_005694), *Oryza sativa* (Os17-1, accession no. XM_468245; Os17-2, accession no. AP005426), *Hordeum vulgare* (Hv17-1, accession no. CA014768; Hv17-2, accession no. CA011631), *Zea mays* (Zm17-1, accession no. DY397513; Zm17-2, accession no. CN844620), *Brassica napus* (Bn17, accession no. CD838662), *Arabidopsis thaliana* (At17-1 and At17-2), *Helianthus annuus* (Ha17, accession no. CD846302), *Glycine max* (Gm17, accession no. AW569190), *Dictyostelium discoideum* (Dd17, accession no. XM_631455), *Chlamydomonas reinhardtii* (Cr17, accession no. AF280543), *Schizosaccharomyces pombe* (Sp17, accession no. NM_001022571) and *Saccharomyces cerevisiae* (Sc17, accession no. L75948). COX, cytochrome *c* oxidase 17.

gus gene and obtained plants stably transformed with these constructs. The regions used for the analysis contain approximately 1 kbp of untranscribed sequences plus transcribed regions down to nucleotide −1 respective to the translation start codon and thus include the intron located in the 5′ leader region. Several independent lines for each construct were analyzed by histochemistry using the chromogenic substrate X-gluc and expression patterns common to most lines are described below.

Seedlings of plants transformed with *AtCOX17-1* upstream regions grown on Petri dishes on Murashige and Skoog medium showed strong staining in roots (Fig. 2A–C). Activity in roots was evident at very early stages of development (1–2 days after imbibition) and was localized to all root tissues. Cotyledons displayed GUS activity mainly in vascular tissues after 5 days postimbibition and also in the lamina (Fig. 2C, D). Upon progression of development staining was also visible in leaf primordia (Fig. 2D). It should be mentioned that staining in cotyledons and leaf primordia required longer incubation times with the reagent, suggesting that GUS activity in these organs is considerably lower than in roots. Partial staining observed in the hypocotyl is most likely because of diffusion of the dye from roots because the pattern is not uniform (i.e. only the region near the root is stained). Adult plants grown on soil displayed activity in roots, flowers and siliques but not in leaves and stems (Fig. 2E–L). Expression patterns in roots changed upon development. In young root tissues, GUS activity was particularly evident in the vascular cylinder and nascent secondary roots (Fig. 2K, L). In older roots, mainly epidermal cells were stained (Fig. 2K). In flowers, strong expression was detected in anthers, especially in pollen grains (Fig. 2J). Activity in anthers increased upon maturation (Fig. 2I, J). Staining was also detected in the stigma, receptacle and petal veins (Fig. 2I, J), and in siliques, in the septum and the funiculus (Fig. 2F, G). In plants transformed with *AtCOX17-2* sequences, staining patterns were essentially the same (Fig. 2M–X) but longer incubation times were required, suggesting that this construct produces considerably lower expression levels. The main differences with this construct were the lack of staining in the lamina of cotyledons and leaf primordia (Fig. 2N, O), but this may be related with the lower activity of the promoter rather than with a change in expression pattern (i.e. these are tissues where the *AtCOX17-1* promoter is also relatively less active).

To estimate the relative expression levels produced by both constructs, fluorometric assays of GUS activity were performed in protein extracts from different organs of mature plants. Both upstream fragments produced relatively low expression levels in leaves and stems compared with roots, flowers and siliques (Fig. 3). These results agree with the histochemical assays of GUS activity described above. Quantitative GUS measurements also indicated that plants transformed with the *AtCOX17-1* upstream fragment displayed 7- to 10-fold higher activities than plants transformed with the *AtCOX17-2* construct. This is also consistent with histochemical assays because shorter incubation times with the reagents were required to obtain similar staining for *AtCOX17-1* respective to *AtCOX17-2*.

AtCOX17 gene promoters are induced by wounding and other biotic and abiotic stress factors

During the course of histochemical studies of GUS expression with isolated organs, especially with leaves and stems, we noticed the presence of discrete regions that were intensely stained. These regions corresponded either to sites where cuts were made to separate organs or to zones occasionally damaged during manipulation with forceps. Accordingly, we investigated if *AtCOX17* genes were inducible by wounding. Fig. 4A–L shows GUS histochemical assays of leaves and stems that were wounded in different ways prior to collection. Intense staining of regions localized adjacent to damaged zones was observed in organs from plants transformed with either *AtCOX17-1* (Fig. 4A–E) or *AtCOX17-2* (Fig. 4G–K) *gus* fusions. As a control, the same treatments were applied to leaves of plants transformed with the promoter of the *AtCOX6b-1* gene, encoding a COX subunit, fused to *gus*. No staining around damaged zones could be observed in these leaves (Fig. 4F, L), suggesting that the effect is specific for *AtCOX17* genes. It can be concluded that *AtCOX17* genes are locally induced as a response to wounding.

Infiltration of leaves with the bacterial pathogen *P. syringae* pv. tomato also produced an increase in GUS expression from both promoters (Fig. 4M, N). Induction was evident after 4 h of infection with either the virulent or the avirulent strain (carrying the *avrRpm1* gene), respective to leaves that were infiltrated with $MgCl_2$ alone (Fig. 4M, N). Fluorometric assays of GUS activity in leaf protein extracts obtained at different times after infection indicated that both strains produced similar levels of induction but the highest activity was obtained earlier with the avirulent strain (Fig. 5), suggesting the existence of a relationship between *AtCOX17* expression and the induction of the hypersensitive response. It must be noticed that with both strains the increase in GUS activity levels was observed at relatively short times (hours, not days). The results suggest that expression of both *AtCOX17* genes is induced in leaves by biotic and abiotic stress factors that ultimately produce damage to organs

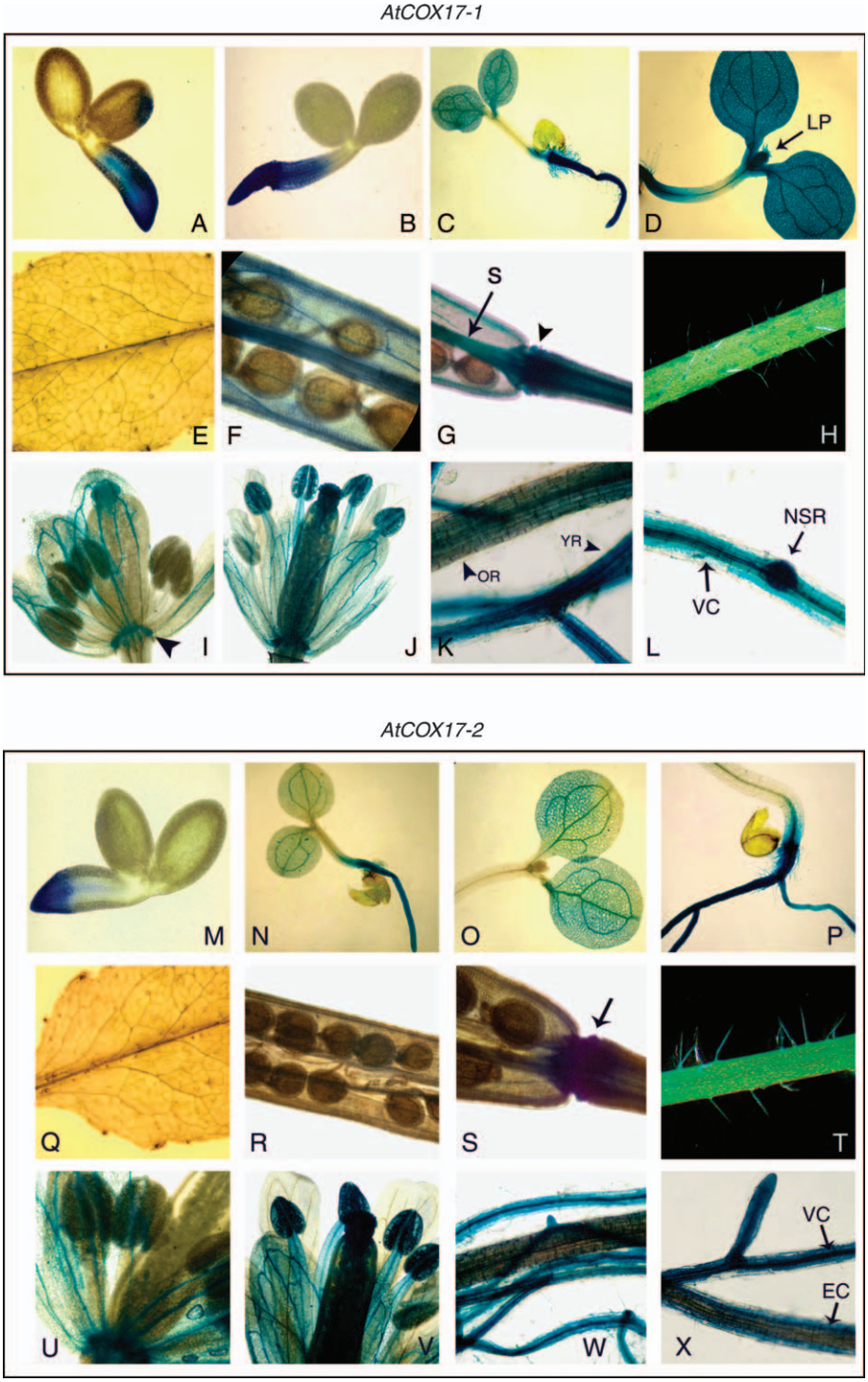

Fig. 2. Histochemical localization of β-glucuronidase (GUS) activity in *Arabidopsis* plants transformed with either the *AtCOX17-1* (A–L) or *AtCOX17-2* (M–X) upstream regions fused to the *gus* reporter gene. Two- (A, M), 3- (B), 5- (C, N), 8- (D, O) or 12-day-old seedlings (P) were incubated in the presence of the chromogenic substrate as described in Materials and methods. Leaves (E, Q), siliques (F, G, R, S), stems (H, T), flowers (I, J, U, V) and roots (K, L, W, X) from adult plants were also analyzed. LP, leaf primordia; S, septum; OR and YR, older and younger root, respectively; VC, vascular cylinder; NSR, nascent secondary root; EC, epidermal cells.

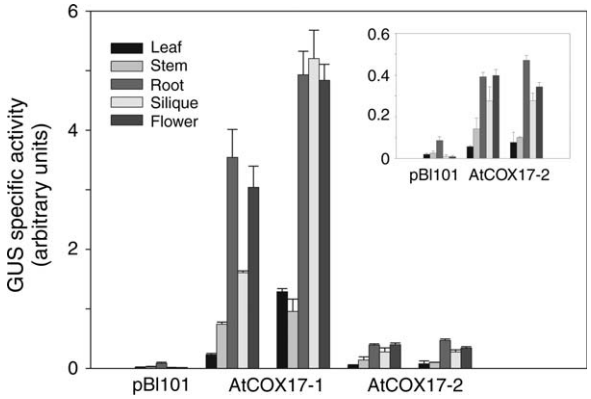

Fig. 3. Analysis of β-glucuronidase (GUS) activity in protein extracts from different organs of plants transformed with *AtCOX17-1* or *AtCOX17-2* upstream regions fused to *gus*. Specific GUS activity was determined using protein extracts prepared from different organs (as indicated) of two independent lines of plants carrying each construct. Activity was also measured in extracts from plants transformed with the promoterless *gus* gene (pBI101). Error bars represent standard deviation of three independent activity measurements. Similar results were obtained with ten different lines for each construct. The inset shows an enlargement of the activity values observed with the *AtCOX17-2* upstream region relative to the promoterless *gus* gene.

or cellular structures. The rapid increase in expression produced by these effectors suggests that induction is part of the primary response to stress or is directly associated with it.

Induction may be related to an increase in reactive oxygen species, which are actively produced under these conditions and act as mediators of cellular responses to these factors. We have then also assayed the effect of treatment of leaves with chemicals that produce oxidative stress, like salicylic acid (SA), sodium nitroprusside (SNP, a nitric oxide donor) and 3-amino-1,2,4-triazole (3-AT, a catalase inhibitor). Histochemical staining of leaves transformed with the *AtCOX17-1* construct showed clear signs of GUS expression after 4 h of infiltration with 1 m*M* SA (Fig. 4Q). A similar result was observed for *AtCOX17-2* (not shown), while leaves infiltrated with water alone displayed no or extremely faint blue color. Increased GUS expression was also observed upon treatment with SNP or 3-AT (Fig. 4Q, R). Fluorometric assays indicated that leaves of plants carrying the *AtCOX17-1* promoter fusion showed a three- to four-fold induction of GUS expression upon treatment with SNP and SA, while the effect of 3-AT was not significant when assayed by this method (Fig. 6A). Induction by SA was observed after 4 h of infiltration, while the effect of SNP was evident only after 8 h. This agrees with histochemical assays because leaves treated with SA during 4 h showed more intense staining than those incubated with SNP or 3-AT (Fig. 4Q, R). *AtCOX17-2*, on the other hand, displayed induction by the three compounds and the response was considerably faster (i.e. highest activity levels were obtained after 4 h of treatment; Fig. 6B). As with *AtCOX17-1*, SA treatment produced the highest expression values among the compounds tested.

Both *AtCOX17* genes were also induced by treatment with copper at several concentrations (Fig. 4O, P). Concentrations of 10–100 μ*M* $CuSO_4$ produced six- to eight-fold increases in expression levels from the *AtCOX17-1* upstream region (Fig. 6A), while 500 μ*M* $CuSO_4$ was less effective. *AtCOX17-2* showed a more uniform response to different copper concentrations (three- to four-fold induction) and was also induced to similar levels by zinc and iron (Figs 4T and 6B). Treatment with zinc was not so effective in plants transformed with the *AtCOX17-1* promoter (Figs 4S and 6A).

A detailed examination of the results obtained after treatment with different compounds indicates that even if both *AtCOX17* gene upstream regions displayed responses to similar factors, their relative effect was not identical for the two genes. *AtCOX17-1* was preferentially induced by relatively low copper concentrations (10–100 μ*M*) and was less responsive to other metals or oxidative stress factors. *AtCOX17-2* showed a more uniform response to all compounds tested.

Discussion

In the present study, we have analyzed the structure of both *Arabidopsis* genes encoding homologs of the metallochaperone COX17, involved in the delivery of copper to mitochondria for COX assembly. The genes share a similar exon–intron structure, with a single intron located in the 5′ untranslated region, close to the start codon. A leader intron is also present in both genes from rice, suggesting that this may be a feature of all plant *COX17* genes. Increasing evidence indicates that introns influence gene expression at different levels (Le Hir et al. 2003, Rose 2004). Accordingly, a role of the leader introns in modulating *COX17* gene expression can be envisaged. It has recently been shown that conserved introns present in the leader regions of *Arabidopsis COX5c* genes (encoding a subunit of COX) greatly influence the expression of these genes (Curi et al. 2005). We have then included the respective introns in the constructs used to study the expression of *AtCOX17* genes.

We have observed that both *AtCOX17* upstream fragments used in this study produce similar tissue-specific expression patterns, with higher reporter gene levels in the case of *AtCOX17-1*. A similar result was obtained when analyzing the upstream regions of two

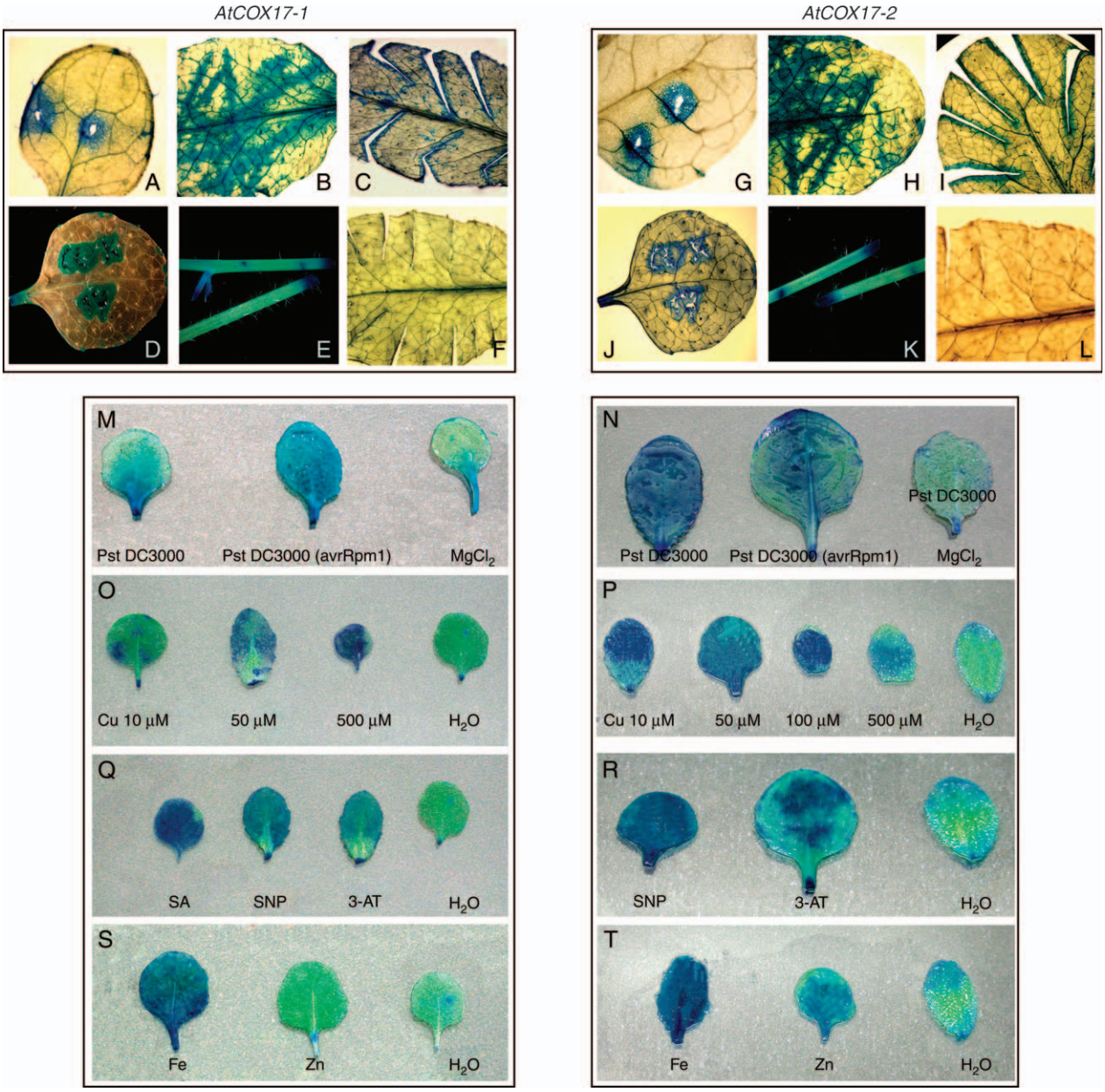

Fig. 4. Induction of *AtCOX17* genes by biotic and abiotic stress factors. Organs from plants transformed with the *AtCOX17-1* or *AtCOX17-2* upstream regions fused to *gus*, as indicated, were wounded in different ways, drilled (A, D, G, J), folded (B, H) or cut (C, E, I, K), 2 h before the histochemical assay of β-glucuronidase (GUS) expression was performed. Plants transformed with an *AtCOX6b-1* promoter–*gus* fusion (F, L) were treated in a similar way. In the lower part of the figure, histochemical detection of GUS activity in leaves infiltrated with different reagents, as indicated (O–T), or with suspensions of the pathogenic bacterium *Pseudomonas syringae* pv. tomato [Pst DC3000, virulent strain; Pst DC3000 (avrRpm1), avirulent strain] (M, N) was performed. Leaves were immersed in the GUS-staining solution 4 h after infiltration and incubated until satisfactory color development was observed. Concentrations of reagents are those described in the legend of Fig. 6.

Arabidopsis COX5c genes (Curi et al. 2005), but not with genes encoding cytochrome *c* or the iron-sulfur subunit of succinate dehydrogenase, which show differential expression patterns (Elorza et al. 2004, Welchen and Gonzalez 2005). It is not clear why two genes with similar expression characteristics persist in the *Arabidopsis* genome in the case that the encoded proteins do not have different functions. This is especially valid for the gene with lower expression levels, *AtCOX17-2*, unless it has acquired the ability to respond to specific factors not recognized in this study. Lister et al. (2004) found that in gene families encoding components of the mitochondrial

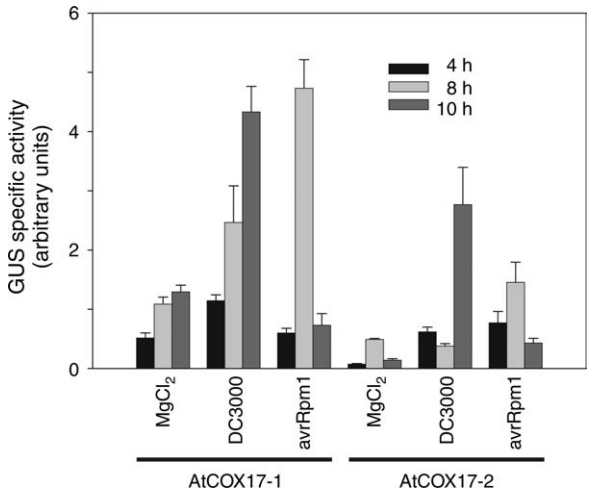

Fig. 5. Induction of *AtCOX17* promoters by pathogen attack. β-Glucuronidase (GUS) activity was measured in leaves of plants transformed with either *AtCOX17-1* or *AtCOX17-2* upstream regions fused to *gus* at different times after exposure to virulent (DC3000) or avirulent (avrRpm1) *Pseudomonas syringae* pv. tomato strains (4×10^8 cfu ml^{-1}). A solution of 1 m*M* $MgCl_2$ was used as a mock control of infiltration. Measurements were performed on a pool of five independent lines for each construct. Error bars represent standard deviation of three independent activity measurements.

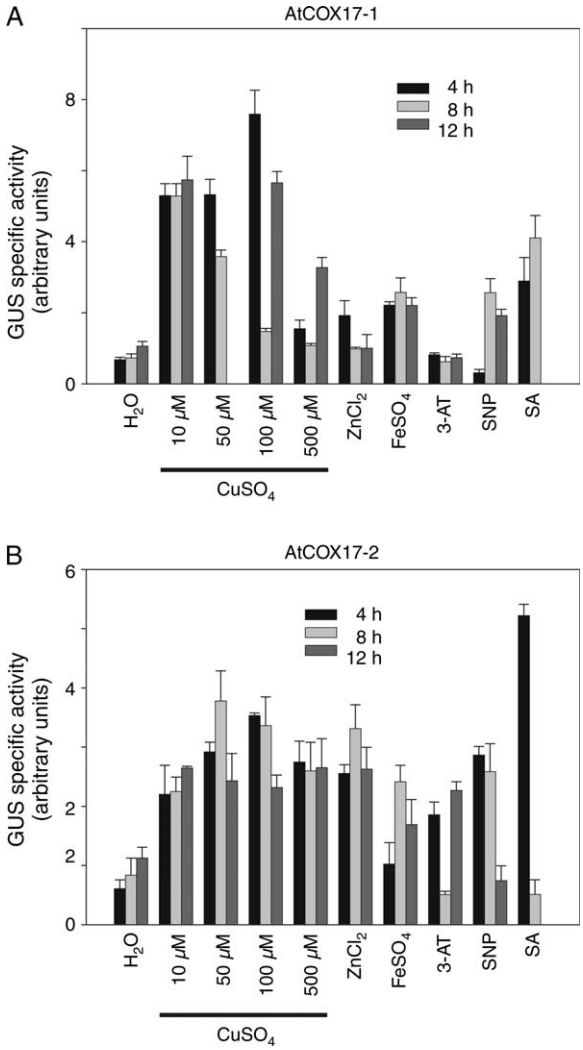

Fig. 6. Induction of *AtCOX17* promoters by metals and compounds that produce oxidative stress. β-Glucuronidase activity was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide, and protein extracts were prepared at different times after infiltration of leaves with H_2O, $CuSO_4$ at different concentrations, 1 m*M* $ZnCl_2$, 1 m*M* $FeSO_4$, 4 m*M* 3-amino-1,2,4-triazole, 1 m*M* sodium nitroprusside or 1 m*M* salicylic acid. Pools of five independent lines transformed with either *AtCOX17-1* (A) or *AtCOX17-2* (B) upstream regions were used for the analysis. Error bars represent standard deviation of three independent activity measurements.

import apparatus, one of the genes was predominantly expressed while the rest showed increased induction by stress factors, tentatively as a response to higher demands for mitochondrial biogenesis to replace damaged proteins. A similar conclusion may not apply for *AtCOX17* genes because both display similar responses (although with some differences, see below). The analysis of mutants in each of the *AtCOX17* genes will be necessary to evaluate if they have redundant or specialized functions.

Because COX17 has a dual intracellular localization in yeast (Beers et al. 1997), one possibility is that one of the *Arabidopsis* genes encodes the mitochondrial form while the other encodes the cytoplasmic form. Predictive analysis of intracellular localization using several available programs does not indicate mitochondrial targeting for any of the *Arabidopsis* COX17 proteins (results not shown). This is not unexpected because COX17 is imported through a disulfide relay system rather than through the common import pathway used by most mitochondrial proteins (Mesecke et al. 2005). The similar expression patterns of the respective genes would indicate, however, that both proteins have similar functions. In addition, it has been observed that both proteins complement the respiratory deficiency of a yeast *cox17* null mutant (Wintz and Vulpe 2002), suggesting that they can reach the intermembrane space.

At early stages of development, GUS expression was mainly localized in roots and low activity levels were also detected in cotyledon veins approximately 1 week after germination. Preferential expression in roots is coincident with previous expression studies using specific antibodies (Wintz and Vulpe 2002). In mature plants, in addition, histochemical staining was especially evident in anthers and was also detected in siliques. Preferential expression in anthers is a feature of most genes encoding mitochondrial components (Elorza et al. 2004, Welchen and Gonzalez 2005, Welchen et al. 2004, Zabaleta et al.

1998) and may be related to the existence of active mitochondrial biogenesis in anther tissues (Huang et al. 1994, Lee and Warmke 1979). Expression in roots was also observed for many respiratory chain genes, but generally localized to the root meristems and/or vascular cylinder (Elorza et al. 2004, Welchen and Gonzalez 2005, Welchen et al. 2004). The high levels of root expression observed for both *AtCOX17* genes may reflect the participation of AtCOX17 in a different process, like the uptake or delivery of copper. It is noteworthy that other metal transporters also show preferential expression in roots (Andrés-Colás et al. 2006, Eckhardt et al. 2001, Mills et al. 2003). Expression in root epidermal cells may also be related to a role in the transport of copper incorporated from the medium. These putative functions of COX17 in roots, not directly related with COX assembly, may be fulfilled by the cytosolic form. This possibility is currently under investigation.

An increase in transcript levels recognized by an *AtCOX17-1* cDNA probe upon treatment of plants with biotic and abiotic stress factors has been reported by Balandin and Castresana (2002). From these results, it was not clear if the observed responses were because of both or only one of the *AtCOX17* genes as the presence of more than one gene was not evidenced in this study. We show here that both *AtCOX17* promoters are responsive to compounds or factors that produce stress, like metals, SA, pathogens and wounding. A common effect of these factors may be the production of reactive oxygen species, which originate damage inside cells (Alvarez et al. 1998, Drazkiewicz et al. 2004, Orozco-Cardenas and Ryan 1999, Rao et al. 1997). High induction levels were observed within a few hours with most treatments, suggesting that this is a direct response to the accumulation of reactive oxygen species and not a secondary effect associated with toxicity of these compounds or cell death. It can be hypothesized that *AtCOX17* promoters respond to factors that may damage or inactivate COX, to increase copper delivery for new COX biogenesis. Whether this is a true retrograde response, that is, a response to signals originated within mitochondria, is not clear from our results. In favor of this, it has been reported that SA and nitric oxide, both inducers of *AtCOX17* expression, act as inhibitors of the respiratory chain (Norman et al. 2004, Zottini et al. 2002). In a similar way, microarray experiments show an induction of *AtCOX17-1* and *-2* by the Complex I inhibitor rotenone (Lister et al. 2004). Inhibition of electron transport causes an increase in the production of reactive oxygen species by mitochondria (Maxwell et al. 1999).

Induction by stress was especially evident in leaves, where reporter gene expression driven by *AtCOX17* promoters was extremely low under basal conditions. The fact that genes encoding COX subunits do not seem to respond to stress in a similar way suggests that COX assembly factors, or at least COX17, may be limiting either because of the availability of newly synthesized subunits or because subunits from damaged COX are reused in the assembly process.

The kind of responses observed for *AtCOX17* promoters resembles those of the *AOX1a* gene, encoding alternative oxidase (Vanlerberghe and McIntosh 1997). It is generally assumed that this gene responds to situations in which the cyanide-sensitive respiratory pathway is compromised, so that the flow of electrons to oxygen continues and the oxidized forms of coenzymes are regenerated. The existence of a similar behavior for genes involved in COX assembly seems to indicate that another level of response to the same situation is an increase in the biogenesis of respiratory complexes. Detailed analysis of the respective promoters will indicate if *COX17* and *AOX1a* genes are targets of similar regulatory pathways. It should be noticed that even if *AtCOX17* promoters respond to similar factors, the relative effects of different factors are not the same. *AtCOX17-1* is preferentially induced by copper at concentrations of 100 μM or less, while *AtCOX17-2* displays a more uniform response to different compounds. This may suggest that induction operates through similar but not identical signaling pathways. The response of *AtCOX17-1* to copper seems to be the result of additional factors besides oxidative stress.

The putative promoter regions of *AtCOX17* genes contain several elements (three in *AtCOX17-1*; four in *AtCOX17-2*) known as site II motifs (Kosugi et al. 1995, Trémousaygue et al. 2003) located proximal to the transcription start site (downstream from −200). These elements have the consensus TGGGCC/T and are frequently observed in genes preferentially expressed in proliferating tissues. A pair of site II motifs is also present in a similar position in *OsCOX17-1*, the rice *COX17* gene that is more related to the *Arabidopsis* ones. Site II elements are associated in many genes with a second motif, named internal telomeric repeat or *telo* box (AAACCCTA), involved in enhancing expression levels (Trémousaygue et al. 2003). Interestingly, a *telo* box is present in both *AtCOX17* leader introns, which were included in the constructs introduced into plants in this study. The presence of these two motifs in both *AtCOX17* genes may have a functional meaning. It has been recently shown that similar elements are responsible for the expression of one of the *Arabidopsis* cytochrome *c* genes (*Cytc-1*) in meristems and anthers (Welchen and Gonzalez 2005), and site II elements are present in a majority of promoters of genes encoding components of the *Arabidopsis* mitochondrial respiratory chain (Welchen and Gonzalez 2005 and unpublished results).

The expression of *AtCOX17* genes may then be linked to that of other respiratory components through these elements. Responses to different types of stress, on the other hand, possibly operate through other elements and factors. A search for motifs involved in induction by biotic and abiotic stress factors allowed the identification of the sequence AGCCACC, termed S box (Kirsch et al. 2000), in the complementary strand of the *AtCOX17-1* gene, 78 nucleotides upstream from the transcription start site. Tetramers of this box were sufficient to confer transcriptional responses to wounding and pathogens in a synthetic promoter (Rushton et al. 2002). In addition, a sequence matching the W box (TGACT), also involved in responses to stress factors (Rushton et al. 1996) is present at −201 from the transcription start site of the same gene. Similar elements were identified in the *AtCOX17-2* gene, but located at very different positions (ACCCACC at −618 and TGACT at +12, within the transcribed untranslated region). Detailed mutagenic analysis of *AtCOX17* promoters will be useful to identify the elements that are responsible for the observed expression characteristics.

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