Altering the Expression of the Chlorophyllase Gene *ATHCOR1* in Transgenic Arabidopsis Caused Changes in the Chlorophyll-to-Chlorophyllide Ratio[1]

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The Arabidopsis gene *ATHCOR1*, which encodes the CORI1 (coronatine-induced) protein, was expressed in bacterial cells. Soluble recombinant CORI1 was purified and shown to possess chlorophyllase (Chlase) activity in vitro. To determine its activity in vivo, wild-type Arabidopsis and *coi1* mutant, which lacks *ATHCOR1* transcripts, were transformed with sense and antisense forms of the gene. Wild-type and *coi1* plants overexpressing *ATHCOR1* showed increased contents of chlorophyllide (Chlide) without a substantial change in the total amount of the extractable chlorophyll (Chl). These plants presented high Chlide to Chl ratios in leaves, whereas antisense plants and nontransformed *coi1* mutant showed undetectable *ATHCOR1* mRNA and significantly lower Chlide to Chl ratios, relative to wild-type control. Overexpression of *ATHCOR1* caused an increased breakdown of Chl *a*, as revealed by the Chlide *a* to *b* ratio, which was significantly higher in sense than wild-type, *coi1* mutant, and antisense plants. This preferential activity of CORI1 toward Chl *a* was further supported by in vitro analyses using the purified protein. Increased Chlase activity was detected in developing flowers, which correlated to the constitutive expression of *ATHCOR1* in this organ. Flowers of the antisense plant showed reduced Chlide to Chl ratio, suggesting a role of CORI1 in Chl breakdown during flower senescence. The results show that *ATHCOR1* has Chlase activity in vivo, however, because *coi1* flowers have no detectable *ATHCOR1* mRNA and present Chlide to Chl ratios comparable with the wild type, an additional Chlase is likely to be active in Arabidopsis. In accordance, transcripts of a second Arabidopsis Chlase gene, *AtCLH2*, were detected in both normal and mutant flowers.

The chlorophyllase (Chlase) enzyme (chlorophyll-chlorophyllido hydrolase, EC 3.1.1.14) catalyzes the hydrolysis of the ester bond of the chlorophyll (Chl) molecule producing chlorophyllide (Chlide) and phytol (for review, see Gossauer and Engel, 1996; Matile et al., 1996, 1999). This reaction is considered to be the first step during Chl catabolism, because the products of the Chl breakdown in different plant species present mainly nonesterified structures (Amir-Shapira et al., 1987; Engel et al., 1991; Shioi et al., 1991).

Chlase has been purified from a variety of plants and shown to be a glycosylated protein associated to plastid membranes (McFeeters, 1975; Terpstra, 1981; Schellenberg and Matile, 1995; Brandis et al., 1996; Matile et al., 1997; Tsuchiya et al., 1997). However, despite the great availability of purified Chlases obtained in the past decades for N-terminal sequencing and antibody production, the identification of Chlase genes was reported only recently (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999; Takamiya et al., 2000).

Chlase activity has been correlated to reduced Chl contents in senescing leaves (Jenkins et al., 1981; Kura-Hotta et al., 1987; Rodríguez et al., 1987) and to respond to ethylene during fruit ripening (Trebitsh et al., 1993). However, Chlase activity has also been found in presenescent leaves, in greened tissues, and during periods of increased Chl synthesis, suggesting a role in Chl turnover (Tanaka et al., 1982; Matile et al., 1996; Minguez-Mosquera and Gallardo-Guerrero, 1996). It has been proposed that Chlase activity is latent because hydrolysis of endogenous Chl does not take place unless chloroplast membranes are disrupted or solubilized with detergents (Terpstra, 1980; Schoch and Brown, 1987). This latency has been attributed to a spatial separation between Chls bound to proteins in the thylakoid membrane and Chlase, which appears to be located in the plastid envelope (Matile et al., 1997). Therefore, Chlase activity in vivo and its regulation and physiological role during Chl catabolism and senescence are still not fully understood.

The Arabidopsis *ATHCOR1* was first identified as a gene induced by coronatine (Benedetti et al., 1998), a chlorosis-inducing phytotoxin produced by various plant pathogenic bacteria (for review, see Bender et al., 1999). Furthermore, the gene was shown to be up-regulated in Arabidopsis leaves by methyl jas-

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monate (MeJA) and wounding, and to require the *COI1* gene for expression (Benedetti et al., 1998). When *ATHCOR1* was identified, there were no hits to similar genes in the public databases, except that its predicted protein (CORI1) had a potential glycosylation site and a conserved motif found in various hydrolase enzymes, particularly Ser lipases (Benedetti et al., 1998).

ATHCOR1 (named *AtCLH1*) was shown recently to be related to a Chlase gene from *Chenopodium album* and to present Chlase activity in cell extracts of *Escherichia coli* transformed with the *AtCLH1* cDNA (Tsuchiya et al., 1999). Here, we show that soluble recombinant CORI1, purified by affinity chromatography, has Chlase activity in vitro, but most importantly, we demonstrate that *ATHCOR1* has Chlase activity in vivo, as revealed by the expression analysis of the gene correlated to the Chl and Chlide contents in the *coi1* mutant, sense, and antisense Arabidopsis.

RESULTS

In Vitro Chlase Activity of Recombinant CORI1 Protein

Recombinant CORI1 was expressed in bacteria as a fusion protein with the maltose-binding protein (MBP) in attempt to obtain soluble and functional Chlase. Although most of the recombinant fusion was retained into the insoluble fraction, a reasonable level of soluble CORI1-MBP was produced (Fig. 1A). The purified fusion protein was tested for in vitro Chlase activity, and when added to a solution containing Chl, the recombinant protein was capable of breaking down Chl into Chlide (Fig. 1B). Purified MBP without fusion did not show Chlase activity (not shown). The contents of Chlide *a* and *b* produced in the reaction were spectrophotometrically quantified. Figure 1C shows that the rate of Chlide formation was higher for Chlide *a*, suggesting an increased activity of CORI1 toward Chl *a*.

Although Chlase appears to be a glycosylated protein (Terpstra, 1981; Tsuchiya et al., 1997), and its activity enhanced by divalent cations and some detergents (Terpstra, 1980), CORI1 produced in *E. coli* was functional and required no lipids or detergents to perform the cleavage of the Chl molecule. In addition, we observed that CORI1 was equally efficient at pH 6.0 to 7.5; it was stable at 4°C but less stable at 37°C. Activity was greatly reduced in the presence of 0.2% to 1.0% (v/v) Triton X-100 or in acetone above 40% (v/v). These properties are remarkably similar to what was found for the native rye Chlase (Tanaka et al., 1982).

The hydrolytic esterase activity of CORI1 appears to be very specific to Chls. The recombinant protein was not capable of hydrolyzing ρ-nitrophenyl-esters of fatty acid or tributyrin (not shown), as is the case of the bacterium oil-degrading enzyme HDE (Mizuguchi et al., 1999), although significant homology is found between CORI1 and HDE within their conserved lipase motifs.

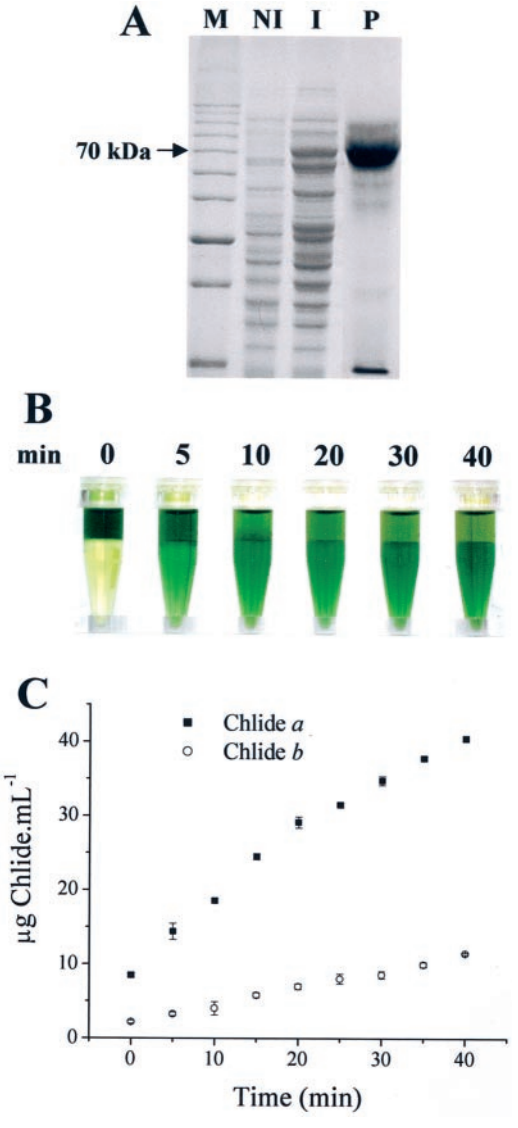

Figure 1. Purification and in vitro activity of recombinant CORI1 protein. A, SDS-PAGE of soluble CORI1 produced as an MBP-fusion protein; M, 10-kD M_r ladder; whole *E. coli* protein extract before (NI) and after (I) induction with isopropylthio-β-galactoside; P, purified CORI1-MBP fusion. B, Aliquots of the purified CORI1-MBP fusion (approximately 50 μg) were incubated in 50 mM MOPS buffer, pH 7.0, containing Chl dissolved in acetone for different time periods. After incubation, 0.5 mL of the reaction was mixed to 0.4 mL of hexane plus 0.4 mL of acetone for partitioning of the remaining Chl to the hexane phase (top). C, Spectrophotometric measurements of the contents of Chlide *a* and *b* in the acetone phase from samples illustrated in B. Values are the mean of three independent measurements plus the SD.

In Vivo Chlase Activity of *ATHCOR1* in Leaves

With the aim of studying the in vivo role of *ATHCOR1*, wild type and *coi1* mutant were transformed with sense and antisense forms of the

ATHCOR1 gene under the control of the cauliflower mosaic virus 35S promoter. After kanamycin selection, wild-type plants transformed with the sense (wtS1 and wtS4) and antisense (wtA1) constructs, and a *coi1* mutant transformed with the sense construct (*coi1*S2) were isolated. The progeny seeds (T2 generation) obtained from wtS4 and wtA1 segregated for kanamycin resistance at a rate of 1:1, as opposed to the 3:1 ratio observed in the progeny of the wtS1 and *coi1*S2.

The phenotypes of the transgenic plants were similar and not distinguishable from nontransformed plants, by visual inspection. Genomic DNA from transgenic and control plants was isolated and used in PCR reactions to evaluate T-DNA insertions. Figure 2A shows that transgenic plants resistant to kanamycin (T2 plants) presented a PCR band corresponding to DNA fragments generated by internal 3′-end primers specific to *ATHCOR1* and a 5′-end primer specific to the 35S promoter. The expected PCR bands were also detected in kanamycin resistant plants of the T3 generations (not shown).

As reported previously, expression of *ATHCOR1* in Arabidopsis is dependent on the activity of the *COI1* gene, and levels of *ATHCOR1* mRNA are low in wild-type leaves but are rapidly increased after wounding or MeJA treatment (Benedetti et al., 1998). As expected, the levels of *ATHCOR1* mRNA (approximately 1.3 kb) were significantly increased in leaves of sense plants wtS1, wtS4, and *coi1*S2, relative to nontransformed wild type and *coi1* mutant (Fig. 2B). In contrast, leaves of the antisense plants showed high levels of an approximately 1-kb message (Fig. 2B), which probably corresponds to the antisense mRNA (an 800-bp construct plus a poly(A) tail). In addition, both wtS4 and wtA1 plants showed lower M_r bands with a "smear" suggestive of mRNA degradation. These lower M_r bands were also observed in the heterozygous *COI1*/*coi1*S2, an F_1 plant from the cross between homozygous *coi1* carrying the sense construct (*coi1*S2) versus the wild type.

To examine the Chlase activity of *ATHCOR1* in vivo, total Chl extracted from leaves of nontransformed and transgenic plants was fractionated into Chl/Chlide. Figure 2C shows that sense plants, including *coi1*S2, visually presented higher contents of Chlide relative to wild type, *coi1* mutant, and antisense wtA1. The amount of extractable Chl/Chlide from leaves of normal and transgenic plants was quantified. Although the amount of Chls *a* and *b* did not vary substantially between nontransformed and transgenic plants or between wild type and *coi1* mutant (Fig. 3A), the contents of Chlide *a* and *b* in leaves of sense plants were significantly increased relative to wild-type, *coi1* mutant, and antisense plants (Fig. 3B). When the relative amount of Chlide per total Chl was measured as an estimate of the Chlase activity, it became clear that antisense and *coi1* mutant had reduced Chlase activities compared with wild-type and sense plants (Fig. 3C). Moreover, the ratio of Chlide *a*/*b* was higher in sense than wild-type, *coi1* mutant, and antisense plants, indicating that Chlase activity by *ATHCOR1* is preferential toward Chl *a* (Fig. 3D).

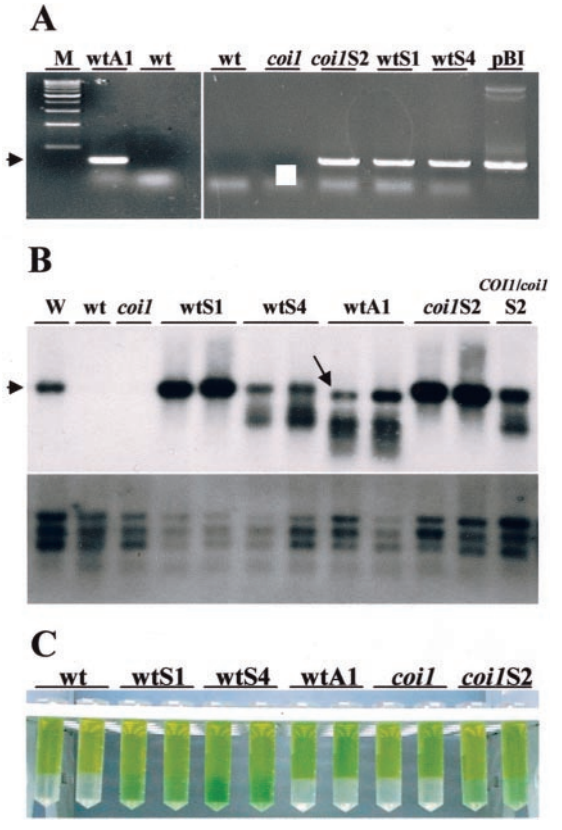

Figure 2. In vivo Chlase activity of CORI1 in wild-type, *coi1*, and transgenic plants. A, PCR products of genomic DNA extracted from Arabidopsis plants transformed with sense (wtS and *coi1*S) and antisense (wtA1) constructs of 35S::*ATHCOR1*, showing the amplification of DNA fragments of approximately 800 bp specific to the transgenes (arrowhead); pBI, control vector containing 35S::*ATHCOR1* used for transformation of sense plants; M, 1-kb M_r ladder. B, Northern-blot analysis of total RNA extracted from leaves of wild type (wt), *coi1* mutant, and two individuals of each transgenic line expressing the sense or antisense *ATHCOR1*, probed with *ATHCOR1* or ubiquitin (below). An mRNA sample from wounded leaves of wild-type plants as positive control for *ATHCOR1* induction is shown (W), and the arrowhead indicates the expected approximately 1.3-kb band; antisense plants showed a major approximately 1-kb band (arrow) plus smaller bands also observed in wtS4 and *COI1/coi1*S2, an F_1 plant from the cross between wild type and homozygous *coi1* transformed with the sense construct. C, Hexane/acetone partitioning of the Chl (top phase) and Chlide (acetone phase) extracted from leaves of wild-type, *coi1* mutant, and transgenic plants.

ATHCOR1 mRNA and Chlase Activity Are Increased in Flowers

It has been shown that, whereas transcripts of *ATHCOR1* are induced upon wounding, coronatine, or MeJA treatments in developing leaves, the gene appears to be constitutively expressed in flowers

(Benedetti et al., 1998). Here, we show that *ATHCOR1* transcripts are differentially expressed during flower development. Higher levels of *ATHCOR1* transcripts were detected at the stage of buds (Fig. 4A), therefore, *ATHCOR1* mRNA of young flowers (stages 1–3 shown in Fig. 4A) of nontransformed and transgenic plants were compared (Fig. 4B). The expression of *ATHCOR1* messages in flowers of normal and transgenic plants was similar to the expression pattern observed in leaves, except that wtS4 flowers showed lower mRNA levels relative to wild type.

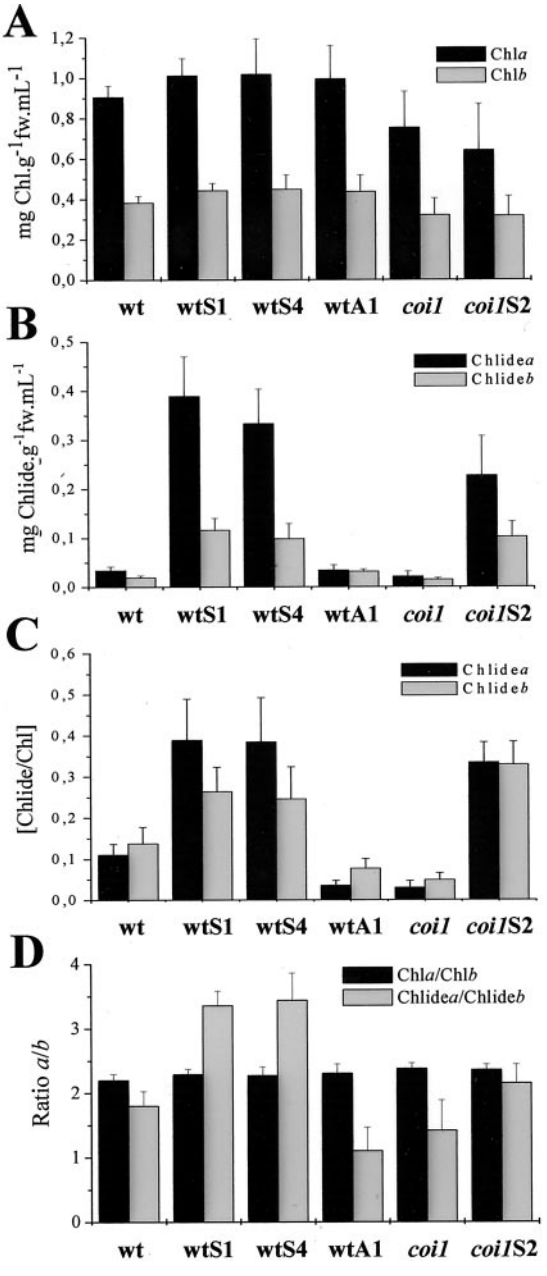

Figure 3. Relative contents of Chl and Chlide in wild-type, *coi1*, and transgenic plants. Contents of Chl and Chlide from leaves of wild-type (wt), *coi1* mutant, sense (wtS, *coi1*S), and antisense (wtA) plants that were extracted in 1 mL of acetone. The amount of extractable Chl per gram of fresh weight did not vary significantly in wild-type, *coi1*, and transgenic plants (A). By contrast, Chide contents in leaves of sense plants were significantly higher than in wild-type, *coi1*, and wtA1 plants (B). C, In vivo Chlase activity (Chlide/Chl) was increased in sense plants, but diminished in the antisense wtA1 and *coi1* mutant. D, The ratio of Chlide *a* to Chlide *b* was higher in sense than in wild-type, wtA1, and *coi1* plants, indicating that CORI1 activity is increased toward Chl *a*. Values are the mean of 10 independent measurements plus the SD.

The $a+b$ Chlide to Chl ratio from young and developed flowers was examined and, contrary to the mRNA accumulation, the estimated Chlase activity was slightly but significantly increased in developed rather than undeveloped flowers, except in the *coi1* mutant, where the difference between the two activities was not statistically significant (Fig. 4C). The values of the $a+b$ Chlide to Chl ratio in flowers were, on average, 2- to 5-fold higher than that of leaves (not shown), which correlates to the *ATHCOR1* mRNA levels found in flowers. Chlase activity in flowers of sense wtS1 was twice of the normal flowers, whereas in the antisense it was significantly reduced (Fig. 4C). Chlase activity in *coi1* flowers was unexpectedly comparable with that of the wild type (Fig. 4C), despite the fact that *coi1* flowers showed no detectable mRNA bands on northerns (Fig. 4B). In addition, Chlase activity in developed flowers of sense wtS4 and *coi1*S2 was not statistically different from the wild type (Fig. 4C), and high levels of *ATHCOR1* transcript in *coi1*S2 flowers (Fig. 4B) did not result in an increment on the Chlide to Chl ratio (Fig. 4C). These results suggested the presence of an additional Chlase activity in flowers not dependent on the *COI1* gene. Therefore, the presence of transcripts of a second Arabidopsis Chlase gene (*AtCLH2*), which is expressed in leaves but is not induced by MeJA (Tsuchiya et al., 1999), was analyzed in normal and mutant flowers.

Transcripts of Chlase Gene *AtCLH2* Is Present in *coi1* Flowers

Transcripts of *AtCLH2* were detected in very low abundance in Arabidopsis flowers; they were present in similar amounts in both wild-type and *coi1* flowers and their relative levels were not altered between normal and transgenic plants (Fig. 5A). Transcripts of *AtCLH2* were poorly detected in leaves of normal, *coi1* mutant, and transgenic plants by northern blot (not shown). To further investigate the expression of *AtCLH2* in different Arabidopsis organs, semiquantitative RT-PCR was employed. Figure 5B shows that *AtCLH2* mRNA is present in leaves, flowers, and flower buds of both wild-type and *coi1* plants, with an apparently higher level in *coi1* flowers. Similar to *ATHCOR1*, *AtCLH2* is not expressed in roots; however, treatment with MeJA did not induce its expression in leaves (Fig. 5).

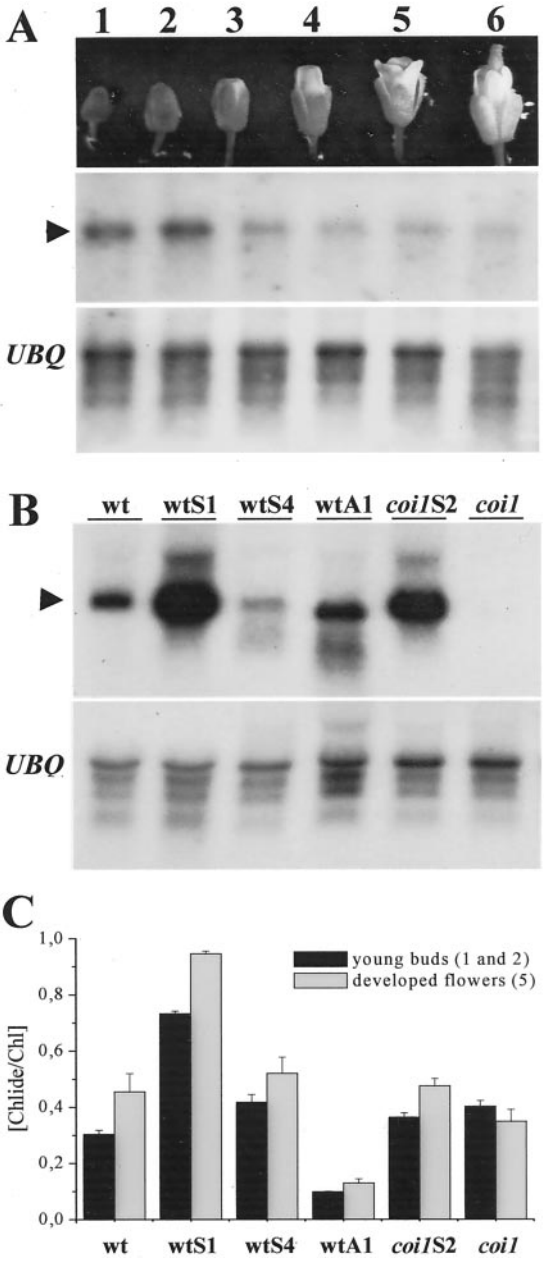

Figure 4. *ATHCOR1* mRNA and Chlide to Chl ratio during flower development. A, *ATHCOR1* mRNA during wild-type flower development (stages 1–6) showing higher levels of transcripts (arrowhead) at the stage of buds, compared with ubiquitin (*UBQ*). B, Northern blot of *ATHCOR1* in flower buds of wild-type (wt), *coi1* mutant, sense (wtS, *coi1*S), and antisense (wtA) plants showing high expression of the sense (wtS1, *coi1*S2) and antisense (wtA1) mRNA relative to normal wild type. Lower levels of sense messages are observed in wtS4, wtA1, and *coi1* plants, as compared with the major approximately 1.3-kb *ATHCOR1* transcript (arrowhead). C, The ratio of Chlide *a*+*b* to Chl *a*+*b* in young (stages 1 and 2) and developed flowers (stage 5) of wild-type, *coi1*, sense, and antisense plants, indicating increased Chlase activities in mature flowers relative to buds, in all plants examined, excepted in *coi1*, where the difference between the means were not statistically significant. Values are the mean of five independent measurements plus the SD.

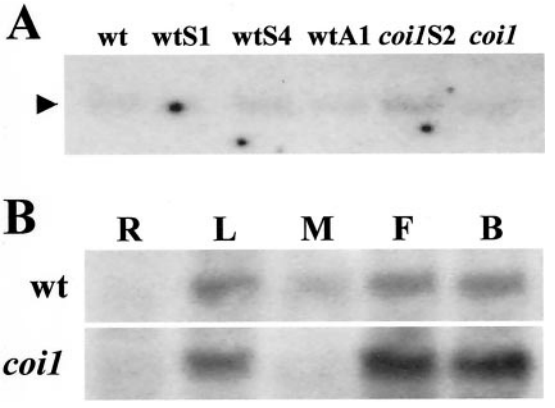

Figure 5. Transcripts of Chlase gene *AtCLH2* is present in *coi1* flowers. A, Northern-blot analyses of the same RNA samples shown in Figure 4B probed with the *AtCLH2* cDNA. Transcripts of *AtCLH2* are detected at low levels in wild-type (wt), *coi1* mutant, transgenic sense (wtS, *coi1*S), and antisense (wtA) plants (arrowhead). B, Southern-blot analysis of *AtCLH2* expression by reverse transcriptase (RT)-PCR. The amplified fragments were blotted, transferred to a nylon membrane, and hybridized to the *AtCLH2* cDNA. *AtCLH2* transcripts were detected in leaves (L), flowers (F), and flower buds (B) of both wild type and *coi1* mutant. The gene was not detected in roots (R) and MeJA did not induce it in leaves (M).

DISCUSSION

In the present study, we provided evidence for the in vitro and in vivo function of the Arabidopsis *ATHCOR1* gene as a Chlase. We showed that soluble CORI1 protein expressed in bacterial cells and purified by affinity chromatography has Chlase activity in vitro. This will help further studies on the molecular and biochemical properties of the enzyme.

Recombinant CORI1 has previously been shown to have in vitro Chlase activity, however, in experiments where whole bacterial cell fractions were used as the source of the enzyme (Tsuchiya et al., 1999). Similarly, recombinant and soluble Chlase from *Citrus* has recently been described, but further purification of the enzyme from bacterial fractions was not reported (Jacob-Wilk et al., 1999).

The in vivo activity of CORI1 as a Chlase was clearly characterized by the expression analysis of the gene associated to the Chlide to Chl ratios obtained from the *coi1* mutant and transgenic Arabidopsis transformed with sense and antisense *ATHCOR1*. Wild-type Arabidopsis and *coi1* mutant overexpressing *ATHCOR1* presented higher Chlase activity in leaves, relative to nontransformed plants, whereas the opposite was observed for the antisense and normal *coi1*, which does not express *ATHCOR1*. In addition, the activity of CORI1 was increased toward Chl *a*, as revealed by the in vitro activities and by the changes in the Chlide *a* to *b* ratio in transgenic plants overexpressing *ATHCOR1*. This is consistent with the in vivo Chlase activities determined during leaf senescence in various plants. For instance, Chl *a* is degraded at a faster rate than Chl *b* in leaves of

soybean (*Glycine max*; Jenkins et al., 1981), rice (*Oryza sativa*; Kura-Hotta et al., 1987), and wheat (*Triticum aestivum*; Patterson and Moss, 1979) and in the algae *Phaeodactylum tricornutum* (Schoch and Brown, 1987).

The Chlide to Chl ratio in flowers was higher than in leaves, which correlates with the constitutive expression of *ATHCOR1* found in developing flowers. Although *ATHCOR1* mRNA accumulates at the early stages of flower development, Chlase activity seems to increase as flowers are fully developed, suggesting that Chl breakdown may take place during sepal senescence. In developed flowers, Chlase activities found in the *coi1* mutant, sense wtS4, and *coi1*S2 were similar to normal wild type. Therefore, although flowers of the antisense plant showed significantly reduced Chlide to Chl ratios, the results suggest that an additional *COI1*-independent Chlase is likely to be active in Arabidopsis flowers. The Arabidopsis gene *AtCLH2*, which is related to *ATHCOR1*, was shown to be constitutively expressed in rosette leaves at a low level and not to respond to MeJA (Tsuchiya et al., 1999). Here, evidence was presented indicating that *AtCLH2* is expressed in *coi1* flowers, therefore, supporting the hypothesis of a second Chlase activity in Arabidopsis not dependent on *COI1*. In addition, *AtCLH2* transcript accumulation did not vary between normal and transgenic *ATHCOR1* plants, indicating that the changes in the Chl to Chlide ratio observed in the transgenic plants were caused by the altered expression of the *ATHCOR1* gene.

Interestingly, *ATHCOR1* and *AtCLH2* transcripts are apparently missing in roots, a strong indication of an association with chloroplast activity. This is consistent with the presence of a typical chloroplast transit peptide found in the amino acid sequence of *AtCLH2* (Tsuchiya et al., 1999). Although the CORI1 protein presents several Ser and Thr residues in its N terminus, a common feature of chloroplast transit peptides, its sub cellular localization cannot be inferred from its sequence.

It was observed that both antisense wtA1 and sense wtS4 presented small M_r mRNA bands on northerns, which is suggestive of a post-transcriptional gene silencing (PTGS) phenomenon (Bass, 2000). In the case of the antisense plant, transcripts corresponding to the endogenous *ATHCOR1* (approximately 1.3 kb) are apparently missing, particularly in flowers where the endogenous gene is normally detected. Interestingly, however, is that the suggested PTGS appears to be more pronounced in wtS4 flowers than in wtS4 leaves, perhaps because endogenous *ATHCOR1* is expressed at a higher level in flowers. In accordance, Chlide to Chl ratio in wtS4 plants is significantly higher in leaves but not statistically different from the wild type in flowers. Similarly, heterozygous *coi1* mutant transformed with sense *ATHCOR1* (*COI1*/*coi1*S2) showed smaller M_r mRNA bands, which are not found in the homozygous *coi1* transformed with sense *ATHCOR1* (*coi1*S2). It is possible that in *COI1*/*coi1*S2, one copy of the *COI1* gene is sufficient to up-regulate endogenous *ATHCOR1* enhancing its transcript level and, thus, inducing PTGS.

The physiological role of Chlases in plants is not entirely clear. It has been shown for instance that *Citrus Chlase1* is constitutively expressed through development, but its transcript level is not altered during fruit ripening, suggesting that CHLASE1 is not the regulating step of Chl breakdown during this process (Jacob-Wilk et al., 1999). In addition, maximum Chlase activities have been correlated to stages of increased Chl synthesis during olive development (Minguez-Mosquera and Gallardo-Guerrero, 1996). The fact that *ATHCOR1* expression is rapidly induced upon wounding and coronatine treatment suggests that it may play a role in tissue repair or defense (Benedetti et al., 1998). Because of the photodynamic nature of Chl and its porphyrin breakdown products, these molecules can induce strong photooxidative damage by transferring electrons to oxygen species (Matile et al., 1996). Therefore, when tissues are damaged, Chlase would rapidly be activated to initiate Chl detoxification at the wounded sites. According to this, it has recently been proposed that accumulation of Chl breakdown products caused by a deficiency in the red Chl catabolite reductase enzyme is the cause of the cell death lesion phenotype of the Arabidopsis *acd2* mutant (Mach et al., 2001). Moreover, light induced the *acd2* phenotype, which could also be trigged by the chlorosis-inducing phytotoxin coronatine through a light-dependent process (Mach et al., 2001). Similarly, disease lesion mimic phenotype resembling those trigged during hypersensitive reactions induced by pathogens is observed in the maize mutant *Les22*, which is defective in uroporphyrinogen decarboxylase, an enzyme involved in Chl biosynthesis (Hu et al., 1998). This mutant also showed necrotic spots on leaves, which developed in a light-dependent manner (Hu et al., 1998). Therefore, if not properly detoxified, photodynamic porphyrins can enhance lesions at sites where they accumulate. During pathogen infection, this could substantially favor pathogens by causing increased plant cell death. It has been shown that in response to infection by a coronatine-producing strain of *Pseudomonas syringae*, transgenic plants overexpressing the ACD2 protein showed reduced disease symptoms (Mach et al., 2001).

Coronatine has been considered to play a critical role as a virulence factor during the early stages of bacterial infection (Mittal and Davis, 1995; Budde and Ullrich, 2000). A proposed mechanism for coronatine action suggests that the toxin suppresses the activation of defense-related genes (Mittal and Davis, 1995). However, coronatine is a mimic of MeJA (Feys et al., 1994), a signal for defense reactions. Thus, it appears that by inducing the expression of *ATHCOR1*, coronatine would increase Chl degradation provok-

ing the formation of phototoxic porphyrins, predisposing the tissue to infection. It would be interesting to challenge the transgenic plants described here with coronatine-producing bacteria. Nevertheless, it has already been demonstrated that leaves of *coi1* mutant showed reduced chlorosis after been infiltrated with *P. syringae* pv *atropurpurea*, which grew significantly less in *coi1* than in wild-type leaves (Feys et al., 1994).

MATERIALS AND METHODS

Plant Growth

Wild-type Arabidopsis ecotype Columbia (Col-0) was obtained from the Nottingham Arabidopsis Stock Centre (UK), whereas the *coi1* mutant was kindly provided by Dr. John G. Turner (University of East Anglia, UK). Seeds were germinated in Murashige and Skoog medium (Murashige and Skoog, 1962) and grown for 2 to 3 weeks before they were transferred to soil. *coi1* seeds from an F_2 population segregating for the Coi phenotype were first germinated in Murashige and Skoog medium containing 10 μM MeJA to select homozygous *coi1* plants (Feys et al., 1994). Seedlings were grown under white light (70 μE m^{-2} s^{-1}) with 16-h-day/8-h-night photoperiod at 20°C.

Plant Transformation

The entire coding sequence of *ATHCOR1* was ligated downstream to the cauliflower mosaic virus 35S promoter, and the resulting 35S::*ATHCORI* construct was inserted into the *Sal*I/*Sst*I sites of pBI101 (CLONTECH, Palo Alto, CA), in which the *GUS* gene had been removed. For the antisense construct, an 800-bp fragment of the *ATHCORI* cDNA, starting from the ATG, was ligated in the inverted orientation into the *Xba*I/*Sst*I sites of the pBI121 (CLONTECH), removing the *GUS* gene. Constructs were verified by sequencing and used to transform *Agrobacterium tumefaciens* LBA4404 cells. Arabidopsis wild-type and heterozygous *coi1* plants were transformed via *A. tumefaciens* inoculation, essentially as described by Chang et al. (1994). Seeds of inoculated plants were sown in Murashige and Skoog medium containing 50 μg mL^{-1} kanamycin to select resistant plants. Seeds of transformed heterozygous *coi1* plants were first germinated in MeJA plates to select homozygous *coi1*, which were then transferred to kanamycin plates. Genomic DNA from normal and transformed plants was isolated and used in PCR reactions to verify the presence of the T-DNA 35S-*ATHCOR1* insertions using 35S promoter primers and *ATHCOR1* internal primers.

Northern Blots

Total RNA from leaves, flowers, and roots was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD). Aliquots of 10 μg of total RNA were fractionated on formaldehyde-agarose gels (Sambrook et al., 1989), transferred onto nylon membranes Hybond N^+ (Amersham, Little Chalfont, UK) by capillary blot, and fixed by UV cross-linking according to the manufacturer's instructions. Blots were hybridized using the *ATHCOR1*, *AtCLH2*, and an ubiquitin cDNA as probes. Membranes were washed twice with 2.0× SSC containing 0.1% (w/v) SDS for 10 min at 42°C and twice with 0.2× SSC containing 0.1% (w/v) SDS for 10 min at 42°C.

Expression and Purification of Recombinant CORI1 Protein

A DNA fragment containing the coding region of ATHCOR1 cDNA was generated by PCR and subcloned into the *Eco*RI/*Sal*I sites of pMalc2 vector, which allows the production of a MBP fusion (New England Biolabs, Beverly, MA). Recombinant constructs were verified by sequencing and used to transform *Escherichia coli* BL21-lysE cells. The recombinant MBP-CORI1 fusion was expressed in these cells in Luria-Bertani medium containing 1 mM isopropylthio-β-galactoside for 4 h and purified by affinity chromatography on an amylose resin, according to the manufacturer's protocol (New England Biolabs). Purified proteins were quantified by a Bradford-based method (Bio-Rad, Hercules, CA) and analyzed on SDS-PAGE (Laemmli, 1970).

Chlase Assay

Aliquots (0.1 mL) of the purified CORI1-MBP fusion (approximately 50 μg) were mixed to 2.0 mL of 0.1 M MOPS buffer, pH 7.0, and to 1.0 mL of purified Chl dissolved in acetone. The mixture was incubated at 25°C for different time periods and the reaction was stopped by transferring 0.5 mL of the reaction mixture to tubes containing to 0.5 mL of hexane plus 0.5 mL of acetone. The reaction was vortexed and centrifuged at 12,000*g* for 2 min for partitioning of the remaining Chl to the hexane phase. Leaves (approximately 0.2 g fresh weight) of wild-type, *coi1* mutant, and transgenic plants were cut and immediately immersed in 6 mL of acetone and incubated at 4°C in the dark for 12 h. Aliquots of total Chl dissolved in acetone were mixed to hexane and 10 mM KOH at a ratio of 4:6:1 (v/v), as described by Jacob-Wilk et al. (1999). After vortexing and centrifugation (12,000*g* for 2 min), Chls *a* and *b* in the hexane phase and Chlides *a* and *b* in the acetone phase were quantified spectrophotometrically according to Arnon (1949): Chl *a* in mg mL^{-1} = 0.0127 A_{663} − 0.00269 A_{645}; Chl *b* in mg mL^{-1} = 0.0229 A_{645} − 0.00468 A_{663}. Total Chl and Chlide from flowers were extracted and quantified as above, except that flowers were carefully excised on the base of the sepals and frozen in liquid nitrogen before they were weighted and immersed in acetone.

Cloning of the *AtCLH2* cDNA and RT-PCR Analysis

Ten micrograms of Dnase I-treated RNA from leaves, seedlings, and flowers of wild-type Arabidopsis was reversed transcribed using SuperScript reverse transcriptase (Invitrogen) and primer CATAAGCAACAAAAGCTGATG complementary to the 3′ end of the *AtCLH2* gene. The

cDNAs were used as templates in PCR reactions with primers 5′-ATGTCCTCTTCTTCATCAAGA-3′ and 5′-CATAAGCAACAAAAGCTGATG-3′, which amplified an approximately 1-kb fragment that was cloned in pGEM-t (Promega, Madison, WI). Three independent clones from each of the RT-PCR reactions were sequenced, and, in all cases, the sequence of the *AtCLH2* cDNA was identified. The *AtCLH2* cDNA was used as a probe in northern and Southern hybridizations.

RT-PCR reactions for expression analysis of the *AtCLH2* gene were performed using Dnase I-treated RNA from roots, leaves, flowers, flower buds, and leaves of plants treated with MeJA for 4h. The PCR reactions were separated in standard agarose gels and the DNA fragments were transferred to a nylon membrane and hybridized with the *AtCLH2* cDNA as probe. The membrane was washed several times at stringent conditions at 65°C, followed by the detection with x-ray films.

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