

Divergent Roles in *Arabidopsis thaliana* Development and Defense of Two Homologous Genes, **ABERRANT GROWTH AND DEATH2** and **AGD2-LIKE DEFENSE RESPONSE PROTEIN1**, Encoding Novel Aminotransferases

Jong Tae Song, Hua Lu, and Jean T. Greenberg¹

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637

The disease-resistant *Arabidopsis thaliana* *aberrant growth and death2* (*agd2-1*) mutant has elevated levels of the defense signal salicylic acid (SA), altered leaf morphology, and mild dwarfism. *AGD2* and its close homolog *ALD1* (for *AGD2-LIKE DEFENSE RESPONSE PROTEIN1*) encode aminotransferases that act on an overlapping set of amino acids *in vitro*. However, kinetic parameters indicate that *AGD2* and *ALD1* may drive the aminotransferase reaction in opposite directions. *ALD1*-deficient mutants have the opposite phenotypes from *agd2-1*, showing reduced SA production and increased disease susceptibility. Furthermore, *ALD1* transcript levels are elevated in *agd2-1* and are induced in the wild type by bacterial pathogen infection. *ALD1* is responsible for some of the elevated SA content and a majority of the disease resistance and dwarfism of *agd2-1*. A complete knockout of *AGD2* renders embryos inviable. We suggest that *AGD2* synthesizes an important amino acid–derived molecule that promotes development and suppresses defenses, whereas *ALD1* generates a related amino acid–derived molecule important for activating defense signaling.

INTRODUCTION

Extracellular bacterial pathogens such as *Pseudomonas syringae* infect a wide variety of plants (Gardan et al., 1999), causing water soaking, cell death, and chlorophyll loss (chlorosis) (Agrios, 1997). Diseases caused by *P. syringae* require bacterial effector proteins that are delivered via a type III secretion apparatus to plants and promote disease by a largely unknown mechanism (Greenberg and Vinatzer, 2003). In some plant genotypes, effectors encoded by *avirulence* genes can be specifically recognized, and a resistance response is mounted to limit the infection in a so-called gene-for-gene interaction (Greenberg and Vinatzer, 2003). However, it is now widely recognized that even without the gene-for-gene interaction, plants mount an active defense response that partially limits virulent *P. syringae* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1996; Rogers and Ausubel, 1997). This general resistance can vary in effectiveness depending on the host-pathogen combination. It is not clear what triggers general resistance, but it may be the perception of damage by the plant, the recognition of pathogen-associated molecular patterns, such as flagella (Asai et al., 2002), and/or the weak recognition of effectors (Chang et al., 2002).

Many lines of evidence point to the small phenolic compound salicylic acid (SA) in playing a prominent role as a signal molecule

in general resistance. First, SA levels rise during *P. syringae* infection (Rasmussen et al., 1991). Second, exogenous application of SA can induce resistance to *P. syringae* (Ryals et al., 1996). Third, numerous mutants compromised for SA accumulation or constitutively producing SA show enhanced susceptibility or resistance, respectively, to *P. syringae*. In particular, *Arabidopsis thaliana* mutants, such as *eds16/sid2* (for *enhanced disease susceptibility16/salicylic acid induction-deficient2*) and *eds5/sid1*, that have very low levels of SA during pathogen infection, probably because of a defect in SA biosynthesis, are disease susceptible (Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). Plants engineered to produce more SA also are more resistant to viral and fungal infections, but resistance to bacterial pathogens has not been specifically tested (Verberne et al., 2000).

Not surprisingly, several mutants with heightened susceptibility to *P. syringae* show altered but not abolished accumulation of SA upon infection. These include *pad4* (for *phytoalexin deficient4*; Zhou et al., 1998), *eds1* (Feys et al., 2001), *eds3* (Glazebrook et al., 1996, 2003), *eds8* (Glazebrook et al., 1996, 2003), *pad1* (Glazebrook et al., 1997), *pad2* (Glazebrook et al., 1997, 2003), *eds4* (Gupta et al., 2000), and *acd6-T* (for *accelerated cell death6-T*; Lu et al., 2003). These mutants affect SA accumulation to varying extents, with some only compromising the early production of SA. *ndr1* (for *nonrace-specific disease resistance1*) mutants also are compromised for SA production after infection with some avirulent *P. syringae* isolates (Shapiro and Zhang, 2001). *PAD4* encodes a possible lipase and may generate a lipid signal (Jirage et al., 1999). *ACD6* is a probable transmembrane protein with an ankyrin repeat region that may act by making protein–protein contacts (Lu et al., 2003). Finally,

¹ To whom correspondence should be addressed. E-mail jgreenbe@midway.uchicago.edu; fax 773-702-9270.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jean T. Greenberg (jgreenbe@midway.uchicago.edu).

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NDR1 is thought to mediate SA induction through the production of reactive oxygen (Shapiro and Zhang, 2001). However, the basis for the reduction in SA accumulation in most mutants is not well understood. In some cases, genes that regulate SA production may regulate other signals as well, as has been hypothesized for *PAD4* (Glazebrook et al., 2003).

Constitutive disease-resistant mutants also have provided a way to identify potential regulators of SA and other defense signals. One such mutant we previously characterized, called *agd2-1* (for *aberrant growth and death2*), showed elevated SA and resistance to *P. syringae* (Rate and Greenberg, 2001). This mutant also had some spontaneous cell death and enlarged cells, and the overall size of the plants was smaller than that of the wild type. The morphology of *agd2-1* was greatly altered by depleting SA with the *nahG* transgene, whose product converts SA to catechol (Gaffney et al., 1993). *agd2-1-nahG* plants had tumor-like growths and cells with highly endoreduplicated DNA, a phenotype that could be suppressed by application of an SA agonist (Rate and Greenberg, 2001; Vanacker et al., 2001). This led us to suggest that some genes that control defenses also play a role in development (Vanacker et al., 2001). Indeed, we found that the *nonexpressor of PR1-1* mutant blocked for SA signaling also had a developmental defect, showing a decrease in cell number and an increase in the ploidy of mesophyll cells when compared with the wild type (Vanacker et al., 2001). Other examples of mutants with developmental and defense defects also have been reported recently (i.e., see Holt et al., 2002; Jin et al., 2002; Barth and Conklin, 2003).

In addition to defense components sometimes affecting cell growth and/or death, they also interact with other pathways. For example, many abiotic stresses, such as ozone, activate SA synthesis and SA-dependent defenses (Sharma et al., 1996). Amino acid starvation or treatment with the abiotic elicitor α -aminobutyric acid can induce SA-dependent and SA-independent defense markers and the phytoalexin camalexin, possibly through a common pathway (Zhao et al., 1998). However, no clear mechanism for how these stimuli are sensed and transduced has yet emerged. Amino acid imbalances also can alter development. Transgenic plants that accumulate 10-fold more Lys than the wild type have striking developmental defects (Frankard et al., 1992). These transgenic *Nicotiana sylvestris* plants have decreased chlorophyll content, lack apical dominance, and show altered leaf size and morphology. Whether these Lys-overexpressing plants also alter the defense response has not been investigated. In plants, amino acids are precursors to hormones or secondary metabolites and serve to transport nitrogen from source to sink organs. As such, the control of amino acid synthesis in plants affects many aspects of growth and development.

In this report, we describe the cloning and characterization of *AGD2*, which encodes a novel aminotransferase. *A. thaliana* has one homolog of *AGD2*, called *ALD1* (for *AGD2-LIKE DEFENSE RESPONSE PROTEIN1*), sharing 62% identity (77% similarity) with *AGD2*. The recombinant *AGD2* and *ALD1* proteins have overlapping substrate specificity on several amino acids but act in different directions in the aminotransferase reaction in vitro. *AGD2* expression is suppressed by dark treatment and during senescence, whereas *ALD1* is induced by bacterial pathogen

infection and senescence. *ald1* loss-of-function mutants had the opposite phenotypes from *agd2-1*, showing increased *P. syringae* susceptibility and reduced SA accumulation. Additionally, most of the *agd2-1* phenotypes were at least partially attributed to *ALD1* function. Finally, we show that complete loss of *AGD2* function results in inviable embryos, suggesting a developmental role for *AGD2*.

RESULTS

Cloning of *AGD2* and Identification of the Homologous *ALD1* Gene

We fine-mapped *AGD2* to a 65-kb interval encompassed on BAC clone T16L1 between the C5-2 and C7-2N markers. *AGD2* was encoded by the At4g33680 gene according to a number of criteria. First, complementation test results using 15- to 20-kb overlapping T16L1 subclones uniquely identified the 7-kb At4g33680 region to be capable of rescuing *agd2-1*. Second, defined genomic and cDNA clones corresponding only to At4g33680 complemented *agd2-1* (Figure 1A; data not shown). Third, the same single nucleotide change was detected in both cDNA and genomic DNA from the At4g33680 gene in *agd2-1*. The *agd2-1* allele contained a C-to-A point mutation resulting in a Pro-to-Ser change at amino acid 398 (Figure 1B).

AGD2 had 9 introns (Figure 1B) and encoded a 461-amino acid protein. Using the simple modular architecture research tool (<http://smart.embl-heidelberg.de/>), we found that residues 148 to 460 had modest similarity ($E = 1.5e-8$) to the aminotransferase class I and class II protein families (Pfam 9.0) and contained a pyridoxal-5'-phosphate attachment site (Mehta et al., 1989). The PSORT and TargetP algorithms (<http://psort.ims.u-tokyo.ac.jp/> and <http://www.cbs.dtu.dk/services/TargetP/>, respectively) both predicted *AGD2* to be chloroplast localized (Figure 1B).

A. thaliana has one close homolog of *AGD2* on chromosome 2 (At2g13810), which we named *ALD1*. *ALD1* had 62% identity and 77% similarity with *AGD2* at the amino acid level. *ALD1* had 10 exons (Figure 1B) and encoded a 456-amino acid protein. It was predicted to localize to the cytoplasm by the PSORT algorithm and to the chloroplasts by the TargetP algorithm. Because of this ambiguity, we have tentatively indicated that *ALD1* lacks a chloroplast transit signal (Figure 1B).

The *Oryza sativa* (rice) genome had two genes: one called *OsJNBa00664E16.9* and one that we annotated from genomic region AC105731 (tentatively named *OsALD1*), whose products had high similarities to both *AGD2* and *ALD1* (Table 1). We isolated full-length *O. sativa* cDNAs for *OsJNBa00664E16.9* (*cOsAGD2*, GenBank accession number AY338235) and *OsADL1* (*cOsALD1*, GenBank accession number AY338236) and found that they had gene structures similar to *AGD2* and *ALD1*, respectively (Figure 1B). Additionally, the product of *cOsAGD2* was predicted to have a subcellular location similar to *AGD2*, whereas the product of *cOsALD1* was predicted to be cytoplasmic. Because of the high similarities of the predicted *O. sativa* proteins to both *AGD2* and *ALD1* (Table 1), localization assignments for these proteins may change when more data is available.

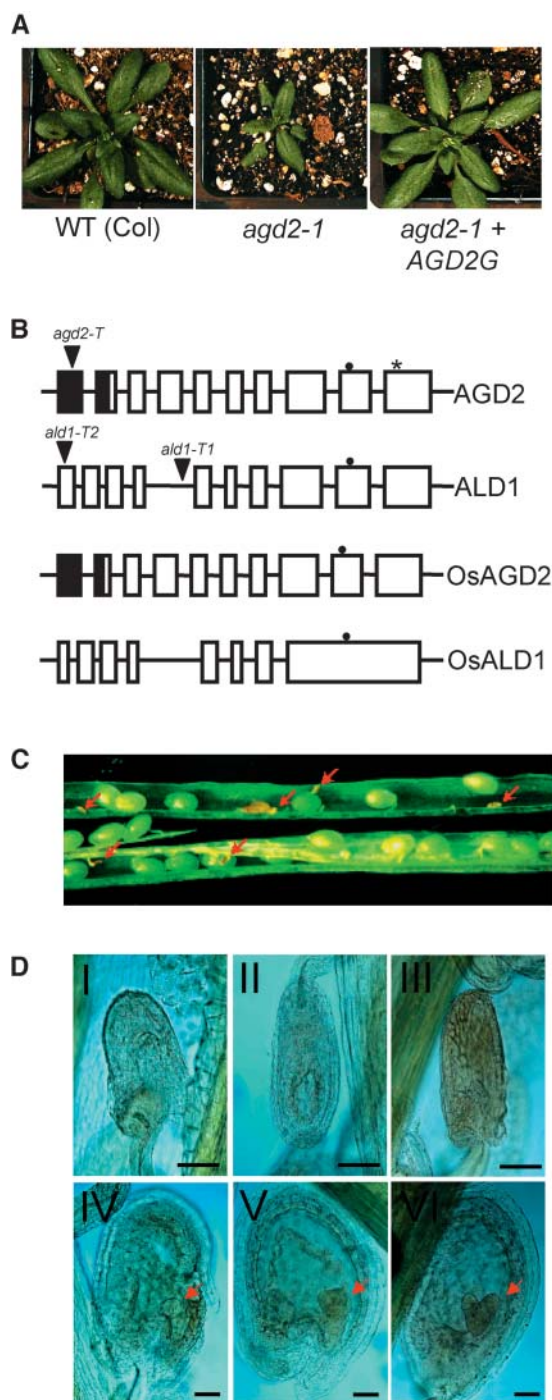


Figure 1. Features of AGD2, ALD1, and *agd2-T*.

(A) Complementation of *agd2-1*. *agd2-1* plants were transformed with the genomic clone of AGD2 (*AGD2G*, at right). The plants shown at the left and in the middle are untransformed wild-type and *agd2-1* control plants. *agd2-1* plants transformed with the genes adjacent to AGD2 were not complemented (data not shown).

(B) Molecular structures of AGD2, ALD1, OsAGD2, and OsALD1. T-DNA insertion sites are indicated by triangles. The asterisk indicates the Pro-to-Ser change caused by the *agd2-1* allele. Closed and open boxes

Loss of AGD2 Causes Embryo Lethality

Analysis of the AGD2^{P398S} mutant recombinant enzyme activity corresponding to the product encoded by *agd2-1* (see below; Figure 6) suggested that the *agd2-1* allele was not null. To ascertain the phenotype of AGD2 null mutant plants, we isolated a heterozygous mutant (*agd2-T/AGD2*) with a kanamycin-resistant (*kan^R*) T-DNA insertion in the first exon of AGD2 (Figure 1B). The progeny of heterozygous *agd2-T/AGD2* plants yielded 20 wild-type, 44 heterozygous, and no homozygous mutant plants, suggesting that AGD2 is an essential gene. Furthermore, of 352 seeds from the heterozygous plant that were germinated on kanamycin plates, 116 kanamycin-sensitive (*kan^S*) and 236 *kan^R* seeds were observed. This is a good fit to the 1:2:0 hypothesis that the homozygous embryos were not viable ($\chi^2 = 0.012$, $P > 0.9$). In accordance with the lack of homozygous seeds, the seed yield per silique in the wild type (54.5 ± 0.9 SD, $n = 4$) and heterozygous *agd2-T* (40.3 ± 2.2 SD, $n = 4$) was significantly different ($P = 0.0009$). The seeds in wild-type plants all looked normal and were of uniform size. By contrast, some seeds of the heterozygous plants were very tiny and dry and looked aborted (Figure 1C). This phenotype was detected in all of the >100 heterozygous plants assayed.

To determine when in development AGD2 might act, we examined at which stage the homozygous embryos died. Before the globular stage, all of the developing embryos in *agd2-T/AGD2* heterozygotes were normal. However, when most of the embryos reached the globular stage in *agd2-T/AGD2* heterozygotes, some seeds already had died or were severely malformed (Figure 1D). The aborted seed size was much smaller than that of normal seeds, and the embryo proper could no longer be seen. It seems likely that after fertilization, homozygous embryos had normal development at the beginning and that later the embryos died. To investigate the ratio of lethal-to-healthy embryos at the heart stage, we examined the contents of individual silique chambers. The heterozygous plant had 20.8 ± 0.66 SD ($n = 5$) normal and 7.0 ± 0.32 SD ($n = 5$) dead seeds per silique chamber, whereas the control wild-type plants had 27.8 ± 0.37 SD ($n = 5$) apparently living seeds. These ratios of live-to-dead seeds were significantly different in the heterozygous mutant and the wild type ($P < 0.0001$). Furthermore, the embryo lethality of the *agd2-T* mutant was complemented by the AGD2 genomic clone. This was evidenced by the fact that numerous viable T2 plants (12) homozygous for *agd2-T* only were recovered when the AGD2 transgene was present. In reciprocal crosses, we found no indication that *agd2-T* haploid pollen or ovules had any transmission defects (data not shown).

represent exons, and closed boxes represent possible chloroplast transit peptides. The closed circles indicate the pyridoxal-5'-phosphate attachment sites.

(C) Immature seeds inside a heterozygous *agd2-T/AGD2* silique. Aborted dead seeds are indicated by red arrows.

(D) Microscopy analysis of aborted and normal seeds in *agd2-T/AGD2* heterozygotes. Embryos are indicated by red arrows. Top panel (I to III), dying and dead seeds; bottom panel (IV to VI), normal seeds. IV, Globular stage; V, triangular stage; VI, heart stage. Bars = 80 μ m.

Table 1. Percentage Identities and Similarities (in Parentheses) among AGD2, ALD1, OsAGD2, and OsALD1 Proteins

	AGD2	ALD1	OsAGD2
ALD1	62 (77)		
OsAGD2	76 (95)	58 (86)	
OsALD1	61 (82)	58 (86)	62 (85)

Therefore, *agd2-T* affected the embryo and resulting diploid seeds but not haploid pollen or ovules. These results also were consistent with the 1:2 segregation of $\text{kan}^{\text{S}}:\text{kan}^{\text{R}}$ of seeds from *agd2-T/AGD2* heterozygotes.

AGD2 Localizes to Chloroplasts

To investigate where in the cell AGD2 might function, we examined the subcellular location of the full-length protein fused to the green fluorescent protein (GFP). We drove expression of the *AGD2* cDNA fused to *gfp* from the 35S promoter of *Cauliflower mosaic virus* (CaMV). This construct complemented the *agd2-1* mutant (data not shown). The GFP fluorescence patterns in protoplasts isolated from *A. thaliana* expressing AGD2-GFP colocalized with the red autofluorescence patterns of the chloroplasts (Figures 2A and 2B). When GFP lacking the AGD2 moiety was expressed, the GFP fluorescence was not specifically colocalized with chloroplast autofluorescence but was distributed throughout the cells (Figures 2C and 2D). The chloroplast localization of AGD2-GFP is consistent with its possible role in amino acid synthesis because many amino acids are known to be synthesized in chloroplasts (Bryan, 1990).

AGD2 and ALD1 Have Divergent Expression Patterns

agd2-1 hypomorphic plants are resistant to *P. syringae* (Rate and Greenberg, 2001) and showed elevated *ALD1* expression (Figure 3A). This suggested that *AGD2* and/or *ALD1* mRNA levels might be modulated during pathogenesis. To test this, we used gene-specific probes to examine their transcript levels after infection of wild-type plants with *P. syringae*. In response to *P. syringae* pv *maculicola* strain DG3 infection, *AGD2* mRNA levels were not significantly changed, whereas *ALD1* was induced in a pattern similar to that of the known defense-related gene *Pathogenesis Related1* (*PR1*; Figure 3B). Mock inoculation did not alter *ALD1* or *AGD2* expression (data not shown). Because some pathogen-induced genes also are senescence regulated (Morris et al., 2000), we followed the expression levels of *AGD2* and *ALD1* in leaf 4 throughout its life span. Visible senescence started on day 21. *AGD2* expression was initially high and decreased with time, whereas *ALD1* was induced after day 24 (Figure 3C). Thus, the expression of both genes was modulated during senescence.

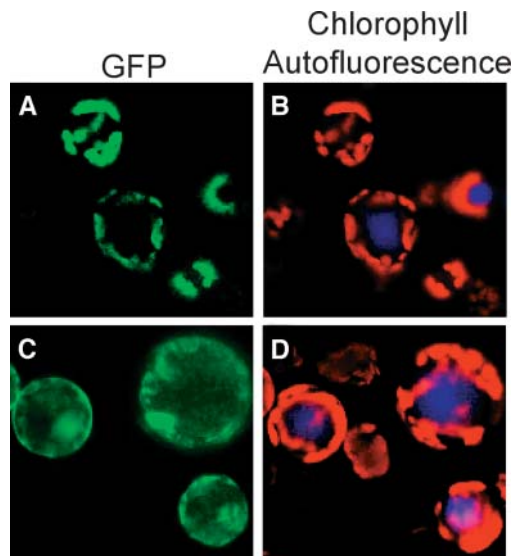
We also examined the expression levels of *AGD2* and *ALD1* in various tissues (Figure 3D). *AGD2* was strongly expressed in all of the tissues, including seedling, root, stem, flower, and leaves. However, *AGD2* expression was relatively lower in siliques, whereas *ALD1* was very high in siliques but relatively low in other tissues. The expression of *ALD1* is reminiscent of that seen for

PR genes that show expression in flowers, fruit, and ripening fruit (Lotan et al., 1989; Salzman et al., 1998). Such expression may confer resistance to pathogens during reproduction.

Amino acid-synthesizing enzymes as well as amino acids (the possible substrates and products of aminotransferases) can vary in abundance depending on whether light is present (Buchanan et al., 2000). Additionally, the expression of some defense genes, such as *ACD6*, is light dependent (Lu et al., 2003). To test the effect of light on *AGD2* and *ALD1* expression, plants maintained in a 16-h-light/8-h-dark cycle were moved to constant dark after 4 h of the normal morning light. These are conditions under which *ACD6* transcript levels are modulated (Lu et al., 2003). In 16-h-light/8-h-dark conditions, *AGD2* expression was unchanged throughout the course of a day (Figure 3E). However, after dark treatment, *AGD2* transcript levels were significantly reduced at 24 and 48 h. When the dark-treated plants were re-exposed to light for 4 h, the *AGD2* transcript level was restored back to the level seen in the control plants maintained in the normal light/dark cycle conditions (Figure 3E). *ALD1* levels were not significantly altered under these conditions. Thus, the expression patterns of *AGD2* and *ALD1* are spatially, developmentally, and environmentally regulated, with different and sometimes complementary patterns.

ALD1 Is Important for Resistance to the Bacterial Pathogen *P. syringae*

The pathogen-induced expression of *ALD1* suggested it could be important for disease resistance. To test this, we isolated a mutant, *ald1-T1*, with a T-DNA insertion in the fourth intron of

**Figure 2.** Subcellular Localization of AGD2.

The pGFP and pAGD2-GFP under control of the CaMV 35S promoter were transformed into *A. thaliana*. GFP fluorescence of the protoplasts was observed using a fluorescence microscope.

(A) and (B) pAGD2-GFP.
(C) and (D) pGFP.

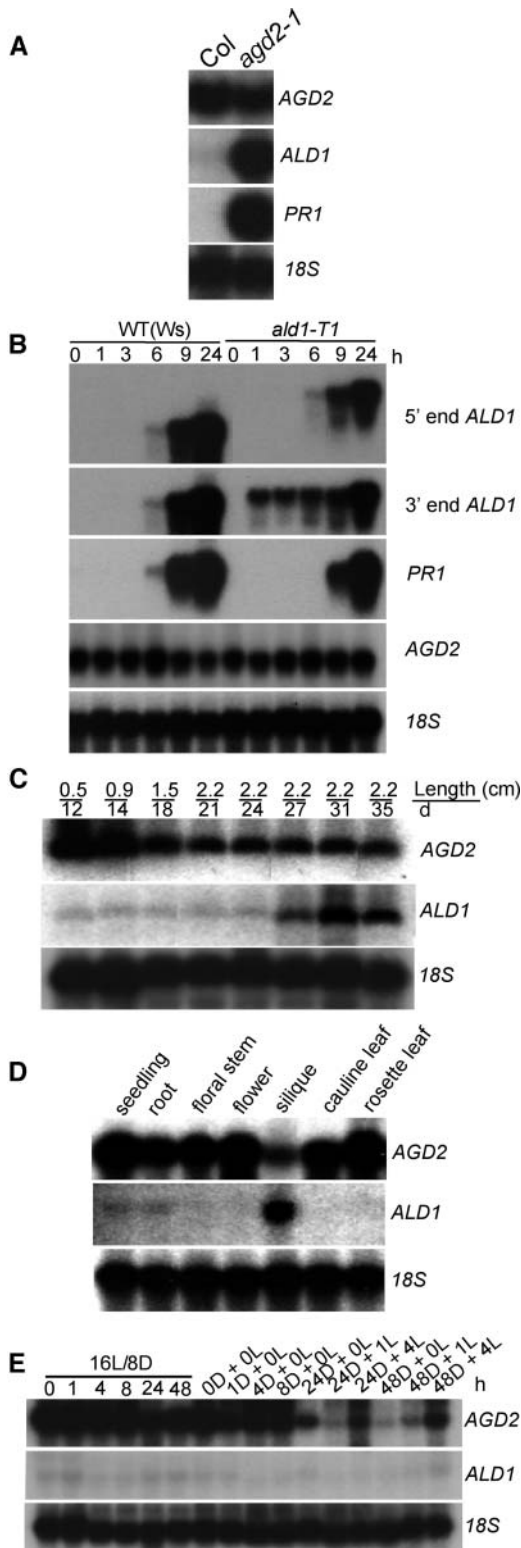


Figure 3. RNA Expression of *AGD2* and *ALD1*. Tissue from the wild type and/or *aldT-1* was used for RNA extraction and RNA gel blot analysis.

ALD1 (Figure 1B) in ecotype Wassilewskija (*Ws*). *ald1-T1* plants showed two aberrantly large *ALD1* transcripts, visualized with 5' and 3' end *ALD1* probes, because of the presence of the T-DNA insertion (Figure 3B). Reverse transcriptase (RT)-PCR and sequence analysis established that *ald1-T1* produced one transcript from the native promoter with the first four *ALD1* exons and T-DNA border sequence. The second transcript included a T-DNA sequence at the 5' end and the six 3' exons of *ALD1* at the 3' end (data not shown). No full-length transcripts were found, suggesting that little if any functional *ALD1* protein was present in the *ald-T1* mutant.

The *ald1-T1* homozygous plants and seeds were morphologically normal (data not shown). Infection of wild-type and *ald1-T1* plants with *P. s. maculicola* DG3 resulted in chlorotic symptoms in the leaves of *ald1-T1* but still green or slight symptom development in wild-type leaves (data not shown). *P. s. maculicola* DG3 bacteria grew at least 10-fold more in *ald1-T1* than in wild-type plants on days 2 and 3 after infection (Figure 4A). As *P. s. maculicola* DG3 was not very virulent in *Ws*, we also assayed the growth of *P. syringae* pv *tomato* strain DC3000, a virulent isolate. *ald1-T1* also was more susceptible than the wild type to *P. s. tomato* DC3000 (Figure 4A). Ecotype Columbia (*Col*) plants with RNA interference-mediated downregulation of *ALD1* or containing a T-DNA insertion in exon one of *ALD1* (*ald1-T2*; Figure 1B) behaved similarly to *ald1-T1* (Figure 5; data not shown). *ald1-T1* was recessive and was complemented by a genomic clone of *ALD1* (Figure 4B).

***ald1-T1* Has Reduced SA Accumulation**

Increased susceptibility of plants to *P. syringae* could result from a defect in SA accumulation. Indeed, the free SA and total SA (including glucoside-conjugated SA) levels in *ald1-T1* plants infected with *P. s. maculicola* DG3 were lower than the levels in infected wild-type plants at all time points tested (Figure 4C). *PR1* defense-related gene expression in *ald1-T1* plants also was delayed and lower than that seen in the wild type (Figure 3B). Similar results were observed when *ald1-T1* and wild-type plants were infected with *P. s. tomato* DC3000 (data not shown). Treatment of plants with the SA agonist benzo (1,2,3) thiazazole-7-carbothioic acid (BTH) before infection resulted in *ald1-T1* and

(A) *ALD1* and *PR1* expression in the fourth and fifth leaves of 20-d-old wild-type (*Col*) and *agd2-1* plants.

(B) *ALD1* and *PR1* expression in wild-type (*Ws*) and *ald1-T1* plants. Leaves (fourth and fifth) from 18-d-old plants were infected with *P. s. maculicola* DG3 ($OD_{600} = 0.01$).

(C) Expression of *AGD2* and *ALD1* during development of the fourth leaf. Leaf length and age of the plant when RNA was extracted are indicated.

(D) Expression of *AGD2* and *ALD1* in the indicated tissues.

(E) A time course of steady state *AGD2* mRNA accumulation. Twenty-day-old plants were kept in a 16-h-light/8-h-dark (16L/8D) cycle or were shifted to continuous dark (D) for the indicated time after 4 h in the light of the normal light cycle (16-h-light/8-h-dark) and then switched back to light (L) for 1 or 4 h. Although the signal was low for the *ALD1* transcript, the probe was verified to be high specific activity. It gave a strong signal on the blots probed in parallel (see [A] and [B]).

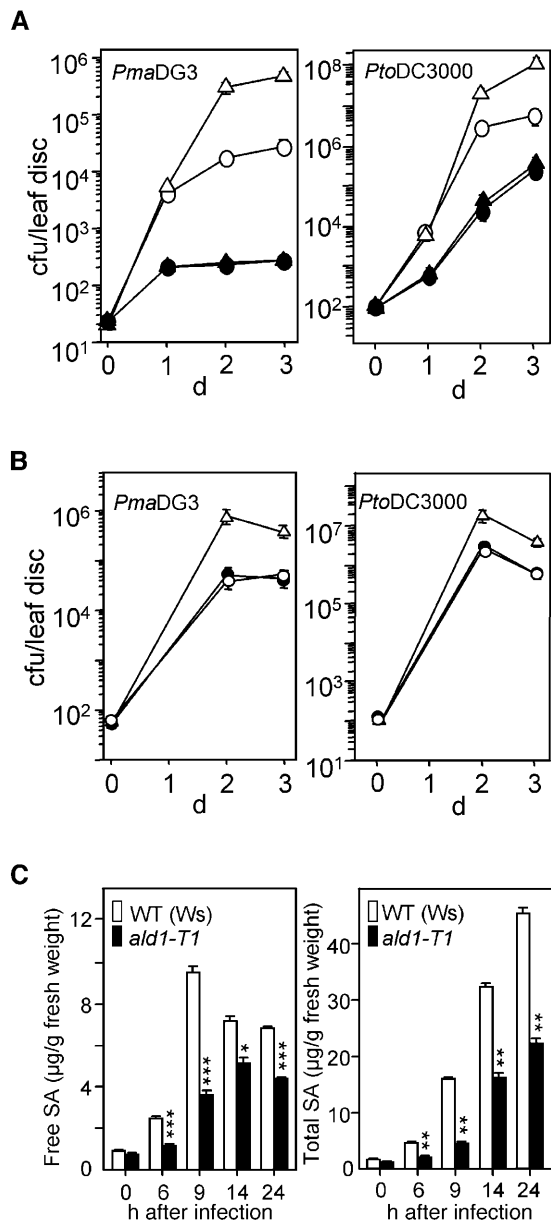


Figure 4. Increased Disease Susceptibility in the *ald1-T1* Mutant.

(A) Disease susceptibility of *ald1-T1* plants. Wild-type Ws (open circles) and *ald1-T1* (open triangles) plants were infected with *P. s. maculicola* DG3 (*Pma*DG3, at left) or with *P. s. tomato* DC3000 (*Pto*DC3000, at right) at $OD_{600} = 0.0001$. Growth of *P. syringae* in Ws and *ald1-T1* was significantly different on days 2 and 3 ($P < 0.01$, *t* test, $n = 8$). Ws and *ald1-T1* plants were pretreated with 100 μ M BTH or water for 2 d and then subjected to infection with *P. s. maculicola* DG3. BTH-treated Ws (closed circles) and *ald1-T1* (closed triangles) were not significantly different for bacterial growth ($P > 0.5$, *t* test, $n = 8$). cfu, colony-forming units. Bars indicate standard error. In some cases, the symbols obscure the error bars.

(B) Complementation of the *ald1-T1* mutant phenotype. Ws (open circles), *ald1-T1* (open triangles), and *ald1-T1* transformed with the *ALD1* genomic clone (closed circles) were infected with *P. s. maculicola* DG3 (*Pma*DG3, at left) or *P. s. tomato* DC3000 (*Pto*DC3000, at right) at $OD_{600} =$

wild-type plants that were equally disease resistant (Figure 4A). Thus, the defect in SA accumulation was likely responsible, at least in part, for the decreased resistance of *ald1-T1*.

ALD1 Is Largely Responsible for the Disease Resistance and Small Size of *agd2-1*

Because *agd2-1* had increased resistance to *P. syringae* and *ald1-T1* and *ald1-T2* had increased susceptibility to *P. syringae*, we tested whether *ALD1* was required for the elevated disease resistance of *agd2-1*. Mutant plants with the *ald1-T2* allele had no detectable *ALD1* transcript (data not shown). *ald1-T2* was in the same genetic background as *agd2-1*, making it suitable for double mutant construction. *agd2-1 ald1-T2* plants had similar disease susceptibility to that seen in the wild type but still less than that seen in *ald1-T2* alone (Figure 5). Thus, the majority of the disease resistance in *agd2-1* was mediated by the *ALD1* gene.

We tested whether the suppression of *agd2-1* disease resistance in *agd2-1 ald1-T2* was correlated with lowered SA levels. Indeed, the *agd2-1 ald1-T2* double mutant had a lower basal level of total SA ($11.2 \pm 0.3 \mu\text{g/g}$ tissue, $n = 3$) compared with the *agd2-1* single mutant ($18.5 \pm 0.4 \mu\text{g/g}$ tissue, $n = 3$, $P = 0.0001$). However, the double mutant still had significantly more SA than the wild type ($1.3 \pm 0.03 \mu\text{g/g}$ tissue, $n = 3$, $P < 0.0001$), even though disease susceptibility was equivalent in wild-type and *agd2-1 ald1-T2* plants. This suggests that SA accumulation and *ALD1* function can only partially explain the disease resistance of *agd2-1*.

Because depletion of SA in *agd2-1* using a *nahG* transgene altered the morphology of *agd2-1* (Rate and Greenberg, 2001; Vanacker et al., 2001), we also examined the morphology of *agd2-1 ald1-T2* plants. *agd2-1 ald1-T2* showed tumor-like growths reminiscent of those seen in *agd2-1-nahG* plants (data not shown), supporting a role for SA in growth control (Vanacker et al., 2001). Strikingly, we also found that the overall size of the *agd2-1 ald1-T2* rosettes was nearly restored to that of the wild type (Table 2). These data suggest that at least some *agd2-1* phenotypes are mediated by the action of *ALD1*.

Recombinant AGD2 and ALD1 Have Aminotransferase Activity in Vitro

Protein basic local alignment search tool (BLAST) searches indicated that AGD2 and ALD1 had the closest similarity to an aromatic aminotransferase from *Procyococcus hirikoshii* with highest activity on the amino acid substrates Tyr, Phe, Glu,

0.0001. Growth of *P. syringae* in Ws and *ald1-T1* was significantly different on days 2 and 3 ($P < 0.01$, *t* test, $n = 8$), whereas growth in Ws and the transgenic plants was not different ($P > 0.7$, *t* test, $n = 8$).

(C) SA levels in Ws and *ald1-T1* plants during pathogen infection. Leaves (fourth and fifth) from 18-d-old plants were infected with *P. s. maculicola* DG3 ($OD_{600} = 0.01$) and were harvested, extracted, and analyzed by HPLC. The free and total SA values \pm SD are averages of three sets of samples. The number of asterisks indicates samples that are significantly different from one another at a given confidence level (one asterisk, $P < 0.004$; two asterisks, $P < 0.0008$; three asterisks, $P < 0.0002$).

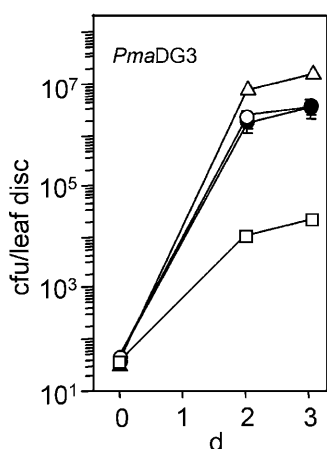


Figure 5. Bacterial Growth in *agd2-1 ald1-T2* Plants.

Wild-type Col (open circles), *ald1-T2* (open triangles), *agd2-1* (open squares), and *agd2-1 ald1-T2* (closed circles) were infected with *P. s. maculicola* DG3 at $OD_{600} = 0.0001$. Growth of *P. syringae* in Col and *agd2-1 ald1-T2* was not significantly different on days 2 and 3 ($P > 0.7$, t test, $n = 8$). Bars indicate standard error. In some cases, the symbols obscure the error bars.

Trp, and His (Matsui et al., 2000). However, the similarity was only modest (25% identity between the aromatic aminotransferase from *P. hirikoshii* and both ALD1 and AGD2). Other much higher BLAST matches were only to putative amino transferases for which activity has not been experimentally validated. To test whether AGD2, AGD2^{P398S}, and ALD1 had amino transferring activity, we overexpressed recombinant AGD2, AGD2^{P398S}, and ALD1 proteins in *Escherichia coli* and performed aminotransferase activity assays. Optimization of the assays is described in Methods. Six His-tagged truncated versions of the proteins lacking potential organelle targeting signals were overexpressed and then purified using nickel-nitrilotriacetic acid agarose (Ni²⁺-NTA) beads. The recombinant AGD2, AGD2^{P398S}, and ALD1 proteins were purified to near homogeneity and migrated on SDS-PAGE at the expected molecular masses (Figure 6A).

We first tested the ability of recombinant AGD2, AGD2^{P398S}, and ALD1 proteins to catalyze the transfer of amino groups onto the amino acceptor molecule 2-oxoglutarate in vitro by monitoring the production of glutamate. This amino acceptor was chosen because many characterized aminotransferases are active with this cosubstrate (Mehta et al., 1993). Recombinant AGD2, AGD2^{P398S}, and ALD1 were incubated with each of the standard 20 amino acids (except Glu, which was used to monitor these aminotransferase reactions), β -Ala, α -aminobutyric acid (AABA), β -aminobutyric acid, or γ -aminobutyric acid (GABA) as the amino donor. As a negative control, we used extract from *E. coli* containing vector alone and purified in the same way as AGD2, AGD2^{P398S}, and ALD1. The highest enzymatic activity was obtained with Lys for both AGD2 and ALD1 (Figure 6B). Both enzymes also exhibited some activity with Ala, Arg, Gln, Met, Leu, and Asn as amino donors. However, they showed no significant activity (the limit of detection was 1% of the activity with Lys) with any other standard amino acids under the conditions tested. It is common for aminotransferases to have

activity with several different substrates (Matsui et al., 2000; Nowicki et al., 2001). ALD1 had activity with AABA but not with β -aminobutyric acid or GABA, indicating that ALD1 needs an α -amino group for its activities (Figure 6B; data not shown).

We also tested AGD2, AGD2^{P398S}, and ALD1 for activity with each of the standard amino acids (except Ala, which was used to monitor these aminotransferase reactions), β -Ala, and AABA using pyruvate as another amino acceptor and monitoring the production of Ala. Pyruvate is the second most common amino acceptor for aminotransferases after 2-oxoglutarate (Mehta et al., 1993). AGD2 had lower activity with pyruvate than with 2-oxoglutarate. However, ALD1 had more activity with pyruvate than with 2-oxoglutarate (Figure 6B). As expected, AGD2^{P398S} had reduced activity relative to the wild-type AGD2 protein with all the substrates tested.

Because *agd2-1* (Rate and Greenberg, 2001) and *ald1-T1* (Figure 4C) mutants have altered SA production, we wondered if ALD2 or AGD2 might act in the SA biosynthetic pathway. One step in this pathway was proposed to convert the oxo-acid prephenate to the amino acid arogenate (Wildermuth et al., 2001). However, we found no detectable activity of AGD2 or ALD1 using prephenate as a cosubstrate with Lys, Ala, or Glu.

In summary, although the BLAST searches with AGD2 and ALD1 revealed modest similarity to an aminotransferase with highest activity with Tyr, Phe, Glu, Trp, and His, the empirical data suggests that AGD2 and ALD1 do not act on these substrates. Rather, AGD2 had its highest activities with Lys, Ala, and Arg, whereas ALD1 had its highest activities with Lys, Ala, Arg, Met, and AABA.

AGD2 and ALD1 Act in Different Directions in the Aminotransferase Reaction in Vitro

To evaluate the kinetic properties of the AGD2 and ALD1 enzymes for three of the best substrates (Lys, Ala, and Arg), the K_m values for the individual substrates were determined (Table 3). The K_m of AGD2 was 100-fold higher and the k_{cat}/K_m value was 100-fold lower for Lys when compared with those of ALD1. The K_m values of AGD2 and AGD2^{P398S} were not significantly different, but the k_{cat} value of AGD2^{P398S} was approximately half that of AGD2, causing the mutant protein to have reduced enzyme efficiency (k_{cat}/K_m). The K_m values for the amino acids Ala and Arg were much lower than that of Lys. They are not good substrates for AGD2 and ALD1, as indicated by the high K_m and low k_{cat}/K_m values. The K_m values of most aminotransferases are within the 0.1 to 5 mM range. For example, the *Homo sapiens* (human) GABA aminotransferase has a K_m of 1.27 mM (Jeon et al., 2000), and a broad substrate specificity aminotransferase

Table 2. Role of ALD1 in *agd2-1* Rosette Size

Genotype	Diameter (mm) \pm SD
Col	73.7 \pm 3.4 ^a , $n = 12$
<i>ald1-T2</i>	73.1 \pm 3.1 ^a , $n = 12$
<i>agd2-1</i>	31.5 \pm 2.7 ^b , $n = 12$
<i>agd2-1 ald1-T2</i>	59.2 \pm 3.4 ^c , $n = 12$

The diameters of 35-d-old plants were measured. Superscript letters represent different statistical values using Student's t test ($P < 0.0001$).

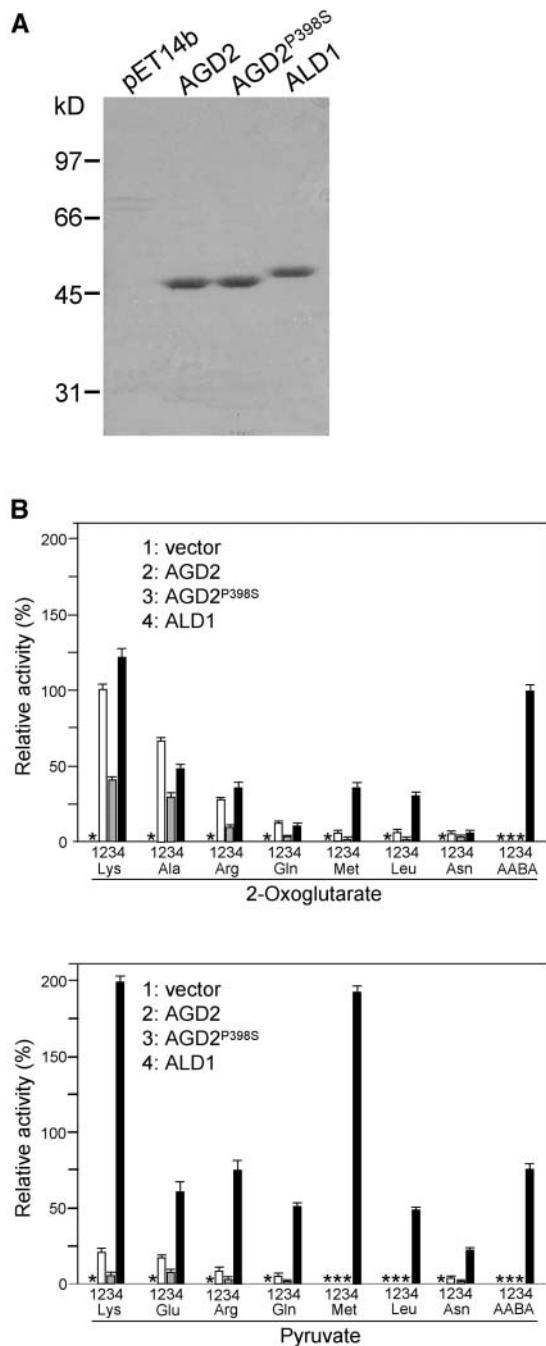


Figure 6. Purification and Relative Activities of AGD2, AGD2^{P398S}, and ALD1.

(A) Electrophoretic patterns of purified recombinant proteins through column elution using Ni²⁺-NTA.

(B) Relative aminotransferase activities of AGD2, AGD2^{P398S}, and ALD1. Substrates were at a 50-mM concentration. Aminotransferase activity was determined by measuring concentration of the reaction product Glu (using 2-oxoglutarate as the cosubstrate) or Ala (using pyruvate as the cosubstrate). The activity of AGD2 with Lys and 2-oxoglutarate was arbitrarily set at 100%. Asterisks indicate no detectable activities. No detectable activity was seen with all of the other standard amino acids tested. Bars indicate standard deviations ($n = 3$).

from *Leishmania mexicana* has 0.5 to 2.5 mM K_m values with aromatic amino acids and aspartate (Vernal et al., 1998).

The high K_m value of AGD2 with Lys suggests that Lys may not be the *in vivo* substrate for AGD2. Rather, the function of AGD2 may be to synthesize Lys or some other amino acid. To test this, we ran the aminotransferase reaction in reverse and measured the K_m values of AGD2 and ALD1 for the oxo-acid pyruvate as the amino acceptor (6-amino-2-oxohexanoate, the predicted oxo-acid produced by the forward reaction using Lys and 2-oxoglutarate, was not commercially available). The K_m value of AGD2 was 3.08 mM, 10-fold lower than that of ALD1 (Table 4). Although AGD2 and AGD2^{P398S} showed similar K_m values, the k_{cat}/K_m value of the wild-type protein was fivefold higher than that of mutant protein. These observations suggest that the reduction of k_{cat}/K_m is the cause of the *agd2-1* phenotype. These data are consistent with AGD2 being required for the synthesis of an amino acid(s) and ALD1 functioning to convert amino acid(s) to an oxo-acid(s) *in vivo*.

Amino Acid Profiles in *agd2-1*, AGD2-Overexpressing Plants, *ald1-T2*, and Wild-Type Plants Are Similar

The aminotransferase activity of AGD2 could indicate a role for AGD2 in the production of Lys and/or other amino acids. To test this, we measured amino acid levels in *agd2-1*, wild-type (Col), and AGD2-overexpressing transgenic plants. Free amino acid levels were determined in leaves of young (10 d) and older (17 d) plants. There were no large differences in the relative amino acid levels of the wild type versus *agd2-1* in the young or older plants, although there were small increases and decreases in some amino acid levels (data not shown). Plants confirmed by RNA gel blot analysis to overexpress AGD2 driven by the CaMV 35S promoter also had similar amino acid levels with wild-type plants (data not shown). Attempts to rescue the *agd2-T* and *agd2-1* plants with major amino acids or mixtures of amino acids using a previously successful approach for rescuing embryo-defective biotin auxotrophs (Patton et al., 1998) were not successful. These data suggest that AGD2 may not be involved in the synthesis of any standard amino acids.

We also tested if *ald1-T1* had abnormal amino acid accumulation after *P. s. maculicola* DG3 infection versus the wild type. Using gas chromatography–mass spectrometry (GC-MS) analysis of methanol extracts, we could see no dramatic differences in the relative levels of the 20 standard amino acids between *ald1-T1* and wild-type plants 9 and 24 h after infection. Most other metabolites were not apparently different except free SA, which was 2.5-fold lower in *ald1-T1* than the wild type (data not shown), consistent with our HPLC analysis (Figure 4C). These data suggest that standard amino acids may not be the targets of ALD1 action *in vivo*. We wondered if AABA could be an endogenous ALD1 substrate because it was a good substrate *in vitro* (Figure 6B). Unfortunately, AABA levels were below the assay detection limit.

DISCUSSION

Gene duplications and gene families often encode or are assumed to encode proteins with similar and/or overlapping

Table 3. Kinetic Parameters of Recombinant AGD2- and ALD1-Catalyzed Reactions for Amino Acid Degradation Using Either Lys, Ala, or Arg with 2-Oxoglutarate

	Substrate	Cosubstrate (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
AGD2	Lys	2-oxoglutarate (100 mM)	58.8	5.33	0.091
AGD2 ^{P398S}	Lys	2-oxoglutarate (100 mM)	71.4	2.87	0.040
ALD1	Lys	2-oxoglutarate (50 mM)	0.53	4.42	8.34
AGD2	2-oxoglutarate	Lys (100 mM)	1.82	2.83	1.55
AGD2 ^{P398S}	2-oxoglutarate	Lys (100 mM)	1.92	1.83	0.95
ALD1	2-oxoglutarate	Lys (100 mM)	2.63	3.75	1.43
AGD2	Ala	2-oxoglutarate (100 mM)	25.6	1.67	0.065
AGD2 ^{P398S}	Ala	2-oxoglutarate (100 mM)	23.3	0.87	0.037
ALD1	Ala	2-oxoglutarate (100 mM)	5.56	2.15	0.39
AGD2	Arg	2-oxoglutarate (100 mM)	154	0.69	0.0045
AGD2 ^{P398S}	Arg	2-oxoglutarate (100 mM)	167	0.35	0.0021
ALD1	Arg	2-oxoglutarate (100 mM)	5.10	2.07	0.41

K_m values were obtained from the initial velocity data and Lineweaver-Burk plots. k_{cat} was calculated using equation $V_{max} = k_{cat}$ [Enzyme]. Three independent measurements were averaged.

functions. However, the *AGD2* and *ALD1* genes of *A. thaliana* have quite divergent roles in development and defense, despite their high sequence similarity. *AGD2* is indispensable for development and also may repress defenses, whereas *ALD1* is important for activating defenses and limiting *P. syringae* growth. The activities of *AGD2* and *ALD1* in vitro significantly differed from each other. Their enzyme kinetic properties suggest that *AGD2* is required for the synthesis of an amino acid(s), whereas *ALD1* may utilize the same or a related amino acid(s) to make a defense-regulating molecule(s) (Figure 7A). The two enzymes may act in different subcellular compartments. Transcripts of *ALD1* were upregulated by pathogen infection and senescence, whereas those of *AGD2* did not accumulate under any of the conditions examined but were downregulated by dark treatment and during senescence.

Specific Physiological Function of AGD2

We established here that many of the *agd2-1* phenotypes are partially caused by the presence of the *ALD1* gene, possibly through its upregulation. In particular, some of the elevated SA, *P. syringae* disease resistance, and dwarfism can be suppressed when *ALD1* function is removed from *agd2-1* plants. However, the fact that in *agd2-1* the effects of *ALD1* are only partial suggests that there are *ALD1*-independent *AGD2* functions important for the control of SA synthesis, defense activation, and/or development.

agd2-1 hypomorphic plants have some dead cells and some cells with altered size and elevated endoreduplication (Rate and Greenberg, 2001; Vanacker et al., 2001). The loss-of-function *agd2-T* plants had an early defect in embryogenesis, suggesting that *AGD2* has an essential function in plant development. What might this function be? An obvious candidate is the synthesis of one or more amino acid. In particular, our in vitro experiments suggest that Lys would be the preferred product. Furthermore, *AGD2* localizes to chloroplasts, the site of synthesis of amino acids (Bryan, 1990). However, several observations lead to questions about how likely this function is for *AGD2*. First, we

saw no significant decrease in the level of Lys (or other amino acids) in *agd2-1*. Second, we were unable to rescue the *agd2-1* or *agd2-T* mutants with Lys or any of the other major amino acids or mixtures of amino acids. Third, plants are known to make Lys from aspartate via meso-diaminopimelate (Galili, 1995), which is decarboxylated to form Lys using the chloroplast-localized diaminopimelate decarboxylase (Azevedo et al., 1997). *A. thaliana* has two putative diaminopimelate decarboxylases encoded by the At3g14390 and At5g11880 genes. These Lys biosynthetic enzymes all should be intact in the *agd2* mutants. Possibly, Lys production in *A. thaliana* is primarily formed by decarboxylation of meso-diaminopimelate, but there is a specific requirement for *AGD2* during embryogenesis and some stage of leaf development. Our assays may have missed differences in Lys (or other amino acids) accumulation at these specific stages.

An alternative suggestion is that *AGD2* is essential for an unknown developmental regulator/signal molecule produced through Lys synthesis or through a nonstandard amino acid. Plant hormones consist of free and conjugated forms. For example, most indole-3-acetic acid (IAA)-based conjugates accumulate as IAA-ester forms to inositol, sugar, or polysaccharides and IAA-amide forms to amino acids or peptides (Cooke et al., 2002; Ljung et al., 2002). The conjugates are generally thought to act as short-term intermediates that can be hydrolyzed to release free forms. Nevertheless, the role of conjugates is still incompletely defined. *AGD2* may provide a precursor for a conjugated hormone, such as IAA-Lys, which is present in *Agrobacterium tumefaciens* and *P. savastanoi* (Glickmann et al., 1998) and may be present in plants. Such a molecule or one derived from a nonstandard amino acid might be important for embryogenesis.

agd2-1 hypomorphic plants also have increased disease resistance (Rate and Greenberg, 2001), which implicates *AGD2* directly or indirectly in repressing defenses. As stated above, this phenotype is partially attributable to *ALD1* function. The *agd2-T* knockout may have a lethal phenotype because of the derepression of defense responses, including programmed cell death. There is precedence for a mutation in a defense

Table 4. Kinetic Parameters of Recombinant AGD2- and ALD1-Catalyzed Reactions for Ala Synthesis Using Glutamate and Pyruvate as Cosubstrates

	Substrate	Cosubstrate (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
AGD2	Glutamate	Pyruvate (50 mM)	3.08	6.47	2.10
AGD2 ^{P398S}	Glutamate	Pyruvate (50 mM)	3.12	1.48	0.47
ALD1	Glutamate	Pyruvate (50 mM)	37.0	5.80	0.16

K_m values were obtained from the initial velocity data and Lineweaver-Burk plots. Three independent measurements were averaged.

component conferring a lethal phenotype. Plants lacking RPM1-interacting protein 4 show lethality (Mackey et al., 2002) that is likely to be because of the direct activation of an *R* gene pathway that activates cell death (Axtell and Staskawicz, 2003; Mackey et al., 2003). A link between developmental control and defense regulation also has been found for several defense regulatory genes (Vanacker et al., 2001; Holt et al., 2002; Jin et al., 2002; Barth and Conklin, 2003).

Specific Physiological Function of ALD1

ALD1 activity is important for *P. syringae* disease resistance. What might account for the increased disease susceptibility of *ald1* mutants? An important clue comes from the observation that SA levels are reduced in *ald1* upon pathogen infection and that *agd2-1 ald1-T2* plants have less SA than *agd2-1* alone. ALD1 possibly synthesizes a signal important for inducing SA synthesis. That the SA agonist BTH can induce disease resistance in *ald1* supports this possibility. We have no evidence for ALD1 acting directly in the SA biosynthetic pathway because the enzyme was not active with any proposed SA biosynthetic intermediates.

The control of SA production may not be the sole function of ALD1. The wild-type level of disease susceptibility of *agd2-1 ald1-T2* plants that still have ~8.6-fold elevated SA relative to the wild type suggests that ALD1 also controls the synthesis of another defense signal. Several other genes in *A. thaliana*, such as *PAD1*, *PAD2*, *PAD4*, *EDS1*, *EDS3*, *EDS4*, *EDS8*, *NDR1*, and *ACD6*, are similar to *ALD1* in that mutations in these genes also lead to reduced SA accumulation during infection by at least one strain of *P. syringae* (Glazebrook et al., 1996, 1997, 2003; Zhou et al., 1998; Gupta et al., 2000; Feys et al., 2001; Shapiro and Zhang, 2001; Lu et al., 2003). However, no demonstrated biochemical activity has been associated with the products of these genes. In the case of *pad4*, the mutation has been suggested to affect both SA-dependent defenses and possibly additional defenses (Glazebrook et al., 2003). Whether ALD1 acts in the same or separate pathways with *PAD4* and/or other SA-regulatory genes remains to be determined.

ALD1 also may have a direct role in synthesizing antimicrobial compounds. Precedent for an aminotransferase having such a function comes from *Streptomyces virginiae*, which uses Lys 2-aminotransferase to synthesize a cyclohexadepsipeptide antibiotic (Namwat et al., 2002).

Relationship and Roles of AGD2 and ALD1 in Development and Defense

The opposing defense phenotypes of *agd2-1* and *ald1* are striking because AGD2 and ALD1 likely drive the aminotransferase reaction on related substrates in opposite directions. A model for how these enzymes function is shown in Figure 7B. AGD2 is shown promoting development based on its requirement for embryogenesis in *agd2-T* and the abnormal leaf morphology in *agd2-1* (Rate and Greenberg, 2001). AGD2 also is shown having a second function repressing defenses. Because *agd2-1* showed constitutive defenses before morphological alterations were apparent (Rate, 2000; Rate and Greenberg, 2001), the effect of *agd2-1* on defense activation is shown as independent from the effect on development (although formally the developmental phenotype could cause the defense phenotype or vice versa). We speculate that in *agd2-1*, an amino acid–derived signal (possibly related to the ALD1 substrate), an amino acid imbalance, or the presence of a developmental regulator results in cross talk to the defense pathway. Amino acid

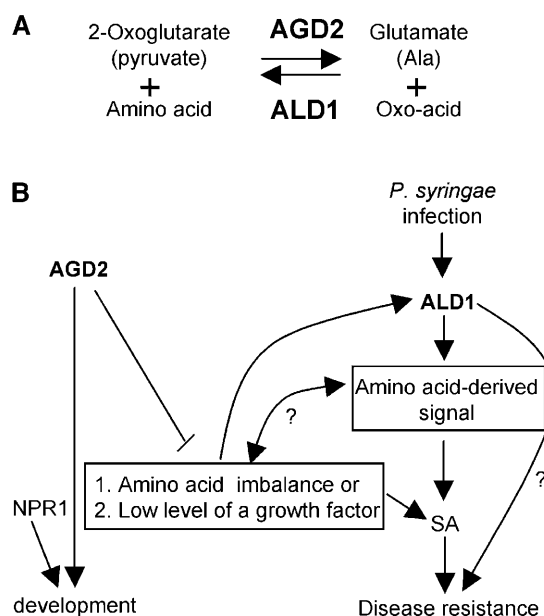


Figure 7. Models for the Enzymatic Functions and Roles of AGD2 and ALD1 in Development and Disease Resistance.

(A) Enzymatic reactions that AGD2 and ALD1 may conduct based on the *in vitro* data. These reactions may be performed in different subcellular compartments.

(B) Integrated model for AGD2 and ALD1 functions *in vivo*. When plants are infected with *P. s. maculicola* DG3, ALD1 is needed for SA accumulation and activation of SA signaling responses, such as *PR1* expression. ALD1 also is probably required for the generation of an SA-independent defense signal. AGD2 is required for normal development. Reduced activity of AGD2 results in developmental cell death and growth phenotypes and SA accumulation possibly because of the generation of an amino acid–derived signal or an amino acid imbalance. NPR1 also is shown as controlling development as an example of a protein that functions in defense and development (Vanacker et al., 2001).

starvation is known to activate plant defenses (Zhao et al., 1998). In support of a relationship between the substrates and products of the AGD2 and ALD1 enzymatic reactions, we found that *ald1-T2* could in large part suppress the reduced rosette size and other phenotypes of *agd2-1*.

We have no direct evidence for any large differences in the amino acid compositions of wild-type and *agd2-1* plants. However, we most likely missed amino acids that are either conjugated to other molecules (such as hormones), are present only transiently, or are low in abundance. Possibly, any amino acid imbalance caused by *agd2-1* is very transient because of the conversion of AGD2 substrates into alternative products. In our model, ALD1 functions to regulate SA synthesis and a second defense signal through an amino acid-derived signal. This is based on the results discussed above showing that *agd2-1 ald1-T2* plants, which still have residual elevated SA, show pathogen susceptibility equal to that of wild-type plants. The second defense signal postulated to be regulated by ALD1 could be directly produced by ALD1.

In summary, we have shown that two related aminotransferases perform divergent yet critical functions to control plant development and defense. Our study implicates amino and/or oxo-acids as having central roles in these processes.

METHODS

Plant Materials, Treatment, and Pathogen Infection

The *agd2-1 A. thaliana* mutant in the Col background was described previously (Rate and Greenberg, 2001). T-DNAs in *AGD2* and *ALD1* (*agd2-T* and *ald1-T1*, respectively) in the Ws background were obtained by screening the T-DNA insertion library at the University of Wisconsin (<http://www.biotech.wisc.edu/>). Another *ALD1* T-DNA allele (*ald1-T2*; SALK_007673) in Col was obtained from the Salk collection (<http://signal.salk.edu/cgi-bin/tdnaexpress/>).

Plants were grown and infections done as described previously (Greenberg et al., 2000). *P. s. maculicola* DG3 derived from *P. s. maculicola* strain ES4326 was described previously (Guttman and Greenberg, 2001). *P. s. tomato* DC3000 was obtained from F.M. Ausubel (Massachusetts General Hospital, Boston, MA).

BTH was a gift from Robert Dietrich (Syngenta, Research Triangle Park, NC). BTH treatments were done by spraying 16- to 18-d-old plants until all the leaves were wet. During dark treatment, plants were covered with an aluminum foil-wrapped plastic dome.

Recombination Mapping and Complementation Tests

To map *AGD2*, *agd2-1* (ecotype Col) was crossed to ecotype Landsberg. Homozygous *agd2-1* F2 plants were used for recombinant analysis using published cleaved amplified polymorphic sequences (CAPS) and simple sequence length polymorphism markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). To identify new CAPS markers, primers were designed using the published sequence (<http://www.arabidopsis.org/ceron/>). *AGD2* was mapped to a 65-kb interval between the CAPS markers C5-2 and C7-2N. The primers used for C5-2 were 5'-GATCGGAGGCATGCATTACAG-3' and 5'-TCCGAACAATCCAGCATTACC-3'. The primers for C7-2N were 5'-TTCTCCTCAGCACCTTGAAC-3' and 5'-CTACCCAGAGGTTTAACGACG-3'. Enzymes used for these CAPS markers were ApeI (C5-2) and DraI (C7-2N). The *A. thaliana* genomic BAC clone T16L1 (The Arabidopsis Information Resource, <http://www.arabidopsis.org>) covering this region was subcloned into the binary vector pAOV-CaMV. This vector is a modified version of pAOV (Mylne and

Botella, 1998) lacking the CaMV 35S promoter, which was removed by digesting pAOV with HindIII and XbaI and filling in the overhangs. Subclones were transformed into *agd2-1* as described (Clough and Bent, 1998). A defined *AGD2* genomic clone (a 7280-bp SacI fragment from BAC clone T16L1) in pAOV-CaMV and the *AGD2* cDNA (AV524925) driven by the CaMV 35S promoter cloned into pAOV were used for complementation of *agd2-1*. For complementation of *ald1-T1* and *ald1-T2*, a genomic clone of *ALD1* was subcloned as a 7970-bp XbaI-ScaI fragment from BAC clone F17L24 into pAOV-CaMV and transformed into *ald1-T1* and *ald1-T2* plants.

RNA Gel Blot Analysis

Total RNA was isolated as described (Kroczek and Siebert, 1990). The RNA (~8 μ g) was separated by electrophoresis on a 1% agarose gel. Hybridization was performed as described (Sambrook et al., 1989). Gene-specific DNA probes for *AGD2* or *ALD1* were made using PCR to amplify a portion (3' end) of each gene from cDNA. The primers used for *AGD2* were 5'-CTGCACCTGTTCATGGTGC-3' (sense primer) and 5'-CAC-TGGTGTCAAAGTGTCTTC-3' (antisense primer). The primers for *ALD1* were 5'-GAGATACGGTCGGTGAACAAC-3' (sense primer) and 5'-CAAGACGATGCACATAACACG-3' (antisense primer). Single-stranded probes were synthesized using the antisense primers.

Purification and Assays of Recombinant AGD2 and ALD1 Proteins

The *AGD2* and *ALD1* genes were amplified using PCR with the following primers using the cDNA clones of *AGD2* (GenBank accession number AV524925), *AGD2^{P398S}* (obtained using RT-PCR), and *ALD1* (GenBank accession number AY057526) as templates. For *AGD2* and *AGD2^{P398S}*, the sequences of primers were 5'-GCAAACCCGGGCAATACTTGC AAATGTGTTGC-3' (underlined sequence indicates the SmaI site) and 5'-AAACAACCCGGGTCATTTGTAAGCTGCTTGA-3' (underlined and boldface sequences indicate the SmaI site and the stop codon, respectively). For *ALD1*, the sequences of primers were 5'-TGGCTCGAGA TTCCAAGGCTAGTTGGAC-3' (underlined sequence indicates the XhoI site) and 5'-TGGCTCGAGATCCTAATTGGTATTAGAAGT-3' (underlined and boldface sequences indicate the XhoI site and the stop codon, respectively). These recombinant *AGD2* (or *AGD2^{P398S}*) and *ALD1* genes used for producing recombinant proteins lacked sequences encoding the first 40 and 20 amino acids, respectively. These N-terminal sequences corresponded to possible plastid-targeting signals. PCR products containing the *AGD2*, *AGD2^{P398S}*, and *ALD1* genes were digested with SmaI and XhoI and then ligated into the expression vector pET14b (Novagen, Madison, WI) cut with the appropriate restriction enzyme. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) pLysS. To induce production of the 6x-His-tagged recombinant enzymes, bacterial cultures grown to an A_{600} of 0.7 to 0.9 in LB medium containing 100 μ g/mL ampicillin at 37°C were treated with isopropyl- β -D-thiogalactopyranoside at a final concentration of 1 mM. Incubation was allowed to continue at 20°C for an additional 12 h, and the cells were harvested. Cell pellets were resuspended in one-tenth the culture volume in a solution containing 50 mM Tris-HCl buffer, pH 8.0, and then subjected to two cycles of freezing and thawing. The crude extracts were treated with DNase and MgCl₂ to final concentrations of 10 μ g/mL and 20 mM, respectively, at room temperature for 30 min. After centrifugation, imidazole and NaCl were added to the supernatant to final concentrations of 5 and 500 mM, respectively. The 6x-His-tagged fusion proteins were purified under native conditions using Ni²⁺-NTA agarose beads (Qiagen, Valencia, CA). The lysates were incubated for at least 30 min with binding buffer (5 mM imidazole, 50 mM NaCl, and 50 mM Tris-HCl, pH 8.0) containing Ni²⁺-NTA agarose beads. Elution was performed by a linear gradient of 5 to 200 mM. The recombinant proteins eluted at nearly 80 mM imidazole. Purified enzymes were stored at 4°C for 2 weeks without

significant loss of the activity. Protein concentrations were determined as described (Bradford, 1976) using BSA as a standard.

The recombinant AGD2, AGD2^{P398S}, and ALD1 activities were determined by measuring the concentration of the reaction product glutamate or Ala. The assay buffer contained 50 mM Tris-HCl (pH 8.0), 100 mM MgCl₂, and 1 μM pyridoxal-5-phosphate. Activities of the recombinant enzymes were found to be highest at 37°C in Tris-HCl, pH 8.0, and 100 mM MgCl₂. The optimum temperature for the recombinant enzymes was determined using assay buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM MgCl₂, and 1 μM pyridoxal-5-phosphate with 50 mM Lys as the amino donor and 50 mM 2-oxoglutarate as the amino acceptor at 4, 15, 25, 30, 37, 45, 50, and 65°C for 4 h. The pH dependence of the enzymes was determined at 37°C using three different buffer systems. Reactions were performed in 50 mM sodium acetate buffer at pH values ranging from 4.0 to 6.0, in 50 mM sodium phosphate buffer at pH values ranging from 6.0 to 7.0, and in 50 mM Tris-HCl buffer at pH values ranging from 7.0 to 10.0. Effects of various cations on activities also were examined. Cations such as Cu²⁺, Mn²⁺, and NH₄⁺ did not significantly activate the activities at 100 mM each, whereas Ca²⁺, Na⁺, and K⁺ showed better activation effects. The highest activities were in 100 mM MgCl₂. After the reaction, samples were applied to a thin-layer chromatographic plate (Si250; J.T. Baker, Phillipsburg, NJ) and developed with *n*-butyl alcohol:acetic acid:water (3:1:1, v/v). The chromatogram, dried at 60°C and cooled to room temperature, was soaked in freshly prepared ninhydrin solution (0.2% in acetone) for 1 min (Bhushan and Martens, 2001). To quantify the signals, the National Institutes of Health Image J program was used (<http://rsb.info.nih.gov/ij/>).

SA Measurements

Free and total SA (the sum of free and glucosyl SA) was extracted and quantified as described previously (Seskar et al., 1998).

Subcellular Localization of AGD2

The *gfp* gene was translationally fused at the 3' end of the cDNA of AGD2 gene in plasmid pAOV (Myline and Botella, 1998) behind the CaMV 35S promoter to give pAGD2-GFP. The plasmid pAGD2-GFP or pGFP (GFP alone driven by the CaMV 35S promoter) was introduced into *A. tumefaciens* (GV3101) and then transformed into *A. thaliana* plants. Protoplasts from leaves of transgenic plants were isolated as described (Asai et al., 2000). GFP localization and chlorophyll autofluorescence were observed using a fluorescence microscope (Axioskop; Carl Zeiss, Jena, Germany) with fluorescein isothiocyanate and UV filters, respectively.

Amino Acid Analysis

Approximately 200 mg of fresh leaves were ground in liquid N₂ in the presence of water:methanol:chloroform (2:3.5:8) (Palanivelu et al., 2003). The homogenate was extracted and centrifuged. The supernatant was dried by passing nitrogen gas over it. The residue was resuspended in water and submitted to the Molecular Structure Facility at University of California (Davis, CA) for amino acid analysis by HPLC (Palanivelu et al., 2003).

GC-MS Analysis

Leaves of *Ws* and *ald1-T1* plants were infected with *P. s. maculicola* DG3 (OD₆₀₀ = 0.01). The infected leaves (50 mg) at 9 and 24 h after infection were ground in liquid N₂ in 1.4 mL of 100% methanol with 50 μL internal standard (2 mg of ribitol/1 mL of water) (Roessner et al., 2000). The mixture was extracted with 1 volume of water and subsequently centrifuged. The supernatant was dried and analyzed by the Michigan State University Mass Spectrometry Facility (East Lansing, MI) using GC-MS.

GC-MS conditions and the system for the analysis of metabolites were performed as described (Roessner et al., 2000).

Characterization of *O. sativa* cDNAs for *OsAGD2* and *OsALD1*

The stop codons of the cDNAs were determined by RT-PCR using primers located at the stop codons of the annotated genomic sequences and/or by homology between the *O. sativa* and *A. thaliana* genes. The 5' ends of the *O. sativa* cDNAs were determined by 5' rapid amplification of cDNA ends PCR according to the Invitrogen manual (Carlsbad, CA). GenBank accession numbers of *cOsAGD2* and *cOsALD1* are AY338235 and AY338236, respectively.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY338235, AY338236, AV524925, and AY057526.

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