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Disruption of the Vacuolar Calcium-ATPases in Arabidopsis Results in the Activation of a Salicylic Acid-Dependent Programmed Cell Death Pathway

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Abstract:

Calcium (Ca\(^{2+}\)) signals regulate many aspects of plant development, including a programmed cell death pathway that protects plants from pathogens (Hypersensitive Response, HR). Cytosolic Ca\(^{2+}\)-signals result from a combined action of Ca\(^{2+}\)-influx through channels, and Ca\(^{2+}\) efflux through pumps and cotransporters. Plants utilize calmodulin activated Ca\(^{2+}\) pumps (ACA, Autoinhibited Ca\(^{2+}\)-ATPase) at the plasma membrane (PM), endoplasmic reticulum (ER) and vacuole. Here we show that a double knockout mutation of the vacuolar Ca\(^{2+}\) pumps ACA4 and ACA11 in Arabidopsis results in a high frequency of HR-like lesions. The appearance of macrolesions could be suppressed by growing plants with increased levels (> 15 mM) of various anions, providing a method for conditional suppression. By removing plants from a conditional suppression, lesion initials were found to originate primarily in leaf mesophyll cells, as detected by aniline blue staining. Initiation and spread of lesions could also be suppressed by disrupting the production or accumulation of salicylic acid (SA), as shown by combining aca4/11 mutations with a sid2 mutation or expression of a SA degradation enzyme (NahG). This indicates that the loss of the vacuolar calcium pumps by itself does not cause a catastrophic defect in ion homeostasis, but rather potentiates the activation of a SA-dependent PCD pathway. Together these results provide evidence linking the activity of the vacuolar Ca\(^{2+}\) pumps to the control of a SA-dependent programmed cell death pathway in plants.
Introduction

Calcium (Ca$^{2+}$) signals have been implicated in regulating many aspects of plant growth and responses to the environment (recently summarized by McAinsh & Pittman, 2009; Dodd et al., 2010; Kudla et al., 2010). Some of the most well studied examples include regulation of guard cells and transpiration (Israelsson et al., 2006; Neill et al., 2008; Pandey et al., 2007; Sirichandra et al., 2009), symbiosis and nodulation in legumes (Kosuta et al., 2008; Oldroyd & Downie, 2004), polarized growth (Bosch & Franklin-Tong, 2008; Bothwell et al., 2008), pathogen response (Lecourieux et al., 2006; Ma et al., 2008; Moeder & Yoshioka, 2008), and salt stress (Song et al., 2008; Mahajan et al., 2008; Qudeimat et al., 2008). Additionally, in both plants and animals, cytosolic Ca$^{2+}$ signals have been linked to the activation of programmed cell death (PCD) (Lecourieux et al., 2006; Verkhratsky, 2007; Ma & Berkowitz, 2007; Clapham, 2007; Bosch & Franklin-Tong, 2008; Reape et al., 2008; Moeder & Yoshioka, 2008; Lee & McNellis, 2009; Zhu et al., 2010).

A cytoplasmic Ca$^{2+}$ ([Ca$^{2+}$]$_{cyt}$) signal is shaped by the balance of activity between Ca$^{2+}$ influx and efflux. Influx occurs down an electrochemical gradient through various ion channels, such as Ca$^{2+}$-permeable cyclic nucleotide gated channels (CNGCs), or voltage gated channels such as TPC (McAinsh & Pittman, 2009; Pottosin & Schönknecht, 2007; Ward et al., 2009). Calcium can enter the cytoplasm from several sources, including the apoplast, ER, vacuole, chloroplast or mitochondria. Efflux requires energy-dependent Ca$^{2+}$ pumps (ACAs, Autoinhibited Ca$^{2+}$-ATPases, and ECAs ER-type Ca$^{2+}$-ATPases), or cotransport systems, such as Ca$^{2+}$/proton exchangers (CAX) (McAinsh & Pittman, 2009). As with channels, different efflux systems are present in different membrane systems, each with various regulatory controls. The different subcellular locations of these Ca$^{2+}$ circuits may contribute to the unique information content of different Ca$^{2+}$ signals, and help provide a mechanism for creating stimulus specific Ca$^{2+}$ signatures.

Plants appear to have three groups of ACAs, located in either the plasma membrane (PM), endoplasmic reticulum (ER), or tonoplast (vacuolar membrane) (Baxter et al., 2003). In Arabidopsis, the vacuoles are equipped with both CAXs (McAinsh & Pittman, 2009) and two closely related ACAs, isoforms ACA4 and ACA11 (Geisler et al., 2000; Lee et al., 2007). While both transport systems are regulated by autoinhibitors, the activation signals for CAXs remain to be determined (McAinsh & Pittman, 2009). However, ACAs are known to be stimulated by Ca$^{2+}$/Calmodulin (CaM), providing a direct feedback pathway for a Ca$^{2+}$ signal to turn itself off. Arabidopsis has 8 additional ACAs, 3 of which are thought to be located in the ER and 5 in the
plasma membrane (Baxter et al., 2003; Boursiac & Harper, 2007). The activity of an ER located ACA has been shown to be inhibited through phosphorylation by a Ca\(^{2+}\) dependent protein kinase (Hwang et al., 2000). This provides a precedent that the activity of some ACAs can be modulated by two different Ca\(^{2+}\) signaling pathways, one that activates, while the other inhibits. Altering the balance between these two pathways may provide a mechanism to fine-tune regulate a stimulus-specific Ca\(^{2+}\) signature.

In animals cells, genetic disruptions of Ca\(^{2+}\) pumps have resulted in multiple phenotypes, including lethality, deafness, muscle and skin disorders, increased frequency of cancer (Okunade et al., 2007; Song et al., 2008), male sterility and apoptosis (Okunade et al., 2007, 2004; Prasad et al., 2007; Vafiadaki et al., 2009). In Arabidopsis, disruptions of ECAs have revealed defects in vegetative development and Mn\(^{2+}\) homeostasis, while disruptions of ACAs have been linked to defects in pollen tube growth, sperm cell discharge, and cell elongation in vegetative development (Li et al., 2008; George et al., 2008; Schiøtt et al., 2004; Wu et al., 2002).

Evidence that ACAs can modulate biotic and abiotic stress response pathways has recently been obtained from experiments with moss and tobacco. In the moss Physcomitrella patens, a knockout of a gene encoding the vacuolar ACA (PCA1) resulted in an increased sensitivity to a NaCl stress, which was correlated with a NaCl triggered cytosolic Ca\(^{2+}\) elevation that was higher in magnitude and longer in duration (Qudeimat et al., 2008). In tobacco, RNAi silencing of NbCA1 resulted in an accelerated pathogen triggered PCD response (Zhu et al., 2010). An ER location for NbCA1 was proposed based on transient expression of a GFP tagged pump. The NbCA1-RNAi plants also showed elicitor triggered Ca\(^{2+}\) signals that were higher in magnitude and longer in duration. These two examples confirm that ACAs can function to modulate the dynamics of Ca\(^{2+}\) signals triggered by multiple environmental signals, as expected for a Ca\(^{2+}\)/calmodulin activated Ca\(^{2+}\) pump.

Here we show that a double disruption of Arabidopsis vacuolar pumps ACA4 and ACA11 results in a high frequency of apoptotic-like lesions. These lesions result from a PCD pathway that is dependent on salicylic acid (SA), similar to PCD pathways associated with a pathogen triggered hypersensitive response (HR), or various lesion mimic mutants (LMMs) (Lecourieux et al., 2006; Ma et al., 2008; Moeder & Yoshioka, 2008; Ma & Berkowitz, 2007; Reape et al., 2008; Gadjev et al., 2008; Kurusu et al., 2005). A role for Ca\(^{2+}\) signals in many PCD pathways has been well established in both animal (Clapham, 2007) and plant systems (Kurusu et al., 2005; Lecourieux et al., 2006; Ma & Berkowitz, 2007; Ma et al., 2008; Moeder & Yoshioka, 2008; Lee & McNellis, 2009; Zhu et al., 2010). However, in plants, the genetic identification of Ca\(^{2+}\) transport systems involved in PCD has been limited to mutations associated with Ca\(^{2+}\)
permeable ion channels thought to be associated with the plasma membrane (e.g. CNGC channels or glutamate receptors Lecourieux et al., 2006; Ma & Berkowitz, 2007), and an RNAi silencing of a proposed ER located Ca\textsuperscript{2+} pump in tobacco (Zhu et al., 2010). Thus, the identification here of ACA4 and ACA11 as genetic suppressors of a PCD pathway, establishes a link between vacuolar modulated Ca\textsuperscript{2+} signals and a PCD pathway in plants. This represents a plant-specific variation on the regulation of PCD, as the large central vacuole in plant cells is a feature not found in typical animal cells.

Results

aca4/11 double mutants exhibit HR-like lesions in leaves- To evaluate the biological functions of ACA4 and ACA11 in Arabidopsis, we created two independent sets of double mutants. Two T-DNA insertion mutants in the WS ecotype (aca4-1 and aca11-1) were isolated from the Arabidopsis Knockout Collection at the University of Wisconsin-Madison (Sussman et al., 2000). Two additional alleles in the Columbia ecotype were obtained from the Salk collection (aca4-3, SALK_029620.50.70.x) (Alonso et al., 2003) and Syngenta/Sail Collection (aca11-5, 269_C07.b.1a.Lb3Fa) (McElver et al., 2001). T-DNA borders for each insertion were PCR amplified and the exact site of insertion reconfirmed by DNA sequencing (positions of the left border are shown in Fig. 1). The T-DNA insertions in aca4-3 and 11-1 contain a basta resistance marker, whereas aca4-1 and 11-5 contain a kanamycin resistance gene.

While the T-DNA insertion sites for the two WS alleles are located in the first intron (and are therefore potentially spliced out at some low frequency), the insertions sites for the two Col background alleles, aca4-3 and 11-5, are located in coding sequence and are expected to result in truncations or deletions that would disrupt the translation of a functional pump (Fig. 1B). Nevertheless, both sets of double mutants showed a very similar lesion mimic phenotype (see below). This phenotype was shown to be rescued by the expression of an ACA11 transgene (see below), indicating that the aca4 and aca11 double mutations result in a loss of function of vacuolar Ca\textsuperscript{2+} pumping activity.

When grown either hydroponically or in soil, single mutants did not display any strong phenotype. On rare occasions, aca4-3 exhibited faint chlorotic spots in leaves (not shown). In contrast, when the double mutants aca4-1/11-1 (ecotype WS) and aca4-3/11-5 (ecotype Columbia) were grown in soil or hydroponically (with commonly used nutrient concentrations), they both developed HR-like necrotic lesions on leaves, normally within a few days after transfer
from germination plates (Fig. 2A). Those lesions appeared as spots that eventually extended to the whole leaf and rosette. At the time of bolting, mutant plants were significantly smaller than wild-type (WT) controls (Fig. 2B), most likely due to the cumulative effects of lesions on the plant’s photosynthetic productivity. Nevertheless, aca4/11 plants can complete their life cycle with no apparent defects in reproductive development. Seeds from double mutant plants showed no detectable alteration in germination rates (not shown).

ACA11-GFP rescues the aca4/11 lesion phenotype- To confirm that the lesion phenotype was due to the absence of ACA4 and ACA11 Ca²⁺ pumps, the aca4-3/11-5 double mutant was transformed with a construct encoding ACA11-GFP under the control of either the natural ACA11 promoter, or 35S promoter from CaMV. This ACA11-GFP was previously shown to be targeted to the vacuolar membrane (Lee et al., 2007). Both constructs provided a complete rescue of the lesion phenotype (Fig. 2B). Expression of the expected 137 kDa protein was confirmed by a Western Blot of each rescued line (Fig. 2C).

Anion supplements can suppress the aca4/11 lesion phenotype- Since young plants often showed no lesions while growing on ½ x MS media, we tested whether some component of this growth media could function to suppress lesion formation. To do this, plants were cultivated in hydroponic solutions with varying concentrations of KNO₃, NH₄NO₃, KCl or KH₂PO₄ (Fig. 3). We observed a suppression of lesion formation for aca4-1/11-1 (Fig. 3) when our standard hydroponic media was supplemented with an additional 15 mM NO₃⁻ in the form of 15 mM KNO₃ or NH₄NO₃ (final [NO₃⁻] = 19.25 mM), 15 mM KH₂PO₄ (final [PO₄³⁻] = 15.5 mM) or 15 mM KCl (final [Cl⁻] = 15 mM). While strong suppression was observed with a 15 mM NH₄NO₃ supplement, the identical concentration of ammonium succinate actually led to an increase in the rate of lesion induction (data not shown). Thus, for suppression by NH₄NO₃, it appears that the NO₃⁻ alone carries the functional suppressor activity. Since there is not a common ion in all three nutrient suppressors (KCl, NH₄NO₃ and KH₂PO₄), and since the NH₄NO₃ /NH₄-succinate experiment indicate that NO₃⁻ alone can function to suppress lesions, these results suggest that the elevated mineral anion component of these nutritional supplements is the functional feature of the nutritional suppression.

While a nutritional suppression was observed for both sets of double KOs (Col and WS backgrounds), the conditional suppression in aca4-3/11-5 (Col background) broke down just after plants initiated a floral bolt. This difference between ecotypes indicates that the observed nutritional suppression can vary as a function of genetic modifiers, some of which might regulate physiological changes that occur during flowering.
**Chronology of lesion induction** - The conditional suppression allowed us to monitor the induction of lesions following a change in nutrient supply. After germination, *aca4-1/11-1* plants were grown without lesions in our standard hydroponic solution supplemented with an additional 15 mM NH₄NO₃. Lesions were then triggered by transferring plants to standard un-supplemented hydroponic solution. Early lesion formation was then monitored during the following 54 to 72h with a diaminobenzidine (DAB) staining for the detection of ROS, and by an aniline blue staining for callose deposition - a standard marker for HR lesions (Dietrich et al., 1994) (Fig. 4, 5 and Fig. SI1).

In controls, WT plants before and after transfer from anion supplemented conditions showed the same low frequency of micro-lesions (callose staining) and low background levels of ROS (Fig. 4). By contrast, *aca4/11* plants even before a transfer showed patches of elevated levels of ROS (Fig. 4A, t = 0h, and Fig. SI1) as well as a detectably higher frequency of microlesions (Fig. 4B). This indicates that while the anion supplement prevented any macro-lesion expansion, it only partially inhibited lesion initiation. However, by 54 hours after transfer from suppression conditions, the surface area covered by patches of ROS had increased more than 2-fold (Fig 4A). In addition, the number of lesions increased 2.5 fold (Fig. 4B), with a typical lesion increasing in surface area by more than 4-fold from 48 to 72 hours (Fig 4C). In most cases, lesions grew to occupy the entire leaf surface within 3 to 4 additional days. Thus, this analysis indicates that a period between 30h-54h after transfer from lesion suppression conditions provides the earliest time at which a transition from micro to macro-lesion formation can be visualized.

To identify the cell types in which lesions originate, we mapped the locations of single-cell sized callose deposits (micro-lesions) during a 72 hour lesion induction experiment (Fig. 5 and Fig. SI2). Under nutritionally suppressed conditions, the micro-lesions detected in *aca4/11* appeared evenly distributed among the internal tissues of the leaf (parenchyma, mesophyll and vessels), whereas none were observed at the epidermis (t=0 in Fig 4b, Fig. 5). Within 40h after transfer to lesion permissive conditions, the number of micro-lesions increased, primarily in locations corresponding to mesophyll cells. In a distribution analysis of 117 lesions, 85 micro-lesions were classified as a mesophyll origin, 25 as parenchyma, and only 7 as epidermal. However, since this staining assay was destructive, it did not allow us to observe individual micro-lesions as they developed into macro-lesions. Nevertheless, this distribution analysis provides strong evidence that lesions in *aca4/11* preferentially initiate in mesophyll cells.

Within the group of lesion mimic mutants (LMMs), some of them such as *vad1* (Lorrain et al., 2004) show a high frequency of lesions near the vasculature. This “vascular”-pattern may result from the spread of a lesion-triggering signal through the vasculature. To test for this in...
aca4/11, the distribution of lesion-initials was evaluated relative to vessels. A lesion was considered vessel-associated if it was directly adjacent or within one cell layer (~25 μm). Between 18h and 30h after lesion induction only 12% of the initials were located in the vicinity of a vessel (Fig SI3). This suggests that micro-lesions initiate independently from a potential signal spreading through the vascular system. Together, this lesion mapping study suggest that lesion initials arise predominately in mesophyll cells due to a stimulus that is intrinsic to the region surrounding the initial.

Calcium levels in aca4/11 plants are similar to WT- To determine if a loss of vacuolar Ca²⁺ pumps would affect the total accumulation of Ca²⁺ (or other ions) in the leaves, we analyzed mutant and WT leaves for differences in Ca, Fe, K, Mg, Mn, Na, P and S (Fig. 6 shows results for Ca, and Fig. SI4 documents other cations). Plants were cultivated hydroponically under conditions of nutritional suppression (i.e. with an addition of 15 mM NO₃⁻) and the rosettes of both WT plants and aca4/11 were harvested before or 30h after transfer into a standard hydroponic solution (lesion permissive condition). Samples were analyzed for levels of mineral nutrients by ICP-AES (inductively coupled plasma atomic emission spectroscopy). Of the cations tested, only K showed a potentially significant difference between mutant and WT controls, with K levels in the aca4/11 mutant approximately 20% less. While a transfer to lesion permissive conditions resulted in an approximately 20% increase in K levels, the relative difference between mutant and a WT control was not altered (Fig. SI4).

For total Ca²⁺ levels, there were no significant differences between WT and aca4/11, although both sets of plants showed a 25% increase in Ca²⁺ when transferred from nutrient suppressed to lesion permissive conditions. Therefore our results indicate that ACA4 and 11 are not required for leaves to achieve normal Ca²⁺ storage levels. This is consistent with a hypothesis that Ca²⁺/proton exchangers (CAX) rather than Ca²⁺ pumps have a primary role in Ca²⁺ loading into plant vacuoles (Hirschi, 1999; Kim et al., 2006).

Cl⁻ and NO₃⁻ levels decrease more rapidly in aca4/11 when switched to lesion triggering conditions- The relative concentrations were also determined for each of the 3 anions used here to suppress aca4/11 lesions (Fig. 7). In plants grown using a 15 mM KCl supplement for lesion suppression (Fig. 7, left panels), chloride content in leaves of both WT and aca4-1/11-1 were elevated nearly 10-fold compared to plants grown with a standard hydroponic solution. This elevated level was maintained in wild type plants during the first 30 hours after transfer to our standard hydroponic conditions. In contrast, the mutants showed a 32% loss of Cl⁻ during this same period.
A similar pattern of anion loss by the mutant was observed for plants transferred from a condition of nutrient suppression using a 15 mM NH$_4$NO$_3$ supplement (Fig. 7, middle panels). When plants were transferred from supplemented to standard hydroponic solution, only the mutant showed a relatively rapid decrease (22%) in NO$_3^-$ during this first 30h period.

In contrast to suppression by KCl and NH$_4$NO$_3$, suppression by 15 mM KH$_2$PO$_4$ was not accompanied by any detectable changes in free concentration of the corresponding anion (i.e., PO$_4^{3-}$, see Fig. 7, right panels). However, it is important to note that our assay was limited to measuring the free concentration of PO$_4^{3-}$, and did not account for other forms of P. Since free PO$_4^{3-}$ levels are expected to be tightly regulated, any difference between the mutant and wild type may have been masked by a rapid homeostasis mechanism that converts PO$_4^{3-}$ to other forms, such as phytate (Loewus & Murthy, 2000).

Despite the inherent difficulty in accounting for the fate of free PO$_4^{3-}$ during these suppression/induction experiments, the relatively rapid loss of NO$_3^-$ and Cl$^-$ in mutants upon moving plants to lesion triggering conditions indicates that homeostasis controls for at least some anions are perturbed by the aca4/11 mutations.

**SA Signaling is activated in aca4/11 mutant**- In plants, SA can function as a signaling molecule to trigger defense responses, including a programmed cell death pathway (Lorrain et al., 2003). To determine if the lesions associated to aca4-1/11-1 involved an SA signal, we examined aca4/11 mutants harboring a sid2-5 mutation that disrupts SA biosynthesis (Nawrath & Métraux, 1999), as well as a NahG transgene that encodes an enzyme that increases the degradation rate of SA (Gaffney et al., 1993; Delaney et al., 1994). Both strategies resulted in suppression of lesions (Fig 8). This genetic suppression was observed in plants grown in soil or under standard hydroponic conditions.

To confirm that endogenous SA levels were up-regulated in aca4/11 mutants, SA was measured in plants before and 30h after moving hydroponically grown plants to lesion inducing conditions. At both time points, mutants showed a 2-fold higher level of SA compared to wild type (Fig. SI5). It is noteworthy that the SA levels were not significantly reduced when growing plants under suppressed conditions with high anion supplements (see Fig. SI5, t=0h). This suggests that the small 2-fold increase in SA is by itself not sufficient for lesion formation, but requires other signaling functions that can somehow be suppressed by factors related to an increase in nutritional supplements.

**Pathogen defense responses occur more quickly in aca4/11 mutants** - Infection by the bacterial pathogen *Pseudomonas syringae* pv Tomato DC3000 was used as a system to monitor a pathogen response in aca4/11 plants. The response was evaluated by measuring bacterial growth
(Figure 9A and B), as well as the expression of a defense-related marker gene \( PR1 \) (Fig. 9C) (Uknes et al., 1992). These experiments were done under lesion suppression conditions to avoid having any pre-existing lesions that could potential alter a pathogen attack.

Under lesion-suppression conditions, the SA-inducible \( PR1 \) gene showed no detectable expression in any of the plants lines tested (Figure 9C, \( t = 0h \)). Nevertheless, when lesion suppression conditions were removed, and \( aca4/11 \) mutants were allowed to develop their SA-dependent lesions, an up-regulation of the \( PR1 \) marker gene was observed (data not shown).

Although our lesion suppression conditions prevented the formation of spontaneous SA-dependent lesions, as well as the up-regulation of an SA-triggered pathogen defense marker gene (e.g. \( PR1 \)), the actual defense response to a \( Pseudomonas syringae \) pathogen attack was significantly faster and more effective in the \( aca4/11 \) mutant, as indicated by lower bacterial growth at 2 and 3 days post inoculation (Fig. 9A), as well as a more rapid induction of a \( PR1 \) marker gene (by at least 12 hours) (Fig. 9C). This accelerated defense response was dependent upon SA, as shown using the \( sid2-5 \) allele to block SA biosynthesis By including the \( sid2-5 \) mutation with \( aca4-3 \) and \( 11-5 \) (Fig. 9B), the \( aca4/11 \) dependent inhibition of bacterial growth was reversed, and the faster pathogen-triggered up-regulation of the \( PR1 \) gene was abolished (Fig. 9C). A visual indication that an \( aca4/11 \) knockout accelerated the defense response was also confirmed by the more rapid development of HR-lesions, which were clearly visible in the \( aca4/11 \) mutant at 54 hours post inoculation, but not yet apparent in the WT control (Figure 9D). These pathogen-triggered lesions were morphologically indistinguishable from the spontaneous lesions originally documented as the characteristic feature of the \( aca4/11 \) lesion mimic phenotype (See Fig. 2)

**Discussion**

*Vacuolar \( Ca^{2+} \) pumps can modulate the initiation and spread of HR-like lesions*- Our analysis provides genetic evidence for a programmed cell death pathway in plants whose initiation and cell-to-cell propagation can be suppressed by the activity of vacuolar \( Ca^{2+} \) pumps. This conclusion is based on the observation that two independent double \( T-DNA \) disruptions of vacuolar \( Ca^{2+} \) pumps \( ACA4 \) and \( ACA11 \) in Arabidopsis, both result in plants that begin developing lesions in rosette leaves early in development. Mutant plants, although smaller, can live for long periods of time and set seed. The lesion phenotype is weak or absent from single mutants, indicating that \( ACA4 \) and \( ACA11 \) provide some level of redundancy. While Arabidopsis
genome encodes 14 different Ca\(^{2+}\) pumps, a lesion phenotype has not yet been uncovered for any other combination of Ca\(^{2+}\) pump disruptions (McAinsh & Pittman, 2009) (Harper, unpublished). Thus, at present, the vacuolar Ca\(^{2+}\) pumps ACA4 and 11 define a specific Ca\(^{2+}\) efflux pathway that can function to suppress a PCD pathway in plants.

It is not yet clear if there is also a specific vacuolar Ca\(^{2+}\) influx channel involved in triggering the aca4/11-PCD pathway. Evidence supporting a role for a TPC-type Ca\(^{2+}\) channel in a pathogen induced HR-response was previously reported for rice and tobacco tissue culture cells (Kurusu et al., 2005; Kadota et al., 2004). However, these studies proposed a plasma membrane location for the TPC being analyzed. In contrast, evidence from Arabidopsis indicates that its single TPC homolog functions as part of the vacuole (Ranf et al., 2008; Peiter et al., 2005). In addition, a knockout of the Arabidopsis homolog (tpc1-2) failed to show an altered phenotype in response to elicitors or a fungal infection (Ranf et al., 2008; Bonaventure et al., 2007). Nevertheless, additional studies will be required to determine if the vacuolar TPC in Arabidopsis can function alone or in conjunction with other putative Ca\(^{2+}\) channels to trigger an aca4/11-dependent PCD pathway.

The aca4/11 mutant can be classified as having a “lesion mimic phenotype” (LMM), since the lesions have features consistent with a classical hypersensitive response (HR), but can initiate in a sterile environment without a pathogen trigger (data not shown). LMMs are often classified as either lesion-initiation or lesion-spreading mutants (Lorrain et al., 2003). The aca4/11 mutation is unusual since both initiation and lesion spreading appear to be enhanced. When lesion suppression conditions were removed (Fig. 4), lesion initiation appeared to increase, and macro-lesions grew rapidly to cover most of the leaf surface within a week.

In plants and animals, 3 different cell death mechanisms have been described: 1) apoptotic-like PCD (AL-PCD), 2) autophagy-mediated PCD, and 3) non-programmed necrosis. Since HR-lesions are considered to be a form of AL-PCD, the aca4/11 lesions can also be classified as a form of AL-PCD. An easily visualized feature of HR-lesions is an increase in callose synthesis at lesion initials. This requires a reprogramming of the cellular machinery, and was observed as a feature of aca4/11 lesions (Fig. 4 and 5). This supports the contention that aca4/11-lesions develop as part of a PCD, as opposed to a spontaneous and rapid cellular necrosis.

Propagation of aca4/11-lesions involves an SA-dependent PCD pathway. Two genetic lines of evidence indicate that aca4/11-dependent lesions propagate through a salicylic acid (SA) dependent programmed cell death pathway (Fig. 7 and SI5). First, lesions were suppressed by a sid2 mutation. The sid2-5 mutation used here disrupts the isochorismate synthase gene ICS1 (Wildermuth et al., 2001) and blocks the production of SA (Nawrath & Métraux, 1999). Second,
lesions were suppressed by expression of a NahG transgene. NahG encodes a bacterial salicylate hydroxylase that degrades SA into catechol (Gaffney et al., 1993; Delaney et al., 1994). This ability to block SA signaling and suppress aca4/11 lesions confirms that lesion development results from a defect in regulating a specific PCD signal transduction pathway, as opposed to an uncontrolled cell death resulting from catastrophic defect in Ca\(^{2+}\) homeostasis or vacuolar degeneration.

*Lesion spread can be suppressed by anion supplements*- Interestingly, growth conditions were found that could separate lesion initiation from its uncontrolled spreading (Fig. 3). When mutant plants were grown with high concentrations of various anions, such as 15 to 20 mM of either NO\(_3^-\), PO\(_4^{3-}\), Cl\(^-\), a high frequency of micro-lesions was still detected by staining leaves with aniline blue (Fig. 4, 5, and SI2). However, these lesions did not spread, indicating that the anion supplements functioned primarily to suppress a second distinct phase of lesion development (i.e. spreading). While the mechanism underlying anion suppression is not clear, suppression by NO\(_3^-\) and Cl\(^-\) did correlate with an increase in their concentrations in rosette leaves, followed by a more rapid loss compared to wild type when transferred to unsupplemented growth conditions (Fig. 7). This supports a model in which the ionic environment at the site of lesion initiation and propagation can be altered to regulate a PCD pathway, potentially through changing ion conductance properties of either the PM or vacuole.

Multiple studies have implicated non-specific anion transporters in membrane depolarization events associated with many ion signaling pathways, including Ca\(^{2+}\) signals and PCD (Ward et al., 1995; Errakhi et al., 2008b, a). For example, an anion efflux in tobacco leaf suspension cells was observed as an early response to a fungal elicitor cryptogein (Pugin et al., 1997). A pharmacological inhibition of this anion release was also observed to prevent the development of an HR in tobacco leaves (Wendehenne et al., 2002).

Using plants that were transferred from anion suppression to lesion inducing conditions, the location of spontaneous lesion initials was found to be predominately in mesophyll cells, without any correlation to being near or far from vascular elements (Fig. 5 and SI4). Since micro-lesions were never seen to appear in cell types of the root (i.e. no aniline blue stained necrotic lesions), it is possible that lesion initiation and propagation are related to physiological triggers associated with photosynthetic pathways, as implicated in several examples of PCD triggered by abiotic stress (Gadjev et al., 2008).

It is noteworthy that ROS and SA levels in both anion-suppressed and non-suppressed plants were approximately 2-fold higher than controls (see Fig. 4 and SI5). Since the anion supplements did not block ROS and SA production, but did prevent the accumulation of PRI (Fig.
9, t = 0h), this suggests that the mechanism for anion suppression is at a point downstream of an initial signaling pathway that generates increased levels of SA or ROS, and upstream of changes in a transcriptional response that upregulates PR1 mRNA levels.

A loss of aca4 and 11 potentiates an accelerated defense response to P. syringae- An enhanced defense response against a bacterial pathogen, *Pseudomonas syringae*, was observed here for aca4/11 mutants (Fig. 9A). The defense response was tested under conditions in which spontaneous lesions in the aca4/11 mutants were suppressed by anion supplements. The initial expression levels for an SA-up-regulated PR1 marker were undetectable in both mutants and WT controls under these conditions, although aca4/11 mutants already showed a moderated elevation in SA (Fig. S15). However, following a pathogen inoculation, the aca4/11 mutants showed a more rapid induction of the PR1 gene, with significant expression within 12 hours (Fig 9C). The enhanced resistance and more rapid induction of a PR1 gene marker were both SA-dependent, as indicated using a sid2 mutation to block SA production (Fig. 9B and C). These results suggest that even under lesion suppressed conditions, the loss of aca4/11 results in a physiologically altered plant that is pre-conditioned to a more rapid defense response, and therefore confirms that ACA4 and 11 act as suppressors of a PCD pathway.

Evidence for Ca\(^{2+}\) signals in regulating PCD- Multiple lines of pharmacological and genetic evidence have implicated Ca\(^{2+}\) signals in regulating AL-PCD pathways in both animals (Clapham, 2007) and plants (Lecourieux et al., 2006; Ma & Berkowitz, 2007; Bosch & Franklin-Tong, 2008; Moeder & Yoshioka, 2008; Zhu et al., 2010). In plants, multiple proteins involved in Ca\(^{2+}\) signaling have been implicated in both positive and negative regulation of PCD. Recent examples include Ca\(^{2+}\)-binding copines (Lee & McNeill, 2009; Liu et al., 2005), the Ca\(^{2+}\)/calmodulin interacting protein MLO in barley (Piffanelli et al., 2002; Kim et al., 2002), and the Ca\(^{2+}\)/calmodulin binding transcription factor AtSR1 (Du et al., 2009). With respect to ion channels, a loss of function mutation in CNGC2 (*dnd1, defense no death*) was found to have a lesion suppressed phenotype (Clough et al., 2000). CNGCs in plants include isoforms that have features of being Ca\(^{2+}\) permeable non-specific ion channels that are gated open by cyclic nucleotides and feed-back inhibited by Ca\(^{2+}\)/calmodulin (Ma & Berkowitz, 2007; Talke et al., 2003; Frietsch et al., 2007). Additionally, a gain of function CNGC mutation (*cpr22, chimera of CNGC11 and 12*) was identified with a lesion phenotype (Moeder & Yoshioka, 2008; Ma & Berkowitz, 2007; Urquhart et al., 2007). With respect to Ca\(^{2+}\) pumps, RNAi silencing of the tobacco NbCA1 showed an acceleration of a pathogen triggered PCD pathway (Zhu et al., 2010).

Evidence from both plant and animal examples provide strong support for the expectation that the activity of Ca\(^{2+}\) efflux pathways can modulate the magnitude and duration of Ca\(^{2+}\) signals.
in specific cellular locations (Hetherington & Brownlee, 2004; Beauvois et al., 2006; Qudeimat et al., 2008; McAinsh & Pittman, 2009; Zhu et al., 2010). For example, in the moss \textit{Physcomitrella patens}, an engineered deletion of a vacuolar Ca\textsuperscript{2+} pump altered a salt stressed induced Ca\textsuperscript{2+} signal to be longer and greater magnitude compared to wild type (Qudeimat et al., 2008).

There are now several examples from plant and animals systems that implicate Ca\textsuperscript{2+} efflux systems as potential regulators of PCD. For example, an apoptosis phenotype was reported in animal smooth muscle cells in which the levels of two plasma membrane Ca\textsuperscript{2+} pumps had been reduced (Okunade et al., 2004; Prasad et al., 2007). In addition, apoptosis in animal cells has also been linked to a genetic disruption of Ca\textsuperscript{2+} pump activities in ER and Golgi locations (Okunade et al., 2007; Vafiadaki et al., 2009). In plants, the RNAi silencing of Ca\textsuperscript{2+} pump \textit{NbCA1} (Zhu et al., 2010) provides an example of an endomembrane Ca\textsuperscript{2+} pump that functions in modulating the kinetics of a pathogen triggered PCD pathway.

The observation here that a loss of ACA4 and ACA11 increases the frequency of SA-dependent lesions is significant because it supports a new model in which the vacuole participates in modulating certain Ca\textsuperscript{2+} signals that can trigger PCD. Future research will be needed to visualize the Ca\textsuperscript{2+} signals that are altered by the loss of ACA4 and 11, and to understand the “upstream” factors that initiate those signals, and the immediate “downstream” targets that link these signals to the activation of PCD. While it is known that Ca\textsuperscript{2+} efflux through ACAs can be turned on and off (e.g. Hwang et al., 2000), it remains to be determined if this activity is actually regulated as part of a natural mechanism by which a pathogen or abiotic stress might trigger the activation of a PCD pathway.

PCD is also involved in many other aspects of plant development, including senescence, sculpting tissues, and the terminal differentiation of tracheids (Jones, 2001; Lam, 2004). While Ca\textsuperscript{2+} signals have been implicated in many of these pathways, it remains to be determined which pathways may involve Ca\textsuperscript{2+} signals modulated by vacuoles. Nevertheless, from a genetic perspective, this study identifies one function of the vacuolar pumps \textit{ACA4} and \textit{ACA11} as a suppressor of an SA-dependent PCD pathway. This function also represents a plant-specific variation on the regulation of PCD, since the large central vacuole in plant cells is a feature not found in typical animal cells.

**Experimental Procedures**
Plant growth conditions- Seeds were surface sterilized for 3h with chlorine gas (Clough & Bent, 1998), then sown on plates on ½ MS strength media complemented by 2% sucrose and 10g/l-1 agar. After 2 days of vernalization in the dark at 4°C, seeds were germinated under continuous light at ~19°C. Nine-day-old seedlings were transferred to soil or hydroponics. Unless otherwise stated, plants in growth chambers were grown at 65% relative humidity, 16h of light at 21°C and 8h of dark at 19°C. Greenhouse grown plants were subject to seasonal variations in light and humidity. Soil used was Special Blend from Sunshine (Sun Gro, USA). For hydroponic cultures, seedlings were transferred onto a floating foam support in a 3 liter bucket filled with a standard hydroponic solution of 1.25 mM KNO₃, 0.75 mM MgSO₄, 1.5 mM Ca(NO₃)₂, 0.5 mM KH₂PO₄, 50 μM FeEDTA, 50 μM H₃BO₃, 12 μM MnSO₄, 0.7 μM CuSO₄, 1 μM ZnSO₄, 0.24 μM MoO₄Na₂, 100 μM Na₂SiO₃. Hydroponic solutions were replaced weekly. For suppression conditions, the standard hydroponic solution above was supplemented as stated in the text.

Plant material and genotyping- Plants were transformed using Agrobacterium tumefaciens (GV3101 line) and a floral dip method (Clough & Bent, 1998). Dry seeds were harvested, and hygromycin-resistant plants (T₀) were identified and grown for seeds. Plant genotypes were determined by PCR. Leaves of approximately 0.5 cm² were harvested, manually ground in an extraction buffer (250 mM NaCl, 200 mM Tris pH 8.0, 25 mM EDTA, 0.1%SDS), and debris pelleted by 10min centrifugation at 10,000g. DNA in the supernatant was recovered by 66% isopropanol precipitation. Touch-down PCR (from 66°C to 60°C in -0.3°C steps, and then 14 additional cycles with an annealing temperature of 60°C) were performed in a 25 μl reaction using ExTAQ DNA polymerase (Takara, Japan) following the manufacturer’s protocol. Oligonucleotides used for the reaction, at a final concentration of 0.2 μM, can be found in the legend of Fig. 1.

Inoculation of plants with P. syringae- The virulent Pseudomonas syringae pv tomato DC3000 were grown at 28°C on King B’s medium (40 g/l Proteose Peptone 3; 20 g/l Glycerin; 10 ml/l MgSO₄ (10% m/v); 10 ml/l K₂HPO₄ (10% m/v)) supplemented with the appropriate antibiotics: 50 mg/mL of rifampicin. To examine the growth of the bacteria, 3-to-4-week-old plants were sprayed with a bacterial suspension containing 5.10⁶ colony-forming units per mL in 10 mM MgCl₂ solution with 0.04% Silwet L-77. Bacterial growth was measured at 0.2 and 3 days after infiltration by extracting bacteria from leaf discs (0.6 cm2 discs per leaf) and plating a series of dilutions on the medium supplemented with appropriate antibiotics.

Plasmid constructs- Plant expression constructs were made in pGreenII vector system (Hellens et al., 2000), with a kanamycin selection marker for bacteria, and a hygromycin marker for plants. The 35S::ACA11-GFP (ps#1658) construct was described previously (Lee et al., 2007). For the ACA11p::ACA11-GFP construct (ps#1657), a 2,127-bp sequence upstream of the ATG
start codon for ACA11 was PCR-amplified from Arabidopsis and replaced the 35S promoter of 35S-ACA11-GFP (construction ps#1658). The DNA sequence of each construct is provided as a supplemental file (Fig SI6).

Northern Blot analysis- Total RNA was isolated from leaves using LiCl – phenol/chloroform extraction method (Chomczynski & Sacchi, 2006). Total RNA (10ug) was separated on 1.5 % agarose-formaldehyde gel and blotted onto nylon membranes. The membranes were hybridized with [α-32P] dATP-labeled gene specific probes for 16 hr at 65°C and washed for 10 min twice with 2 X SSC (0,15 M NaCl, 15 mM trisodium-citrate), once with 1 X SSC, and 10 min with 0.5 X SSC, 1%(W/V) SDS at 65°C.

Western blot analysis- Membrane proteins (30 μg) were isolated from ACA11-GFP transgenic mutant plants and separated on 8% SDS-PAGE. A rabbit anti-GFP antibody (Santa Cruz Biotechnology, USA) was used to probe a Western blot, and visualized using a goat anti-rabbit IgG antibody (Calbiochem, USA) and Enhanced Chemical Luminescence detection according to the manufacturers protocol (GE healthcare, USA).

Detection of Reactive Oxygen Species and Callose- Excised leaves were vacuum infiltrated with a solution of 1 mg.ml^-1 of 3,3’-Diaminobenzidine (DAB, Sigma) and then stored for 3h on wet paper under lights. Leaves were then fixed and bleached overnight with a solution of ethanol/lactic acid/glycerol (3/1/1 in volume), washed with decreasing ethanol concentration (75, 50, 25%) and equilibrated in water. Pictures were taken with a digital camera under a dissecting microscope. DAB staining appeared as a brown deposit.

Detection of callose in leaves was performed essentially as described (Dietrich et al., 1994). The 2 largest leaves were fixed for 2h in 10% formaldehyde, 5% acetic acid, 45% ethanol, cleared for 2 min in boiling alcholic lactophenol (95%ethanol:lactophenol, 2:1) and stained overnight in a solution of 150 mM K_2HPO_4, pH 9.5 with 0.01% aniline blue. Leaves were rinsed in distilled water before observation. Callose deposition was observed with an Olympus FV1000 confocal microscope with 405nm excitation and 440-480nm emission window. Six Z-sections (640μm x 640μm) spanning the whole leaf thickness were taken per leaf. Lesions surface and number were obtained from an analysis of every section in the stack.

Ion Concentration Measurements- Four to 5-week old rosette leaves were harvested and their dry weight (DW) was determined after incubation for 3 days at 110°C. Dried tissue was digested overnight with 3ml of concentrated HNO_3 and cation content was determined using an inductively coupled plasma atomic emission spectrocope or ICP-AES (Varian, USA). To determine the concentrations of anions, 4- to 5-week old rosette leaves were harvested, weighed
and processed by one of two procedures. Samples were either ground in liquid nitrogen and resuspended in 3ml chloroform, or frozen samples were ground directly in 3ml HPLC grade chloroform using a mixer mill (Retsch, Newtown, PA, USA). Samples were then incubated in 15ml polypropylene tubes for 1h at 50°C. Ultrapure water (5ml) was added and samples were incubated for an additional hour. Tubes were centrifuged for 15min at 2900g to clear debris from the aqueous phase. The supernatant was analyzed by anion-exchange chromatography. Aliquots of the supernatant (10μl) were run on a Dionex high-performance liquid chromatography (Dionex, USA) through a Dionex AS11-HC column with a gradient of 1 mM to 60 mM NaOH over 40 min. The column was at room temperature with a flow rate of 0.27 ml.min⁻¹. Anions were detected by suppressed conductivity method and NO₃⁻ was specifically detected by absorbance at 210 nm. Peaks were identified using pure anion salt standards purchased from Sigma.

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Figure legends

Figure 1. Identification of 2 independent knock-out lines for each AtACA4 and AtACA11.

A- Location and direction of the T-DNA insertions on the genomic sequence for the alleles aca4-1, aca4-3, aca11-1 and aca11-5. Alleles 4-1 and 11-1 are in the WS ecotype, 4-3 and 11-5 are in Col ecotype. Exons (bars) and introns (lines) are presented according to gene models at TAIR8 website (www.arabidopsis.org) for At2g41560 (ACA4) and At3g57330 (ACA11). The primers corresponding to the T-DNA left border are 245 (5’-CATTTTATAATAACGCTGCGACATCTAC-3’), 1682 (5’-ATTTTGCCGATTTCCGGAAC-3’) or 638 (5’-AGGTGAATCTTAATGGTGTG-3’). Primers used to genotype the alleles are the following: 1346rk (5’-CCCATCTAGCCACATTTACTATTGTTTGAAGT-3’), 1346h (5’-GAGGGAAGAAAGAACCTTTAGTTTGAA-3’), 1347d (5’-ACGTCCCCATTTTGGCCACAT-3’), 1347c (5’-GCCTTTTCAGAATGATAATAGCTTTGCTTCC-3’), 621 (5’-AGTAACCCATGACACCAAGAG-3’), 1346g (5’-TCTAATCCACACTTTTGCATAC-3’), 1347rg (5’-CTTCTGTGTTTGCATTTCCTTCTTCT-3’), and 1347e (5’-CATTTTATAATAACGCTGCGACATCTA-3’).

B- Location of insertions in the context of the protein topology. Both ACA4 and ACA11 are highly similar and have the same general topology. Grey arrows indicate the site at which T-DNA insertions would be expected to create truncated transcripts and proteins. The positions of cytosolic and vacuolar loops (black and grey bars, respectively) and transmembrane segments (white bars with stripes) are shown.

Figure 2. Lesion phenotype in aca4/11 can be suppressed by transgene expression of an ACA11-GFP fusion.

A- Lesions are shown for 20 days-old leaves from plants cultivated hydroponically. Representative images are shown for knockouts and controls in the Col (upper panels) and WS (lower panels) ecotypes.

B- Pictures of soil-grown plants. Col Wild Type (WT), aca4-3/11-5 and aca4-3/11-5 plants transformed by ACA11-GFP construct under the 35S (35S-ACA11-GFP) or the native promoter (ACA11p-ACA11-GFP) are compared. Rescued lines were recovered at a frequency of 21 out of 28 for the 35S-ACA11-GFP construct (e.g. ss1355-1358), and 2 out of 8 for the ACA11p-ACA11-GFP construct (ss1353 and 1354).
C- Immunodetection of ACA11-GFP fusion. 30μg of membrane proteins from WT, aca4-3/11-5, and two lines of aca4-3/11-5 transformed by ACA11-GFP construct under the 35S (35S-ACA11-GFP) or the native promoter (ACA11p-ACA11-GFP) were analyzed by immunoblot for their reaction with an antibody raised against GFP. Bands corresponding to the expected size of the GFP tagged ACA11 were detected at 137kDa.

Figure 3. Nutritional supplements suppress the lesion phenotype of aca4/11. Nine day-old seedlings were transferred from in vitro culture to a standard hydroponic solution supplemented with either an additional 15 mM NH₄NO₃, 15 mM KCl or 15 mM KH₂PO₄. Pictures taken 7 days later show the development of lesions in aca4-1/11-1 plants under standard conditions, but not in plants with anion supplements.

Figure 4. ROS production and callose deposition increase rapidly following a removal of a lesion suppression media. Plants (WT in black circles, aca4/11 in open circles) were grown for 10 days under “suppressing conditions” provided by a 15 mM NH₄NO₃ supplement to the standard hydroponic solution. Plants were then transferred to the standard hydroponic solution for the indicated time before harvest. A- ROS production was monitored by staining with diamino benzidine (DAB). B - Callose deposition was monitored by staining with aniline blue. C-The area of lesions was calculated from aniline blue stained leaves. Values and are error bars are reported as the mean ± SEM (Fig. 4C: median ± 75 percentile). n=8 individual leaves (except t=0, n=4).

Figure 5. Lesion initials occur preferentially in mesophyll cells, as detected by aniline blue staining. Plants were grown and lesions were induced as explained in figure 4. Each micro-lesion was detected as a callose deposit and recorded according to its relative position in the leaf. Diagram to the right shows a transverse cut of Arabidopsis leaf. with labels corresponding to different cell types (S: stomate, E: epidermis, P: parenchyma cells, M: mesophyll cells).

Figure 6. The aca4-1/11-1 mutants have total Ca²⁺ levels that are similar to wild type (WS) under both suppressed and lesion triggered conditions. Plants were grown hydroponically for 20 days under lesion suppressed conditions (i.e., with 15 mM NH₄NO₃ supplement) and either harvested directly (lesion suppressed samples) or transferred to standard hydroponic solution for an additional 30h before harvest (lesions triggered samples). Ca²⁺ concentrations were determined by ICP-AES (n=12 plants) and are reported as the mean ± SEM.
Figure 7. The *aca4/11* mutants show total anion levels similar to wild type under lesion-suppressed conditions, but a more rapid loss of Cl⁻ and NO₃⁻ when transferred to lesion induction conditions. 20 day old plants were grown hydroponically under 15 mM KCl (left), 15 mM NH₄NO₃ (middle) or 15 mM KH₂PO₄ (right) before harvesting (clear columns, “supp.”) or transferred into regular hydroponic solution 30h prior to harvesting (grey columns, “lesions trig.”). Anion content was determined after chloroform/water extraction using liquid chromatography and normalized to the fresh weight (FW) of extracted leaves. Average results (±SEM) for 2 independent experiments (n>16 for KCl and NH₄NO₃ suppression experiments, and n = 6 for KH₂PO₄ suppression experiments) are presented for WS wild type plants (black bars) and *aca4-1/11-1* (white bars). Within a subgraph, conditions sharing common labels (letters) are not significantly different from each other (p>0.05).

Figure 8. Lesions in *aca4/11* mutants can be suppressed by reducing the levels of SA. Pictures are shown of *aca4-1/11-1* with and without a *sid2-5* mutation 10 days after transfer into hydroponic solution. A similar phenotype is observed with *NahG* expressed in *aca4-3/11-5*. Refer to figure 2A and B for comparison to *aca4/11* mutants.

Figure 9: An SA dependent pathogen defense response is accelerated by an *aca4/11* knockout. Leaves of 4-week-old plants (wild-type Col-0, *sid2*, *sid2/aca4/11* and *aca4/11*) grown under lesion-suppressed conditions (50 mM KH₂PO₄) were sprayed with suspensions of *P. syringae* DC3000 (OD₆₀₀=0.01). A and B - Bacterial growth determinations were performed at the times indicated. Data points are the average of three replicate samples (±SD). C - Total RNA was isolated from leaves harvested at the indicated time points after bacterial inoculation. The panel section labelled PR1 shows an autoradiogram of a Northern blot probed for the defense related gene *PR1*. The section labelled rRNA shows a control for equal loading of RNA, as visualized by ethidium bromide staining of rRNAs. The Northern blot shown is representative of two independent experiments showing equivalent results. D – Representative leaves from the different plant lines assayed are shown 54h after bacterial inoculation. Note the fully developed lesions in the *aca4/11* leaf, whereas the other leaves show only yellow chlorotic patches.
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