Characterization of a Novel, Defense-Related *Arabidopsis* Mutant, *cir1*, Isolated By Luciferase Imaging

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In order to identify components of the defense signaling network engaged following attempted pathogen invasion, we generated a novel *PR-1::luciferase* (*LUC*) transgenic line that was deployed in an imaging-based screen to uncover defense-related mutants. The recessive mutant designated *cir1* exhibited constitutive expression of salicylic acid (SA), jasmonic acid (JA)/ethylene, and reactive oxygen intermediate-dependent genes. Moreover, this mutation conferred resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and a virulent oomycete pathogen *Peronospora parasitica* Noco2. Epistasis analyses were undertaken between *cir1* and mutants that disrupt the SA (*npr1*, *nahG*), JA (*jar1*), and ethylene (ET) (*ein2*) signaling pathways. While resistance against both *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica* Noco2 was partially reduced by *npr1*, resistance against both of these pathogens was lost in an *nahG* genetic background. Hence, *cir1*-mediated resistance is established via *NPRI*-dependent and -independent signaling pathways and SA accumulation is essential for the function of both pathways. While *jar1* and *ein2* reduced resistance against *P. syringae* pv. *tomato* DC3000, these mutations appeared not to impact *cir1*-mediated resistance against *Peronospora parasitica* Noco2. Thus, JA and ET sensitivity are required for *cir1*-mediated resistance against *P. syringae* pv. *tomato* DC3000 but not *Peronospora parasitica* Noco2. Therefore, the *cir1* mutation may define a negative regulator of disease resistance that operates upstream of SA, JA, and ET accumulation.

Additional keywords: systemic acquired resistance.

Plants have evolved an extensive array of sophisticated defense mechanisms that are employed to resist the establishment of disease (Glazebrook et al. 1997; Yang et al. 1997). Preformed physical and chemical barriers constitute the first line of defense (Osbourne 1996; Showalter 1993), upon which are superimposed a battery of inducible defense responses, including a rapidly activated oxidative burst (Grant and Loake 2000), the cross-linking of cell wall structural components (Wallace and Fry 1999), and the accumulation of a variety of antimicrobial proteins (Bol et al. 1990; Penninckx et al. 1998). The expeditious engagement of these responses is dependent upon successful pathogen recognition, which is thought to occur fol-

lowing the interaction of a specific pathogen avirulence (*avr*) gene product with a corresponding plant resistance (*R*) gene product (Bent 1996; Dangl 1995). A nearly ubiquitous feature of successful pathogen recognition is the visible development of hypersensitive cell death (hypersensitive response [HR]) surrounding the area of attempted pathogen ingress (Greenberg 1997). Recent evidence suggests that the HR may share functional and mechanistic similarities with cell death programs found in animals (Ryerson and Heath 1996). However, in some plant–pathogen interactions, cell death may be neither necessary nor sufficient for effective disease resistance (Bendahmane et al. 1999; Yu et al. 1998).

Following the successful establishment of local resistance is the development, over several days, of resistance throughout the plant to infections by a broad range of virulent pathogens, termed systemic acquired resistance (SAR) (Ryals et al. 1996). The development of SAR is marked by an increase in lignin deposition, a rise in peroxidase activity, and the coordinate activation of so-called SAR genes, many of which encode pathogenesis-related (PR) proteins (Bol et al. 1990). The establishment of SAR is preceded by a local and systemic increase in salicylic acid (SA) concentration, which is thought to play a central role in the establishment of this resistance mechanism (Malamy et al. 1990). Both tobacco and *Arabidopsis* plants containing a salicylate hydroxylase gene (*nahG*) from *Pseudomonas putida*, which degrades SA to inactive catechol, show reduced expression of SAR genes and accumulation of PR proteins and are unable to establish SAR (Delaney et al. 1994; Gaffney et al. 1993). Currently, the accumulating data supports a paradigm in which SA functions at multiple nodes in the defense signaling network (Weyman et al. 1995), possibly by acting as a signal amplifier (Conrath et al. 1995; Fauth et al. 1996; Mur et al. 1996; Shirasu et al. 1997).

The accumulation of jasmonic acid (JA) and ethylene (ET) following pathogen recognition are also thought to play key roles in orchestrating the deployment of specific sets of inducible defense responses (Epple et al. 1995; Penninckx et al. 1996). While ET insensitivity was initially found to result in increased pathogen tolerance (Bent et al. 1992), more recent studies have suggested that ET may be required for resistance against some microbial pathogens (Hoffman et al. 1999). This presumably reflects a requirement for the expression of ET-dependent genes, such as those encoding basic PR proteins (Eyal et al. 1993; Santamaria et al. 2001). The biosynthesis of the octadecanoid signaling molecule JA has been shown to be stimulated in both local and systemic tissues following pathogen infection (Penninckx et al. 1996), and the accumulation of JA is known to engage the expression of distinct sets of genes, including those encoding antimicrobial defensins and thionins (Epple et al. 1995; Penninckx et al. 1996). Interestingly, sepa-

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rate JA- and SA-dependent defense response pathways in *Arabidopsis* are thought to convey resistance against discrete microbial pathogens (Thomma et al. 1998).

In order to dissect the signaling networks underlying the establishment of disease resistance, a number of *Arabidopsis* defense-related mutants have recently been isolated and analyzed. The loss-of-function mutant *npr1* (also termed *nim1* and *sail*) failed to establish SAR in response to the exogenous application of SA and also exhibited increased susceptibility to virulent microbial pathogens (Cao et al. 1994;

Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Mutants have also been isolated that show increased endogenous SA levels, constitutive SAR gene expression, and subsequently enhanced resistance to virulent pathogens; these include the *cim*, *cpr*, *dnd*, *cep*, *acd*, *lsd*, and *mpk4* mutants (Clarke et al. 1998; Dietrich et al. 1994; Greenberg et al. 1994; Lawton et al. 1993; Petersen et al. 2000; Silva et al. 1999; Yu et al. 1998).

Studies utilizing ET- and JA-insensitive *Arabidopsis* mutants have further highlighted a role for these hormones in the establishment of disease resistance. The ET-insensitive mutants *etr1* and *ein2* and the JA-insensitive mutants *jar1* and *coi1* are defective in the expression of *PDF1.2* and *Thi2.1*, which encode antimicrobial defensin and thionin, respectively (Epple et al. 1995; Penninckx et al. 1996). Moreover, the exogenous application of JA induced resistance against *Botrytis cinerea* and *Alternaria brassicicola* in *Arabidopsis*, and this induced resistance was compromised in the JA-insensitive *coi1* mutant (Thomma et al. 1998). Further evidence of a functional role for JA and ET in the establishment of disease resistance in *Arabidopsis* has emerged from studies of JA- and ET-insensitive plants, which were found to exhibit increased susceptibility to isolates of the soilborne fungus genus *Pythium* (Staswick et al. 1998).

To gain further insight into the signaling networks underlying the establishment of plant disease resistance, we generated a novel *Arabidopsis* line containing a *PR-1::luciferase* (*LUC*) transgene to image, in living plants, the temporal and spatial dynamics of *PR-1* gene expression following attempted pathogen invasion. Moreover, we exploited this genetic background to undertake screens for *Arabidopsis* mutants exhibiting perturbations in resistance against microbial pathogens. With this approach, the *cir* (constitutively induced resistance) 1 mutant was isolated, which displayed the constitutive expression of SA-dependent, JA-dependent, and reactive oxygen intermediate (ROI)-dependent genes and exhibited enhanced resistance against the oomycete *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato*.

RESULTS

A *PR-1::LUC* transgene robustly reports endogenous *PR-1* gene expression.

To accurately determine, in living plant tissue, the temporal and spatial expression program established by the *PR-1* gene in local tissues following attempted pathogen infection, we generated transgenic *Arabidopsis* plants containing a chimeric *PR-1::LUC* gene fusion. The *PR-1* promoter was transcriptionally fused to the firefly luciferase (*LUC*) reporter gene (Fig. 1A), and the resulting construct was transformed into *Arabidopsis* accession Col-0. The profile of *PR-1::LUC* gene expression in the resulting lines was compared with that of the endogenous *PR-1* gene following inoculation with *P. syringae* pv. *tomato* DC3000 expressing the *avrB* gene (Bisgrove et al. 1994) (Fig. 1B). From 12 h postinoculation, expression of the *PR-1::LUC* gene steadily increased, reaching a maximum value between 36 and 48 h postinoculation, after which time *PR-1::LUC* expression slowly decreased. The temporal expression of the endogenous *PR-1* gene was found to be congruent with expression of the *PR-1::LUC* transgene. We also determined the temporal profile of LUC activity following inoculation with *P. syringae* pv. *tomato* DC3000(*avrB*) (Fig. 1C). This also exhibited kinetics similar to that of the endogenous *PR-1* gene, with maximum LUC activity recorded at 36 h postinoculation. These experiments therefore confirmed that the *PR-1::LUC* transgene functioned as a robust reporter for the engagement of endogenous *PR-1* gene expression.

Fig. 1. *PR-1::luciferase* (*LUC*) transgenic plants accurately report endogenous *PR-1* gene expression following inoculation with *Pseudomonas syringae* pv. *tomato* DC3000(*avrB*). **A**, A schematic diagram of the *PR-1::LUC* gene cassette: the *PR-1* promoter was transcriptionally fused with the *LUC* gene upstream of an *OCS* terminator region. The *NPTII* gene conveys kanamycin resistance in transgenic plants. **B**, Profile of *PR-1* and *LUC* gene expression over time, following *P. syringae* pv. *tomato* DC3000(*avrB*) infiltration. Accumulated *PR-1* and *LUC* transcripts were normalized to that of the constitutively expressed *r18* gene. **C**, Profile of LUC activity established in *PR-1::LUC* transgenic plants over time, following inoculation with *P. syringae* pv. *tomato* DC3000(*avrB*).

Temporal and spatial *PR-1* expression program established during HR formation.

To image LUC activity following attempted pathogen invasion, a single leaf of the selected *PR-1::LUC* transgenic line was infiltrated within a limited area with *P. syringae* pv. *tomato* DC3000(*avrB*). The infiltrated leaf was immediately sprayed with a 1-mM-luciferin solution and placed within a dark box, and the temporal and spatial distribution of LUC activity was recorded at 6-h intervals for a period of 3 days with an ultra-low-light imaging camera system. Selected images at given time points are shown from a typical time course experiment (Fig. 2). LUC activity was routinely first detected from 12 to 18 h postinoculation. This activity was initially localized to the vascular tissue adjacent to the inoculation zone: first being detected in the major vein and often spreading in this tissue beyond the initial area of inoculation. Typically, by 18 h postinoculation, the magnitude and spatial distribution of LUC activity had increased to include both perivascular and leaf epidermal cells at a distance from the major vein. By 24 h postinoculation, both the magnitude and spatial distribution of LUC activity had increased further and surrounded the developed HR lesion. Maximum LUC activity was routinely detected between 36 and 48 h postinoculation, after which time activity steadily decreased. Inoculation of the *PR-1::LUC* transgenic line with virulent *P. syringae* pv. *tomato* DC3000 induced significantly less LUC activity during a similar time course experiment (data not shown).

Identification of *Arabidopsis* mutants perturbed in *PR-1::LUC* expression.

To identify components of the signaling network regulating *PR* gene expression, we sought mutations that caused miss-expression of the *PR-1* gene. *PR-1::LUC* transgenic plants were mutagenized with ethylmethane sulfonate (EMS), and approximately 5,000 of the resulting M₂ plants were screened via LUC imaging for mutants showing *PR-1* miss-expression following challenge with *P. syringae* pv. *tomato* DC3000(*avrB*). Mutant candidates were identified that showed (i) no activation of LUC activity; (ii) potentiated LUC activity; (iii) a spatially modified distribution of LUC activity; and (iv) constitutive LUC activity in the absence of *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation. One mutant, designated *cir1*, was selected for further study. The mutant *cir1* was found to exhibit both an increased level and modified distribution of LUC activity compared with that of *PR-1::LUC* Col-0 plants following inoculation with *P. syringae* pv. *tomato* DC3000(*avrB*) (Fig. 3B and C, respectively). This mutation may, therefore, result in either the constitutive expression of *PR-1* in the absence of pathogen challenge or a superinduction of this gene in response to *P. syringae* pv. *tomato* DC3000(*avrB*). To address this question, *cir1* plants were imaged in the absence of pathogen challenge. The *cir1* plants exhibited significant LUC activity preceding *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation (Fig. 3D), suggesting that the *cir1* mutation may result in the constitutive expression of *PR-1*. To confirm that the LUC imaging data was congruent with the expression of the endogenous *PR-1* gene, we carried out northern blot analysis (Fig. 3E). In the absence of *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation, *cir1* plants were found to accumulate *PR-1* transcripts, thus confirming the constitutive expression of this gene. However, the constitutive level of *PR-1* gene expression was less than that produced following inoculation of wild-type Col-0 plants with *P. syringae* pv. *tomato* DC3000(*avrB*). Interestingly, *PR-1* transcript levels were also consistently found to accumulate to an increased level in *cir1* plants compared with that of Col-0 wild-type plants following *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation. Thus, in addition to resulting in the constitutive

expression of *PR-1*, the *cir1* mutation may also result in potentiated *PR-1* gene expression following attempted *P. syringae* pv. *tomato* DC3000(*avrB*) infection. This may be *PR-1*-specific, because neither *PDF1.2*, *PR-2*, *PR-5*, nor *GST1* gene expression exhibited the same phenomenon (data not shown). Under short-day conditions, *cir1* plants exhibited a small reduction in stature compared with wild-type Col-0 plants, but they did not exhibit any significant morphological perturbations.

Genetic analysis of the *cir1* mutation.

To determine the genetic segregation of the *cir1* phenotype, a backcross was performed between *cir1/cir1* plants and the wild-type parent (*CIR1/CIR1*). The resulting F₁ progeny (*CIR1/cir1*; 20 plants) all showed the absence of constitutive LUC activity, indicating that the *cir1* mutation was recessive. From the F₂ population, 72 F₂ plants were examined by northern blot analysis by scoring for *PR* gene expression. Sixteen constitutive *PR-1*-expressing F₂ plants were identified from this population, confirming the expected ratio of 1:3 for a recessive gene ($\chi^2 = 0.296$; $0.5 < P < 0.8$).

To determine the map position of the *cir1* mutation, *cir1* plants were crossed to Landsberg *erecta* (*Ler*) plants. The F₁ progeny did not exhibit constitutive *PR-1* gene expression, as was expected for a recessive trait. These plants were allowed to self-pollinate, and the F₂ progeny were analyzed for constitutive *PR-1* gene expression by northern analysis. DNA was isolated from 48 homozygous *cir1* F₂ plants and examined using codominant cleaved amplified polymorphic sequences (CAPS) and simple satellite-length polymorphism (SSLP) markers (Bell and Ecker 1994; Konieczny and Ausubel 1993). Segregation of the analyzed markers indicated that *cir1* mapped approximately 9.4 cM below nga111 on the lower arm of chromosome 4. As the *cpr1* mutation mapped to this general region (Bowling et al. 1994), we carried out complementation analysis between *cir1* and *cpr1*. Following a cross between these two mutants, with *cir1* as the pollen donor, all of the resulting 27 F₁ plants examined exhibited a wild-type phenotype with respect to *PR-1* expression. Therefore, *cir1* is not an allele of *cpr1*.

The *cir1* mutant shows constitutive expression of multiple defense genes.

As *cir1* plants showed constitutive expression of the *PR-1* gene in the absence of pathogen challenge, we also carried out northern blot analysis to determine if other plant defense genes

Fig. 2. Ultra-low-light camera imaging of luciferase (LUC) activity. Images showing LUC activity in the leaves of transgenic *PR-1::LUC* plants over time, in hours, following infiltration with *Pseudomonas syringae* pv. *tomato* DC3000(*avrB*). Bar = 1 cm.

were constitutively expressed in this mutant (Fig. 4). First, we tested other SA-dependent genes, including *PR-2* and *PR-5*, which encode a β -1,3-glucanase and a thaumatinlike protein, respectively (Uknes et al. 1992). We detected significant expression of both *PR-2* and *PR-5*. Hence, the *cir1* mutation promotes the constitutive expression of other SA-dependent genes in addition to *PR-1*. Furthermore, we also examined the expression of the *PDF1.2* gene, which is locally and systemically induced following attempted pathogen infection. The *PDF1.2* gene is activated independently of SA, and its expression is thought to be engaged following the accumulation of JA/ET (Penninckx et al. 1996, 1998). Hence, this gene has been deployed as a marker for the activation of JA/ET-dependent defense responses. While *PDF1.2* was not significantly expressed in wild-type Col-0 plants, this gene was constitutively expressed in *cir1* plants. Thus, both SA- and JA-dependent gene expression were constitutively engaged in *cir1* plants.

We also examined the expression of the *GST1* gene, which is activated in response to ROI accumulation following engagement of the oxidative burst during HR formation (Grant et al. 2000). This gene was also found to be constitutively expressed in *cir1* plants. However, neither visible spontaneous HR-like lesions nor "micro-HR" lesions were observed on *cir1* plants following trypan blue staining (data not shown). Thus, the *cir1* mutation results in the activation of multiple defense signaling

networks, leading to the expression of distinct sets of defense-related genes.

The *cir1* plants show broad-spectrum resistance against virulent pathogens.

Since *cir1* plants were found to constitutively express a number of key defense marker genes, we examined whether this gene expression profile could convey resistance against ordinarily virulent microbial pathogens. First, we tested *cir1* plants for resistance against *P. syringae* pv. *tomato* DC3000. In order to quantify *P. syringae* pv. *tomato* DC3000 growth in planta, bacteria were extracted from leaves 3 days postinoculation. This time point was chosen since *P. syringae* pv. *tomato* DC3000 growth has been shown to reach a maximum value 3 days postinoculation of a suspension with an optical density at 600 nm (OD_{600}) of 0.002 (Whalen et al. 1991). The growth of *P. syringae* pv. *tomato* DC3000 detected in the leaves of *cir1* plants was more than 10-fold less than that present in wild-type plants (Fig. 5A), suggesting that the growth of this phytopathogen was suppressed in *cir1* plants. Similar results have been documented for other mutants that ectopically express defense-related genes (Clarke et al. 1998; Lawton et al. 1993; Petersen et al. 2000; Silva et al. 1999; Yu et al. 1998). In contrast, as previously reported (Delaney et al. 1994), immunosuppressed *nahG* plants supported greater than a 10-fold increase in *P. syringae* pv. *tomato* DC3000 growth compared with that in wild-type Col-0 plants. Furthermore, Col-0 plants sprayed with 1 mM SA 3 days prior to inoculation limited *P. syringae* pv. *tomato* DC3000 growth to a magnitude similar to that recorded for *cir1* plants. Thus, the level of *cir1*-mediated resistance against *P. syringae* pv. *tomato* DC3000 is similar to that induced following the establishment of SAR by the exogenous application of SA.

We also tested *cir1* plants for resistance against *Peronospora parasitica* Noco2, a biotrophic oomycete pathogen that is virulent on the *Arabidopsis* accession Col-0 (Parker et al. 1993). A spore suspension of *Peronospora parasitica* Noco2 was sprayed onto 4-week-old *Arabidopsis* plants, and conidiophore formation was visually assessed 10 days later. The *cir1* plants showed reduced conidiophore formation in comparison to that in wild-type Col-0 plants, suggesting that *cir1* also mediates resistance against *Peronospora parasitica* Noco2 (Fig. 5B). The magnitude of resistance established was similar to that induced in wild-type Col-0 plants following exogenous SA application. As expected, *nahG* plants exhibited increased susceptibility to this pathogen (Delaney et al. 1994).

Characterization of the *cir1* double mutants.

The accumulation of SA has been proposed to play a central role in the development of plant disease resistance (Delaney et al. 1994; Gaffney et al. 1993). To determine if the *cir1* phenotype was dependent on SA accumulation, crosses were performed between homozygous *cir1* plants and a homozygous Col-0 line expressing the *nahG* transgene (Delaney et al. 1994). As expected, because of the recessive nature of *cir1*, LUC activity was absent in all of the 25 F_1 progeny tested. If SA accumulation was required for the *cir1* phenotype, the expected ratio of *cir1* plants showing constitutive LUC activity in the F_2 progeny would be 1:20. Following examination of the resulting F_2 progeny, 2 of 19 plants showed LUC activity, suggesting that the accumulation of SA is required for *cir1*-mediated *PR-1* expression ($\chi^2 = 1.23$; $0.2 < P < 0.5$). Furthermore, F_3 seedlings derived from self-pollination of these F_2 plants were tested for the presence of the *nahG* transgene (described below). Neither F_3 family showed the presence of the *nahG* gene, further indicating that *PR-1* expression in *cir1* is dependent on SA accumulation.

Fig. 3. The *cir1* mutant miss-expresses the *PR-1* gene. Images showing **A**, Luciferase (LUC) activity of an unchallenged leaf; **B**, the leaf of a wild-type plant infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000(avrB), with LUC activity surrounding the area of hypersensitive response (HR) development (lower, middle of leaf); **C**, a *cir1* leaf infiltrated with *P. syringae* pv. *tomato* DC3000(avrB), showing HR-mediated cell death (lower, middle of leaf); and **D**, an unchallenged *cir1* leaf. **E**, Expression of the endogenous *PR-1* gene in *P. syringae* pv. *tomato* DC3000(avrB) challenged and unchallenged wild-type Col-0 and *cir1* plants.

The elaboration of SA-induced SAR is thought to be dependent on NPR1, which was identified via a number of distinct genetic screens (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Moreover, *npr1* plants also exhibit an increased susceptibility to virulent pathogens. To address whether NPR1 is required for *cir1*-mediated resistance, we created *cir1 npr1* double mutants. Northern blot analysis of these plants suggested that *npr1* reduced *PR-1* gene expression (Fig. 6A). Hence, NPR1 appears to be required for *cir1*-mediated *PR-1* expression. We also examined the expression of the *PDF1.2* gene in this double mutant, which is a well-characterized marker for the expression of JA- and ET-responsive genes (Thomma et al. 1998). Interestingly, *npr1* also reduced the expression of this JA- and ET-responsive gene in a *cir1* genetic background (Fig. 6A).

The plant hormones ET and JA are also thought to play important roles in plant defense signaling responses (Clarke et al. 2000; Hoffman et al. 1999; Knoester et al. 1998; Thomma et al. 1998). To address the possible role of these hormones in the establishment of the *cir1* phenotype, we crossed *cir1* plants with the JA- and ET-insensitive mutants *jar1* and *ein2*, respectively (Guzman and Ecker 1990; Staswick et al. 1992). Before generating *jar1* and *ein2* double mutants, *cir1* plants were examined to confirm that they did not exhibit phenotypes associated with the overproduction of or insensitivity toward ET or JA (described below). The impact of *jar1* and *ein2* on the *cir1*-mediated expression of *PR-1* and *PDF1.2* was examined in the *cir1 jar1* and *cir1 ein2* double mutants by northern blot analysis (Fig. 6A). As expected, both *jar1* and *ein2* reduced the expression of the *PDF1.2* gene. However, both of these mutations also appeared to decrease the expression of the SA-dependent gene *PR-1*. Thus, while JAR1 and EIN2 are absolutely required for *cir1*-mediated *PDF1.2* expression, these signaling components may also contribute to the expression of the *PR-1* gene in a *cir1* genetic background. Finally, we also examined whether any of the *cir1* double mutants were compromised in potentiated *PR-1* gene expression following *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation. In *cir1 ein2* and *cir1 jar1* mutants, *PR-1* expression was potentiated in a fashion similar to that observed in *cir1* plants (Fig. 6B). In contrast, potentiated *PR-1* gene expression was abolished in *cir1 npr1* plants.

Impact of *nahG*, *npr1*, *jar1*, and *ein2* on *cir1*-induced disease resistance.

To investigate the effects of *npr1*, *jar1*, and *ein2* on *cir1*-mediated disease resistance, we challenged our panel of double mutants with *P. syringae* pv. *tomato* DC3000. The bacterial growth in these plants was determined 3 days postinoculation with *P. syringae* pv. *tomato* DC3000 (Fig. 7A). While similar numbers of *P. syringae* pv. *tomato* DC3000 were observed in *jar1* and *ein2* compared with wild-type Col-0 plants, increased growth of *P. syringae* pv. *tomato* DC3000 was found in *npr1* plants, supporting previous observations (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Inoculation of *jar1 cir1* and *ein2 cir1* double mutants revealed that either *jar1* or *ein2* abolished the resistance conveyed by *cir1* against this bacterial pathogen. Moreover, in *cir1 npr1* double mutants, the *npr1* mutation was found to partially suppress *cir1*-mediated resistance, suggesting that both *npr1*-dependent and *npr1*-independent resistance contribute to limit the growth of *P. syringae* pv. *tomato* DC3000 in *cir1* plants.

The same panel of single and double mutants was also tested for resistance against *Peronospora parasitica* Noco2. Plants were sprayed with a conidiospore suspension, conidiophore growth was visually assessed after 10 days, and disease indices were calculated (Fig. 7B). As observed for *P. syringae* pv. *tomato* DC3000, disease indices were similar for Col-0, *ein2*,

and *jar1* plants, while the disease index for *npr1* plants was significantly increased in comparison to that of Col-0, as reported previously (Cao et al. 1994; Delaney et al. 1995). As expected, *cir1* plants exhibited significant resistance against this pathogen. Interestingly, *cir1*-induced resistance was lost in the *cir1 npr1* mutant but was maintained in the *cir1 ein2* and *cir1 jar1* double mutants, indicating that *cir1*-induced resistance to *Peronospora parasitica* Noco2 is dependent on NPR1 but is established independently of EIN2 and JAR1. Additionally, *cir1 npr1* plants were not as susceptible to *Peronospora parasitica* Noco2 as were *npr1* plants, suggesting that both an NPR1-dependent and an NPR1-independent pathway also contribute to the establishment of *cir1*-induced resistance against this pathogen.

We also examined whether SA accumulation was required for *cir1*-induced resistance against both *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica*. It has been previously demonstrated that *Arabidopsis* plants expressing the *nahG* transgene show increased susceptibility to both of these microbial pathogens (Delaney et al. 1994). Crosses were performed between homozygous *cir1* plants and a homozygous Col-0 line expressing the *nahG* transgene (Delaney et al. 1994; Gaffney et al. 1993). If a single copy of the *nahG* transgene produces sufficient salicylate hydroxylase activity to suppress *cir1*-mediated disease resistance, then three-quarters of the plants in the F₂ population would be expected to show increased disease susceptibility for *P. syringae* pv. *tomato* DC3000. Out of a total of 26 F₂ plants, 19 exhibited increased susceptibility to this pathogen, following leaf inoculation with an OD₆₀₀ *P. syringae* pv. *tomato* DC3000 suspension, suggesting that SA accumulation is required for *cir1*-mediated resistance against *P. syringae* pv. *tomato* DC3000 ($\chi^2 = 0.051$; $0.8 < P < 0.9$). Similarly, out of 30 F₂ plants examined, 22 exhibited increased susceptibility to *Peronospora parasitica* Noco2 following spray inoculation with a spore suspension. Thus, SA accumulation is also required for *cir1*-mediated resistance against this oomycete pathogen ($\chi^2 = 0.044$; $0.8 < P < 0.9$).

DISCUSSION

The utility of the *PR-1::LUC* transgenic line was exploited in a screen designed to uncover mutants that regulated *PR-1* gene expression following *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation. It has recently been reported that the application of

Fig. 4. The *cir1* mutant constitutively expresses salicylic acid, jasmonic acid/ethylene, and reactive oxygen intermediate (ROI)-dependent defense-related genes. RNA gel blot analysis of *PR-1*, *PDF1.2*, *PR-2*, *PR-5*, and *GST1* expression in the *cir1* mutant in comparison with wild-type Col-0 plants. The resulting blot was also hybridized against the constitutively expressed *r18* gene as a control for equal loading and transfer.

luciferin, the substrate of luciferase, may induce *PR-1* gene expression (Jorda and Vera 2000). However, during the time frame of our experiments, we never observed LUC activity in luciferin-sprayed leaves of transgenic *PR-1::LUC* plants in the absence of pathogen challenge. We found the *LUC* reporter gene to be well suited for a screen designed to uncover defense-related mutants. The *cir1* mutation described in this report conveyed significant constitutive expression of both the *PR-1::LUC* transgene and the endogenous *PR-1* gene. In addition, the *cir1* mutant also showed constitutive expression of other SA-dependent genes, including *PR-2* and *PR-5*. Interestingly, *P. syringae* pv. *tomato* DC3000(*avrB*) inoculation of *cir1* plants appeared to result in a relatively small but reproducible *PR-1*-specific superinduction. Moreover, this phenomenon was

NPR1 dependent but JAR1 and EIN2 independent. The *Arabidopsis edr1* mutant, which showed enhanced expression of *PR-1* in response to *Erysiphe cichoracearum*, is similar in this respect (Frye and Innes 1998). In contrast to *cir1*, however, the *edr1* mutation resulted in no basal *PR-1* expression in the absence of pathogen challenge (Frye and Innes 1998).

In addition to SA-dependent genes, northern analysis also indicated that *cir1* plants constitutively expressed the JA/ET-dependent gene *PDF1.2*. Thus, *cir1* was found to be distinct from the *cpr1* mutant (Bowling et al. 1994), which does not engage JA-dependent gene expression (Penninckx et al. 1996). The recessive *cpr5* and dominant *cpr6* mutations (Bowling et al. 1997; Clarke et al. 1998) also expressed the *PDF1.2* gene. However, *cpr6* plants exhibited a pronounced loss of apical dominance and *cpr5* plants develop spontaneous HR-like lesions, phenotypes that were absent in *cir1* plants. Based on genetic analysis, the *cir1* trait was inherited as a single recessive mutation that mapped to the lower arm of chromosome 4. Therefore, *CIR1* may encode a negative regulator of SAR that suppresses the expression of SA-, JA/ET-, and ROI-dependent defense-related genes.

To help determine the point of *cir1* function within the SAR signaling network, we undertook epistasis studies utilizing the well-characterized *Arabidopsis* mutants *npr1*, *jar1*, and *ein2* (Cao et al. 1994; Guzman and Ecker 1990; Staswick et al. 1992). To uncover a potential role for SA in the establishment of the *cir1* phenotype, we crossed *cir1* plants with those possessing the *nahG* transgene (Delaney et al. 1994). Analysis of the F₂ progeny from this cross indicated that removing SA strongly reduced *cir1*-mediated *PR-1* gene expression and abolished resistance against *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica*. Therefore, in a *cir1* genetic background, SA is required for both NPR1- and JA/ET-dependent disease resistance. Moreover, this data suggested that *CIR1* may operate upstream of SA accumulation in the establishment of *cir1*-mediated resistance. Alternatively, *CIR1* could function downstream of SA but activate the accumulation of this metabolite via a feedback loop. The existence of such a mechanism has been proposed from studies utilizing a number of *lsd* mutants (Weyman et al. 1995). Moreover, feedback loops have been identified in the pathways that regulate a number of distinct plant defense responses (Fauth et al. 1996; Mur et al. 1996; Shirasu et al. 1997; Thulke and Conrath 1998). SA may also be required to activate *CIR1* or a downstream signaling component. Interestingly, application of exogenous SA inhibited constitutive *PDF1.2* expression in *cir1* plants and inoculation with *P. syringae* pv. *tomato* DC3000(*avrB*) also reduced expression of this gene (S. L. Murray and G. J. Loake, unpublished data). Hence, increased flux through the SA-dependent network appears to reduce *PDF1.2* expression. In *cir1* plants, while SA is essential for both NPR1- and JA/ET-dependent disease resistance, further elevation of the SA concentration appears to reduce expression of the JA/ET-dependent *PDF1.2* gene. Therefore, in this genetic background, increasing the level of SA may engage a negative feedback loop that functions to suppress JA/ET-dependent signaling. This process could occur via either network evolution, producing a new link between signaling nodes, or progressively increasing the weight of a preformed link. In this context, SA has been shown to inhibit the synthesis of both JA and ET (Leslie and Romani 1988; Peña-Cortés et al. 1993; Pennazio et al. 1985).

In *cir1 npr1* double mutants, *PR-1* gene expression was reduced, this may reflect the fact that *cir1* appears to function upstream of SA accumulation and NPR1 is thought to be a key component in SA-mediated resistance (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). *PR-1* gene expression has been shown to depend on NPR1 in

Fig. 5. The *cir1* plants exhibit resistance against virulent pathogens. **A**, *Arabidopsis* leaves of selected genotypes were infiltrated with virulent *Pseudomonas syringae* pv. *tomato* DC3000. Leaf disks were collected 3 days postinfection for the determination of bacterial growth. Wild-type Col-0 plants were sprayed with 0.1 mM salicylic acid 3 days prior to pathogen challenge. Error bars represent 95% confidence limits of log-transformed data. **B**, Growth of *Peronospora parasitica* Noco2 on selected genotypes. Infection by *Peronospora parasitica* was accomplished by spraying a 1×10^5 conidiospore suspension and assaying for pathogen growth 7 days later. Scoring was as follows: 0 = no infection, 1 = less than 25% of one leaf covered with conidiophore growth, 2 = 25 to 50% of one or two leaves covered with conidiophore growth, 3 = 25 to 50% of three or four leaves covered with conidiophore growth, 4 = 25 to 50% of all leaves covered with conidiophore growth, and 5 = all leaves covered with conidiophores. Plants were assigned a disease index (DI) as follows: $DI = \sum i \times j/n$, in which i = infection class, j = the number of plants scored for that infection class, and n = the total number of plants in the replicate (Epple et al. 1997). The *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica* experiments were each repeated three times with similar results.

the *cpr5* mutant (Bowling et al. 1997) but not in the *cpr6* and *ssi1* mutants (Clarke et al. 1998; Shah et al. 1999). Moreover, in *acd6* plants, *PR-1* gene expression was found to be partially dependent on NPR1, because it was still possible to detect weak *PR-1* expression in an *npr1* background (Rate et al. 1999). An interesting feature of the *cir1 npr1* double mutants was that the expression of the JA-dependent *PDF1.2* gene was also suppressed in these plants. Recent evidence suggests that *npr1* may negatively regulate SA accumulation (Clarke et al. 2000). Therefore, SA levels may be significantly increased in *cir1 npr1* plants, which may antagonize JA-dependent signaling, leading to reduced *PDF1.2* expression. The accumulation of *PDF1.2* transcripts was significantly suppressed in *cir1 jar1* and *cir1 ein2* double mutants, suggesting that JA and ET sensitivity is essential for *PDF1.2* expression in *cir1* plants. Similar observations have been reported for *cpr5 jar1* and *cpr6 ein2* double mutants (Clarke et al. 2000). The expression of the SA-dependent *PR-1* gene was also slightly reduced in *cir1 jar1* and *cir1 ein2* double mutants, implicating a requirement for JA and ET sensitivity for maximal *PR-1* gene expression in *cir1* plants. Recent analysis of *cpr6 npr1 jar1* and *cpr6 npr1 ein2* triple mutants has uncovered an NPR1-independent pathway regulating *PR-1* gene expression that is dependent on ET, and possibly JA, sensitivity (Clarke et al. 2000). Hence, this pathway may be engaged in *cir1* plants, in addition to the NPR1-dependent pathway, to produce maximal *PR-1* transcript accumulation in this genetic background.

We also analyzed the response of this collection of double mutants to the virulent microbial pathogens *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica* Noco2. In an *npr1* genetic background, *cir1*-mediated resistance against *P. syringae* pv. *tomato* DC3000 was partially suppressed. Therefore, resistance in *cir1* plants against this pathogen may be established via NPR1-dependent and NPR1-independent signaling. A similar observation was made following inoculation of virulent *P. syringae* pv. *maculicola* into *acd6 npr1* plants, which showed an intermediate level of bacterial growth when compared with either *acd6* or *npr1* plants (Rate et al. 1999). In contrast, resistance against *P. syringae* pv. *maculicola* in the *cpr5* and *cpr6* mutants is abolished by *npr1* (Bowling et al. 1997; Clarke et al. 1998, 2000), while the *ssi1-1* mutation completely restores resistance against *P. syringae* pv. *tomato* DC3000(*avrRpt2*) in an *npr1* genetic background (Shah et al. 1999). The expression of *PR* genes is partially induced in *npr1* plants following attempted pathogen infection but not by SA alone, suggesting that engagement of an SA-dependent but NPR1-independent pathway may require SA plus a signal produced or induced by the pathogen (Clarke et al. 2000; Shah et al. 1999). In *cir1* plants, the requirement for this unknown second signal could be bypassed, although SA would still be required.

Interestingly, both *cir1 jar1* and *cir1 ein2* double mutants abolished resistance against *P. syringae* pv. *tomato* DC3000, suggesting that JA and ET sensitivity was essential for resistance against this pathogen in *cir1* plants. Thus, the NPR1-independent pathway engaged by the *cir1* mutation may require JA and ET sensitivity or JAR1 and EIN2 function. In this context, JA/ET-based signaling has recently been found to be required for NPR1-independent resistance in *cpr5* and *cpr6* plants (Clarke et al. 2000). Furthermore, the SA-independent resistance induced by certain root-colonizing bacteria against foliar infection by *P. syringae* pv. *tomato* DC3000 was also found to be dependent on sensitivity to these plant hormones, in addition to NPR1 function (Pieterse et al. 1998).

The response of our panel of double mutants to *Peronospora parasitica* was found to be distinct to that of *P. syringae* pv. *tomato* DC3000. In an *npr1* genetic background, *cir1*-mediated resistance against *Peronospora parasitica* was partially suppressed,

as observed for *P. syringae* pv. *tomato* DC3000, suggesting that resistance against this pathogen may also be established via an NPR1-dependent and SA-dependent but NPR1-independent mechanism. This is an interesting feature of the *cir1* mutant, because these results contrasted with resistance against *Peronospora parasitica* in the *cpr1*, *cpr5*, and *cpr6* mutants, in which *npr1* was found not to reduce resistance against this oomycete pathogen (Bowling et al. 1994, 1997; Clarke et al. 1998). Interestingly, analysis of the *cir1 jar1* and *cir1 ein2* double mutants showed that JA or ET sensitivity was not required for resistance against *Peronospora parasitica* in *cir1* plants. Therefore, in a *cir1* genetic background, either JA- or ET-dependent signaling or JAR1 or EIN2 function are dispensable for resistance against this pathogen. Similar observations have been reported for *cpr5* plants (Clarke et al. 2000). This contrasts strikingly with that observed for *P. syringae* pv. *tomato* DC3000, in which resistance was abolished in *cir1 ein2* and *cir1 jar1* plants. Therefore, distinct defense response signaling pathways engaged in the *cir1* mutant are required for resistance against different microbial pathogens.

In summary, we have demonstrated the utility of LUC imaging for targeted genetic screens designed to uncover novel defense-related mutants. Using this approach, we isolated and subsequently characterized the *cir1* mutant. Future studies will focus on extending the double mutant analysis, the identification of *cir1*-regulated genes, and the molecular cloning of *CIR1*.

Fig. 6. Impact of *npr1*, *ein2*, and *jar1* on *cir1*-mediated *PR-1* and *PDF1.2* gene expression. **A**, RNA was isolated from wild-type Col-0, *cir1*, *npr1*, *cir1 npr1*, *ein2*, *cir1 ein2*, *jar1*, and *cir1 jar1* plants and subjected to northern blot analysis with hybridization against *PR-1* and *PDF1.2* probes. **B**, Col-0, *cir1*, and *cir1* double mutants were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000(*avrB*) and total RNA was isolated. The corresponding northern blot was probed with the *Arabidopsis PR-1* gene. In each case, the resulting blots were also hybridized against the constitutively expressed *r18* gene as a control for equal loading and transfer.

MATERIALS AND METHODS

Generation of *PR-1::LUC* transgenic *Arabidopsis* plants.

A 1,663-bp *HindIII-XbaI* fragment of the firefly luciferase (*LUC*) reporter gene from plasmid pSP-luc+ (Promega, Madison, WI, U.S.A.) was cloned into pART7 (Gleave 1992), creating plasmid p7LUC. A 903-bp *KpnI-NcoI* fragment of the *PR-1* promoter (Uknes et al. 1992) was cloned into pSL1180 (Amersham Pharmacia, London). Subsequently, the *PR-1* promoter sequence was released from pSL1180 as an *SacI-NcoI* fragment and was cloned directly into p7LUC, replacing the endogenous CaMV35S promoter. The *PR-1::LUC::OCS* chimeric gene resulting from this step was subsequently cloned into pART27 (Gleave 1992) as an *NotI* fragment. The generated binary vector containing the *PR-1::LUC::OCS* gene was then transformed into *Agrobacterium tumefaciens* strain GV3101(pMP90RK) by

freeze-thaw transformation, and transformants were selected on Luria-Bertani agar media containing the following antibiotics: spectinomycin (100 µg ml⁻¹); rifampicin (100 µg ml⁻¹), and gentamicin (100 µg ml⁻¹). Transformed bacterial colonies were identified after 2 days incubation at 30°C. *Arabidopsis* ecotype Col-0 was transformed by vacuum infiltration (Bechtold et al. 1993) and transgenic plants were identified following growth in Murashige-Skoog (MS) medium containing 50 µg of kanamycin per ml.

LUC activity measurements, in planta imaging, and mutant screening.

LUC activity in vitro was measured with an EG&G Berthold MicroLumat LB96P luminometer (Berthold, London). Total protein content was measured with the Bradford micro-assay, with bovine serum albumin as the protein standard. LUC activity was calculated as relative light units (RLU) per microgram of extractable protein. Leaves of 5-week-old plants were infiltrated with *P. syringae* pv. *tomato* DC3000(*avrB*) at an OD₆₀₀ of 0.2, which under our conditions, produced visible cell death approximately 20 h postinoculation. Infiltrated leaves were sprayed with a 1-mM-luciferin solution and imaged for LUC activity with an ultra-low-light imaging camera system (Berthold), as described (Grant et al. 2000). Images were routinely collected over a 10-s time period and integrated for 5 s. Images were processed with Confocal Assistant and PaintShop Pro software.

Approximately 10,000 seeds homozygous for the *PR-1::LUC* transgene were mutagenized in 0.3% EMS. Mutagenized M₁ seeds were allowed to grow, self-fertilize, and set seed. Plants from approximately 5,000 M₂ seeds were screened for perturbations in LUC activity following leaf inoculation with *P. syringae* pv. *tomato* DC3000(*avrB*).

RNA analysis.

Total RNA was routinely extracted from 5-week-old plants using a guanadinium thiocyanate phenol chloroform extraction method. For northern blot analysis, 10-µg samples were separated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Amersham Pharmacia). ³²P-labeled DNA probes were prepared with a Prime-a-Gene labeling kit (Promega) and hybridized as described (Grant et al. 2000).

Genetic analysis.

Genetic crosses were undertaken by dissecting and emasculating unopened flower buds and then using the remaining pistils as the recipients for pollen from opened flowers. Pollen from *cir1/cir1* plants was used to fertilize wild-type Col-0 *PR-1::LUC* plants for backcrossing. Pollen from *nahG* plants was used to fertilize *cir1/cir1* plants, and F₁ progeny containing *nahG* were confirmed by virtue of brown deposits in root tissue when grown on MS media plus 1 mM SA (Bowling et al. 1994). Pollen from homozygous *cir1* plants was used to pollinate *npr1*, *jar1*, and *ein2* plants. Double mutants containing these mutations in the F₂ generation were identified as described previously (Cao et al. 1997; Guzman and Ecker 1990; Staswick et al. 1992).

Polymerase chain reaction-based mapping.

The *cir1* plants were crossed to Landsberg *erecta* (*Ler*) plants and the progeny that segregated homozygous for the *cir1* mutation were selfed and used for mapping. DNA was isolated from 48 homozygous *cir1* plants. Recombinant seedlings were identified using CAPS (Konieczny and Ausabel 1993) and SSLP (Bell and Ecker 1994) reported on the *Arabidopsis* database (available online).

Fig. 7. Impact of *npr1*, *ein2*, and *jar1* on *cir1*-mediated disease resistance. **A**, Growth of *Pseudomonas syringae* pv. *tomato* DC3000 on wild-type, *cir1*, *npr1*, *cir1 npr1*, *ein2*, *cir1 ein2*, *jar1*, and *cir1 jar1* plants. Leaf disks were collected 3 days postinfection for the determination of bacterial growth. Wild-type Col-0 plants were sprayed with 0.1 mM salicylic acid 3 days prior to pathogen challenge. Error bars represent 95% confidence limits of log-transformed data. **B**, Growth of *Peronospora parasitica* Noco2 on wild-type, *cir1*, *npr1*, *cir1 npr1*, *ein2*, *cir1 ein2*, *jar1*, and *cir1 jar1* plants. Infection by *Peronospora parasitica* was accomplished by spraying a 1×10^5 conidiospore suspension and assaying for pathogen growth 7 days later. Scoring was as follows: 0 = no infection, 1 = less than 25% of one leaf covered with conidiophore growth, 2 = 25 to 50% of one or two leaves covered with conidiophore growth, 3 = 25 to 50% of three or four leaves covered with conidiophore growth, 4 = 25 to 50% of all leaves covered with conidiophore growth, and 5 = all leaves covered with conidiophores. Plants were assigned a disease index (DI) as follows: $DI = \sum i \times j/n$, in which i = infection class, j = the number of plants scored for that infection class, and n = the total number of plants in the replicate (Epple et al. 1997). The *P. syringae* pv. *tomato* DC3000 and *Peronospora parasitica* experiments were each repeated three times with similar results.

Infection with *P. syringae* pv. *tomato* DC3000.

P. syringae pv. *tomato* DC3000 (Whalen et al. 1991) was grown in King's broth liquid media supplemented with 50 mg of rifampicin per liter. Five-week-old soil-grown plants were infected with a *P. syringae* pv. *tomato* DC3000 suspension ($OD_{600} = 0.002$) in 10 mM $MgCl_2$ by completely infiltrating the abaxial side of the leaf with a 1-ml syringe (Cao et al. 1994). After 3 days, plants were inspected for development of symptoms. Leaves were also harvested for analysis of bacterial growth. Leaf disks of uniform size (0.5 cm^2) were made from the leaf samples using a cork borer. Three random leaf disks from six independent plants were ground in 10 mM $MgCl_2$ with a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension, and 100 μ l of each dilution was spread onto King's B medium plates containing 50 mg of rifampicin per liter. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies for each sample was recorded. Bacterial counts were statistically analyzed using the Mann-Whitney test.

Infection with *Peronospora parasitica*.

The *Peronospora parasitica* Noco2 oomycete pathogen (Parker et al. 1993) was maintained in Col-0 seedlings grown under humid conditions, and conidiospores were transferred to new seedlings weekly by dusting infected seedlings onto healthy seedlings. For the *Peronospora parasitica* disease resistance assays, conidiospores were harvested by vortexing infected seedlings in water. The conidiospore concentration was determined using a haemocytometer and adjusted to 1×10^5 spores per ml. Four-week-old plants grown under short-day conditions (10 h of light, 14 h of dark) were sprayed with the conidiospore solution and maintained under humid conditions. Results were taken after 7 days, and infected plants were scored for the extent of conidiophore development (Bowling et al. 1997). Scoring was as follows: 0 = no infection, 1 = less than 25% of one leaf covered with conidiophore growth, 2 = 25 to 50% of one or two leaves covered with conidiophores, 3 = 25 to 50% of three or four leaves covered with conidiophore growth, 4 = 25 to 50% of all leaves covered with conidiophore growth, and 5 = all leaves covered with conidiophores. Plants in different replicates were assigned a disease index (DI) as follows: $DI = \sum i \times j/n$, in which i = infection class, j = the number of plants scored for that infection class, and n = the total number of plants in the replicate (Epple et al. 1997).

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