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Human GLTP and mutant forms of ACD11 suppress cell death in the Arabidopsis acd11 mutant

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Abstract

The *Arabidopsis acd11* mutant exhibits runaway, programmed cell death (PCD) due to loss of a putative sphingosine transfer protein (ACD11) with homology to mammalian glycolipid transfer protein (GLTP). We demonstrate that transgenic expression in *Arabidopsis thaliana* of human GLTP partially suppressed the phenotype of the *acd11* null mutant, resulting in delayed PCD development and plant survival. Surprisingly, a GLTP mutant form impaired in glycolipid transfer activity also complemented the *acd11* mutants. To understand the relationship between functional complementarity and transfer activity, we generated site-specific mutants in ACD11 based on homologous GLTP residues required for glycolipid transfer. We show that these ACD11 mutant forms are impaired in their *in vitro* transfer activity of sphingolipids. However, transgenic expression of these mutant forms fully complemented *acd11* mutant cell death, and transgenic plants showed normal induction of hypersensitive cell death upon infection with avirulent strains of *Pseudomonas syringae*. The significance of these findings with respect to the function(s) of ACD11 in sphingolipid transport and cell death regulation is discussed.

Introduction

The recessive *Arabidopsis acd11* mutant initiates defense-related programmed cell death (PCD) at the early seedling stage, leading to a lethal phenotype before flowering [1]. The ACD11 protein has homology to glycolipid transfer proteins (GLTPs) found in most eukaryotes (Figure 1A). The founding member of this protein family is the mammalian GLTP that facilitates intermembrane transfer of glycosphingolipids (GSLs) [2,3]. The biological role of mammalian GLTP remains unknown, but the protein is well characterized structurally and biophysically [4]. The crystal structure of GLTP in complex with several different GSLs identified amino acid residues required for substrate binding [5-7]. A biological role of a GLTP-like protein has recently been found by studying mammalian FAPP2, which contains a c-terminal domain homologous to GLTP as well as an N-terminal PH domain. FAPP2 was originally identified as a protein that interacted with phosphatidylinositol-4- phosphate (PtdIns4-P; [8]), and has been shown to be involved in transport of lipids to the plasma membrane [9,10]. FAPP2 localizes to the Golgi apparatus

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through interaction between the PH domain and PtdIns4-P in an ARF-dependent manner [11], and it mediates glycolipid transfer via its GLTP domain [12]. Furthermore, FAPP2 is required for the synthesis of complex GSLs in the Golgi [12,13].

In addition, the *Podospora anserina* GLTP homologue HET-C2 is involved in non-allelic vegetative incompatibility together with the HET-E and HET-D loci, in a cell death reaction accompanied by autophagy [14-16]. HET-C2 has been shown to transport glycosphingolipids in a manner similar to GLTP [17]. To test the potential lipid transfer activity of ACD11 in a manner similar to GLTP, HET-C2 and FAPP2, a lipid transfer assay was performed with galactosylceramide, ceramide and sphingosine as potential substrates. This showed that ACD11 was able to accelerate transfer of sphingosine, but not ceramide and galactosylceramide between membranes, indicating substrate or cargo specificity different from the previously described GLTPs [1]. In order to further elucidate the role of lipid transfer in control of *acd11* cell death, we decided to investigate whether GLTP could substitute for ACD11 *in planta*. We show here that wtGLTP as well as a mutant form impaired in lipid transfer partially complements *acd11*. We also show that ACD11 is likely to adopt a GLTP-like structure, and that conserved residues between ACD11 and GLTP are required for ACD11 lipid transfer activity. In addition, these ACD11 activity mutant forms fully complement *acd11* cell death, and induce a normal hypersensitive response (HR) when challenged by avirulent bacteria.

Results

Several of the residues required for GLTP activity are conserved in ACD11 (Figure 1A), including D48 and H140 that participates in hydrogen bonding between GLTP and the amide group of the sphingoid long chain base (LCB). D48 and H140 are therefore likely determinants of the specificity of sphingolipid binding by GLTP [7]. Importantly, sequence alignment indicates that GLTP W96, which is important for lipid binding and for membrane interaction [7, 18], appears to be replaced by R103 in ACD11. In order to further illustrate the structural similarity between ACD11 and GLTP, the crystal structure of the glycolipid-free form (PDB: 1swx) of GLTP was used as a template for structural modeling of the ACD11 amino acid sequence. Three different public online services were tested (CPH models, Swiss Model & 3D Jigsaw) which all generated similar structures. 3D jigsaw [19] was used for further studies, and the validity of the structure was evaluated in a Ramachandran plot visualizing Phi-Psi angles [20]. As shown in Figure 1B, the predicted structure of ACD11 is very close to that of GLTP, and the environment around the putative binding center has a similar configuration (Figure 1C-D), as has been noted previously [21]. These data indicate that ACD11 is capable of forming a GLTP-like structure.

Another way to confirm the functional relevance of homology between two proteins across kingdoms is to express one of the proteins from a transgene and assess its ability to complement the phenotype of the loss-of-function mutant of the other protein. This strategy has been extensively used in yeast, but has also been applied in *Arabidopsis* with a human transgene [22]. Since the runaway cell death phenotype of the *acd11* loss-of-function mutant is easy to observe, we undertook transgenic complementation experiments expressing human GLTP in *Arabidopsis acd11* mutants. To this end, wild type wtGLTP, as well as four point mutants, were cloned into a plant expression vector containing an N-terminal Myc-tag under control of a constitutive Cauliflower mosaic virus 35S promoter (Figure 2B), and transformed into heterozygous *acd11* mutant plants. The four GLTP point mutants were D48V, W96A, H140L and F148S, which have previously been shown to be affected in their transfer activities [5,7]. Several independent transgenic lines for each construct were examined. All transgenic T2 lines of GLTP forms with one of three point mutations W96A, H140L or F148S segregated in a 1:3 Mendelian fashion for the recessive *acd11* PCD

phenotype, indicating non-complementation by these forms. Western blotting confirmed the expression of these Myc-Tagged protein forms in the non-complementing lines (figure 2C). In contrast, transgenic expression of wtGLTP and of the D48V form resulted in growth affected, but nevertheless surviving plants. This indicates that wtGLTP and the D48V form partially complement the loss of ACD11. Two independent transgenic lines expressing wtGLTP or GLTP D48V were chosen for further analysis. In the first weeks after germination, *acd11* homozygous plants expressing either transgene were indistinguishable from wild type seedlings or *acd11* plants complemented with a corresponding constitutively expressed wild type ACD11 construct (Figure 2A, top). Even after *acd11* homozygous plants were completely dead, complemented plants set new, green leaves. In addition, the onset of PCD was invariably more delayed in the two lines expressing D48V than in those expressing wtGLTP (Figure 2A, bottom). PCR analysis showed that the transgenic plants were indeed homozygous for the *acd11* mutation (Figure 2D), and Western blotting confirmed that Myc-tagged wtGLTP and D48V mutant forms were expressed in these plants (Figure 2E). In addition, sequencing of transgene specific PCR products reconfirmed the identity of transgenes. Transgenic wtGLTP was apparently expressed to a higher level than D48V, which might account for the presumptive degradation products that are not seen in D48V. In addition, the D48V GLTP form migrated slightly faster than wtGLTP in SDS-PAGE. The reason for this difference is not known.

In order to obtain a quantitative measure of the degree of complementation, an inducible *acd11*/nahG death system was used. PCD in *acd11* is dependent upon the plant hormone salicylic acid (SA), and transgenic introduction of the bacterial salicylate hydroxylase nahG completely rescues *acd11* mutants. However, application of BTH, a functional analog of SA not catabolized by nahG, restores cell death in *acd11*/nahG [23]. The kinetics of BTH-induced cell death can be followed by measuring the leakage of ions from dying and dead cells as a rise in conductivity of a water bath [24,25]. As shown in Figure 3A, there was almost no measurable ion leakage in wild type L*er*, nahG or *acd11*/nahG plants complemented with constitutively expressed ACD11, while *acd11*/nahG began to exhibit significant ion leakage approximately 70 hours after BTH application. Three different transgenic lines expressing wtGLTP in the *acd11*/nahG genetic background significantly delayed this BTH-induced cell death up to 30 hours, and the level of complementation corresponded to the transgenic protein level (not shown). The complemented D48V-2 line (Figure 2A) was crossed to *acd11*/nahG, yielding homozygous *acd11* F1 plants dominantly heterozygous for both the nahG and D48V transgenes. These plants were used together with the best complementing wtGLTP line in the experiment shown in Figure 3B. This shows that the D48V GLTP form suppressed cell death to an extent similar to wtGLTP, when using the BTH inducible *acd11*/nahG death assay.

As noted above, a number of amino acid residues in GLTP have been shown to be essential for lipid transfer [5-7]. In order to gain insight into the structural background of ACD11 function, we decided to mutate the corresponding residues in ACD11. H143 and D60 were changed to Leucine and Valine respectively, in line with the published GLTP mutants, while R103 was mutated ‘back’ to Tryptophan to assess whether this ACD11 form behaved more similar to GLTP. The mutated forms were generated using the genomic ACD11 region, including introns, 1.5 kb 5’ upstream promoter sequence, and 500 bp downstream of the 3’ UTR. Both wild-type ACD11 (gACD11 for genomic ACD11) as well as the three mutants exhibited transgenic complementation at the T2 stage, scored by identifying several lines carrying each construct that were homozygous for the *acd11* mutation. Two *acd11* homozygous transgenic lines of each genotype were selected, and T3 lines homozygous for both the transgene and *acd11* were isolated (Figure 4A-B). The ACD11 protein level was investigated using a polyclonal rabbit anti-ACD11 antibody. This showed that all lines, except H143L, expressed comparable protein amounts that were slightly higher than seen in

wild type L*er* (Figure 4C). The reason for the lower levels of ACD11 H143L in the transgenic lines could be attributable to lower recognition efficiency of the ACD11 antibody for this mutant. All transgenic lines were confirmed multiple times by sequencing PCR products generated using transgene specific primers (not shown).

In order to study whether the generated ACD11 point mutants were affected in their lipid transfer capabilities, recombinant protein was used in established lipid transfer assays using radiolabeled lipids [1,2,7]. ACD11 was capable of transferring sphingosine while GLTP was more or less inactive towards this lipid (Figure S1), confirming previously published data [1]. D60V, R103W & H143L were all affected in their activity in varying degrees (Figure S1). The significant solubility of sphingosine [26] results in relatively fast spontaneous transfer of this labeled lipids between donor and acceptor vesicles. The resulting high background activities hamper detailed studies of transfer activity. Different, available substrates were tested to counter this experimental drawback, and it was found that ACD11 also has activity towards sphingomyelin (Figure 5A). Although sphingomyelin (SM) is not detectable in plant tissues [27], it may serve as a model for studying ACD11 transfer activity. In assays for SM transfer, the H143L and D60V ACD11 forms were completely unable to transfer lipid, while wtACD11 and the R103W form had some activity (Figure 5A). This strongly indicates that H143L and D60V are deficient in transfer. The R103W form did not 'gain' transfer activity towards galactosylceramide (not shown), indicating that this mutation alone is not sufficient to change ACD11 into a glycolipid transfer protein. Elaborating on this, we studied transfer activity of increasing amounts of protein at a fixed timepoint. H143L and D60V seem to begin to show a limited capacity to transfer SM at high protein levels, supporting the idea that they are not globally misfolded, but that the point mutations result in specific defects in the lipid recognition site (Figure 5B).

The PCD seen in dying *acd11* plants has many features in common with the hypersensitive response (HR), which is initiated upon pathogen infection recognized by a host plant resistance (*R*) gene [28,29]. *acd11* mutants can be rescued by mutations in the well-known defense regulators EDS1 and PAD4 [1], which are required for resistance mediated by the resistance gene RPS4 but not by RPM1 [30,31]. Despite the convincing complementation data, it still remains possible that ACD11 mutant forms would behave differently from the wild type upon induction of HR. To examine this, complemented transgenic lines were infiltrated with avirulent strains of the pathogenic bacteria *Pseudomonas syringae* expressing the avirulence factors avrRPM1 or avrRPS4, which induce host HR dependent on RPM1 or RPS4, respectively. The HR was measured using ion leakage and, as shown in Figure 6, infiltration with avrRPM1 and avrRPS4 produced a similar HR in all tested lines.

Discussion

Previous links between sphingolipids and PCD in *Arabidopsis* include identification of the *acd5* mutant, which exhibits spontaneous PCD in a manner similar to *acd11*. *ACD5* encodes a protein with ceramide kinase activity, and non-phosphorylated ceramides accumulate in the dying plant [32]. Also, treating plants with the fungal inhibitors of ceramide synthase, AAL toxin and fumonisin B1, leads to cell death and accumulation of sphinganine and phytosphingosine [33]. A plant homologue of yeast LAG1 (Longevity assurance gene) called Asc1 encoding a putative ceramide synthase was shown to confer resistance to FB1 and AAL in tomato [34,35]. Here we show that transgenic expression of human wild type wtGLTP and point-mutated D48V GLTP partially rescued the lethal phenotype of *acd11* mutant plants. D48V GLTP was previously shown to be impaired in transfer activity [5,7]. In contrast to D48V, three other previously described point mutants (W96A, H140L, F148S) affected in lipid transfer activity failed to rescue *acd11* death. We also show that mutating residues conserved between ACD11 and GLTP leads to losses in transfer activity, and that

mutated ACD11 forms are capable of fully complementing the *acd11* mutant. These results indicate that although sphingolipids may be involved in regulating defense related PCD, sphingolipid transfer activity is, at least in part, dispensable for PCD suppression by ACD11.

The following speculative model, based on two sets of inferences, may be proposed for ACD11 function and its role in PCD induction. First, ACD11 maintains or regulates a non-essential function related to sphingolipids. Although 4-unsaturated sphingosine in *Arabidopsis* is present in very low amounts [36], sphingosine and sphingosine-1-phosphate, as well as phytosphingosine and phytosphingosine-1-phosphate, have been implicated in the regulation of stomatal aperture [37-39]. This confirms that sphingolipids have signaling properties in plants, as they have in mammalian cells [26]. Thus, any of these compounds might be bound and/or transferred by ACD11. In addition, we have recently found that ACD11 interacts with proteins involved in vesicular trafficking between the ER and Golgi (unpublished), suggesting that ACD11 is involved in the transport or sensing of sphingolipids in or associated with these subcellular compartments.

Second, it is well documented that some plant proteins are ‘guarded’ such that the plant immune system is capable of directly or indirectly sensing their perturbation by pathogen effector proteins [40]. In this way, plants can sense the effects of diverse invading pathogens, and induce defense responses including the HR. Thus, the cellular complexes in which ACD11 functions might be targeted by one or more pathogen effectors to promote colonization and infection, and in addition be ‘guarded’ by a host plant defense mechanism, in a situation similar to that of RIN4 whose knockout is embryo lethal in an *RPS2*-dependent manner [41]. If so, *acd11* knockout mutants could sense the absence of ACD11 protein and trigger downstream defense responses leading to accelerated cell death. This might be achieved through direct interaction of ACD11 with an unknown R protein, or via a protein normally associated with ACD11. In the case of mutated forms of ACD11 that complement *acd11* loss-of-function, the plant still perceives the ACD11 related mechanism as being intact, leading to a wild type appearance. GLTP on the other hand is only capable of partially substituting for ACD11 and different physical properties of the D48V GLTP form, such as stability or complexing capability, might account for the different degrees to which wtGLTP and D48V rescue *acd11* null mutants. The reason for the non-complementation of W96A, H140L and F148S mutants are not known. The known suppressors of *acd11* PCD (nahG, *pad4* and *eds1*) would all function downstream of ACD11, inhibiting general defense responses and the HR.

An alternative explanation of the PCD seen in *acd11* may be that absence of ACD11 induces a compensatory over-reaction through a similar mechanism, which may lead to accumulation of PCD inducing sphingoid species. In line with this model, one of the most highly upregulated genes in *acd11* mutants encodes the closest ACD11 homolog in the *Arabidopsis* genome (*At4g39670*, unpublished results). This homolog shares 45% identical residues with ACD11 including R103, suggesting similar substrate or cargo specificities. If this is the case, then certain sphingolipid species might be erroneously transported to locations where they are perceived as, or promote, pro-death signals. Again, mutated ACD11 forms and transgenic GLTP would be fully or patially percieved as functional ACD11.

In keeping with such a function, it is interesting that some forms of ecotype incompatibility in *Arabidopsis*, known as hybrid necrosis, have features similar to defense related HR, and are dependent upon at least one NB-LRR resistance gene [42]. This is intriguing considering the homology between ACD11 and the glycolipid transfer protein HET-C, as polymorphic forms of HET-C are involved in an incompatibility reaction in *P. anseria* together with

highly polymorphic HET-D and HET-E alleles [43]. Whether ACD11 has also been subject to rapid changes during evolution has yet to be investigated, but it is possible that ACD11 is involved in a self / non-self recognition system yet to be identified in *Arabidopsis*.

Materials and methods

Cloning and generation of transgenic plants

The entire 35S:Myc:Nos cassette from pMYC2 was sub-cloned using EcoR1 into pCAMBIA1300. The wtGLTP cDNA, as well as the D48V, W96A, H140L & F148S mutants were amplified from constructs in the pET30 expression vector, using primers *5'GLTP Sst1: GTGGAGCTCTTATGGCGCTGCTGGCCGAAC* and *3'GLTP Sst1: GTGGAGCTCCTACACCTTGTAGTTAAGCTC* and cloned into pCAMBIA1300:Myc using Sst1. The genomic ACD11 region was amplified from wild type L*er* DNA using primers *ACD11 prom: TGAATCTAGACTCCGGTGG CGAGAATCCCGGCG and ACD11 3'UTR: TTTGAGCTCTAACGAAGACGCTT TTGTCCG*, subcloned into pGEM t-easy and then mutated using PCR based site directed mutagenesis. The resulting products were cloned into the binary pCAMBIA3300 vector using XbaI/SacI. All constructs were checked by sequencing, and subsequently transformed into heterozygous *acd11* L*er* plants by *Agrobacterium*-mediated transformation. wtGLTP was also transformed directly into *acd11*/nahG plants, while D48V was crossed into *acd11*/nahG as described in the text. Transgenic plants, selected on hygromycin, were isolated and selected on kanamycin in T2 to select for plants harboring the *acd11 Ds* transposon insertion [44]. Lines containing the transgene as well as the *acd11* transposon were set out as T3 seeds, reconfirmed by sequencing transgene specific PCR products, and observed closely during the first weeks after germination.

Western blotting

Leaf discs (26.3 mm^2) were taken from 5 week-old seedlings and extracted with SDS-containing loading buffer. Samples were boiled 8 minutes and centrifuged at 13000rpm for 15 min to remove cell debris. 10μl aliquots were loaded on 15% SDS polyacrylamide gels, which were subsequently blotted onto nitrocellulose membranes at 150v/gel for 1 hour using a Whatman Biometra semidry blotter (Fastblot B44). Primary antibody (anti-c-Myc 9E10 (Santa Cruz Biotechnology)) or rabbit polyclonal anti-ACD11, 1:2000 dilution) was used overnight in PBS containing 5% BSA, and secondary AP conjugated, anti-mouse (Myc) or anti-rabbit (ACD11) antibody (1:2000) was used for 1 hour. Blots were developed using NBT/BCIP in AP buffer (100mM NaCl, 5mM $MgCl_2$, 100 mM Tris pH 9.5) for 15-30 minutes. The reaction was stopped by washing in water.

BTH induced ion leakage

6-8 week-old, short day grown plants were sprayed with 100μM BTH once. After 48h incubation, leaf discs (19.1 mm^2) were harvested, and 4 discs submerged in 3 ml miliQ H_2O. Ion leakage was measured using an Ecoscan Con 5 from Eutech Instruments. The results shown are averaged from 3 samples, and standard deviations indicated.

Pseudomonas infiltration assays

Over-night cultures of *Pseudomonas syringae* was grown in NYG medium to an OD_{600} of 0.2, washed and resuspended in 5mM $MgCl_2$. Using a 5 ml syringe, bacteria was injected directly into leaves of short day grown plants of approx. 6 weeks of age. Leaf discs (26.3 mm^2) were punched out, washed in miliQ H_2O for 30 min, and then incubated in 4 ml miliQ H_2O for measurement of ion leakage as above.

Recombinant protein purification

The ACD11 cDNA was cloned into the pET30 His-tagged expression vector from Novagen, and site-directed mutagenesis used to generate D60V and H143L. ACD11 R103W in pET30 was produced by site-directed mutagenesis in pGEM T-easy, and subsequently cloned into pET30. Expression plasmids were transformed into BL21(DE3) cells (Novagen). Large-scale protein production was performed as follows: 500ml culture was grown to OD_{600}= 0.5, cooled to 20°C, induced with IPTG (final concentration 1 mM) and grown over night at 20°C. The buffer (B) used for all other solutions contained 100 mM Tris-HCl (pH 7.0), 150 mM NaCl and 10 % Glycerol. Cell pellets were resuspended in 20 ml lysis buffer (B + 10 mM imidiazole, 1 mg/ml Lysozyme, 10 mM ß-mercaptoethanol), sonicated in 10 bursts of 45 seconds, and centrifuged for 20 min at 10.000 rpm in a SLA-1500 Sorval rotor. The lysate was applied to a Qiagene Ni-NTA column pre-equilibrated with B + 10 mM imidiazole. The column was washed with 10 ml (B + 10 mM imidiazole), and then eluted with 5 ml each of 50 mM, 100 mM, 150 mM & 200 mM imidiazole. 1 ml fractions were collected and measured for protein concentration.

Sphingolipid intervesicular transfer

Protein-mediated transfer of radiolabeled sphingolipid was performed as described previously [2,5]. Lipids for the desired vesicle composition were mixed in chloroform/methanol and dried under a stream of nitrogen. Residual solvent was removed under high vacuum for 4 h. The dry lipid films were hydrated in 20 mM NaH_2PO_4 (pH 7.4) containing 0.5 mM EDTA and 0.02% NaN_3 by vigorous vortexing and brief bath sonication. Donor vesicle mixtures consisted of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC; 88 mole%), negatively-charged dipalmitoylphosphatidic acid (10 mole%), [^{3}H]-sphingolipid (2 mole%), and a trace of nontransferable marker ([^{14}C]-tripalmitin). Acceptor vesicles consisted only of POPC. Probe sonication (Ultrasonic Processor W-225) was used to generate unilamellar vesicles, which were isolated by ultracentrifugation (100,000g × 60 min; 80% recovery) to remove contaminating titanium particles from the probe and partially-sonicated liposomes. In a typical sphingolipid transfer assay (0.5 ml), the donors (0.4 mM) and acceptors (4 mM) were incubated with the indicated protein amount (0.2 to 20 μg) at 37°C for the desired time interval. Recovery of acceptor vesicles was achieved by elution through DEAE-Sephacel minicolumns (1.5 ml bed vol.). Sphingolipid intervesicular transfer was quantified by liquid scintillation counting of [^{3}H]-sphingolipid in the recovered acceptor vesicles as previously described [2]. Control assays without protein enabled monitoring of spontaneous (non-protein mediated) intervesicular transfer. [^{3}H]-sphingolipid, consisting of either sphingomyelin (bovine) [choline-methyl-^{3}H] or sphingosine (D-erythro-[3-^{3}H]), were obtained from American Radiolabeled Chemicals Inc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BTH	benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
FB1	fumonisin B1
GSL	glycosphingolipid
HR	hypersensitive response
LCB	long chain base (sphingolipid backbone)
PCD	programmed cell death
SA	salicylic acid
SM	sphingomyelin

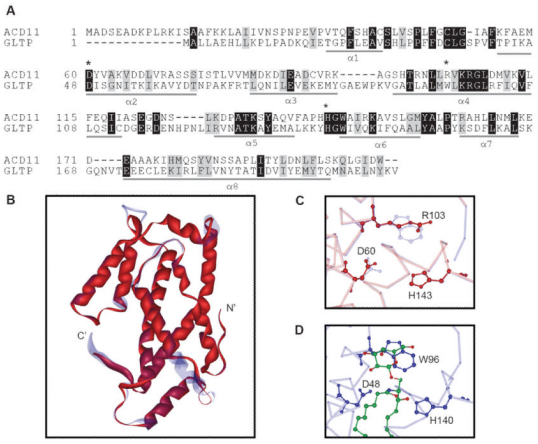

Fig .1.

ACD11 and GLTP protein alignment and modeling. (A) Gray lines depict alpha helices in GLTP (PDB: 1swx). Asterisks indicate residues mutated in this study. (B) Comparison of modeled structures of ACD11 (red) and the glycolipid-free form of GLTP (blue). (C) Comparison of the putative lipid recognition centers of the two structures. (D) Lipid recognition of GLTP in complex with GalCer (PDB: 2euk; 2evl)

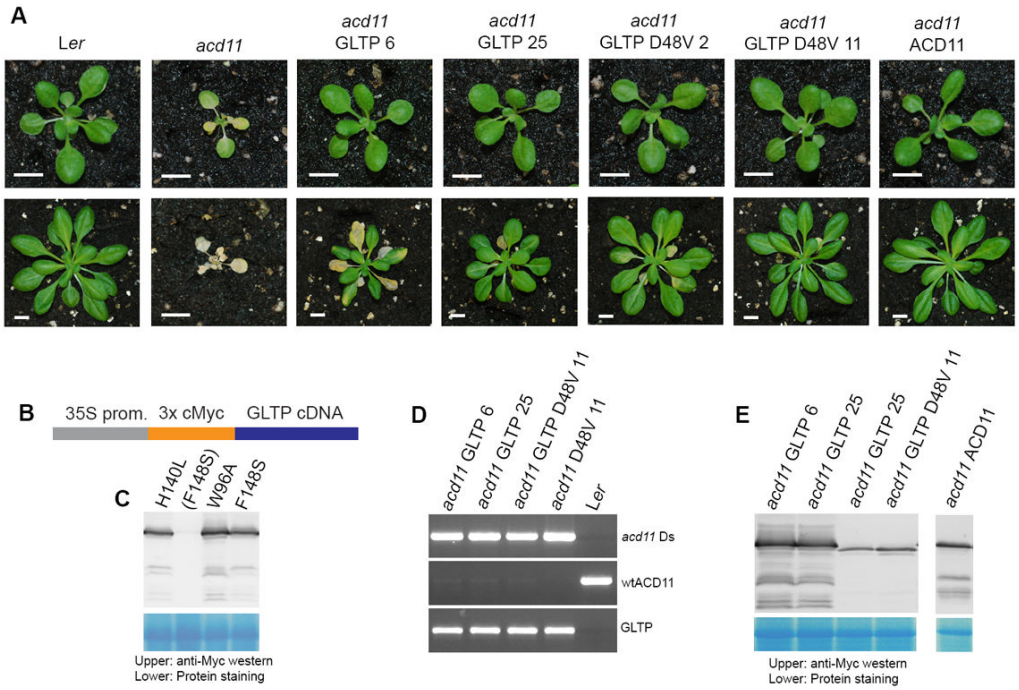

Fig. 2.

GLTP partially complements *acd11* PCD. (A) 4 week-old *acd11* mutants expressing GLTP transgenes are indistinguishable from wild type or plants harboring a wild type ACD11 transgene (top row). After 6 weeks wtGLTP plants are severely affected, while GLTP D48V plants show some early senescence (bottom). Scalebar = 0.75 cm. (B) Scheme of a transgene construct. (C) Non-complementing GLTP mutant forms were also expressed *in planta*. (F148S) indicates a transgenic line with low expression. (D) Complemented plants contain the *acd11 Ds* transposon insertion (top), no detectable *ACD11* wild type allele (middle) and a human GLTP transgene (bottom). (E) Complemented plants express Myc-tagged proteins. Primary antibody: anti-myc 9E10. Page blue staining shows equal loading.

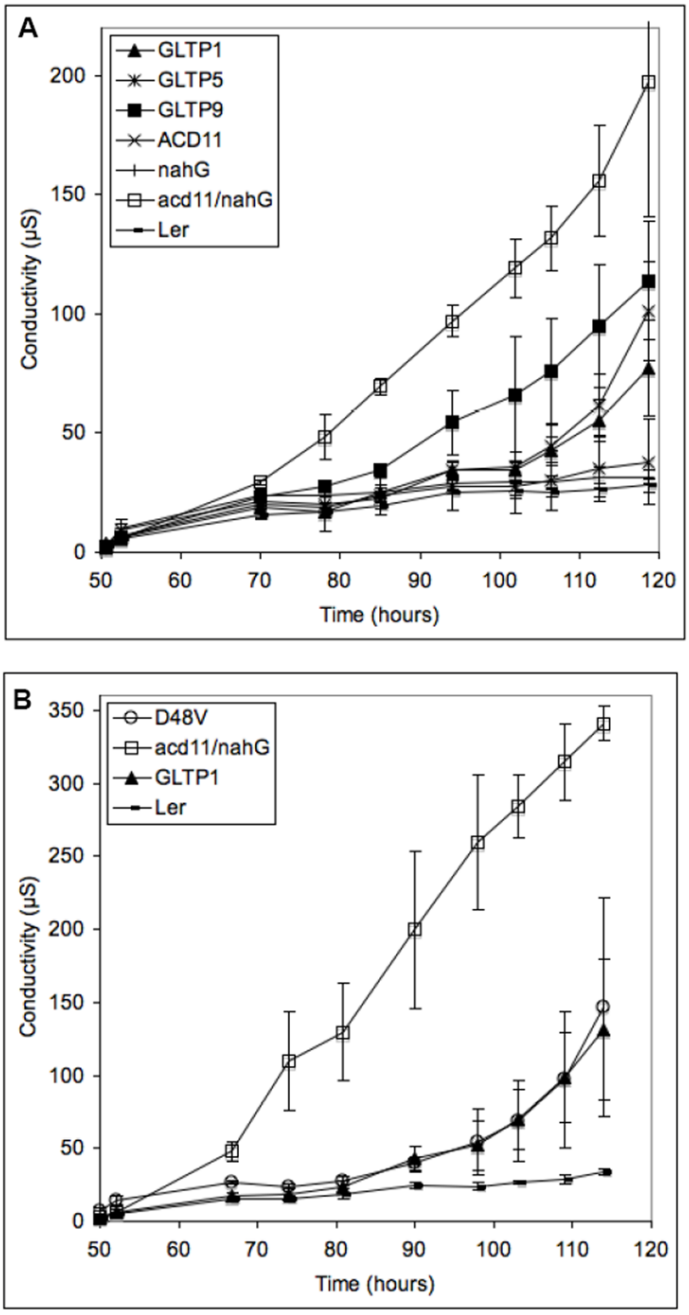

Fig. 3.

GLTP transgenes delay cell death measured by ion leakage. (A) Three independent transgenic wtGLTP lines significantly delay the onset of death, while ACD11, nahG & L*er* are unaffected. (B) Independent experiment showing that D48V crossed into *acd11*/nahG delays death to an extent similar to GTLP1.

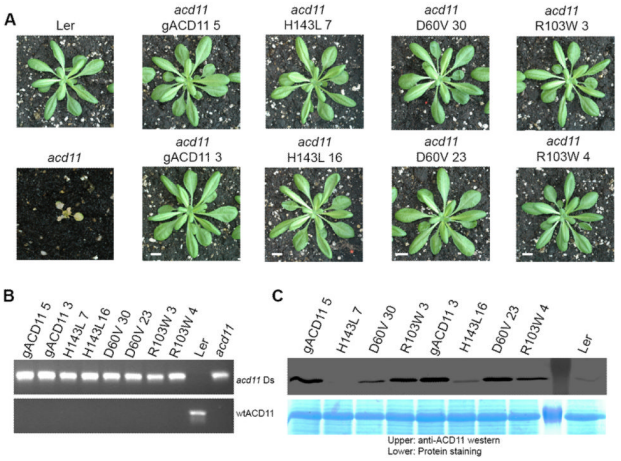

Fig. 4.

ACD11 activity mutants complement *acd11* PCD. (A) 8 week-old plants grown under short day conditions. gACD11 designates the wild type genomic ACD11 fragment, and H143L, D60V & R103W the three different lipid-binding mutants. Scalebar = 0.75 cm. (B) All transgenic lines are in the *acd11* mutant background containing the ACD11 *Ds* transposon insertion (top) but not the wild type *ACD11* locus (bottom). (C) Transgenic lines express ACD11 protein. All lines except H143L express higher protein levels than wild type L*er*.

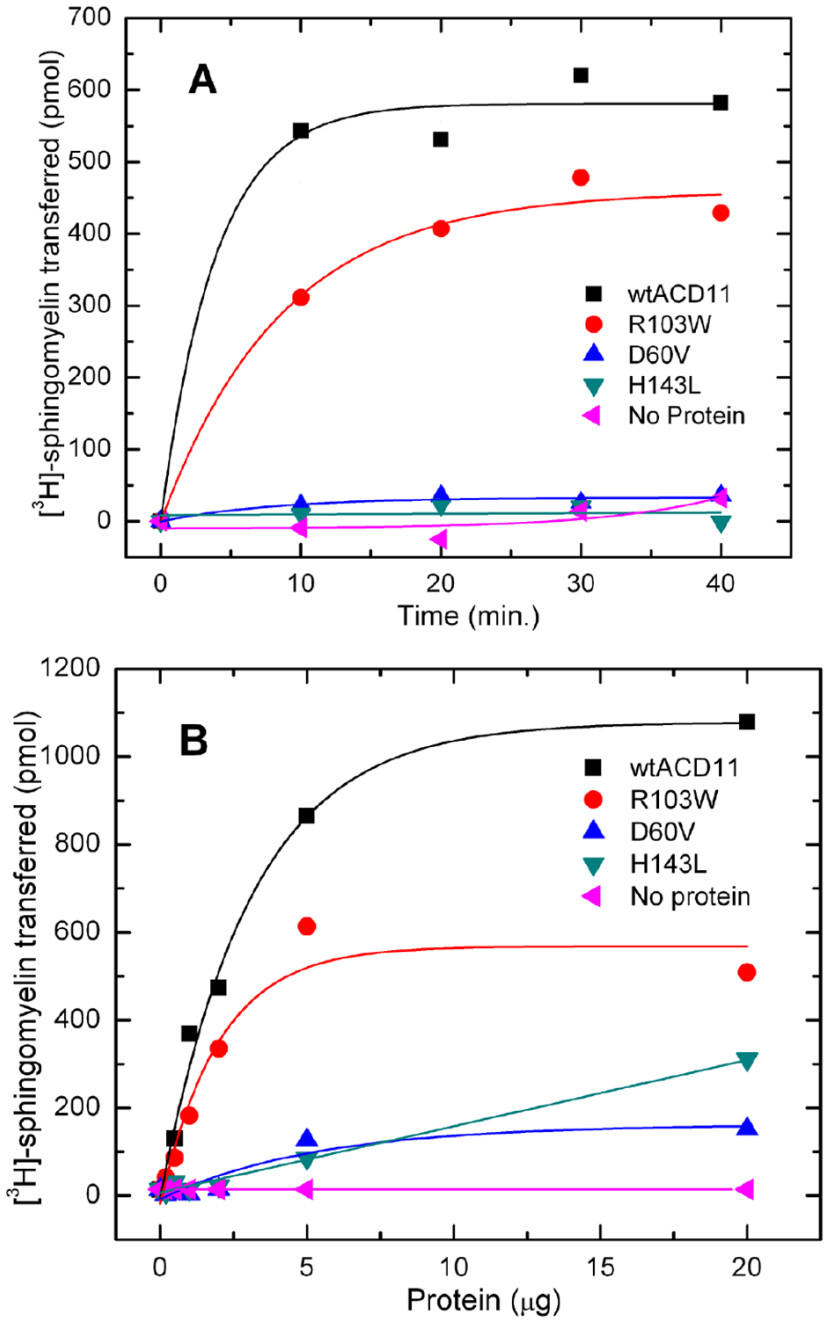

Fig. 5.

ACD11 point mutants are altered in their lipid transfer activities. (A) Sphingomyelin (SM) time course transfer assay using fixed protein amount (2 μg). ACD11 has SM transfer activity while H143L & D60V are completely inactive. R103W has intermediate activity. (B) SM transfer assay using increasing amounts of protein. The data points were fit using a first order exponential fitting routine. All data points represent duplicate assays (average values).

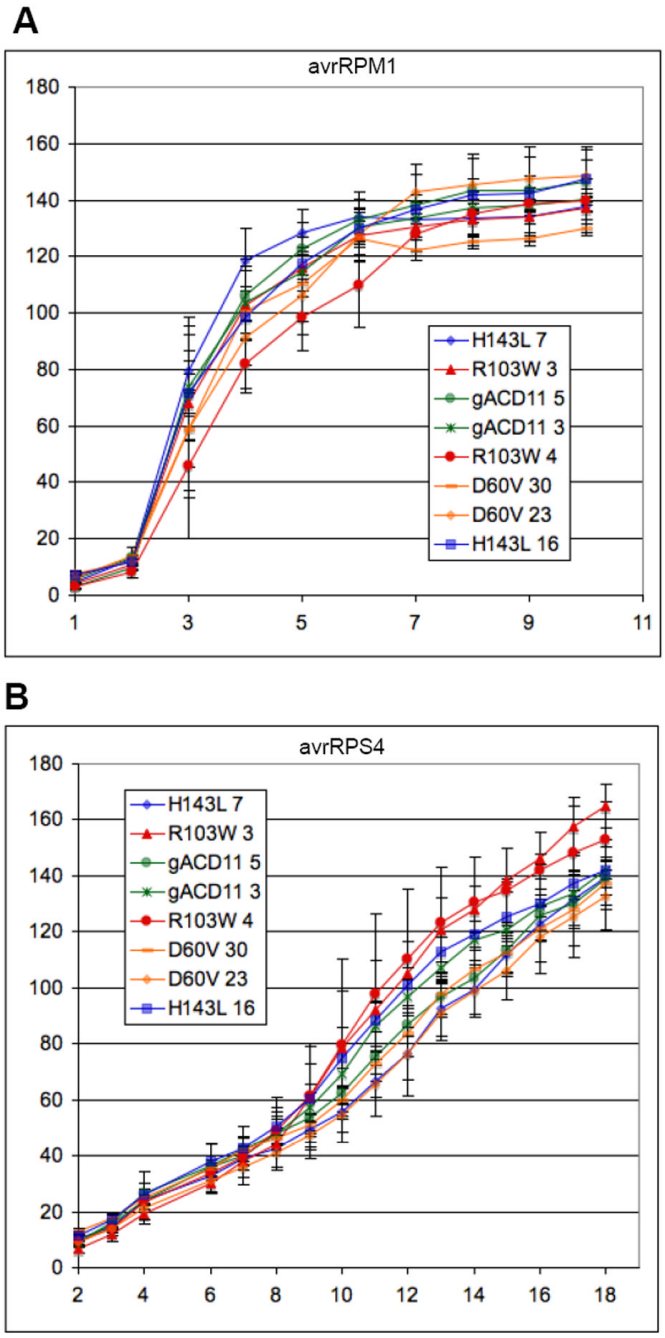

Fig. 6.

Plants expressing ACD11 point mutants infected with avirulent bacteria. (A) No significant difference is seen in infections with avrRPM1 expressing bacteria. All lines exhibit HR with similar kinetics. (B) avrRPS4 induced HR measured by ion leakage. A slightly higher leakage level is seen in the two R103W lines compared to the rest.