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Activation tagging of ADR2 conveys a spreading lesion phenotype and resistance to biotrophic pathogens

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Summary

- An Arabidopsis PR1::luciferase (LUC) transgenic line was transformed with activation T-DNA and the resulting population screened for dominant gain-of-function mutants exhibiting constitutive LUC activity.
- LUC imaging identified activated disease resistance 2 (adr2), which exhibited slowly spreading lesions in the absence of pathogen challenge. Molecular, genetic and histochemical analysis was employed to characterize this mutant in detail.
- adr2 plants constitutively expressed defence-related and antioxidant genes. Moreover, this line accrued increased quantities of salicylic acid (SA) and exhibited heightened mitogen-activated protein kinase activity. adr2 plants exhibited increased resistance against numerous biotrophic but not necrotrophic pathogens. The adr2 phenotype resulted from the overexpression of a Toll interleukin receptor (TIR) nucleotide binding site (NBS) leucine rich repeat (LRR) gene (At1g56510). Constitutive PR1 expression was completely abolished in adr2 nha1, adr2 npr1 and adr2 eds1 double mutants. Furthermore, heightened resistance against Hyaloperonospora arabidopsis NoCo2 was compromised in adr2 nha1 and adr2 eds1 double mutants but not in adr2 npr1, adr2 coi1 or adr2 etr1 plants.
- These data imply that adr2-mediated resistance operates through an Enhanced Disease Susceptibility (EDS) and SA-dependent defence signalling network which functions independently from COI1 or ETR1.

Introduction

Plants have evolved an array of sophisticated mechanisms to detect and respond to attempted pathogen ingress. Preformed physical and chemical barriers constitute the first line of defence (Haralampidis et al., 2001) upon which are superimposed a battery of inducible defence responses (Yun et al., 2003; Nürnberger & Lipka, 2005). Prominent in the triggering of these protective mechanisms is a repertoire of resistance (R) gene products, which recognize either directly or indirectly pathogen effector proteins (Dangl & Jones, 2001). An effector protein whose presence is detected by a resistant plant is termed an AVIRULENCE (AVR) gene product although its likely function is to aid pathogenesis on a susceptible host (Nomura et al., 2006). The largest class of R genes encode NUCLEOTIDE BINDING SITE (NBS) LEUCINE RICH REPEAT (LRR) proteins, which can be divided into subgroups defined by the presence of either COILED-COIL (CC) or TOLL INTERLEUKIN RECEPTOR (TIR) domains in their N-termini (Meyers et al., 2003).

R gene-dependent pathogen recognition is usually associated with a form of genetically programmed plant cell death termed the hypersensitive response (HR) surrounding the site of attempted infection (Greenberg, 1997; Gilchrist, 1998). This cell death event has been shown to induce a phenomenon known as systemic acquired resistance (SAR) which establishes relatively durable, broad-spectrum protection from ordinarily virulent pathogens, throughout the plant (Durrant & Dong, 2004; Grant & Loake, 2007). The development of SAR correlates with a notable rise in peroxidase activity, increased lignin deposition and the expression of PATHOGENESIS-RELATED (PR) genes (Ryals et al., 1996).

One of the most rapid biochemical changes engaged following pathogen recognition is the so-called oxidative burst

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producing reactive oxygen intermediates (ROIs), primarily superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), at the site of attempted invasion (Lamb & Dixon, 1997; Bolwell, 1999; Grant & Loake, 2000). These may serve both as antimicrobial agents and signalling molecules. Nitric oxide (NO), (Delledonne et al., 1998) and related S-nitrosothiols (SNOs) (Feechan et al., 2005; Hong et al., 2007; Wang et al., 2009) also function as important signals in plant disease resistance against pathogens and may interact with ROIs to mediate the HR (Delledonne et al., 2001). Salicylic acid (SA) accumulates in plant tissue responding to pathogen infection and is essential for the induction of SAR as well as being required for some R gene-mediated responses (Delaney et al., 1994). Currently, the accumulating data supports a role in which SA acts at multiple nodes in the defence signalling network, possibly by functioning as a signal amplifier (Fauth et al., 1996; Mur et al., 1996; Shirasu et al., 1997). Accumulating evidence suggests that the balance and cooperation between NO, ROI and SA produced early in the plant resistance response is required for the full expression of the HR (Kumar & Klessig, 2000; Delledonne et al., 2001).

A number of Arabidopsis mutants have been described that exhibit constitutive activation of PR gene expression and resistance to biotrophic pathogens, such as cim, cpr, cir1, cep and dnd1 mutants (Bowling et al., 1994; Clarke et al., 1998; Yu et al., 1998; Silva et al., 1999; Maleck et al., 2002; Murray et al., 2002). In other mutants, including cpr5, adr1, hrl1, lsd and acd (Dietrich et al., 1994; Greenberg, 1994; Bowling et al., 1997; Rate et al., 1999; Devadas et al., 2002; Grant et al., 2003) these traits are also associated with the spontaneous development of HR-like cell death lesions. Another related mutant, lsd1, exhibits normal HR after infection by various incompatible pathogens, but runaway cell death (RCD) is initiated subsequently at the margins of these sites (Dietrich et al., 1994).

Activation tagging has successfully been employed to identify novel defence signalling components in Arabidopsis (Grant et al., 2003; Tani et al., 2004; Nurmberg et al., 2006). We have carried out an activation tagging approach in a designer PR1::LUCIFERASE (LUC) genetic background (Murray et al., 2002; Grant et al., 2003) to further uncover genes whose enhanced expression leads to the establishment of broad-spectrum disease resistance against virulent pathogens. In this report, we describe the characterization of adr2, which exhibits a spreading cell death phenotype and broad-spectrum resistance against biotrophic pathogens. Furthermore, our findings show that the adr2 phenotype results from over-expression of a TIR NBS LRR gene.

Materials and Methods

Plant growth

Arabidopsis thaliana (L.) Heyn. plants were placed in a phytochamber under short daylength conditions (8 h light, 16 h dark at 20°C) at a light intensity of 150 µmol m$^{-2}$ s$^{-1}$. Plants destined for Agrobacterium transformation, were placed under long daylength conditions (16 h light, 8 h dark) at a high light intensity of 15 000 lux, 90 W m$^{-2}$ or low intensity 3000 lux, 14 W m$^{-2}$. All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Experiments were routinely undertaken with 23-d-old plants during the lesion initiation stage.

Activation tagging and LUC imaging

T-DNA activation tagging and generation of transgenic Arabidopsis plants were essentially carried out as described by Grant et al. (2003) with transformation via the floral dip procedure (Clough & Bent, 1998). The LUC imaging was performed as described (Grant et al., 2000).

In vitro LUC activity

Measurement of in vitro LUC activity was carried out using LUC Assay System kit (Promega) according to the manufacturer’s instructions. A microplate luminometer EG&G Micro-Lumat LB96P (Berthold, London, UK) was used and LUC activity expressed as relative light units (RLU) over a 0.5 s time-period. For specific LUC activity, the protein concentration of each sample was determined by Bradford Micro-Assay (Bio-Rad), using BSA as protein standard (Bradford, 1976). Specific LUC activity was calculated as RLU µg$^{-1}$ total protein.

Histochemistry and northern blot analysis

Trypan blue staining of leaves, for the examination of microlesions or fungal hyphae and spores, was carried out according to Bowling et al. (1997). In situ H$_2$O$_2$ detection, in epidermal leaf tissue, was performed using 3,3’-diaminobenzidine (DAB; Sigma) according to the protocol of Thordal-Christensen et al. (1997). Leaf autofluorescence was examined under UV illumination using UV epifluorescence microscopy (excitation filter 365 nm, dichroic mirror, 395 nm and barrier filter 420 nm; Dietrich et al., 1994). In all cases, treated leaves were mounted on glass slides in 70% glycerol, subsequently examined using Leica Wild M3C microscope, and photographed. Northern analysis was undertaken as described by Chini & Loake (2005).

Biochemical analysis

A pyrogallol-based method was conducted to measure peroxidase (POD) activity (Kwak et al., 1995). Catalase (CAT) activity was assayed according to the method of Abei (1984). In-gel kinase assay was essentially conducted according to Romeis et al. (1999). Leaf SA measurements were performed in 5-wk-old plants using a microscale high-pressure liquid chromatography (HPLC)-based procedure as described by Aboul-Soud et al. (2004). Total chlorophyll content was determined in 5-wk-old plants according to Lichtenthaler (1987).
Pathogen inoculations

The bacterial pathogen Pst DC3000 was grown and maintained as described by Whalen et al. (1991). Fungal and oomycete infection assays were performed according to Grant et al. (2003).

Mapping and cloning of ADR2

The homozygous adr2 mutant, a Col-0 background carrying the PR1::LUC transgene, was crossed to Ler. Next, 50 F2 plants showing characteristic adr2 HR-like lesions were used to obtain a rough map position for the wild-type ADR2 gene by bulked segregant analysis (Lukowitz et al., 2000). This was carried out using standard simple sequence-length polymorphism (SSLP) (Bell & Ecker, 1994) markers as reported on the Arabidopsis Information Resource database website (http://www.arabidopsis.org/). The position of the T-DNA activation tag within adr2 plants was determined by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (Singer & Burke, 2003).

Results

Identification of adr2 by activation tagging

A population of c. 9000 activation tagged plants which contained a PR1::LUC transgene were screened for enhanced LUC activity in the absence of pathogen challenge using an ultra low light imaging (Murray et al., 2002; Grant et al., 2003). The activated disease resistance 2 (adr2) mutant line had sustained high levels of PR1::LUC expression throughout development. The LUC activity was particularly strong in cotyledons and all true leaves (Fig. 1a). Furthermore, in vitro enzymatic assays confirmed that adr2 plants constitutively expressed significant LUC activity (Fig. 1b).

Phenotypic and biochemical characterisation of adr2 plants

The adr2 line is significantly smaller compared with wild-type Col-0 plants, displaying reduced stature and the development of macroscopic HR-like lesions (Fig. 2a). Growth of this mutant under sterile conditions confirmed that the appearance of these lesions is both stress- and pathogen-independent. Lesion development was more pronounced on homozygous compared with heterozygous adr2 plants (Fig. 2b). The lesion distribution pattern was found to be uniform, emerging first at the tip of the leaf and progressing gradually towards the leaf origin, ultimately covering the total leaf surface, resulting in a mosaic-like appearance. At c. 6 wk postgermination these lesions had coalesced consuming the whole of older leaves (Fig. 2c).

Lesion development in both adr2 adr2 and adr2 ADR2 plants was not dictated by daylength. Microscopic analysis, performed on wild-type and adr2 adr2 leaves by Trypan blue staining, revealed an absence of HR-like lesions in wild-type plants while in adr2 adr2 plants macroscopic HR-like lesions were predominantly formed of clusters of dead cells that were spread over the leaf surface (Fig. 2d,e). As ROIs are thought to help cue lesion development (Grant et al., 2000; Delledonne et al., 2001), we also examined ROI accumulation by the DAB peroxidase-based method (Thorold-Christensen et al., 1997). While DAB staining was largely absent in wild-type Col-0 plants (Fig. 2f), adr2 adr2 leaves accumulated lesion-localized H2O2 (Fig. 2g).

Although UV fluorescence was largely absent from wild-type leaves that had first been cleared of their chlorophyll (Fig. 2h), it could be detected following challenge with Pseudomonas syringae pv. tomato (Pst DC3000) expressing the avrB avirulence gene (Bisgrove et al., 1994; Grant et al., 1995) (Fig. 2i). The accumulation of autofluorescent material in adr2 adr2 plants appeared significantly stronger than that which had accrued in an HR-lesion triggered following recognition PstDC3000(avrB) and was mainly confined to HR-like lesion sites (Fig. 2j). Hence, the accumulation of phenolic compounds in adr2 adr2 leaves appeared to be correlated with the exhibition of cell death.

Increases in the local concentrations of salicylic acid (SA) underpin the establishment of basal disease resistance against

Fig. 1 In planta luciferase (LUC) imaging and in vitro LUC activity in adr2 leaves. (a) A pseudo-coloured image of a homozygous adr2 plant exhibiting bioluminescent leaves, indicative of high LUC activity. Each of the three leaves was treated with luciferin before imaging. The colour index to the right of the image denotes light intensity, where light colours are high and dark colours are low. (b) Graph depicting in vitro LUC activity in adr2 leaves compared to PR-1::LUC control. Each data point represents the mean of LUC activity measured in three independent experiments. Error bars represent the standard error of the mean, n = 3.
microbial pathogens (Delaney et al., 1994). We therefore investigated whether SA and its glucoside conjugate (SAG) accumulated in adr2 adr2 plants and an adr2 adr2 nahG double mutant. The nahG transgene encodes a salicylate hydroxylase which depletes cellular SA levels (Delaney et al., 1994). Leaves of adr2 adr2 plants were found to accumulate c. 8 times and 15 times more SA and SAG, respectively, than wild-type Col-0 (Fig. 3a,b). This result is consistent with reports of other mutants, which show spontaneous accumulation of SA and constitutive expression of PR genes (Durrant & Dong, 2004; Grant & Loake, 2007). Moreover, 95% of SA in adr2 adr2 plants was present as SAG and accumulation of both SA and SAG was significantly reduced in adr2 adr2 nahG plants compared with the adr2 adr2 line (Fig. 3a,b).

Quantification of soluble POD activity in crude protein extracts of adr2 adr2 compared with wild-type Col-0 plants revealed a significant difference. Interestingly, adr2 adr2 leaves exhibited c. 14 times more soluble POD activity than wild-type (Fig. 3c). Moreover, the activity of ionically bound POD was c. 10 times higher in adr2 adr2 plants compared with that of wild type (Fig. 3c). Catalase was another antioxidant enzyme whose activity was examined in adr2 adr2 plants. Surprisingly, there was no significant difference of either in vitro or in-gel CAT activity between adr2 adr2 plant extracts compared with wild-type (data not shown).

Mitogen-activated protein kinase (MAPK) activity has been shown to be elevated in response to ROI accumulation following the oxidative burst triggered by attempted pathogen infection (Grant et al., 2000). We therefore examined whether the accumulated H2O2 present in the adr2 adr2 mutant could cue a similar profile of MAPK activity. In this context, adr2 adr2 protein extracts were found to contain elevated MAPK activity, as determined by in-gel kinase assays (Fig. 3d). Two conspicuous myelin-basic protein (MBP)-phosphorylating activities of 46 kDa and 48 kDa were detected in extracts from adr2 adr2 plants. By contrast, there was little MAPK-related activity detected in wild-type plants. Moreover, the profile of adr2 adr2 MAPK activity could be mimicked by infiltrating wild-type plants.
leaves with an H$_2$O$_2$-generating solution containing glucose/glucose oxidase (G/GO) within 15 min post infiltration (Fig. 3d). Furthermore, the MAPK activity induced by H$_2$O$_2$ could be blocked by co-infiltrating PD98059, a well-known mammalian MAPK inhibitor (Fig. 3d) (Grant et al., 2000) confirming the source of the MBP-phosphorylating activity.

Homozygous $adr2$ plants grown under low light intensity (LLI) did not go on to develop macroscopic HR-like lesions (Fig. 4a). By contrast, homozygous $adr2$ plants grown under either continuous high light intensity (HLI) or HLI for 14 d and then subsequently shifted to low light intensity (LLI) subsequently developed prominent yellow lesions that coalesced to cover most of the leaf surface (Fig. 4b). We next examined the effect of changes in light intensity on chlorophyll content in $adr2$ plants compared with wild type in response to either LLI (open bars) or HLI for first 14 d (closed bars) and then transferred to LLI. Chlorophyll content is represented as total chlorophyll per µg fresh leaf tissue. The experiment was repeated twice with similar results ($n = 3$). Error bars represent the standard error of the mean.
adr2 conveys constitutive expression of defence and antioxidant-related genes

Leaves of homozygous adr2 plants were found to accumulate PR1 transcripts, as revealed by northern blot analysis (Fig. 5), thus confirming previous LUC activity data (Fig. 1b). Furthermore, other SA-dependent genes including PR-2, PR-5, GER3 and PAL1 (Davis et al., 1991; Ryals et al., 1996; Berna & Bernier, 1999) were also activated, however, PR-5 was induced to a greater extent in homozygous adr2 plants compared with wild-type challenged with PstDC3000(avrB) (Fig. 5). The ROI responsive gene GST1 was also induced (Grant et al., 2000). By contrast, the jasmonic acid (JA)/ethylene (ET)-regulated gene PDF1.2 (Penninckx et al., 1996) was not strongly expressed in the homozygous adr2 line compared with wild-type plants challenged with PstDC3000(avrB) (Fig. 5). Another antioxidant gene, CAT3 (McClung, 1997), was also expressed in homozygous adr2 plants.

adr2 plants exhibit broad-spectrum resistance against biotrophic but not necrotrophic pathogens

As homozygous adr2 plants accumulate the key defence signalling molecule SA and display constitutive expression of numerous defence genes, we examined whether this line displayed resistance against usually virulent microbial pathogens. Therefore, we challenged these plants with one hemi-biotrophic and two biotrophic pathogens: the bacterium PstDC3000 (Whalen et al., 1991), the oomycete pathogen Hyaloperonospora arabidopsis Noco2, formerly H. arabidopsis (Parker et al., 1996) and the fungal pathogen Golovinomyces cichoracearum UED1 (Yun et al., 2003), respectively. We also challenged adr2 adr2 plants with the necrotrophic fungal pathogen, Botrytis cinerea (Nurnberg et al., 2006).

The homozygous adr2 plants exhibited conspicuous resistance against the virulent pathogen PstDC3000 (Fig. 6a). Bacterial growth in this line was approx. 0.5 log less than that present in wild-type Col-0 plants 3-d postinoculation (dpi) (Fig. 6a). Moreover, the growth of H. arabidopsis Noco2 was also significantly suppressed in adr2 adr2 leaves compared with wild-type at 10 dpi (Fig. 6b). Interestingly, the level of resistance exhibited by homozygous adr2 leaves mirrored that of wild-type leaves treated with SA (Fig. 6b). As expected, NahG plants exhibited increased susceptibility to this pathogen (Delauney et al., 1994). Furthermore, adr2 adr2 leaves also displayed increased basal resistance against G. cichoracearum UED1 (Fig. 6c) and this observation was confirmed by Trypan blue staining (Fig. 6c). By contrast, the response of homozygous adr2 plants to Botrytis cinerea was indistinguishable from wild-type (data not shown).

Characterization of defence responses in adr2 double mutants

To further investigate the individual role of known defence signalling pathways to the establishment of the adr2 phenotype, we also crossed adr2 adr2 plants with the coi1 mutant, which is insensitive to JA (Feys et al., 1994), the ET insensitive mutant etr1-1 (Blecker et al., 1988), the SA-insensitive mutant npr1 (Cao et al., 1994), the enhanced disease susceptibility mutant, eds1 (Parker et al., 1996), and ndr1, which compromises signalling by CC NBS LRR genes (Century et al., 1995; Aarts et al., 1998). The expression of the SA-dependent PR1 and JA-dependent PDF1.2 genes were subsequently studied by northern blot analysis in the resulting panel of adr2 adr2 double mutants (Fig. 7a).

The level of PR1 gene expression in adr2 adr2 plants was similar to that detected in SA-treated wild-type plants (Fig. 7a). Furthermore, significant PR1 transcript accumulation in adr2 adr2 plants was also observed in the presence of ndr1, etr1 or coi1. However, PR1 expression was reduced in adr2 adr2 eds1 plants and absent in adr2 adr2 nahG or adr2 adr2 npr1 plants. There was a small but significant accumulation of
PDF1.2 transcripts in adr2 adr2 plants and this was strikingly increased in the adr2 adr2 nahG line. By contrast, PDF1.2 expression was abolished in adr2 adr2 plants in the presence of coi1, ndr1 and etr1.

We also monitored lesion development in this set of double mutants. The depletion of SA by nahG or the presence of npr1 or eds1 in adr2 plants abolished lesion formation (data not shown). By contrast, ndr1, coi1 or etr1 failed to affect lesion formation in the adr2 line. This panel of double mutants was also challenged with H. arabidopsis. As expected, adr2 adr2 plants exhibited significant resistance against this pathogen (Fig. 7b). Interestingly, ndr1 did not affect resistance against H. arabidopsis in adr2 adr2 plants. By contrast, the presence of npr1 diminished adr2-mediated resistance to a level similar to that expressed by wild-type plants. However, the presence of either nahG or eds1 supported strikingly increased growth of H. arabidopsis in the adr2 adr2 line. Resistance against this pathogen in adr2 adr2 plants was not compromised by coi1 or etr1 (data not shown).

Mapping and cloning of ADR2

In order to determine the mode of inheritance of the adr2 mutation, homozygous adr2 plants were backcrossed to the
The dominant *adr2*-associated traits cosegregated with insensitivity to the ammonium gluconate herbicide encoded within the T-DNA. Sequences flanking this T-DNA insert were recovered by TAIL-PCR (Singer & Burke, 2003). By interrogating the TAIR database (http://www.arabidopsis.org), the T-DNA insert was found to reside on chromosome 1 between two *TIR NBS LRR* genes (*At1g56520* and *At1g56510*), with a third *TIR NBS LRR* (*At1g56540*) approx. 6 kb away from the T-DNA insert beyond *At1g56520* (Fig. 8a). Expression analysis of genes around the T-DNA tag by reverse-transcriptase PCR revealed that only the three *TIR NBS LRR* genes were ectopically overexpressed in *adr2* plants (Fig. 8b). To identify *ADR2* we employed a transgenic reconstitution approach. Thus, full-length cDNAs were generated for each of these genes and their integrity confirmed by DNA sequencing. The resulting cDNA sequences were subsequently cloned behind the CaMV35S promoter and the constructs generated were transformed individually into wild-type Col-0 plants using floral dip transformation (Clough & Bent, 1998). Overexpression of *At1g56510* but not either *At1g56520* or *At1g56540* reconstituted the *adr2* phenotype in T1 and T2 plants. To confirm that basal disease resistance was increased, plants were challenged with virulent *Pst DC3000* and scored for pathogen titre over time. Overexpression of *At1g56510* conveyed cell death development and robust resistance against attempted *Pst DC3000* infection in a similar fashion to *adr2* (Fig. 9a). Furthermore, increased expression of this gene also resulted in enhanced protection against *G. cichoracearum UED1* (Fig. 9b). Collectively, these findings imply that *At1g56510* is *ADR2*.

**Discussion**

We have employed activation tagging in an *Arabidopsis* line containing a *PR1::LUC* transgene to uncover genes whose enhanced expression leads to the establishment of broad-spectrum disease resistance. One mutant, designated *adr2*, has been described in this report. The *adr2* mutant conveyed significant constitutive expression of both LUC activity and the endogenous *PR1* gene. *adr2* is a dominant, gain-of-function, monogenic mutation that conveys spreading HR-like lesions, accumulation of *H₂O₂* and auto-fluorescent phenolic compounds. Significantly, *adr2* plants also exhibit broad-spectrum disease resistance.

Several *Arabidopsis* mutant classes displaying constitutive lesion formation have previously been described including *lsd*, *acd*, *cpr5* and *cpr6* mutants (Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995; Bowling et al., 1997; Clarke et al., 1998). Of the previously described lesions mimics, only *acd6*, *lsd2*, *lsd4*, *lsd6* and *lsd7* are dominant. Notably, *acd6* leaves display a few punctate cell death patches as opposed to the uniformly-distributed mosaic-like lesions that are associated with the *adr2* phenotype. Unlike *adr2*, the spontaneous lesion formation phenotypes associated with the *lsd2* and *lsd4* mutations have previously been shown to be SA-independent.

![Fig. 7 Impact of nahG, npr1, ndr1, eds1, etr1 and coi1 on adr2-mediated PR1 and PDF1.2 gene expression and resistance against *Hyaloperonospora arabidopsis*. (a) RNA was isolated from the indicated genotypes and subjected to northern blot analysis with PR1 and PDF1.2 probes. The RNA in each lane was also stained with methylene blue as a control for equal loading and RNA transfer. (b) Infection of the given *adr2* double mutants with *H. arabidopsis*. Infection was carried out by spraying a *10⁵ conidia* suspension of *H. arabidopsis* and assaying for pathogen growth at 7 dpi.](https://www.newphytologist.org/issue/9/3/article/1170 abrir)
Moreover, when compared with adr2, both lsd2 and lsd4 display distinct HR-like lesion phenotypes and distribution. Therefore, despite the fact that no map position has previously been assigned to these mutations, adr2 is unlikely to be allelic to either lsd2 or lsd4. By contrast, unlike lsd2 and lsd4, lesion formation on lsd6 and lsd7 leaves has previously been reported to be SA-dependent (Weymann et al., 1995). The lsd7 phenotype is associated with very small lesions that became evident when visualized microscopically by Trypan blue staining. Moreover, lsd7 has been shown not to be linked to chromosome I (Weymann et al., 1995). Hence, we conclude that adr2 is also not allelic to lsd7. The lsd6 phenotype is characterized by spontaneous formation of punctate necrotic lesions that are not affected by daylength. Interestingly, the spontaneous lesion formation on lsd6 leaves was blocked under high humidity. The lsd6 mutation has been mapped to the lower arm of chromosome I, within an c. 25 cM region, between the SSLP markers nga111 and nga128 (Weymann et al., 1995). However, adr2 was not found to reside in this region. Thus, adr2 is also not allelic to lsd6. Together, our data imply that mutations in ADR2 leading to a spreading lesion phenotype have not been described previously.

The adr2 line was found to exhibit broad-spectrum resistance against two biotrophic microbial pathogens, *H. arabidopsis* Noco2 and *G. cichoracearum* UED1 and a hemibiotrophic pathogen, *Pst* DC3000. Depletion of SA blunted PR1 expression and abolished resistance against *H. arabidopsis* Noco2, suggesting that SA accumulation is essential for adr2-mediated resistance against these pathogens. Moreover, *npr1* or *eds1* also abolished PR1 gene expression in adr2 plants. Functional EDS1 but not NPR1 was also required for *adr2*-mediated resistance against *H. arabidopsis* Noco2, as resistance was dramatically reduced in the respective double mutants. NPR1 was also found to be dispensable for *H. arabidopsis* Noco2 resistance in the *cpr1*, *cpr5* and *cpr6* mutants (Bowling et al., 1994, 1997; Clarke et al., 1998).

Interestingly, there was a small but significant accumulation of *PDF1.2* transcripts in *adr2 adr2* plants, suggesting that *adr2* also weakly activates expression of this JA/ET-dependent

![Fig. 8](image-url) Position of the activation tag in *adr2* plants. (a) Schematic showing the location of the T-DNA insertion, containing four copies of the 35S enhancer sequence, within the *adr2* line. Adjacent genes along with their corresponding identification numbers are shown. (b) Reverse transcriptase polymerase chain reaction analysis of the indicated genes in the given plant genotypes.

![Fig. 9](image-url) CaMV35S::*At1g56510* plants exhibit increased disease resistance. (a) Plants containing a CaMV35S::*At1g56510* transgene show heightened resistance against virulent *Pst* DC3000. Closed, hatched, open and dotted bars indicate bacterial titre at 0, 2, 3 and 4 d, respectively, postinoculation in the indicated genotypes. (b) The given plant lines were challenged with *Golovinomyces cichoracearum* UED1 and scored for disease resistance. The experiments were repeated twice with similar results. Error bars represent the standard error of the mean.
marker gene. This was unexpected because SA accumulation is thought to routinely suppress JA signalling (Petersen et al., 2000; Glazebrook, 2005). In the presence of either coi1 or ett1 this adr2-mediated expression of PDF1.2 was abolished, suggesting that adr2 activates both weak ET- and JA-dependent signalling. Similar observations were made for the adr1 mutant line which also expressed PDF1.2 in addition to PR1 (Grant et al., 2003). Unexpectedly, this low level of PDF1.2 expression is also lost in adr2 plants in the absence of NPR1 function. NPR1 has previously been proposed to suppress JA signalling (Spoel et al., 2003), at least in lines without adr2. Strikingly, in adr2 adr2 nprG and adr2 adr2 eds1 plants the accumulation of PDF1.2 transcripts is markedly increased relative to the adr2 adr2 line. This further supports the idea that SA signalling is antagonistic to JA signalling. Despite the relatively low level of constitutive PDF1.2 expression in adr2 adr2 plants do not exhibit increased resistance against the necrotrophic pathogen B. cinerea. Engagement of the defence response following attempted infection by this class of pathogen is thought to be dependent upon expression of JA- and ET-dependent defence genes (Thomma et al., 1998). However, the magnitude of JA signalling marked by PDF1.2 expression in adr2 adr2 plants may be insufficient to convey protection against B. cinerea. Furthermore, adr2 triggered COI1- and ETR1-dependent PDF1.2 gene expression was unnecessary for resistance against H. arabidopsis NoCo2 because the growth of this pathogen on adr2 adr2 plants was not affected by either coi1 or ett1.

The adr2 line accumulates H₂O₂ and exhibits a marked increase in MAPK activity. This is a common plant response to a variety of different biotic and abiotic stresses (Inze & van Montagu, 1995). In many eukaryotes, the transduction of oxidative signals is controlled by protein phosphorylation involving MAPKs. These MAPKs and their immediate upstream activators, MAPKKs and MAPKKKs, constitute a functionally interlinked MAPK cascade (Jonak et al., 1994). However, our current knowledge as to the precise role(s) of MAPK signalling in disease resistance remains rather limited. Elevation of MAPK activity has been detected in plants after exposure to various stimuli including AVR proteins (Romeis et al., 1999), H₂O₂ (Grant et al., 2000), flg22, a peptide derived from bacterial flagellin (Asai et al., 2002), and SA (Kumar & Klessig, 2000). Our findings indicate that adr2 constitutively activates two MAPK activities of 46 kDa and 48 kDa. We speculate that this constitutive MAPK activity detected in adr2 plants is possibly initially cued by the high H₂O₂ concentrations in this line, which subsequently leads to the synthesis and accrual of SA. This key plant immune activator may then govern a positive feedback loop that promotes further H₂O₂ production during the defence response. In this context, SA has been shown to potentiate the oxidative burst during the development of disease resistance (Shirasu et al., 1997). Thus, MAPK signalling in adr2 plants may be engaged by ROIs and subsequently amplified by SA.

The insertion of the T-DNA activation tag in adr2 plants was found to reside between two TIR NBS LRR genes (At1g56520 and At1g56510), with a third TIR NBS LRR (At1g56540) approx. 6 kb away from the T-DNA insert beyond At1g56520. A RT-PCR analysis revealed that all these TIR NBS LRR genes were ectopically overexpressed in adr2 plants, suggesting the 35S enhancers present within the inserted T-DNA increased the basal expression levels of these genes. Thus, implying that the adr2 phenotype is the result of overexpression of At1g56520, At1g56510 or At1g56540, or a combination of these three TIR NBS LRR genes. Furthermore, this idea is also supported by the double mutant analysis because while adr2 adr2 npr1 plants exhibited broad-spectrum disease resistance the adr2 adr2 eds1 line did not. NDR1 is known to be essential for defence signalling following pathogen recognition by CC NBS LRR proteins but is dispensable for defence signalling activated by TIR NBS LRR gene products. By contrast, EDS1 is essential for defence signalling following pathogen recognition by TIR NBS LRR proteins but is dispensable for CC NBS LRR-mediated disease resistance (Aarts et al., 1998). Therefore, the loss of broad-spectrum disease resistance in adr2 adr2 eds1 but not adr2 adr2 npr1 plants is consistent with the notion that overexpression of a TIR NBS LRR gene is responsible for this phenotype. Our findings from transgenic reconstitution experiments identified At1g56510 as ADR2.

A prior example of TIR NBS LRR overexpression resulting in defence activation has been reported previously (Stokes et al., 2002). Here, the metastable epigenetic variant bal led to overexpression of a single TIR NBS LRR gene (At1g16890) from a cluster of R genes on chromosome 4. This resulted in plants of reduced stature that showed constitutive activation of SA-dependent defence responses (Stokes et al., 2002). However, spreading lesion development was not associated with this line. Also, in an Ethyl Methane Sulphonate (EMS) screen for suppressors of npr1-5, the ssi4 mutation was uncovered which resulted in increased expression of SSI4, a TIR NBS LRR gene (Shirasu et al., 2002). The ssi4 line exhibited lesion development, constitutive activation of SA signalling and broad-spectrum disease resistance. Interestingly, ssi4 was found to be a substitution within the NBS domain of SSI4 and furthermore overexpression of SSI4 in transgenic plants did not recapitulate the ssi4 phenotype. In contrast to bal, this mutation was therefore thought to cause activation of SSI4 resulting in the subsequent engagement of SA-dependent signalling that ultimately led to the increased expression of SSI4, which was shown to be a SA-responsive gene. Therefore, only the ectopic expression of a subset of TIR NBS LRR genes is likely to result in the activation of plant defence responses.

Collectively, our data suggests that overexpression of At1g56510 triggers an EDS1, NPR1 and SA-dependent defence signalling pathway that establishes broad-spectrum disease resistance against biotrophic pathogens in Arabidopsis. This may occur because an increase of At1g56510 might titrate out a guard protein that ordinarily sequesters the defence
signalling function of this TIR-NBS-LRR protein in the absence of pathogen-derived cues. A biological manifestation of this phenomenon is the recent demonstration that intraspecies and possibly interspecies hybrid necrosis may be underpinned by specific epistatic interactions that trigger R-protein signalling (Bomblies et al., 2007). This is also thought to result from a breakdown in the interaction between a given NBS LRR and its guard protein.

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