An Arabidopsis Homeodomain Transcription Factor, \textit{OVEREXPRESSOR OF CATIONIC PEROXIDASE 3}, Mediates Resistance to Infection by Necrotrophic Pathogens

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The mechanisms controlling plant resistance to necrotrophic fungal pathogens are poorly understood. We previously reported on Ep5C, a gene shown to be induced by the H$_2$O$_2$ generated during a plant–pathogen interaction. To identify novel plant components operating in pathogen-induced signaling cascades, we initiated a large-scale screen using \textit{Arabidopsis thaliana} plants carrying the β-glucuronidase reporter gene under control of the H$_2$O$_2$-responsive Ep5C promoter. Here, we report the identification and characterization of a mutant, ocp3 (for \textit{overexpressor of cationic peroxidase 3}), in which the reporter construct is constitutively expressed. Healthy ocp3 plants show increased accumulation of H$_2$O$_2$ and express constitutively the \textit{Glutathione S-transferase1} and \textit{Plant Defense 1.2} marker genes, but not the salicylic acid (SA)–dependent pathogenesis-related \textit{PR-1} gene. Strikingly, the ocp3 mutant shows enhanced resistance to the necrotrophic pathogens \textit{Botrytis cinerea} and \textit{Plectosphaerella cucumerina}. Conversely, resistance to virulent forms of the biotrophic oomycete \textit{Hyaloperonospora parasitica} and the bacterial pathogen \textit{Pseudomonas syringae pv tomato} DC3000 remains unaffected in ocp3 plants when compared with wild-type plants. Consistently with this, ocp3 plants are not affected in SA perception and express normal levels of \textit{PR} genes after pathogen attack. To analyze signal transduction pathways where ocp3 operates, epistasis analyses between ocp3 and pad4, nahG, npr1, ein2, jin1, or coi1 were performed. These studies revealed that the resistance signaling to necrotrophic infection in ocp3 is fully dependent on appropriate perception of jasmonic acid through COI1 and does not require SA or ethylene perception through NPR1 or EIN2, respectively. The OCP3 gene encodes a homeodomain transcription factor that is constitutively expressed in healthy plants but repressed in response to infection by necrotrophic fungi. Together, these results suggest that OCP3 is an important factor for the COI1-dependent resistance of plants to infection by necrotrophic pathogens.

\textbf{INTRODUCTION}

Plants react to attack by phytopathogenic microorganisms with an array of inducible responses that lead to local and systemic expression of a broad spectrum of antimicrobial defenses. These include the strengthening of mechanical barriers, an oxidative burst, de novo production of antimicrobial compounds, and the induction of the hypersensitive response (HR) mechanism, where the tissue at the infection site dies and in turn confines the pathogen growth and prevents its spreading (Hammond-Kosack and Parker, 2003). Our understanding of how plants activate defense responses has grown substantially, and this in part has been facilitated by the cloning and characterization of plant disease resistance factors that recognize the corresponding avirulence factors from the pathogen to trigger the HR (Dangl and Jones, 2001). The induction of HR is often associated with the development of systemic acquired resistance, another well-studied defense response that provides long-lasting protection throughout the plant against a broad spectrum of pathogens (Durrant and Dong, 2004).

The characterization of cellular components involved in signal transduction and the understanding of the role of plant defense signal molecules is being aided by the isolation and analysis of mutants with altered defense responses (Kunkel and Brooks, 2002; Durrant and Dong, 2004). These studies are of paramount importance for understanding the coupling of pathogen recognition to the activation of defense responses in the plant. Salicylic acid (SA), a benzoic acid derivative, has emerged as a pivotal signal molecule mediating different aspects of HR and systemic acquired resistance responses. SA synthesis and accumulation have long been shown to be indispensable for mounting an efficient defense response against oomycete and bacterial pathogens (Gaffney et al., 1993), and its signaling is mostly mediated by an ankyrin repeat protein, NPR1/NIM1/SAI1 (Cao et al., 1997), albeit NPR1-independent pathways for funneling SA signaling have been proposed and genetically identified (Clarke et al., 1998, 2000; Shah et al., 1999; Mayda et al., 2000).
In addition to SA, some other signaling molecules, such as jasmonic acid (JA) and ethylene (ET), either alone or in concerted combination, have been shown to regulate other distinct aspects of the plant defense responses (Kunkel and Brooks, 2002; Turner et al., 2002), and genetic evidence for the implication of JA/ET in the response to fungal pathogens also has been provided. For example, Arabidopsis thaliana mutants impaired in production of JA (e.g., thefad3fad7fad8triple mutant) or perception of this hormone (e.g.,coi1,jin1, orjar1/jin4) resulted in an altered susceptibility of Arabidopsis plants to different necrotrophic pathogens (Staswick et al., 1998; Thomma et al., 1998, 2001; Vijayan et al., 1998; Kunkel and Brooks, 2002; Lorenzo et al., 2004). Moreover, during the disease resistance response, mutual antagonistic relationships between SA and JA signaling pathways have been described (Kunkel and Brooks, 2002). In this respect, Arabidopsis mutants either deficient in SA accumulation (e.g.,pad4andeds1) or with an impaired response to SA (e.g.,npr1) all exhibit enhanced induction of JA-responsive genes (Penninckx et al., 1996; Clarke et al., 1998; Gupta et al., 2000). The suppression of JA response genes by SA has been postulated to be regulated by the differential cellular localization of the NPR1 protein (Spoon et al., 2003). Likewise, genetic studies provide evidence indicating that JA signaling can also negatively control the expression of SA-responsive genes in Arabidopsis (Petersen et al., 2000; Li et al., 2004). The molecular mechanism explaining such pathway crosstalk remains poorly understood. Therefore, the characterization of molecular components that ultimately coordinate the SA and JA signaling pathways is paramount for understanding, and eventually engineering, highly regulated mechanisms of resistance that provide sufficient protection to specific subsets of pathogens.

In addition to the signal molecules mentioned above, the production and accumulation of reactive oxygen species (ROS), primarily superoxide (O2-) and hydrogen peroxide (H2O2), during the course of a plant-pathogen interaction has long been recognized (Apostol et al., 1989; Baker and Orlandi, 1995). Evidence suggests that the oxidative burst and the cognate redox signaling engaged subsequently may play a central role in the integration of a diverse array of plant defense responses (Alvarez et al., 1998; Grant and Loake, 2000). Furthermore, crosstalk between ROS and SA-dependent defense responses has also been documented in plants (Kauss and Jeblick, 1995; Shirasu et al., 1997; Mur et al., 2000; Tierens et al., 2002), but the exact mechanisms and components linking redox signaling to the induced defense response remain poorly understood.

Recently, the Ep5C gene from tomato (Lycopersicon esculentum) plants, encoding a cationic peroxidase, has been identified and used as a marker for early transcription-dependent responses controlled by H2O2 after perception of a pathogen, with a mode of gene activation conserved both in tomato and Arabidopsis plants (Coego et al., 2005). Because pathogen-induced expression of Ep5C relies on the production and accumulation of H2O2 by the afflicted plant cell, this points to Ep5C as a marker to search for novel defense components participating in the still poorly understood defense-related pathways in plants.

Toward this end, we describe here the isolation and characterization of the overexpression of cationic peroxidase 3 (ocp3) mutant from Arabidopsis, which is deregulated in the expression of Ep5C. We show that OCP3 encodes a predicted homeobox-like transcription factor that regulates different aspects of the defense response. Through the analysis of ocp3 mutant plants and epistasis analysis with other defense-related mutants, we propose that OCP3 controls critical aspects of the JA-mediated pathway to necrotrophic pathogens.

RESULTS

Isolation and Characterization of the Arabidopsis ocp3 Mutant

Ep5C encodes an extracellular cationic peroxidase and is transcriptionally activated by the H2O2 generated during the course of plant–pathogen interactions (Coego et al., 2005). To identify signals and mechanisms involved in the induction of the Ep5C gene and study the impact this pathway may have on disease resistance, we searched for mutants using transgenic Arabidopsis plants that harbor an Ep5C–β-glucuronidase (GUS) gene fusion. Our screening rationale was that by looking for mutants showing constitutive expression of the reporter gene in plants grown under noninductive conditions, we would identify mutations affecting the regulation of this signal pathway. We therefore mutagenized one of our previously characterized Ep5C-GUS transgenic Arabidopsis Columbia (Col-0) lines with ethyl methanesulfonate, and M2 plants were screened for constitutive expression of GUS in the absence of any pathogenic insult. From ~10,000 M2 plants screened, 18 constitutive GUS expressers were identified that could be selfed. GUS activity was assayed again in progeny of all these putative mutants to confirm whether the phenotype was heritable. Eight lines, corresponding to six complementation groups (data not shown), maintained constitutive GUS activity in subsequent generations. We named these mutants ocp (for overexpression of cationic peroxidase gene promoter), and the mutant selected for further analysis was ocp3 because it was the one to show the highest GUS activity. Macroscopically, ocp3 plants are not very dissimilar to wild-type plants both in terms of plant architecture and growth habit (Figure 1A). However, at early stages of plant development, ocp3 plants show retardation in growth rate compared with wild-type plants. This retardation in the growth rate is also accompanied by a less intense green color in young leaves (data not shown).

Histochemical staining was performed to investigate the pattern of constitutive reporter gene expression in the ocp3 mutants compared with the parental nonmutagenized wild-type plants. As shown in Figure 1B, in the parental seedlings, no GUS activity was detected except in a discrete zone at the root–stem junction (see arrow in the left panel of Figure 1B). Conversely, in ocp3 seedlings, GUS activity was detected in expanding leaves as well as in the cotyledons and the stem, but very poorly in roots. In rosette leaves of ocp3 plants, GUS activity was distributed throughout the leaf blade, whereas leaves from the parental plants did not show detectable GUS expression (Figure 1C).

Because H2O2 was proposed to be the signal molecule that sets in motion the transcriptional activation of Ep5C after pathogen perception (Coego et al., 2005), we hypothesized that
either H2O2 accumulation is increased in ocp3 plants or, alternatively, the ocp3 mutant is hypersensitive to this ROS molecule. To examine if ocp3 plants showed any phenotype in relation to this, we studied the sensitivity to H2O2 and to reagents that generate directly or indirectly H2O2. Seeds from ocp3 and from wild-type plants were germinated on MS media containing different amounts of H2O2 (ranging from 2 to 20 mM), and growth was recorded at different time intervals. No significant differences in growth inhibition were found for ocp3 with respect to wild-type seedlings (data not shown). Likewise, growth inhibition was similar in wild-type and ocp3 seedlings when assayed in the light either in the presence of the ROS-generating molecules Rose Bengal (4,5,6,7-tetrachloro-2’,4’,5’,7’-tetraiodofluorescein; 0.1 to 2 µM) or paraquat (methyl viologen; 0.1 to 2 µM) (data not shown). Thus, from these assays, the ocp3 mutation does not seem to confer increased sensitivity or enhanced resistance to oxidative stress. However, RNA gel blot analyses with mRNA from wild-type and ocp3 plants revealed (Figure 1F; lanes on the left) that mutant seedlings expressed constitutively Glutathione S-transferase1 (GST1), a gene previously shown to be controlled by H2O2 (Levine et al., 1994; Alvarez et al., 1998). This reflects that ocp3 plants may be producing and/or accumulating higher levels of H2O2 than those normally found in wild-type plants. To test this, leaves were stained in situ with 3,3’-diaminobenzidine (DAB), a histochemical reagent that polymerizes in the presence of H2O2, forming reddish-brown precipitates (Thordal-Christensen et al., 1997). Little DAB staining was evident in the leaves of wild-type plants (Figure 1D, left). Conversely, leaves from ocp3 plants showed distinct foci of DAB staining scattered throughout the leaf blade (Figure 1D, right). Moreover, ocp3 does not show any sign of cell death or cell collapse as revealed by staining with trypan blue (Figure 1E) nor does it show any differences with the wild type when assayed for the production of superoxide anions (O2·−) by staining with nitroblue tetrazolium (data not shown). Thus, the observed increased accumulation of H2O2 and induction of GST transcripts in ocp3 plants suggests that the mutation presumably cues a signal related to oxidative stress but not to prime a cell death response. This explains previous observations in which H2O2, but none of the other reactive oxygen intermediate (ROI) species generated during pathogenesis or by in situ infiltration with different H2O2-generating systems, is the signal that sets in motion the characteristic transcriptional activation of Ep5C-GUS in transgenic Arabidopsis plants (Coego et al., 2005). Therefore, both the generation of H2O2 and the activation of the signaling

**Figure 1.** Characterization of ocp3 Plants and Comparison with Wild-Type Plants.

(A) A comparison of the macroscopic appearance of a 3.5-week-old parental wild-type plant (left) and ocp3 plant (right).

(B) Histochemical staining of GUS activity driven by the Ep5C promoter in a 10-d-old wild-type transgenic seedling (left) and an ocp3 seedlings (right) grown on MS agar medium. The arrow points to a discrete tissue zone in the junction of the hypocotyl and the root where GUS activity is observed in wild-type seedlings.

(C) Fully expanded rosette leaf from a wild-type transgenic plant (left) and from an ocp3 plant (right) stained for GUS activity.

(D) Production of H2O2 in wild-type (left) and ocp3 (right) plants. H2O2 production was assayed using 3,3’-diaminobenzidine. The reddish-brown coloration indicates the polymerization of 3,3’-diaminobenzidine at the site of H2O2 production.

(E) Staining of leaf tissue from wild-type (left) and ocp3 (right) plants with trypan blue in search for signs of cell death. The absence of cell collapse is revealed by the lack of intense blue spots after staining with trypan blue.

(F) Expression of PR-1, Plant Defensine 1.2 (PDF1.2), and GST1 marker genes in wild-type and ocp3 plants 36 h after the plants were sprayed with (+SA) or without (−SA) a buffer solution containing 0.3 mM SA.
mechanism leading to transcriptional activation of Ep5C concurred in the ocp3 mutant.

The ocp3 Mutant Has Enhanced Resistance to Necrotrophic but Not to Biotrophic Pathogens

To study a causal link between the signal pathway mediating the activation of Ep5C-GUS in ocp3 and that mediating disease susceptibility, we tested the response of this mutant to different pathogens that generate disease in Arabidopsis. The response of ocp3 plants to the obligate biotroph oomycete Hyaloperonospora parasitica and its comparison to the response of wild-type plants is shown in Figure 2. Growth of the pathogen was assayed by direct observation of stained hyphae in infected leaves (Figure 2A) and by counting the spores produced on infected leaves (Figures 2B). Using both measurements, there was no significant difference in pathogen growth between wild-type and ocp3 plants. Sporulation occurred on 50% of the leaves from either wild-type plants or ocp3 plants. Therefore, the ocp3 mutation did not affect the susceptibility of the plant to colonization by H. parasitica.

Changes in the susceptibility of ocp3 plants to pathogens were investigated further using the virulent bacterial pathogen Pseudomonas syringae pv tomato DC3000 (Pst DC3000) and monitoring the growth rate of these bacteria in extracts from inoculated leaves; the resulting growth curves are shown in

![Image](image_url)

**Figure 2. ocp3 Plants Are Resistant to Necrotrophic but Not Biotic Pathogens.**

(A) Resistance response of wild-type and ocp3 mutant Arabidopsis plants to virulent H. parasitica. Seven days after spray inoculation of 2-week-old plants with $10^5$ conidiospores per milliliter of water, leaves were stained with lactophenol-trypan blue and viewed under a microscope to reveal the characteristic extensive growth of hyphae.

(B) To quantify resistance to H. parasitica, production of conidia was counted 7 d after inoculation with the aid of a haemocytometer. Plants carrying the ocp3 mutation were as resistant to this pathogen as wild-type plants. FW, fresh weight.

(C) Growth of Pst DC3000 in wild-type and ocp3 plants. Four-week-old plants were infiltrated with bacterial suspension, and the bacterial titer, measured as colony forming units (c.f.u) per fresh weight, was determined at 0, 3, and 5 d after infection for the wild type (red lines) and ocp3 (blue lines). Eight samples were taken for each genotype at each time point. The experiment was repeated three times with similar results. dpi, days postinoculation.

(D) Representative leaves from wild-type and ocp3 plants at 4 d after inoculation with a 6-μL droplet of B. cinerea spores (2.5 × 10⁶ conidia/mL).

(E) Lesion size as generated by B. cinerea was measured at 6 d after inoculation. Data points represent average lesion size ± SE of measurements from a minimum of 30 lesions.

(F) Representative leaves from wild-type and ocp3 plants at 4 d after inoculation with a 6-μL droplet of a spore suspension (5 × 10⁶ spores/mL) of P. cucumerina.

(G) Disease symptoms measured as lesion size were evaluated 6 d after inoculation with P. cucumerina by determining the average lesion diameters on three leaves of eight plants each. Data points represent average lesion size ± SE of measurements.

All the experiments were repeated at least three times with similar results.
The constitutive expression in *Fungi* Requires JA but Not SA or ET

Figure 2C. As with *H. parasitica*, the rate of growth of *Pst* DC3000 in *ocp3* plants was not significantly different to that observed in wild-type plants. Therefore, the susceptibility of the wild type and the *ocp3* mutant remains also nearly the same upon local inoculation with this pathogen.

To determine if the *ocp3* mutation could provoke changes in the susceptibility to necrotrophic pathogens, we inoculated plants with *Botrytis cinerea*. Disease was scored between 5 and 10 d after inoculation by following the extent of necrosis appearing in the inoculated leaves. As expected, wild-type plants were highly susceptible to *Botrytis*, and all inoculated plants showed necrosis accompanied by extensive proliferation of the fungal mycelia (Figures 2D and 2E). However, and by marked contrast, none of the *ocp3* plants that were inoculated with the same fungi showed extended necrosis in the inoculated leaves (Figures 2D and 2E). Furthermore, the proliferation of the fungal mycelia was drastically inhibited in *ocp3* plants (data not shown). This indicates that resistance to this necrotrophic pathogen was dramatically enhanced, or susceptibility blocked, in the *ocp3* mutant.

To test whether the altered disease susceptibility of *ocp3* is specific to *Botrytis*, we challenged plants with *Plectosphaerella cucumerina*, another necrotroph. Infection of wild-type plants with *P. cucumerina* lead also to a strong degradation of the leaf tissue, manifested by extended lesions and chlorosis that increased in diameter as the infection progressed along the inoculated leaf (Figure 2F). Conversely, *ocp3* plants showed a high degree of resistance to this fungal pathogen as the visible tissue deterioration in the inoculated leaves was drastically reduced (Figures 2F and 2G). Here also the proliferation of the fungal mycelia was drastically inhibited (data not shown).

On the basis of all these findings, it can be concluded that susceptibility to necrotrophic fungi is a characteristic trait linked to the *OCP3* locus, and the identified mutation in this locus renders enhanced resistance to the same pathogens. This is in accordance with the observation that PDF1.2, an inducible marker for the ET/JA-responsive defense pathway against necrotrophic fungal pathogens (Tumer et al., 2002), is constitutively expressed in *ocp3* plants (Figure 1F).

**The Enhanced Resistance of *ocp3* Plants to Necrotrophic Fungi Requires JA but Not SA or ET**

The constitutive expression in *ocp3* plants of the H$_2$O$_2$-inducible GST and the JA-inducible PDF1.2, but not of the SA-inducible PR-1 (Figure 1F), suggests a link between oxidative stress and JA signaling that apparently is SA independent. In the complex network of interactions operating during plant resistance responses, an antagonistic relationship between the SA and JA/ET pathway has been well documented (Kunkel and Brooks, 2002) and indicates that, in *ocp3* plants, the constitutive activation of the pathway leading to PDF1.2 gene expression could be negating expression of the SA-dependent genes. However, the exogenous application of SA promotes expression of the marker PR-1 gene in both *ocp3* and wild-type plants (Figure 1F), indicating that *ocp3* plants are not compromised in perception of SA, and is in accordance with the observation that the resistance to biotrophic pathogens is also intact in this mutant (Figures 2A to 2C). Furthermore, the exogenous application of SA abrogates the constitutive expression of PDF1.2 that occurs in *ocp3* plants (Figure 1F). This antagonistic effect of SA was specific for PDF1.2 expression because GST1 expression was not repressed in *ocp3* upon treatment with SA. Instead, SA promoted activation of GST1 in wild-type plants (Figure 1F). This latter observation reinforces the link existing between SA and ROS as previously documented by others (Shirasu et al., 1997; Mur et al., 2000; Tierens et al., 2002) but also indicates that the oxidative stress mediated expression of GST1 in *ocp3* plants and concurred expression of PDF1.2 might be SA independent.

To more directly assess if SA could be contributing to the phenotype of *ocp3* plants in relation to the observed resistance to necrotrophic pathogens, we crossed the nahG transgene into the *ocp3* background. nahG encodes a bacterial salicylate hydroxylase that blocks the SA pathway by degrading SA (Delaney et al., 1994). The *ocp3* nahG plants retained the resistance to *B. cinerea* (Figures 3A and 3B) and *P. cucumerina* (Figure 3C) to levels similar to those of *ocp3* plants. Likewise, the pad4 mutation compromises SA accumulation after pathogen attack (Zhou et al., 1998). When *pad4* mutation was introgressed in the *ocp3* background, the resulting *ocp3 pad4* plants remained as resistant to *B. cinerea* (Figures 3A and 3B) or to *P. cucumerina* (Figure 3C) as *ocp3* plants.

To further extend these studies, we created an *ocp3* *npr1* double mutant. The *npr1* mutant was originally identified by its insensitivity to SA and is considered the master regulator of SA-mediated responses (Durrant and Dong, 2004). As observed for *ocp3* nahG and *ocp3 pad4* plants, the resistance of *ocp3 npr1* plants to necrotrophic fungi also remained the same as observed in *ocp3* plants (Figures 3A to 3C). All these results thus indicate that SA seems not to be required for the enhanced resistance attributable to the *ocp3* mutation against necrotrophic pathogens.

We also assessed the importance of JA in contributing to the phenotype of *ocp3* plants. We tested whether a defect in the perception of this hormone might affect the observed enhanced resistance of *ocp3* plants to necrotrophic fungi. The Arabidopsis *coi1* mutant is fully insensitive to JA, and the COI1 protein is required for all JA-dependent responses so far identified. COI1 encodes an F-box protein involved in the ubiquitin-mediated degradation in JA signaling by means of forming functional E3-type ubiquitin ligase complexes (Xie et al., 1998; Devoto et al., 2002). Furthermore, *coi1* plants are impaired in expression of PDF1.2 and show increased sensitivity to necrotrophic fungi (Thomma et al., 1998; Tumer et al., 2002). All this supports the importance of JA in the resistance of plants to this type of pathogen and justified the introgression of *coi1* in the *ocp3* background to generate *ocp3 coi1* double mutant plants (Figure 4). Importantly, the enhanced resistance observed in *ocp3* plants to both *B. cinerea* and *P. cucumerina* is abrogated when the *coi1* mutation is present (Figures 4A to 4C). The *ocp3 coi1* plants behave as *coi1* plants upon infection of either fungi, with necrotic lesions spreading throughout the inoculated leaves as exemplified in Figure 4C for the response to *P. cucumerina*.

In addition to *coi1*, we studied *jin1*, another JA-insensitive mutant (Berger et al., 1996), in relation to the *ocp3* mutant. *JIN1* is a MYC-like transcription factor that functions in a
COI1-dependent manner (Lorenzo et al., 2004). In contrast with coi1 and despite the defect in JA signaling, jin1 plants show increased resistance to necrotrophic pathogens, indicating that JIN1 may function as a repressor of the resistance to this type of pathogen. Interestingly, the ocp3 jin1 double mutant plants remained highly resistant when assayed against infection by B. cinerea (Figure 4A) and to levels comparable to those attained by either ocp3 plants or jin1 plants. It is worth mentioning here that the ocp3 mutation neither confers insensitivity to JA (according to the root-growth inhibition assay in the presence of JA; data not shown) nor is allelic to jin1. The lack of additive effect thus indicates that there might be a certain functional overlap between ocp3 and jin1 for the enhancement of resistance to B. cinerea that ultimately is primed by JA and controlled by COI1.

ET has also been shown to mediate certain aspects of the plant response to pathogens (Thomma et al., 2001; Berrocal-Lobo et al., 2002). However, ET signaling can also function independently of JA, or even inhibit JA-dependent responses (Ellis and

Figure 3. Effect of SA-Related Mutations on the Disease Resistance Response of ocp3 Plants.

(A) Resistance response of ocp3 nahG, ocp3 npr1, and ocp3 pad4 double mutants to B. cinerea compared with that of single mutant genotypes and wild-type plants. Plants were inoculated and disease symptoms were evaluated as described in Figure 2 by determining the average lesion diameters on three leaves of eight plants each.

(B) Representative leaves of each genotype showing symptoms of disease observed 5 d after inoculation with a 6-μL droplet of B. cinerea spores (2.5 × 10^4 conidia/mL).

(C) Resistance response of ocp3 nahG, ocp3 npr1, and ocp3 pad4 double mutants to P. cucumerina compared with that of the single mutant genotypes and wild-type plants. Lesion measurements were performed by determining the average lesion diameters on three leaves of eight plants each.

Data points represent average lesion size ± SE of measurements.

Figure 4. Effect of JA and ET-Related Mutations on the Disease Resistance Response of ocp3 Plants.

(A) Resistance response of ocp3 coi1 and ocp3 jin1 double mutants to B. cinerea compared with that of single mutant genotypes and wild-type plants. Plants were inoculated and disease symptoms were evaluated as described in Figure 2 by determining the average lesion diameters on three leaves of eight plants each.

(B) Resistance response of ocp3 coi1 and ocp3 ein2 double mutants to P. cucumerina compared with that of single mutant genotypes and wild-type plants. Disease symptoms were evaluated by determining the average lesion diameters on three leaves of eight plants each.

(C) Representative leaves of each genotype showing symptoms of disease observed 7 d after inoculation with a 6-μL droplet of P. cucumerina spores (5 × 10^6 spores/mL).

Data points represent average lesion size ± SE of measurements.
Turner, 2001; Thomma et al., 2001). To test the importance of ET in the resistance response mediated by the ocp3 mutation, we crossed ocp3 plants with the ET-insensitive ein2 mutant (Alonso et al., 1999) to generate the ocp3 ein2 double mutant. As observed in Figures 2B and 2C, the resistance of ocp3 ein2 plants to P. cucumerina remained the same compared with that observed in ocp3 plants (Figures 4A to 4C), thus indicating that for the observed resistance mediated by ocp3, the plant hormone ET is dispensable.

Isolation of OCP3

To determine the nature of the ocp3 mutation, a backcross was performed between ocp3/ocp3 plants and wild-type OCP3/OCP3 plants containing the Ep5C-GUS transgene and the progeny analyzed. In the F1 plants resulting from this cross, constitutive expression of GUS activity was absent in all 21 seedlings tested, and in the F2 plants, expression was present in 31 of 118 seedlings. The F2 segregation ratio of the phenotype conferred by ocp3 was 1:3 (constitutive expressers:nonexpressers, $\chi^2 = 1.48; 0.1 > P > 0.5$), indicative of a single recessive mutation. The ocp3 mutant was backcrossed with wild-type Landsberg erecta to generate an F2 mapping population, and recombinant seedlings were identified with the use of simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). DNA was isolated, initially, from 38 ocp3 homozygous plants, and the segregation of SSLP markers indicated that ocp3 showed linkage to the Nga249 marker on chromosome 5 where all 76 alleles analyzed were Col-0 (data not shown). Further analysis of the ocp3 selected plants with additional available markers for chromosome 5 identified the SSLP markers Nga 249 and ca72 as the closest markers flanking the ocp3 mutation on each side (Figure 5A). Screening of 1100 randomly chosen plants from a Landsberg erecta × ocp3 F2 mapping population with the SSLP markers Nga249 and ca72 identified 29 plants having a recombination in the interval. Using these 29 recombinant plants, OCP3 was found to be located 4 centimorgans from Nga249 and 1.9 centimorgans from ca72. We designed further polymorphic markers for the region between Nga249 and ca72, and the position of OCP3 was narrowed down to a genomic region that included the end of BAC clone T5K6 and the beginning of BAC clone F2I11. Nineteen genes are present on this region rich in acidic residues (positions 84 to 181), a feature that could be mediating targeting of the protein to the nuclei. These features could be mediating targeting of the protein to the nuclei. Another salient feature of OCP3 is the presence of an extended region in acidic residues (positions 84 to 181), a feature common to several transcriptional activators (Cress and

OCP3 Encodes a Homeobox Transcription Factor

DNA sequencing showed that the OCP3 cDNA encodes a protein of 354 amino acid residues (Figures 7A and 7B), of 39,111 D, and a pI of 4.53. OCP3 contains various salient features. Close to the C terminus, a 60–amino acid domain (position 284 to 344) resembling that of a homeodomain encoded by homeobox genes of various organisms (Gehring et al., 1994) can be identified. The homeodomain of OCP3 shares most of the highly conserved amino acids characteristic of the 60–amino acid homeodomain module. The conservation of these critical residues (e.g., L-16, Y-20 instead of F-20, I/L-34, I/L/M-40, W-48, F-49, and R-53) is easily identified when compared with different Arabidopsis homeodomain-containing proteins that belong to different protein subgroups (Figure 7C). Inspection of the amino acid sequence of OCP3 also revealed the presence of two canonical bipartite nuclear localization signals (Dingwall and Laskey, 1991; Nigg, 1997): RK-(X)_{10}-KKKKKK at positions 64 to 81 and KK-(X)_{10}-RRKK-KKKKK at positions 294 to 310, with the latter being buried within the homeodomain (Figures 7A and 7B). These features could be mediating targeting of the protein to the nuclei.
The last identifiable feature within OCP3 is the presence of the canonical LxxLL motif at positions 101 to 105 (Figures 7A and 7B). This motif is a signature sequence that facilitates the interaction of different transcriptional coactivators to nuclear receptors and is thus a defining feature identified in several nuclear proteins (Heery et al., 1997). All these structural motifs strongly indicate that OCP3 is a nuclear protein presumably involved in transcriptional regulation in Arabidopsis.

According to a general classification scheme for homeobox genes (http://www.homeobox.cjb.net/) OCP3 is unique as it is set apart from the major classes of homeodomain-containing proteins found in plants, including KNOX or HD-Zip. In addition OCP3 is present as a single copy gene in the Arabidopsis genome. Sequence searches in databases revealed extensive identity of OCP3 with six other proteins—from tomato (GenBank accession number AW223899, 48.9% identity), potato (GenBank BQ112211, 48.3% identity), grape (GenBank CD003732, 51.1% identity), rice (GenBank AY224485, 49.5% identity), wheat (GenBank CK205563, 49.4% identity), and maize (GenBank BGB40814, 51.3% identity)—which were found to have a high degree of sequence similarity with OCP3 and with conservation of all the major structural motifs discussed above (data not shown). This indicates that the function of this type of transcriptional regulator has been highly conserved in plants during evolution.

Subcellular Localization of OCP3

The subcellular localization of OCP3 was investigated using C-terminal green fluorescent protein (GFP) fusions of full-length OCP3. Expression of this construct, as driven by the 35S promoter of Cauliflower mosaic virus in stable transgenic Arabidopsis plants and monitored in epidermal cells of leaves using confocal microscopy, demonstrated that, consistent with a role for OCP3 as a transcription factor, the fusion protein localized predominantly to the nucleus (Figure 7D). Parallel expression of native GFP under control of the 35S promoter in transgenic Arabidopsis plants did not reveal any preferential localization to sequence. Lowercase letters mark nucleotide sequences at the beginning of exon III. The G-to-A transition is indicated in bold uppercase letters. The deduced amino acid sequences are indicated as uppercase single letter code below each nucleotide triplet, and the boldface letters mark the amino acid changes (Ala to Thr) in the protein sequences.

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The nucleus (Figure 7D). Thus, OCP3 carries all major determinants for a nuclear localization.

**OCP3 Expression Is Partially Repressed by Fungal Infection**

The expression of OCP3 in response to infection with a necrotrophic fungal pathogen was analyzed in wild-type plants at different time intervals after infection. OCP3 mRNA levels were undetectable by RNA gel blot analysis in any tissue analyzed, indicating that the OCP3 gene is transcribed at a very low rate. To circumvent this difficulty, the presence of OCP3 mRNAs was studied by RT-PCR. These analyses revealed that OCP3 is constitutively expressed in leaf tissue from healthy plants. Figure 8 shows that after infection with *P. cucumerina*, there is a decrease in the level of accumulation of the OCP3 mRNAs, being most evident at 72 h after infection. Concomitantly, and inversely correlated with this reduction, the JA and fungal-inducible marker gene PDF1.2 is upregulated upon infection with *P. cucumerina*. At latter stages of infection, induced expression of the defense-related gene *PR1* also takes place and is indicative of the tissue deterioration occurring as a result of the growth habit of the fungi.

The downregulation of OCP3 upon fungal infection, its inverse correlation with the induced expression of PDF1.2, and the recessive nature of the ocp3 mutation favors the interpretation that OCP3 may be functioning as a repressor of the resistance response to fungal pathogens in wild-type plants.

**DISCUSSION**

The data presented in this article provide evidence for a role of OCP3 in regulating disease resistance to necrotrophic pathogens. A recessive mutation in the OCP3 gene resulted in enhanced resistance of ocp3 plants to the fungal necrotrophs *B. cinerea* and *P. cucumerina*, whereas resistance toward infection by biotrophs, including the oomycete *H. parasitica* and the bacteria *Pst* DC3000, remained invariant in the same plants.
Figure 7. OCP3 Protein and Comparison with Other Arabidopsis Homeodomain-Containing Proteins.

(A) Predicted amino acid sequence of OCP3. The homeodomain is shown in boldface letters. The two conserved signatures for nuclear localization are underlined. The acidic domain is shown in italics and the nuclear protein interacting domain (LxxLL), embedded within this acidic region, is shown underlined.

(B) Predicted protein structure of OCP3. The relative position of nuclear localization signals (NLS), nuclear protein interacting domain (LxxLL), acidic domain, and homeodomain are indicated.

(C) Sequence alignment showing the C-terminal amino acid sequence of OCP3 with the homeodomain of different homeobox genes from Arabidopsis, including members of the KN and HD-Zip families. Asterisks above the alignments correspond to amino acid positions in the homeodomain that are highly conserved in all organisms and define the homeodomain signature. Black shading indicates amino acids conserved in all entries, and gray shading indicates amino acids with similar physicochemical characteristics.

(D) Leaves of transgenic Arabidopsis plants expressing a 35S:GFP or a 35S:OCP3-GFP fusion were observed using confocal microscopy. Shown are projections of the fluorescent images of epidermal cells. A predominant nuclear localization of the OCP3-GFP fusion protein is observed when it is compared with the general cellular distribution of the GFP protein alone.
Interestingly, the OCP3 gene is expressed at very low levels in healthy plants, and this constitutive expression is partially repressed during the course of infection by a fungal necrotroph. In addition, the resistance phenotype conferred by the ocp3 mutation is blocked when assayed in the coi1 mutant background, with the ocp3 coi1 double mutant plants retaining the increased sensitivity to necrotrophs attributable to coi1. These findings suggest that OCP3 may play a role in the defense response regulated by JA. In fact, the recessive ocp3 mutation confers constitutive expression of the PDF1.2 gene, which encodes a defensin protein with a defined role in the JA-mediated plant defense response (Thomma et al., 1998). Because PDF1.2 expression is fully dependent on COI1 (Turner et al., 2002) and is up in healthy ocp3 mutant plants, our finding reinforces the consideration that OCP3 might be functioning in a COI1-dependent manner by acting as a negative regulator of the JA-mediated defense response to necrotrophic pathogens.

Another salient feature of ocp3 plants is the increased accumulation of H$_2$O$_2$ observed to occur under resting conditions that is accompanied by the constitutive expression of the H$_2$O$_2$-inducible marker gene GST1 (Levine et al., 1994; Alvarez et al., 1998) but not by symptoms indicative of cell death. H$_2$O$_2$ and other ROI molecules are normally produced to high levels during infection by both biotrophic and necrotrophic pathogens and have been implicated as regulatory signals for the basal disease resistance response to these pathogens (Tiedemann, 1997; Mengiste et al., 2003). However, of the pathogen tested on ocp3 plants, only enhanced resistance was observed toward necrotrophic pathogens, whereas the resistance to biotrophic pathogens remained invariant. This significant difference might indicate that the ocp3 mutation may affect specific functions related to ROIs by regulating certain effector molecules directed toward the sensing and identification of a necrotroph. Alternatively, OCP3 may be functioning as a specific regulator of the redox homeostasis, and any alteration in this role, such as that anticipated to occur in the recessive ocp3 mutant, may result in increased accumulation of ROIs, and in particular of H$_2$O$_2$. This in turn may activate specific signal components that may predispose the plant to react more effectively to an infection by a necrotrophic pathogen. Interestingly, SA and H$_2$O$_2$ have been demonstrated to form a feedback loop circuit during the course of a plant–pathogen interaction (Draper, 1997; Shirasu et al., 1997), and there is evidence suggesting that SA may be required for a local response to a necrotroph such as Botrytis at the point of infection (Govrin and Levine, 2000; Ferrari et al., 2003). However, SA synthesis and accumulation are neither increased nor repressed in ocp3 plants (data not shown). Moreover, the analysis of double mutant plants for ocp3 and key regulators of SA accumulation and perception, such as the ocp3 pad4, ocp3 nahG, or ocp3 npr1 double mutants generated in this work, indicate that SA is not required for the observed ocp3-mediated resistance to necrotrophs. Likewise, the plant hormone ET neither seems to be required for the enhanced resistance of ocp3. Here, the lack of perception of this hormone, as studied with the ocp3 ein2 double mutant plants, does not suppress or reduce the characteristic resistance of ocp3 plants to P. cucumerina. Because JA and ET can work either in concert or independently for the activation of specific signaling pathways (Ellis and Turner, 2001; Thomma et al., 2001), the ET-independent resistance of ocp3 may indicate that OCP3 regulates a specific branch of the JA pathway. Moreover, this branch appears to be the same as that ascribed to the JA-regulated and ET-independent JIN1 transcription factor (Lorenzo et al., 2004), as deducted from the lack of additive effect observed in the ocp3 jin1 double mutant plants. All these observations point to a role of OCP3 in specifically regulating a COI1-dependent resistance to necrotrophic pathogens.

OCP3 is a member of the homebox gene family. Homeobox proteins are ubiquitous in higher organisms and represent master control switches involved in developmental processes and cellular adaptation to changes in the environment. They function as transcriptional regulators that are characterized by the presence of an evolutionarily conserved homeodomain responsible for specific DNA binding (Gehring et al., 1994). In plants, two major classes of homeodomain–encoding genes have been identified: the homeodomain class represented by KNOTTED1 (Vollbrecht et al., 1991) and the family of HD-Zip proteins (Schena and Davis, 1992). The latter is characterized by an additional Leu zipper motif adjacent to the homeodomain that facilitates homodimerization and heterodimerization of the transcriptional regulators. Functional characterization of some members of the homeobox family supports a role for some of them as key regulators of hormone signaling (Himmelbach et al., 2002), adaptive responses to environmental cues (Steindler et al., 1999; Zhu et al., 2004), and pathogen-derived signaling processes (Mayda et al., 1999).

The single point mutation identified in the ocp3 allele results in abnormal splicing of the corresponding transcript that provokes
...an internal deletion of the first 36 nucleotides of exon III. This short deletion leads to a frame shift in the ocp3 open reading frame that results in the generation of a premature stop codon. This mutation thus leads to a truncated ocp3 protein consisting of 210 amino acid residues instead of the 354 amino acid residues predicted for OCP3. The predicted 60-amino acid domain corresponding to the homeodomain is located within the 144-amino acid C-terminal domain that is missing in ocp3. This domain is required for homeobox proteins to function as transcriptional regulators because it is the place where contact with DNA is established, primarily through helix 3 of the homeo-domain, which targets the major groove of the DNA helix present in the promoter region of downstream genes (Gehring et al., 1994). Therefore, it is conceivable that the mutated ocp3 protein no longer functions as a transcriptional regulator. It is thus our current working hypothesis that OCP3 functions as a specific transcription factor of a JA-mediated and COI1-dependent plant cell signal transduction pathway and modulates transcription of genes important for the defense response(s) to necrotrophic pathogens.

The identification of target genes of OCP3 and interacting protein partners is our challenge for the future. Furthermore, the possible interaction of OCP3 with other transcriptional regulators involved in the defense response to necrotrophic pathogens, such as the MYC-related JIN1 protein (Lorenzo et al., 2004), the AP2-like ERF1 protein (Lorenzo et al., 2003), the MYB-related BOS1 protein (Mengiste et al., 2003), or the WRKY70 transcription factor (Li et al., 2004), and how they operate in a concerted manner to regulate transcription are interesting challenges for the future. All these approaches should help understand the mechanistic basis of the regulatory function of OCP3 and how we can exploit this function to generate plants more resistant to fungal pathogens without affecting the defense responses to other types of pathogens.

METHODS

Plants, Growth Conditions, and Treatments

Arabidopsis thaliana plants were grown in soil or on plates containing MS media, as described previously (Mayda et al., 2000). The ocp3 mutant was isolated in a screen for constitutive expressers of the Ep5C-GUS reporter gene in transgenic Col-0 plants mutagenized with ethyl methanesulfonate, as described previously for another mutant (Mayda et al., 2000). The transgenic line used (line 5.2) was homozygous and contained a single insertion of the Ep5C-GUS transgene. The ocp3 mutant line used in these experiments has been backcrossed three times to the wild-type parental line. Plants were grown in a growth chamber (19 to 23°C, 85% relative humidity, 100 μE·m⁻²·s⁻¹ fluorescent illumination) in a 10-h-light and 14-h-dark cycle. Unless otherwise indicated, fully expanded leaves of 4-week-old plants were used for all experiments. Staining for the presence of H₂O₂ via the DAB uptake method was performed as described previously (Mayda et al., 2000). On the seventh day, leaf samples were stained with lactophenol-trypsin blue at different days after inoculation and examined under the microscope as described previously (Mayda et al., 2000). For resistance to Plectosphaerella and Botrytis, 3-week-old seedlings were transplanted to single pots and cultivated at a 22°C day/18°C night temperature with 12 h of light per 24 h. When plants were 6 weeks old, they were inoculated by applying 6-μL droplets of spore suspension of either Plectosphaerella cucumerina (5 × 10⁵ spores mL⁻¹) or Botrytis cinerea (2.5 × 10⁴ conidia mL⁻¹) to three fully expanded leaves per plant. P. cucumerina was isolated from naturally infected Arabidopsis (accession Landsberg erecta) (Ton and Mauch-Mani, 2004) and grown on 19.5 g/L of potato dextrose agar (Difco, Detroit, MI) at room temperature for 2 weeks before spores were collected and suspended in 10 mM MgSO₄. B. cinerea (strain BM1, isolated from Pelargonium zonale; Zimmerli et al., 2001) was grown on 19.5 g/L of potato dextrose agar (Difco) at 20°C for 10 d. The conidia were collected and suspended in sterile PDS (12 g L⁻¹; Difco). The plants were maintained at 100% RH, and disease symptoms were evaluated 4 to 10 d after inoculation by determining the average lesion diameter on three leaves of five plants each.

Genetic Analysis

Crosses were performed by emasculating unopened buds and using the pistils as recipients for pollen. Backcrosses with the parental transgenic line were performed using Ep5C-GUS plants as the pollen donor. The reciprocal crosses were also performed. F1 and F2 plants were grown on MS plates and tested for the presence of the GUS activity. Segregation of phenotype in the F2 generation was analyzed for goodness of fit with the χ² test.

PCR-Based Mapping

An ocp3 plant (in the Col background) was crossed with Landsberg erecta, and amongst the segregating F2 progeny, homozygous ocp3 mutants were selected for mapping. Recombinant seedlings were identified using SSLP markers by the protocol described by Bell and Ecker (1994) and with new markers as reported on the Arabidopsis database Web site (www.arabidopsis.org).

Generation of Double Mutants

The mutant alleles used throughout this study were npr1-1 (Cao et al., 1997), pad4-1 (Zhou et al., 1998), coli-1-1 (Xie et al., 1998), ein2-5 (Alonso et al., 1999), and jin1-1 (Lorenzo et al., 2004). All the mutants and transgenic plants used in these studies were in ecotype Col-0. The ocp3 npr1, ocp3 pad4, ocp3 col1, ocp3 ein2, ocp3 jin1, and ocp3 nahG double mutants were generated using ocp3 as recipient for pollen. The homozygosity of the loci was confirmed using a molecular marker for each of the alleles in segregating populations. All the double mutants were confirmed in the F3 generation, except ocp3 col1 plants that were sterile and could only be propagated as heterozygotes for col1. For the double mutant containing ein2-5, F2 seed was plated on MS plates containing 20 μM 1-amino-cyclopropane-1-carboxylic acid and placed in a growth...
Genomic and cDNA Cloning

The genomic sequence was used as the basis for cloning of cDNAs and genomic clones. Poly(A+) RNA was isolated from different wild-type and ocp3 plants and was reverse-transcribed using oligo(dT) primers as described (Mayda et al., 1999). These were used as templates to amplify OCP3 and ocp3 cDNAs using different combinations of the sense and antisense gene-specific primers: pfullD1 (5'-GCCATGG-C-3'), pfullR1 (5'-GACACCTCATGATCTTTCGAGA-3'), pD1 (5'-GGTGATGTTGATGTTGATTG-3'), pR1 (5'-CTTAGGTTCGACCAC- AACATCTTCAG-3'), and pD2 (5'-ATCTGGCAGCTGAGGTTTGTTGCTG-3').

Reverse Complementation

The OCP3 genomic region was amplified by PCR using gene-specific primers designed to include the 1.5-kb region upstream of the start codon and a part of the 3' region that follows the stop codon. The sequences of the OCP3 genomic forward and reverse primers used were 5'-GAG- ATTGAAGCTTGGCTGATCTTTCGAG-3' and 5'-TTCTGTAATCTTACATT- TATCATAG-3', respectively. A 3.2-kb genomic fragment containing the wild-type Atg511270 gene was obtained by PCR and cloned into pCAMBIA1300 to render clone pCAMBIAOCP3 that was transferred to Agrobacterium tumefaciens and used to transform ocp3 plants by the floral-dip method (Bechtold et al., 1993).

Expression Analysis

To analyze the level of gene expression by RT-PCR, total RNA samples were prepared from leaf tissues using the Total RNA kit from Ambion (Austin, TX). Reverse transcription was performed using the RT-for-PCR kit from Clontech (Palo Alto, CA). The oligonucleotide primer sets (50 pmol each) used to amplify OCP3 were OCP3PCR1 (5'-GCTTAAAAGAC- TGGCTTAAAAGAC-3') and OCP3PCR2 (5'-TTCCTGAATTCATACTT- ACCAGATAC-3'). The primers used to amplify PDF1.2 were PDF1.2PCR1 (5'-AGG-ATGCACTAGATCTTTCCGGAG-3') and PDF1.2PCR2 (5'-CTTAGGTTCGACCAC- AACATCTTCAG-3').

Confocal Laser Microscopy

A Leica TCS SL confocal microscope (Mannheim, Germany) was used in these studies. To detect GFP fluorescence, the excitation wavelength was 488 nm, and a band-pass filter of 510 to 525 nm was used for emission. Confocal images were taken from leaves from 15-d-old transgenic plants expressing 3SS-GFP or 3SS-OCP3-GFP that were mounted on standard microscope slides in the presence of water.

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