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# Chlorophyllase 1, a Damage Control Enzyme, Affects the Balance between Defense Pathways in Plants

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**Accumulation of reactive oxygen species (ROS) is central to plant response to several pathogens. One of the sources of ROS is the chloroplast because of the photoactive nature of the chlorophylls. Chlorophyllase 1 (encoded by *AtCLH1*) of *Arabidopsis thaliana* is quickly induced after tissue damage (e.g., caused by the bacterial necrotroph *Erwinia carotovora* or the necrotrophic fungus *Alternaria brassicicola*). RNA interference silencing of *AtCLH1* resulted in failure to degrade free chlorophyll after tissue damage and in resistance to *E. carotovora*. Both inoculation with *E. carotovora* and exposure to high light caused elevated accumulation of hydrogen peroxide in *AtCLH1* silenced plants. This was accompanied by expression of marker genes for systemic acquired resistance and induction of antioxidant defenses. Interestingly, downregulation of *AtCLH1* resulted in increased susceptibility to *A. brassicicola*, resistance to which requires jasmonate signaling. We propose that AtCLH1 is involved in plant damage control and can modulate the balance between different plant defense pathways.**

## INTRODUCTION

In all organisms, reactive oxygen species (ROS), such as $O_2^{\cdot-}$ and hydrogen peroxide ($H_2O_2$), are formed as by-products of normal, unstressed cellular metabolism. In plants, respiratory and photosynthetic processes responsible for this production take place in several organelles, including mitochondria and chloroplasts (Wojtaszek, 1997; Grene, 2002). The photosynthetic electron transport system, a major source of ROS in plants, resides in the thylakoid membranes of chloroplasts (Foyer et al., 1994).

Several forms of biotic and abiotic stress, such as pathogen attack or excess light (Karpinski et al., 2003), can damage plant tissues. This in turn may result in the release of chlorophyll from the thylakoid membranes. In such a situation, the chlorophylls need to be degraded quickly to avoid cellular damage by their photodynamic action (Takamiya et al., 2000). Thus, failure in chlorophyll degradation can increase the amount of ROS produced to an extent where the detoxification capacity of the antioxidant systems may be overridden. The toxic molecules formed may result in damage of the organelle and in cell death, or they may act as cellular signals (Foyer et al., 1994; Wojtaszek, 1997). It is therefore crucial that the breakdown of chlorophyll is both efficient and tightly regulated (Hendry et al., 1987; Matile and Hörtensteiner, 1999; Takamiya et al., 2000).

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Chlorophyll degradation is initiated with the dissociation of the phytol residue and the porphyrin ring of the chlorophyll molecule catalyzed by the first enzyme in the degradation pathway, chlorophyllase (chlorophyll-chlorophyllido hydrolase; EC 3.1.1.14; Matile and Hörtensteiner, 1999; Takamiya et al., 2000). In *Arabidopsis thaliana*, two genes encoding chlorophyllases, *AtCLH1* (originally described as *ATHCOR1*) and *AtCLH2* (At5g43860), have been characterized (Benedetti et al., 1998; Tsuchiya et al., 1999; Benedetti and Arruda, 2002). Rapid induction of *AtCLH1* but not of *AtCLH2* has been shown in response to wounding, methyl jasmonate (MeJA), and the bacterial jasmonate (JA)-mimicking toxin coronatine. Moreover, the expression of *AtCLH1* is reduced in the JA-insensitive *coi1* mutant plants (Benedetti et al., 1998; Tsuchiya et al., 1999; Benedetti and Arruda, 2002). Specific defects either in the degradation or biosynthesis of chlorophyll have been shown to result in the accumulation of photosensitive porphyrin rings, causing increased oxidative stress and lesion development in both Arabidopsis and tobacco (*Nicotiana tabacum*; Matile and Hörtensteiner, 1999; Mock et al., 1999; Mach et al., 2001).

Although potentially damaging, ROS can also be beneficial for the plant (e.g., by triggering protective responses). ROS has been shown to promote plant resistance to pathogens in several ways, including cross-linking of the plant cell wall polymers, promoting hypersensitive response (a form of programmed cell death often associated with incompatible plant–pathogen interactions), or by being directly harmful to pathogens (Baker and Orlandi, 1995; Greenberg, 1997; Lamb and Dixon, 1997; Bolwell, 1999). Moreover, the induction of plant defense and, hence, plant resistance, such as salicylic acid (SA)–dependent systemic acquired resistance (SAR; Ryals et al., 1996; Sticher et al., 1997) or JA/ethylene (ET)-dependent resistance mechanisms (Thomma et al., 2001), can be influenced by ROS accumulation (Baker et al., 1997; Lamb and Dixon, 1997; Bolwell, 1999). For example, elevated ROS levels have been shown to enhance the

accumulation of SA and pathogenesis-related (PR) proteins like PR1 and PR2, markers of the SAR pathway (Uknes et al., 1992; Chen et al., 1995; Maleck and Dietrich, 1999; Van Loon and Van Strien, 1999).

The bacterial pathogen *Erwinia carotovora* is a broad host range necrotroph that secretes an array of plant cell wall–degrading enzymes, and it causes soft rot in wide variety of plants, including Arabidopsis (Pérombelon and Kelman, 1980; Norman-Setterblad et al., 2000). Resistance to *E. carotovora* can be generated either by induction of JA/ET-mediated (Vidal et al., 1998; Norman-Setterblad et al., 2000; Kariola et al., 2003) or SA-mediated defenses (Palva et al., 1994; Kariola et al., 2003; Li et al., 2004), both of which can be triggered by distinct *E. carotovora*–derived elicitors (Palva et al., 1993; Norman-Setterblad et al., 2000; Brader et al., 2001; Kariola et al., 2003). By contrast, resistance against the necrotrophic fungus *Alternaria brassicicola* has been shown to be dependent mainly on functional JA signaling (Thomma et al., 1998). Here, using RNA interference (RNAi), we show that the specific silencing of *AtCLH1* encoding the first enzyme in the chlorophyll degradation pathway, chlorophyllase 1, leads to increased accumulation of ROS and enhanced induction of SAR marker genes in response to pathogen infection. Moreover, the induction of the JA/ET-induced gene *PDF1.2* (Penninckx et al., 1996) is downregulated in these plants. This results in resistance to *E. carotovora* but increased susceptibility to *A. brassicicola*, suggesting that *AtCLH1* may modulate the balance between different defense pathways in plants.

## RESULTS

### Arabidopsis *AtCLH1* Gene Is Induced in Response to Pathogens

To elucidate plant responses to pathogens, we identified Arabidopsis genes induced in response to *E. carotovora* using a subtracted cDNA library (Brader et al., 2001). One of the genes that was rapidly upregulated by *E. carotovora* elicitors was identified as *AtCLH1* encoding chlorophyllase 1. In addition to *E. carotovora*, *AtCLH1* was also induced by another necrotrophic pathogen, the fungus *A. brassicicola* (Thomma et al., 1998, 1999; Figure 1A). In agreement with previous studies (Benedetti et al., 1998; Tsuchiya et al., 1999; Benedetti and Arruda, 2002), the gene was also strongly induced in response to wounding or treatment with MeJA. By contrast, neither SA (Figure 1A) nor the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (data not shown) significantly induced the expression of *AtCLH1* at early time points. These results show that *AtCLH1* is induced by pathogens and suggest that it might be a component of JA-dependent defense.

### Arabidopsis Plants with RNAi Silenced *AtCLH1* Gene Are Resistant to *E. carotovora*

To elucidate the possible involvement of the *AtCLH1* gene in plant defense, Arabidopsis Columbia (Col-0) plants were transformed with RNAi and overexpression constructs of this gene. The effect of RNAi silencing and overexpression on *AtCLH1* transcript accumulation was evaluated by gel-blot hybridization using a gene-specific RNA probe for *AtCLH1*. Three RNAi lines (numbered 12, 46, and 51) with clearly decreased and one overexpression line with increased *AtCLH1* expression were chosen for further studies (Figure 1B). *AtCLH1* overexpression plants were morphologically similar to wild-type Arabidopsis, whereas the RNAi plants were slightly smaller (Figure 1C). To assess the effect of RNAi silencing or overexpression of *AtCLH1* on chlorophyll breakdown, we measured chlorophyll and chlorophyllide levels from the leaves of wild-type, vector control, and transgenic plants. The measurements were done both from nontreated plants and from plants in which *AtCLH1* expression was induced by MeJA (Figure 1D). The chlorophyllide level was clearly increased in overexpression plants relative to wild-type, vector control, and *AtCLH1* RNAi silenced plants, suggesting more efficient chlorophyll degradation in these plants. This increase was enhanced by MeJA treatment. By contrast, silencing of *AtCLH1* by RNAi resulted in a clear decrease in relative chlorophyllide levels even after MeJA treatment, suggesting that silencing of the gene has indeed impaired chlorophyll degradation in these plants (Figure 1D).

To determine the contribution of the *AtCLH1* gene to disease resistance of Arabidopsis, RNAi silenced and overexpression lines as well as vector control plants were inoculated with *E. carotovora*, and the symptom development and bacterial growth were followed. *AtCLH1* RNAi silenced plants displayed greatly increased resistance to *E. carotovora* infection. Whereas disease symptoms, such as tissue maceration, were clearly evident 24 h after inoculation in the *AtCLH1* overexpression and vector control plants, the inoculated leaves of RNAi silenced plants hardly showed any symptoms (Figure 2A). After 72 h, the infection had spread in the wild-type, vector control, and *AtCLH1* overexpression lines, and the treated leaves of these plants were totally macerated. By contrast, most of the *E. carotovora*–inoculated leaves of RNAi silenced plants still showed no signs of infection (Figure 2A, Table 1). In the few leaves of *AtCLH1* RNAi silenced plants in which symptom development was seen, the infection did not spread from the site of bacterial inoculation, and the area with beginning maceration soon dried. To quantify the apparent difference in disease development, the bacterial growth was determined from the plants after inoculation. The results show a clear reduction in pathogen growth in *AtCLH1* RNAi plants when compared with *AtCLH1* overexpressor and vector control plants (Figure 2B) and suggest that AtCLH1 might be involved in plant defense.

### Development of Disease Symptoms in *AtCLH1* RNAi Plants Is Light Dependent

If the pathogen resistance observed in *AtCLH1* RNAi silenced plants after *E. carotovora* infection was indeed as a result of the phototoxic nature of chlorophyll, the development of tissue maceration in these plants should also be light dependent. To test this hypothesis, *AtCLH1* RNAi silenced and vector control plants were infected with *E. carotovora* in different light conditions and the development of disease was monitored. When infected in normal light (200 to 250 $\mu$mol $m^{-2}$ $s^{-1}$ photons), *AtCLH1* RNAi silenced plants showed hardly any disease

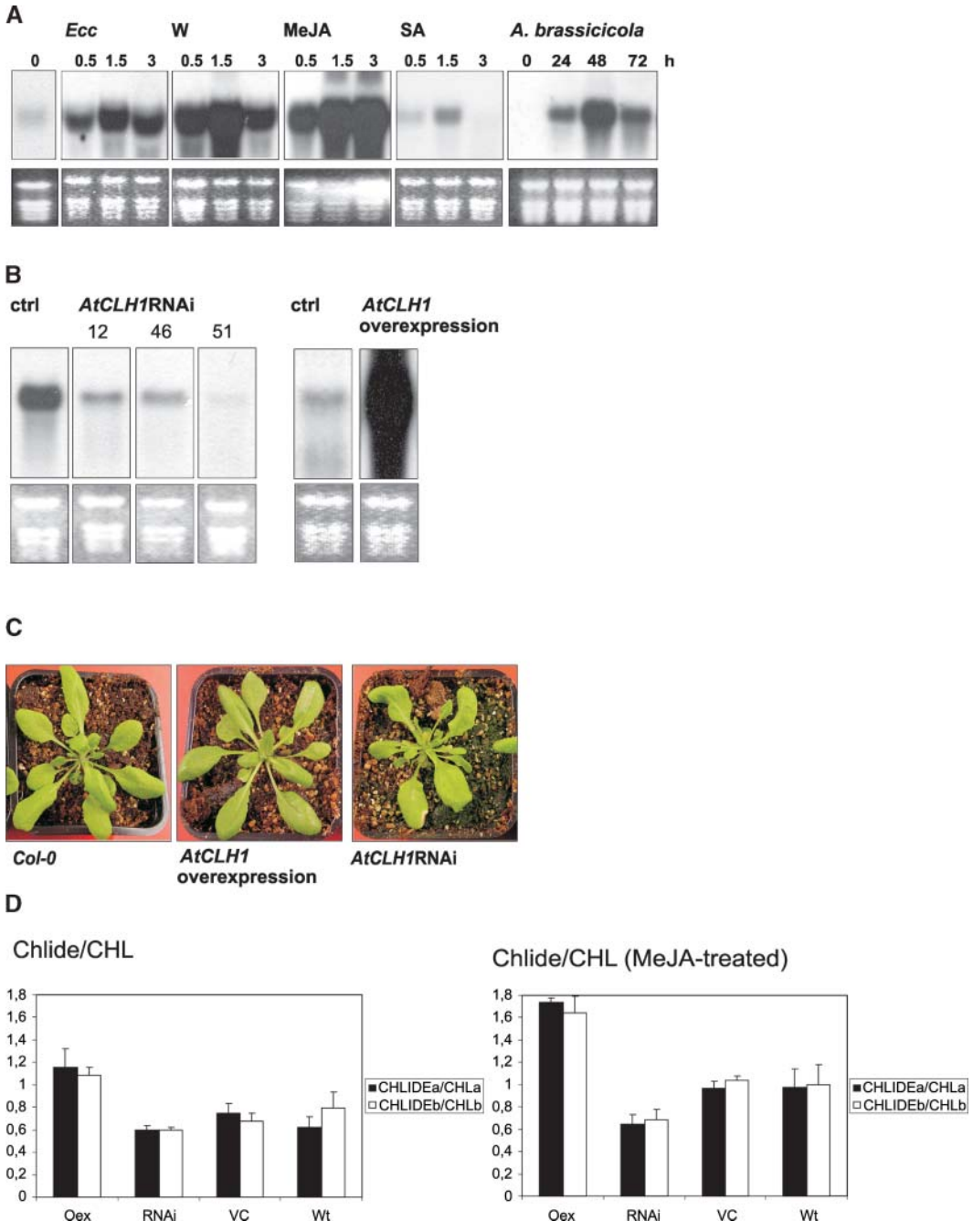


**Figure 1.** Induction of the *AtCLH1* Gene and Characterization of Transgenic Lines.

**(A)** Wild-type Arabidopsis was treated with *E. carotovora* (*Ecc*), wounding (W), MeJA, SA, and *A. brassicicola*. Local samples were collected 0, 0.5, 1.5, and 3 h after treatment with *Ecc*, W, MeJA, and SA and 0, 24, 48, and 72 h after *A. brassicicola*. The samples were analyzed in RNA gel blot hybridization with gene-specific RNA probe for *AtCLH1*.

**(B)** The transgenic lines chosen from Arabidopsis carrying *AtCLH1* RNAi and overexpression constructs. Sample for evaluating the efficiency of RNAi silencing on *AtCLH1* as well as the vector control sample (ctrl) were collected 3 h after treating the plants with MeJA. Evaluation of overexpression efficiency was done from nontreated samples. All samples were analyzed by RNA gel blot hybridization with gene-specific RNA probe for *AtCLH1*. As a control for equal loading ethidium bromide staining of RNA is shown at the bottom.

**(C)** Phenotypic differences of Arabidopsis Col-0, *AtCLH1* overexpression, and *AtCLH1* RNAi silenced plants.

**(D)** Total chlorophyll extracted from the leaves of *AtCLH1* RNAi (RNAi), *AtCLH1* overexpression (Oex), vector control (VC), and wild-type (wt) plants was fractionated into chlorophyll (CHL) and chlorophyllide (chlide). The relative amount of chlide per total CHL was measured as an estimate of AtCLH1 activity and the values are mean of eight independent measurements ± SE. The extraction was done in 1 mL of 80% acetone.

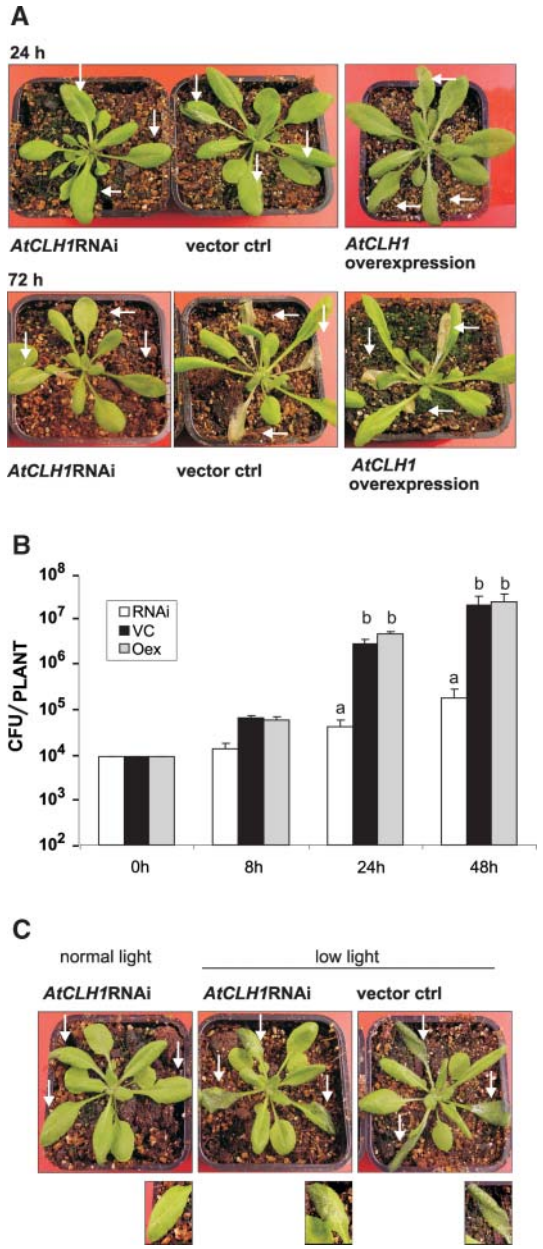


**Figure 2.** *AtCLH1* RNAi Silenced Plants Are Resistant to *E. carotovora* Infection in Normal Light.

Three leaves of Arabidopsis *AtCLH1* RNAi silenced and vector control plants were inoculated by infiltration with *E. carotovora*. Infiltrated leaves are indicated with arrows.

**(A)** *AtCLH1* RNAi silenced, vector control, and *AtCLH1* overexpression plants 24 and 72 h after bacterial inoculation.

**(B)** Growth of *E. carotovora* in planta 0, 8, 24, and 48 h after inoculation. Colony-forming units of five to seven individual plants were determined from each time point in two independent experiments. Different letters within 24- and 48-h bars point to significant differences ($P < 0.05$) calculated with one-way analysis of variance (ANOVA) and Tukey honestly significantly different (HSD) test in all data points.

**(C)** *AtCLH1* RNAi silenced and vector control plants were inoculated with *E. carotovora* in normal (200 to 250 μmol $m^{-2}$ $s^{-1}$ photons) and low (50 to 60 μmol $m^{-2}$ $s^{-1}$ photons) light conditions and photographed 24 h later. The pathogen inoculated leaves are indicated with arrows and one of the leaves is shown as a close up for each plant. Similar results were obtained from three independent experiments (**[A]** and **[C]**).

symptoms. In low light (50 μmol $m^{-2}$ $s^{-1}$ photons), however, the maceration also proceeded in these transgenic plants (Figure 2C). In vector control plants, the infection proceeded similarly or even somewhat faster in low light when compared with normal light conditions (Figure 2C). After 72 h, there was no detectable difference between *AtCLH1* RNAi silenced and vector control plants, as all the local leaves were totally macerated (Table 1).

Interestingly, the enhanced development of disease symptoms introduced by the lack of light was conditional. When plants inoculated in darkness were transferred to high light after 14 h, the maceration that had started in all plants was stopped and did not spread further in the majority of *AtCLH1* silenced plants. A similar change in the light conditions did not decrease disease symptoms of *AtCLH1* overexpression or vector control plants, and they were still macerated despite the increased light intensity (Table 1). This data demonstrates that the lack of disease symptoms seen in *AtCLH1* RNAi silenced plants after *E. carotovora* infection is clearly dependent on light and argues that this could be because of the phototoxic nature of chlorophyll.

### *E. carotovora* Triggers Enhanced ROS Accumulation and Antioxidant Defenses in *AtCLH1* RNAi Silenced Plants

How does the downregulation of chlorophyll degradation cause enhanced resistance to *E. carotovora* in Arabidopsis? Infection by this necrotroph causes tissue damage resulting in release of the chlorophylls from the thylakoid membranes. Inability to efficiently remove these free, light-absorbing molecules could result in increased ROS production (Takamiya et al., 2000). Consequently, decreased AtCLH1 activity would impair the ability of the plant to quickly start the chlorophyll degradation process after tissue damage and would result in increased ROS levels. To test if this indeed was the case, *AtCLH1* RNAi silenced, *AtCLH1* overexpression, wild-type, and vector control plants were infiltrated with *E. carotovora* in normal light (200 to 250 μmol $m^{-2}$ $s^{-1}$ photons), and the leaves of these plants were stained with 3,3′-diaminobenzidine (DAB) to detect $H_2O_2$ accumulation. RNAi silenced plants showed strong accumulation of $H_2O_2$ 2 h after bacterial inoculation throughout the whole inoculated leaf, whereas in the wild-type and vector control plants $H_2O_2$ was only detected at the site of infiltration. By contrast, no $H_2O_2$ accumulation was detected in *AtCLH1* overexpression plants in response to *E. carotovora* infection (Figure 3A).

Antioxidant defenses are usually induced as a consequence of increased production of ROS. To test whether this was the case in *AtCLH1* RNAi silenced plants, we determined the expression of the gene for glutathione *S*-transferase (*GST1*; Conklin and Last, 1995). The induction of *GST1* transcripts after *E. carotovora* infection was higher in these plants than in *AtCLH1* overexpression, wild-type, or vector control plants (Figure 3B). Taken together, these results show that in plants in which *AtCLH1* is

**Table 1.** Effect of *AtCLH1* on Plant Resistance to *E. carotovora* under Different Light Regimes

| Light Conditions | *AtCLH1* RNAi Line 12 | *AtCLH1* RNAi Line 46 | *AtCLH1* RNAi Line 51 | *AtCLH1* Overexpression Line | Vector Control |
|---|---|---|---|---|---|
| Normal light | 16.3 **a** ± 6.3 | 15.2 **a** ± 2.2 | 13.8 **a** ± 8.7 | 94.3 **b** ± 6.9 | 84.6 **b** ± 1.2 |
| Low light | 100 | 100 | 100 | 100 | 100 |
| Darkness followed by high light | n.t. | 14.9 **a** ± 6.5 | n.t. | 91.1 **b** ± 15.4 | 92.3 **b** ± 13.3 |

*AtCLH1* RNAi silenced, *AtCLH1* overexpression, and vector control plants were inoculated with *E. carotovora* in normal light (200 to 250 μmol $m^{-2}$ $s^{-1}$ photons) or low light (50 μmol $m^{-2}$ $s^{-1}$ photons). In another experiment, plants were inoculated with *E. carotovora* in darkness and transferred to high light (900 μmol $m^{-2}$ $s^{-1}$ photons) 14 h after this. Percentage ± SD of leaves completely macerated after 3 d was calculated from three independent experiments with 10 to 30 individual leaves. Different letters (in bold) indicate significant differences ($P < 0.05$) calculated with one-way ANOVA followed by Tukey HSD test. n.t., Not tested.

silenced, pathogen infection generates increased ROS production manifested in enhanced $H_2O_2$ accumulation and activation of antioxidant defenses.

### Silencing of *AtCLH1* Sensitizes Plants to High Light

Excess light (2000 μmol $m^{-2}$ $s^{-1}$ photons) has been shown to induce oxidative stress and defense gene expression in wild-type Arabidopsis plants (Karpinski et al., 1999; Mullineaux et al., 2000; Mullineaux and Karpinski, 2002). We were interested to examine whether silencing of *AtCLH1* would result in increased ROS production under high light in the absence of pathogen infection. DAB staining of plants exposed to high light (900 μmol $m^{-2}$ $s^{-1}$ photons) revealed that already 2 d of exposure was sufficient to trigger strong accumulation of $H_2O_2$ in the leaves of *AtCLH1* RNAi silenced plants. Considerably less $H_2O_2$ was detected in the leaves of the wild-type and vector control plants, whereas *AtCLH1* overexpression plants showed no detectable accumulation at all (Figure 4A).

To elucidate the consequences of this ROS accumulation on downstream gene expression, we characterized induction of antioxidant defenses. The accumulation of *GST1* transcripts was significantly enhanced in *AtCLH1* RNAi silenced plants when compared with *AtCLH1* overexpression, wild-type, and vector control plants (Figure 4B). Our data indicate that the decreased ability of chlorophyll degradation as a result of downregulation of the *AtCLH1* gene makes the plants more responsive to high light manifested in increased accumulation of ROS and antioxidant defense gene transcripts.

### Altered Levels of SA and JA in *AtCLH1* RNAi Silenced Plants

Accumulation of ROS has been implicated as a signal for activation of plant defense responses and has been shown to modulate SA- and JA-dependent defenses (Lamb and Dixon, 1997; Bolwell, 1999; Overmyer et al., 2000; Karpinski et al., 2003). To elucidate the involvement of these defense pathways in the enhanced pathogen resistance of *AtCLH1* silenced plants, we characterized the levels of SA and JA in the transgenic and control lines after infection with *E. carotovora* under normal light conditions (Figure 5). SA levels were higher in *AtCLH1* RNAi silenced plants than in either the *AtCLH1* overexpression or control plants 24 h after infection, but otherwise the SA content did not vary considerably between the lines (Figure 5A). By contrast, the JA levels were clearly reduced in *AtCLH1* RNAi silenced plants from 3 to 24 h (Figure 5B) but upregulated to some extent in the *AtCLH1* overexpression line 24 h after the infection (Figure 5B). These results indicate that *AtCLH1* can modulate the levels of these hormones and suggest that silencing of *AtCLH1* enhances SA and represses JA levels.

### *AtCLH1* RNAi Silenced Plants Show Enhanced Induction of SA-Dependent but Decreased Induction of JA-Dependent Defense Genes

The altered hormone levels in *AtCLH1* RNAi silenced plants prompted us to elucidate the role of different defense pathways in the resistance to *E. carotovora* observed in these plants. To achieve this, we characterized the expression of marker genes specific for these pathways. Pathogen-induced expression of SAR marker genes *PR1* and *PR2* was followed by RNA gel-blot analysis in both normal and low light conditions. Under normal light conditions, where *AtCLH1* RNAi silenced plants demonstrated resistance to *E. carotovora*, the expression of *PR1* and *PR2* was induced 6 h and *PR1* strongly enhanced 24 h after inoculation, whereas a much weaker and delayed induction was evident in vector control plants (Figure 6A). This enhanced induction was considerably decreased under low light conditions, in which the accumulation of both *PR1* and *PR2* transcripts was substantially reduced (Figure 6B). By contrast, exposure to high light induced considerable ROS accumulation (Figure 4A) and clearly enhanced expression of the SAR marker gene *PR2* in *AtCLH1* RNAi silenced plants even without pathogen inoculation (Figure 6C).

Because SA- and JA-dependent defenses can be mutually antagonistic (Vidal et al., 1997; Thomma et al., 2001; Spoel et al., 2003; Li et al., 2004), we wanted to elucidate the effect of *AtCLH1* RNAi silencing on JA-dependent defense. To this aim, we followed the expression of *PDF1.2* in plants treated with MeJA up to 48 h under normal light conditions. The expression of *PDF1.2* was clearly suppressed in *AtCLH1* RNAi silenced plants, especially at early time points, when compared with the vector control plants (Figure 6D). Taken together, these data argue that silencing of the *AtCLH1* gene promotes SA-dependent but downregulates JA-dependent defense responses.

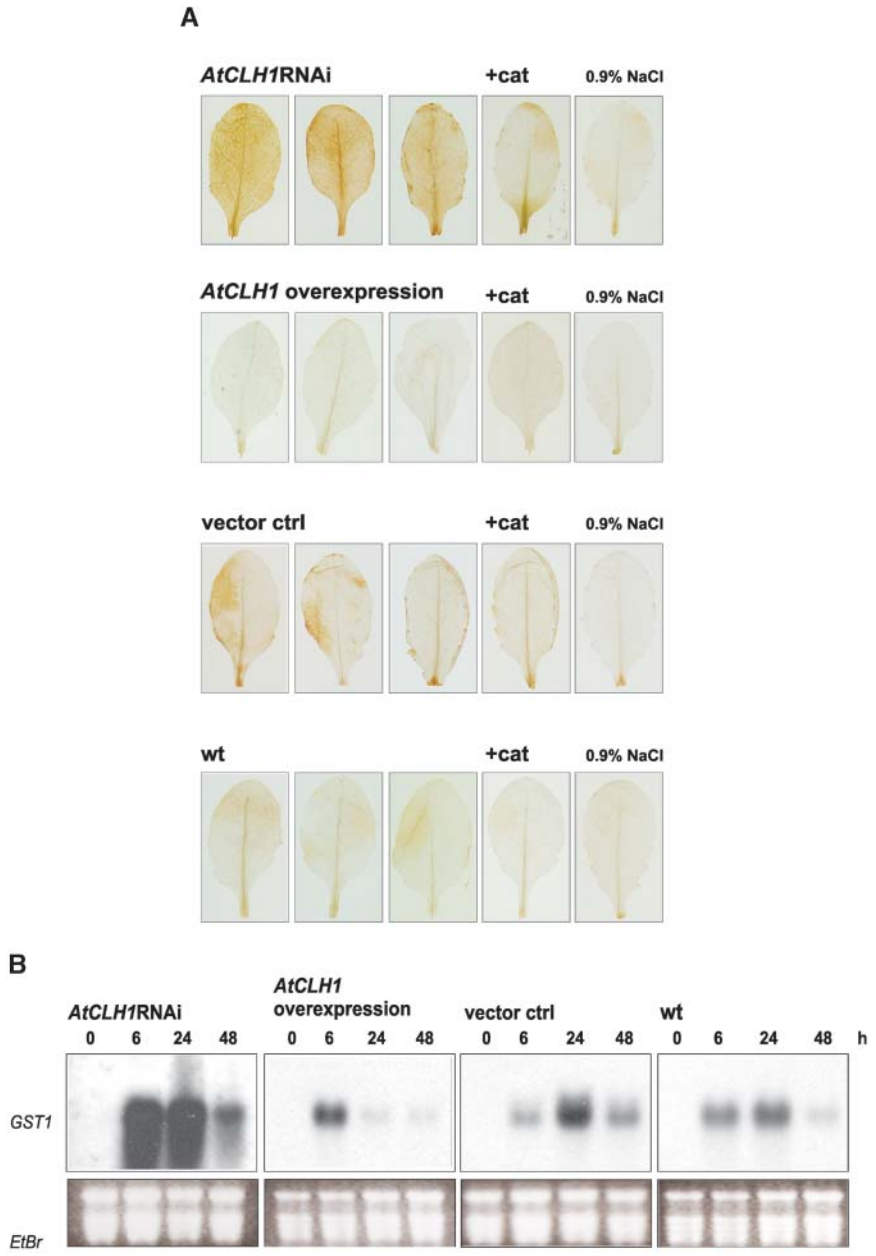


**Figure 3.** *E. carotovora* Triggers Enhanced Accumulation of $H_2O_2$ and *GST1* Transcripts in *AtCLH1* RNAi Silenced Plants.

*AtCLH1* RNAi silenced and vector control plants were inoculated with *E. carotovora* or as a control with 0.9% NaCl.
**(A)** The local (inoculated) leaves were stained with DAB 2 h later to detect possible $H_2O_2$ accumulation. The specificity of the staining was verified by infiltration of leaves with catalase (100 units/mL) before staining with DAB (+cat).
**(B)** Local leaf samples were collected from *AtCLH1* RNAi silenced, *AtCLH1* overexpression, vector control, and wild-type (wt) plants 0, 6, 24, and 48 h after the inoculation. Total RNA was extracted and analyzed by RNA gel blot hybridization with gene-specific probe for *GST1*. As a control for equal loading ethidium bromide (*EtBr*) staining of RNA is shown at the bottom. In both cases, similar results were obtained from three independent experiments.

### *AtCLH1* RNAi Silenced Plants Are Susceptible to *A. brassicicola* Infection

The observed reduction in JA levels and downregulation of JA-dependent defense gene expression suggested that this pathway might be compromised in *AtCLH1* RNAi silenced plants. Because *E. carotovora* triggers both SA- and JA-dependent defenses (Kariola et al., 2003), we employed the necrotrophic fungus *A. brassicicola* as another model to test this hypothesis. In Arabidopsis, resistance to *A. brassicicola* relies on JA-dependent defense mechanisms, and the JA-insensitive *coi1* mutant, but not wild-type plants, is susceptible to it (Thomma

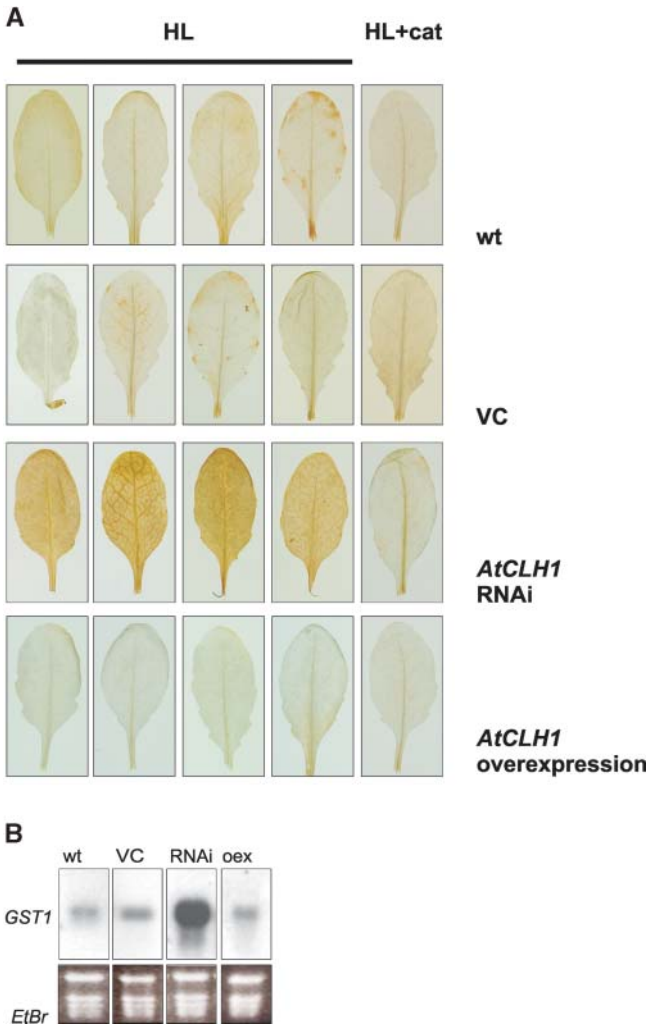


**Figure 4.** High Light Induces $H_2O_2$ Accumulation and *GST1* Expression in *AtCLH1* Silenced Plants.

*AtCLH1* RNAi silenced, *AtCLH1* overexpression, wild-type (wt), and vector control (VC) plants were exposed to high light conditions (900 μmol $m^{-2}$ $s^{-1}$ photons).

**(A)** Leaves of the plants were stained with DAB for monitoring $H_2O_2$ accumulation after 2 d in wt, VC, *AtCLH1* RNAi, and *AtCLH1* overexpression line plants. The specificity of the staining was verified by infiltration with catalase (100 units/mL) before staining with DAB (HL+cat). The samples were taken from at least six individual plants of each transgenic line in every experiment.

**(B)** Leaf samples were collected from wt, VC, *AtCLH1* RNAi silenced (RNAi), and *AtCLH1* overexpression (oex) line plants after the high light treatment, and total RNA was extracted and analyzed by RNA gel blot hybridization with a gene-specific probe for *GST1*. As a control for equal loading ethidium bromide (*EtBr*) staining of RNA is shown at the bottom. In both cases, similar results were obtained from three independent experiments.

et al., 1998, 1999). *AtCLH1* RNAi silenced, *AtCLH1* overexpression, wild-type, and vector control plants were infected with *A. brassicicola* under normal light conditions. No signs of infection were observed in overexpression, wild-type, or vector control plants (Figure 7A). However, ~70% of *A. brassicicola*–inoculated leaves of *AtCLH1* RNAi silenced plants were successfully infected with the fungus (Figure 7A). The fungal growth was also evaluated by determining the amount of fungal DNA from the infected leaves with quantitative PCR (Figure 7B). This further verifies the susceptibility of *AtCLH1* RNAi plants to *A. brassicicola*. These results show that the enhanced resistance to *E. carotovora* seen in *AtCLH1* RNAi silenced plants is not effective against a different type of a pathogen, the necrotrophic fungus *A. brassicicola*.

To confirm the role of *AtCLH1* in the resistance to *A. brassicicola*, we assessed whether compromising the JA-dependent defense increased the susceptibility of the transgenic and control plants to this pathogen. To this aim, axenically grown *AtCLH1* RNAi silenced, *AtCLH1* overexpression, and vector control plants were treated with SA 1 d before infection with *A. brassicicola*. Similar to results with soil grown plants, *AtCLH1* silenced lines were most susceptible to the infection (Figure 7). This susceptibility was clearly increased by the SA treatment, resulting in infection of almost all the local leaves (Figure 7C). There was a comparable increase in susceptibility by SA in vector control plants, and *AtCLH1* overexpression plants were slightly less affected by the SA treatment (Figure 7C). These results indicate that *AtCLH1* appears to positively influence JA-mediated defense against *A. brassicicola*.

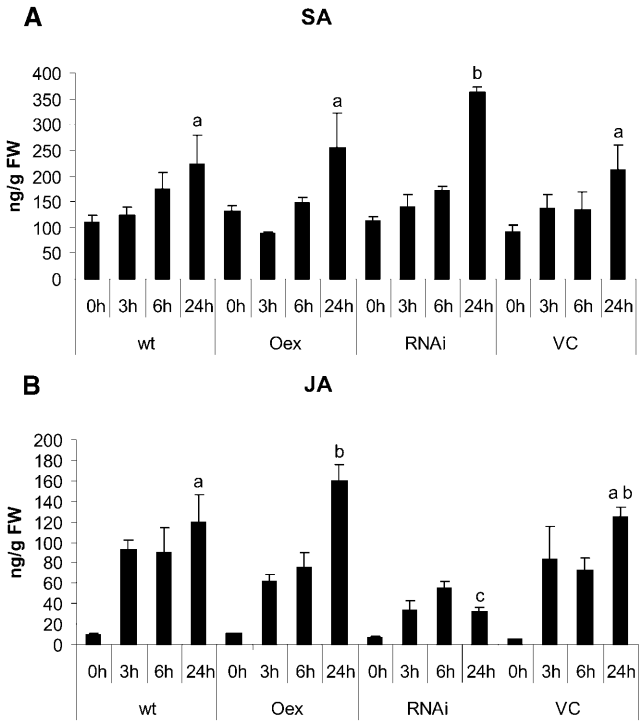


**Figure 5.** *AtCLH1* Modulates the Balance between SA and JA.

Inoculated leaf samples were collected 0, 3, 6, and 24 h after inoculation with *E. carotovora*, and the levels of free SA **(A)** and JA **(B)** were measured from wild-type (wt), *AtCLH1* overexpressor line (Oex), *AtCLH1* RNAi line (RNAi), and vector control (VC) plants. The values represent the average of three replicates ± SD. Different letters indicate significant differences ($P < 0.05$) 24 h after inoculation and were calculated with one-way ANOVA and Fisher's LSD test.

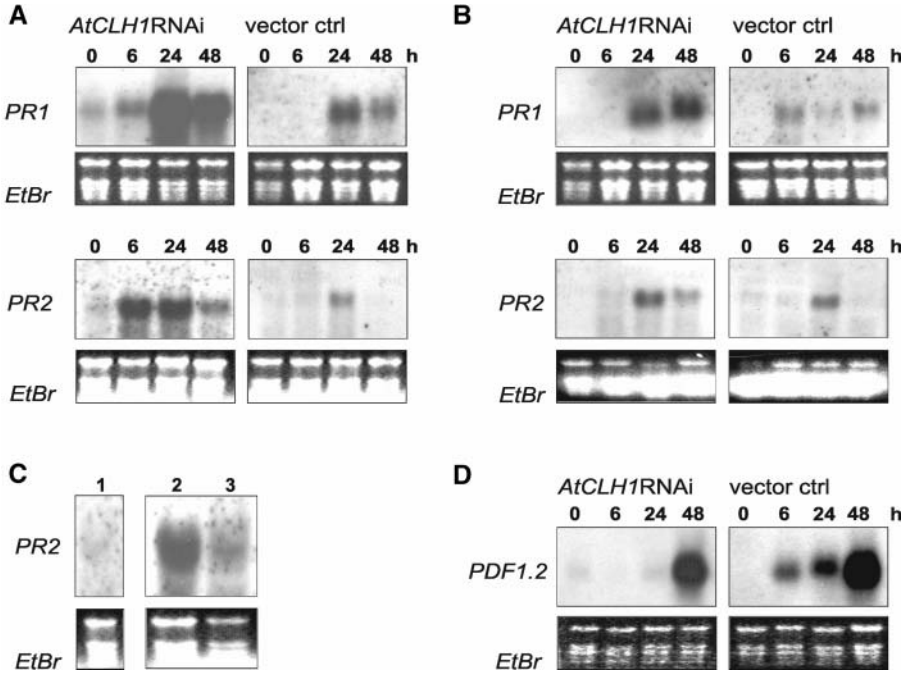


**Figure 6.** *E. carotovora* Induces Enhanced SAR Marker Gene Expression in *AtCLH1* Silenced Plants.

**(A)** and **(B)** Local leaf samples were collected from the *AtCLH1* RNAi silenced and vector control plants 0, 6, 24, and 48 h after *E. carotovora* infection ($4 \times 10^5$ cfu/plant) in normal **(A)** and low **(B)** light conditions. Total RNA was extracted and analyzed by RNA gel blot hybridization with gene-specific probes for *PR1* and *PR2*.

**(C)** Local leaf samples were collected from the *AtCLH1* RNAi silenced and vector control plants after 2 d of high light exposure. Total RNA was extracted and analyzed by RNA gel blot hybridization with a gene-specific probe for *PR2*. 1, Vector control; 2, *AtCLH1* RNAi line 46; and 3, *AtCLH1* RNAi line 51.

**(D)** Local leaf samples were collected from the *AtCLH1* RNAi silenced and vector control plants 0, 6, 24, and 48 h after treating the plants with MeJA. Total RNA was extracted and analyzed by RNA gel blot hybridization with gene-specific probe for *PDF1.2*. As a control for equal loading ethidium bromide (*EtBr*) staining of RNA is shown at the bottom. In each case, similar results were obtained from two independent experiments.

## DISCUSSION

*AtCLH1*, coding for one of the two chlorophyllases characterized from Arabidopsis, is rapidly induced not only in response to the chlorosis-inducing phytotoxin coronatine, wounding, and MeJA (Benedetti et al., 1998; Tsuchiya et al., 1999), but also by necrotrophic plant pathogens such as *E. carotovora* and *A. brassicicola* (Figure 1A). The rapid inducibility of *AtCLH1* in response to wounding and pathogen attack suggests a role for chlorophyllase 1 in damage control after tissue injury and possibly in plant defense. Our results demonstrate that this is indeed the case; silencing of the *AtCLH1* gene resulted in (1) changed tolerance to high light, (2) altered expression of defense-related genes, and (3) modulated plant resistance/susceptibility to different pathogens.

We propose a central role for AtCLH1 as an emergency chlorophyllase, a quick initiator of the chlorophyll degradation process after its release from the thylakoid membranes. Benedetti et al. (1998) originally suggested, based on the expression pattern of *AtCLH1*, that chlorophyllase 1 might be involved in tissue repair. Our data support this notion and show that AtCLH1 is involved in tolerance of high light stress. This is evidenced by the increased ROS levels and the induction of antioxidant defenses in *AtCLH1* silenced plants in high light, whereas similar light conditions were not sufficient to induce these responses in wild-type plants. Previous work has shown that the degradation of the major light-harvesting chlorophyll *a*/*b*-binding proteins of photosystem II occurs when plants are transferred from low to high light conditions (Yang et al., 1998). Most probably this is accompanied by chlorophyll release from these complexes that would create a need for rapid degradation to minimize photodamage.

Interestingly, RNAi silencing of *AtCLH1* does not lead to lesion formation. This is somewhat unexpected when compared, for example, with *acd2* plants that have a mutation in the *red chlorophyll catabolite reductase* gene and are compromised in chlorophyll degradation (Mach et al., 2001). However, unlike *AtCLH1*, which is only expressed when the plant is injured or stressed, the constitutively expressed *ACD2* presumably participates in all chlorophyll degradation occurring in the plant cell (Mach et al., 2001). Thus, the lack of lesions in *AtCLH1* silenced plants can be partly explained by the transient and local nature of the injury. Furthermore, Benedetti and Arruda (2002) showed that the expression of the second Arabidopsis chlorophyllase gene, *AtCLH2*, did not vary in *AtCLH1* (*ATHCOR1*) antisense and overexpression plants, and might partly substitute the missing chlorophyllase 1 activity. It is also likely that there is some residual activity of AtCLH1 in RNAi silenced plants (Figure 1B).

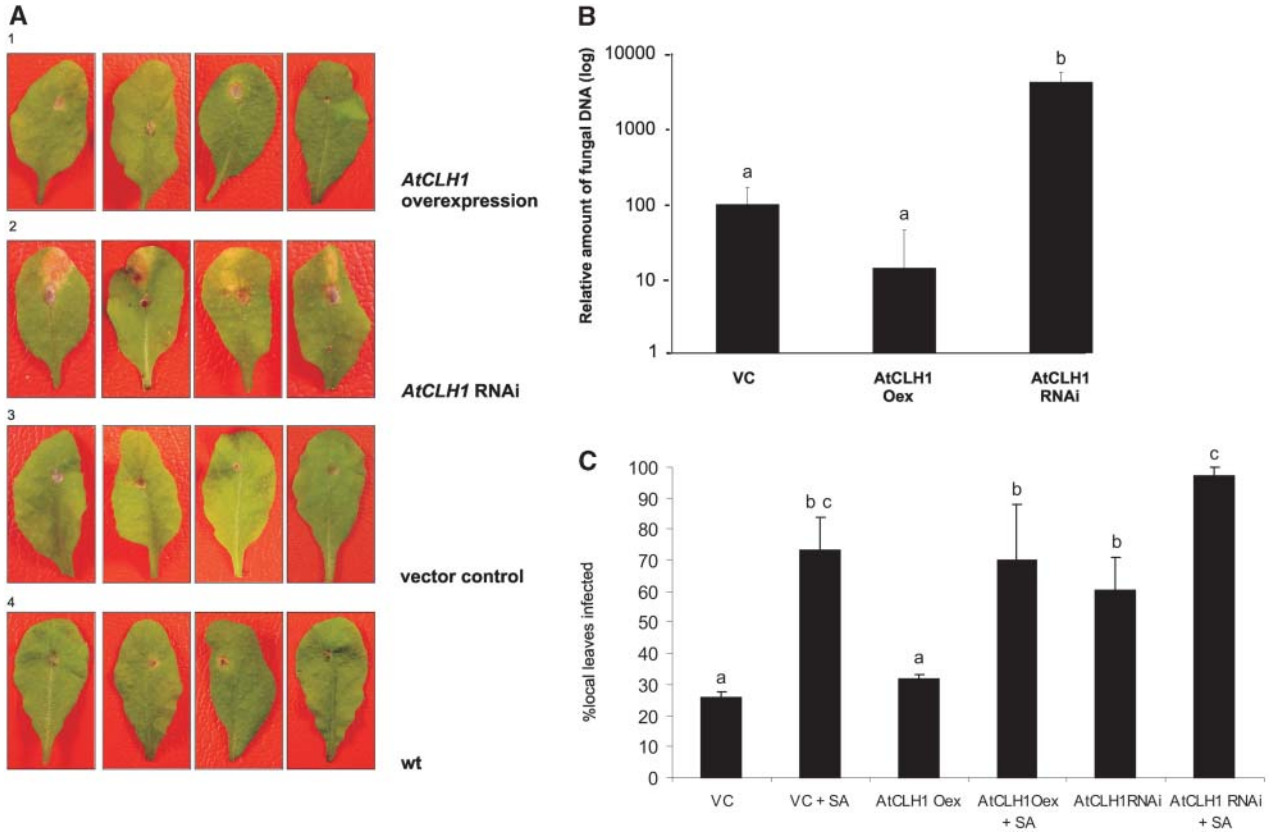


**Figure 7.** *AtCLH1* Silenced Plants Are Susceptible to the Fungus *A. brassicicola*.

**(A)** Second major rosette leaves of *AtCLH1* overexpression, *AtCLH1* RNAi silenced, vector control, and wild-type (wt) plants were inoculated with *A. brassicicola* spores in normal light conditions (200 to 250 μmol $m^{-2}$ $s^{-1}$ photons). The growth of the fungus was monitored visually for 1 week, after which the treated leaves were cut off and photographed. At least 12 plants of each transgenic line were used in every experiment.

**(B)** Quantification of *A. brassicicola* biomass in *AtCLH1* overexpressor (Oex), *AtCLH1* RNAi silenced, and vector control (VC) plants 7 d after inoculation. Relative fluorimetric values ± SE were obtained by quantitative PCR using six independent samples. The relative fungal DNA content was compared with the plants' DNA content and set to 100 in the VC. Different letters indicate significant differences ($P < 0.05$) and were calculated with one-way ANOVA and Tukey HSD test.

**(C)** Oex, *AtCLH1* RNAi, and VC plants were infected with *A. brassicicola* in vitro after both with and without pretreatment 450 μM SA (+SA). Percentage of infected leaves of 24 to 36 axenic plants was calculated 7 d after the infection. Standard deviation was calculated from three independent experiments. Different letters indicate significant differences ($P < 0.05$) and were calculated with one-way ANOVA and Tukey HSD test.

Thus, the local and transient nature of the stress and the residual chlorophyllase 1 activity might help to contain the possible lesions.

Our results suggest a role for *AtCLH1* in plant defense. This is clearly indicated in the altered resistance/susceptibility of plants to two different types of pathogens, *E. carotovora* and *A. brassicicola*, when the *AtCLH1* gene was silenced. Surprisingly, RNAi silencing of *AtCLH1* made Arabidopsis resistant to the virulent pathogen *E. carotovora*. Light appeared to play a major role in this, as the disease symptoms were evident, if these plants were inoculated with the pathogen in low light conditions. Why would downregulation of chlorophyllase, the first enzyme in the chlorophyll degradation pathway, enhance plant disease resistance? The answer appears to lie in the photosensitive nature of chlorophyll. Tissue damage caused by this necrotrophic pathogen will detach chlorophylls from thylakoid membranes of the chloroplasts at the site of injury. Such a release of photoactive molecules would further increase the ROS formation already arising in response to pathogen attack. This is evidenced by DAB staining that revealed clearly increased accumulation of $H_2O_2$ in *AtCLH1* silenced plants as well as enhanced induction of antioxidant defenses in response to *E. carotovora*. The accumulated $H_2O_2$ could, according to earlier studies, have antimicrobial activity (Peng and Kuc, 1992; Wu et al., 1995), and the increased resistance to *E. carotovora* could be a direct consequence of the high ROS levels in *AtCLH1* silenced plants. However, it has been shown that ROS can influence the defense signaling (Bolwell, 1999), which would be a more likely explanation in this case. This is evidenced by the enhanced induction of the defense-related *PR1* and *PR2* genes in *AtCLH1* silenced plants and suggests involvement of SA-dependent defenses in the observed resistance to *E. carotovora*. In low light conditions, development

of disease symptoms was observed also in *AtCLH1* silenced plants, and the concomitant reduction in expression of *PR1* and *PR2* argues for a combined role of chlorophyll and light in this phenotype (Figure 8A). Previous studies of plants defective in chlorophyll metabolism show that accumulation of phototoxic intermediates may result in activation of defense responses (Mock et al., 1999; Molina et al., 1999; Mach et al., 2001).

If plants benefit from having a silenced chlorophyllase in defense against pathogens, why do they even possess a chlorophyllase such as AtCLH1 if they were better off without it? Our results indicate that although silencing of the *AtCLH1* gene results in enhanced resistance to one pathogen, this will compromise other defense pathways needed to defeat another type of pathogen. That this is indeed the case was demonstrated by enhanced sensitivity to *A. brassicicola* in *AtCLH1* silenced plants. Resistance to this necrotrophic fungus requires intact JA-dependent defenses in Arabidopsis (Thomma et al., 1998). Whereas pathogen injury to *AtCLH1* silenced plants will trigger enhanced induction of SAR and resistance to *E. carotovora*, the outcome for a pathogen like *A. brassicicola*, in which plant resistance is JA mediated, is different. The downregulation of JA-dependent defense seen as reduced *PDF1.2* expression in *AtCLH1* silenced plants could be explained by the demonstrated antagonism between SA- and JA/ET-dependent defense pathways. This is supported by the altered SA/JA ratio in the transgenic lines (Figure 5). Previous studies have shown that SA and its functional analogs prevent the expression of JA-dependent defense genes (Peña-Cortés et al., 1993; Vidal et al., 1997; Gupta et al., 2000; Norman-Setterblad et al., 2000; Kunkel and Brooks, 2002; Spoel et al., 2003). We propose a model according to which the RNAi silencing of *AtCLH1* gene increases the amount of oxidative stress in a light-dependent manner that in turn

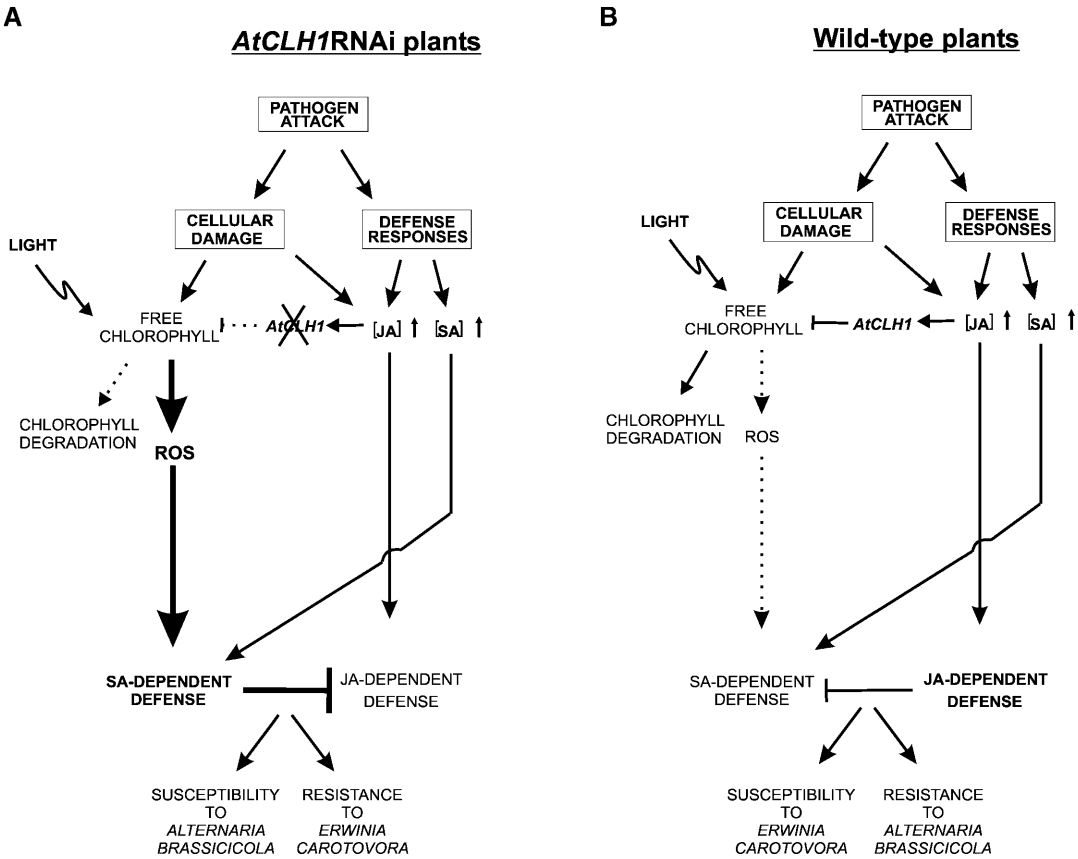


**Figure 8.** A Hypothetical Model for the Role of *AtCLH1* in Plant Defense.

The activation of defense pathways in response to two necrotrophic pathogens, the bacterium *E. carotovora* and the fungus *A. brassicicola*, is shown both in *AtCLH1* RNAi silenced and wild-type plants. Positive effect is shown with arrow line and suppressing effect with end line. The strength of either positive effect or suppression is presented from low (dotted line) to strong (thick solid line). Pathogen attack results in activation of defense responses and increase in the levels of SA and JA. Pathogens also damage tissues, and this in turn releases chlorophylls from the membranes. In *AtCLH1* silenced plants **(A)**, the degradation of free chlorophyll is not initiated rapidly, and this results in enhanced ROS accumulation as a result of the phototoxic nature of chlorophylls. ROS enhances strongly SA-dependent defense that in turn suppresses JA-dependent defense. This builds up the resistance to *E. carotovora*, but simultaneously the plants become susceptible to *A. brassicicola.* In wild-type plants **(B)**, *AtCLH1* is rapidly induced after pathogen attack and initiates the degradation of free chlorophyll molecules. The emphasis of defense is directed to the JA-dependent pathway that suppresses SA-dependent defense to some extent. This results in susceptibility to *E. carotovora* but resistance to *A. brassicicola*.

enhances the SAR-response. This builds up the observed resistance to *E. carotovora* but simultaneously downregulates JA-dependent defense and increases susceptibility to *A. brassicicola* (Figure 8).

Taken together, our data indicates that AtCLH1 has a role in plant defense and argues that this enzyme might be involved in the employment of different defense pathways. Being able to modulate the balance between alternative response strategies is essential for plants. Depending on the invading pathogen, plants can fine-tune the activation of defense genes to achieve the best possible result (Reymond and Farmer, 1998). In Arabidopsis, one way of accomplishing this could be the use of *AtCLH1* as a switch in shifting the emphasis of defense to the direction of JA-dependent pathways (Figure 8). In wild-type plants, tissue damage induces *AtCLH1*, and free chlorophyll is degraded. Without increased ROS production, SAR response is not activated and the defense is directed toward the JA-dependent pathway. This will result in resistance to *A. brassicicola* (Figure 8B). However, some pathogens could also take advantage of such a switch and interfere with the plant defense signaling (Reymond and Farmer, 1998). It seems that *E. carotovora*, a virulent broad host range pathogen that triggers both SA- and JA-mediated responses, has evolved to exploit the activation of chlorophyll degradation. By triggering *AtCLH1* expression, the pathogen decreases the amount of oxidative stress arising in the plant as a result of tissue damage at the very early stage of the infection. This in turn delays the induction of the SAR response, which could otherwise contain the infection and lead to resistance to *E. carotovora*.

Despite the fact that the manipulation of chlorophyll catabolism by silencing *AtCLH1* is not beneficial for the plant in every situation, it still could be an elegant way to enhance the tolerance of agronomically important plant species to pathogens, like *E. carotovora*, that can be controlled by SAR. The increase in oxidative stress and the activation of defense genes only occurs when needed—after pathogen invasion. The plant will not suffer from continuous imbalance in the cellular metabolism because the ROS levels are returned to normal after the pathogen causing the tissue damage has been eliminated.

## METHODS

### Plant Material and Growth Conditions

*Arabidopsis thaliana* plants used in all experiments were derived from ecotype Col-0. Seeds were germinated on MS medium (Sigma-Aldrich, St. Louis, MO) plates and seedlings transferred either to soil or to MS in 12-well plates (Cellstar; Greiner Bio-One, Frickenhausen, Germany) after 1 week. Plants were grown in 1:1 peat:vermiculite (Finnpeat B2; Kekkilä Oyj, Tuusula, Finland) with a 12-h light period at 22°C. Four- to 5-week-old plants were used for experiments.

### Generation of Transgenic Plants

A 983-bp fragment of *AtCLH1* (*AthCOR1*; GenBank accession number AF021244) was amplified from an Arabidopsis leaf cDNA library by PCR using the primers 5′-TACAAATGGCGGCGATAGA-3′ and 5′-AATCTAGACGAAGATACCAGAAGCT-3′, cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), and the *Spe*I/*Eco*RV fragment ligated with the *Xba*I/*Sma*I sites of the binary vector pCP60 (pCP60 is derived from pBIN19 and contains the 35S promoter of the *Cauliflower mosaic virus*, unique multiple cloning sites *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Not*I, *Eco*RI, and *Sac*I, and a nos terminator) to obtain a full-length fragment in sense orientation driven by the 35S promoter. For obtaining the RNAi construct, a 769-bp fragment was amplified from cDNA by PCR with the primers 5′-TACAAATGGCGGCGATAGA-3′ and 5′-ACAAAACCGGGCAAATCATCG-3′, cloned into pCR2.1, and put in antisense orientation into the pCP60 construct containing the 983-bp fragment described above using *Not*I and *Sac*I restriction sites. After sequence verification, the *Agrobacterium tumefaciens* strain GV2260 transformed with these constructs and the empty pCP60 vector was used for plant transformation by floral dip (Clough and Bent, 1998). Seeds were germinated on MS half-strength medium with 100 $\mu$g mL$^{-1}$ kanamycin, 1% sucrose, and 0.9% agar, and transgenic progenies containing single insertions were carried to homozygosity by repeating this procedure to the T3 generation.

### Chlorophyll Extraction

Chlorophyll extraction and chlorophyll/chlorophyllide partitioning was done as described by Benedetti and Arruda (2002).

### Pathogen Strains and Plant Treatments

*Erwinia carotovora* subsp *carotovora* strain SCC1 (Rantakari et al., 2001) was propagated in Luria medium (Miller, 1972) at 28°C. An overnight culture was centrifuged for 7 min (6500*g*), the pellet resuspended in 1 mL of 0.9% NaCl, and diluted to the appropriate concentration. The plants were infected by infiltrating *E. carotovora* subsp *carotovora* SCC1 culture ($\sim$10$^4$ to 10$^5$ cfu/plant) with a needleless syringe. The plants were infected at 50 (low light) and 200 to 250 (normal light) $\mu$mol m$^{-2}$ s$^{-1}$ photon flux density at $\sim$80% humidity in a growth chamber with a 12-h light period. The bacterial growth was determined by homogenizing the infected plants in 10 mL of 0.9% NaCl and plating serial dilutions on Luria plates containing ampicillin at 50 $\mu$g mL$^{-1}$. *Alternaria brassicicola* strain CBSnr 567.77 was obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The fungus was cultivated on potato carrot extract agar plates as recommended by the supplier. After a growth period of at least 10 d, spores were washed from plates with water, and the concentration was adjusted to $5 \times 10^5$ mL$^{-1}$ after counting the spores microscopically. Fourth to sixth true leaves of soil-grown plants were infected with 5 $\mu$L of spore suspension after making a small wound with a pipette tip. Axenic plants were wounded with a pipette tip, and 2 $\mu$L of spore suspension was added to two leaves per plant. SA treatment of axenic plants was done by adding SA to the growth medium to 450 $\mu$M and inoculating the plants with *A. brassicicola* 24 h later. Infected plants were kept in 200 to 250 (normal light) $\mu$mol m$^{-2}$ s$^{-1}$ photon flux density at $\sim$80% humidity in a growth chamber with a 12-h light period. To the soil-grown plants MeJA was applied to the plants as 500 $\mu$M, SA as 5 mM, and 1-aminocyclopropane-1-carboxylic acid as 100 $\mu$M solution, all by pipetting 5 $\times$ 5-$\mu$L droplets on the leaves. Wounding was done by pressing two leaves per plant with forceps. The light intensity used in high light experiments was 850 to 900 $\mu$mol photons m$^{-2}$ s$^{-1}$, and the experiment was performed in a phytotron.

### RNA Gel Blot Analyses

Isolation of total RNA, labeling of DNA probes with digoxigenin (DIG) and RNA gel blot analysis was performed as described previously (Kariola et al., 2003), and the membranes were hybridized with PCR-labeled gene-specific DNA or RNA DIG probes. DIG labeling of RNA, hybridization, and detection were done according to the manufacturer's instruction (Roche, Basel, Switzerland). A 1000-bp cDNA fragment cloned to pCR2.1 (Invitrogen) was used as a template for an *AtCLH1* (At1g19670)-specific

RNA probe synthesized with T7 RNA-polymerase (Promega, Madison, WI). DNA probes were amplified by PCR from the cDNA of *PR1* (At2g14610; Uknes et al., 1992) and *PR2* (At3g57260; Chen et al., 1995). *PDF1.2* (At5g44420) and *GST1* (At1g02930) probes were obtained from the Arabidopsis Biological Resource Center (GenBank accession numbers T04323 and N37195).

### Diaminobenzidine Staining

Accumulation of $H_2O_2$ was detected by staining Arabidopsis leaves with DAB (Sigma-Aldrich). The leaves were vacuum infiltrated with 0.1% DAB solution (10 mM Mes, pH 6.5) for 15 min, and after 30 min in the light the leaves were cleared by boiling in alcohol:lactophenol (2:1) for 5 min and rinsed twice with 50% ethanol. Catalase was used in the concentration of 100 units/μL in the control reactions.

### Quantification of JA and SA

JA and SA were extracted and quantified with (±)-9,10-dihydro-JA and $^{13}C_1$-SA as internal standards using the protocol of Baldwin et al. (1997).

### Quantitative PCR Assay for the Determination of Fungal Biomass

Fungal DNA levels were determined on the seventh day of control and *A. brassicicola*–infected leaves relative to the Arabidopsis DNA levels by quantitative PCR using primers for the genomic *Alternaria* sp *5.8S* rRNA region (GenBank accession number U05198; 5′-CGGATC-TCTTGGTTCTGGCA-3′ and 5′-AATGACGCTCGAACAGGCAT-3′) and primers for Arabidopsis genomic *Actin2* (At3g18780; 5′-CTCCCGCT-ATGTATGTCGCC-3′ and 5′-CGGTTGTACGACCACTGGC-3′). DNA extraction was performed as described by Tierens et al. (2001). Real-time quantitative PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each reaction (25 μL) contained 12.5 μL of SYBR Green PCR Master Mix, 0.75 μL of both primers (10 μM), and 100 ng of DNA template. Forty cycles of amplification (15 s at 95°C, 60 s at 60°C) after an initial 10 min at 95°C were performed in 96-well optical reaction plates (Applied Biosystems). The relative amount of fungal DNA in relation to plant DNA in a given sample (vector control is set to 100%) corresponded to $2^{-\Delta\Delta T}$ with $\Delta_T$ as the difference in cycle numbers to reach a given fluorescence threshold level between *A. brassicicola* and Arabidopsis specific amplification reactions. The combined error is calculated with $\Delta\Delta_{T+s}$ and $\Delta\Delta_{T-s}$, where s is the standard deviation of the $\Delta_t$ values of the reactions of independent samples ($n = 6$).

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