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Identification and phylogenetic analyses of VAS_t, an uncharacterized protein domain associated with lipid-binding domains in Eukaryotes

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Abstract

Background: Several regulators of programmed cell death (PCD) in plants encode proteins with putative lipid-binding domains. Among them, VAD1 is a regulator of PCD propagation harboring a GRAM putative lipid-binding domain. However the function of VAD1 at the subcellular level is unknown and the domain architecture of VAD1 has not been analyzed in details.

Results: We analyzed sequence conservation across the plant kingdom in the VAD1 protein and identified an uncharacterized VAS_t (VAD1 Analog of StAR-related lipid transfer) domain. Using profile hidden Markov models (profile HMMs) and phylogenetic analysis we found that this domain is conserved among eukaryotes and generally associates with various lipid-binding domains. Proteins containing both a GRAM and a VAS_t domain include notably the yeast Ysp2 cell death regulator and numerous uncharacterized proteins. Using structure-based phylogeny, we found that the VAS_t domain is structurally related to Bet v1-like domains.

Conclusion: We identified a novel protein domain ubiquitous in Eukaryotic genomes and belonging to the Bet v1-like superfamily. Our findings open perspectives for the functional analysis of VAS_t-containing proteins and the characterization of novel mechanisms regulating PCD.

Keywords: VAS_t, VAD1, Protein domain, Programmed cell death, GRAM domain, Bet v1-like

Background

Protein domain predictions are a starting point for a range of functional analyses and can either newly predict or further refine functional predictions [1]. Indeed, domains form structural, evolutionary and functional units of proteins [2]. The combination and order of domains in a protein is frequently considered as a fundamental level of protein functional complexity. The majority of proteins is composed of multidomain proteins and the domain composition of multidomain proteins is critical for their specialized functions [3]. Furthermore, domain combinations are not random, which may indicate functional cooperation [4].

Plant “lesion mimic mutants” (LMMs) show spontaneous necrotic lesion resembling the so-called Hypersensitive Response (HR), a form of programmed cell death associated with plant defense [5,6]. The *Arabidopsis thaliana vad1* (*vascular associated death1*) mutant is a LMM altered in a negative regulator of PCD and defense responses harboring a GRAM domain predicted to bind lipids [7,8]. Contrary to most LMM genes characterized to date, *VAD1* is expected to control the cell-to-cell propagation of PCD instead of its initiation [5]. GRAM is a ~70 amino-acids domain predicted to mediate intracellular protein binding or lipid binding during membrane-associated processes [9]. This domain is related to the PH domains and is found in animal glucosyltransferases, Rab-like GTPase activators, myotubularins and other membrane-associated proteins. The GRAM domain of human myotubularins is able to bind phospholipids and is involved in membrane signaling [10-12], but its exact function often remains

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enigmatic [13]. The presence of a GRAM domain in VAD1 protein suggests that lipid binding could be required for VAD1 function, but the role of VAD1 at the subcellular level is currently unknown.

Remarkably, a significant proportion of plant LMMs show mutations in genes associated with lipid biosynthesis and homeostasis [14-17]. This notably concerns sphingolipid metabolism: the *acd5* and *acd11* mutants carry mutations in a ceramide kinase and a putative sphingosine transfer protein, respectively [15,16]. Conversely, *ERH1* is a positive regulator of the HR encoding a functional inositolphosphoryl-ceramide (IPC) synthase which converts ceramide to IPC [18]. Furthermore, *EDR2* was isolated as a negative regulator of PCD and defense responses encoding a multi-domain protein featuring a DUF1336 domain, a PH domain and a START domain [17,19]. Like CERT PH domain, EDR2 PH domain preferentially binds to PI4P [19]. Nevertheless, the mechanisms by which lipid-binding domain containing proteins regulate PCD in plants are largely unknown.

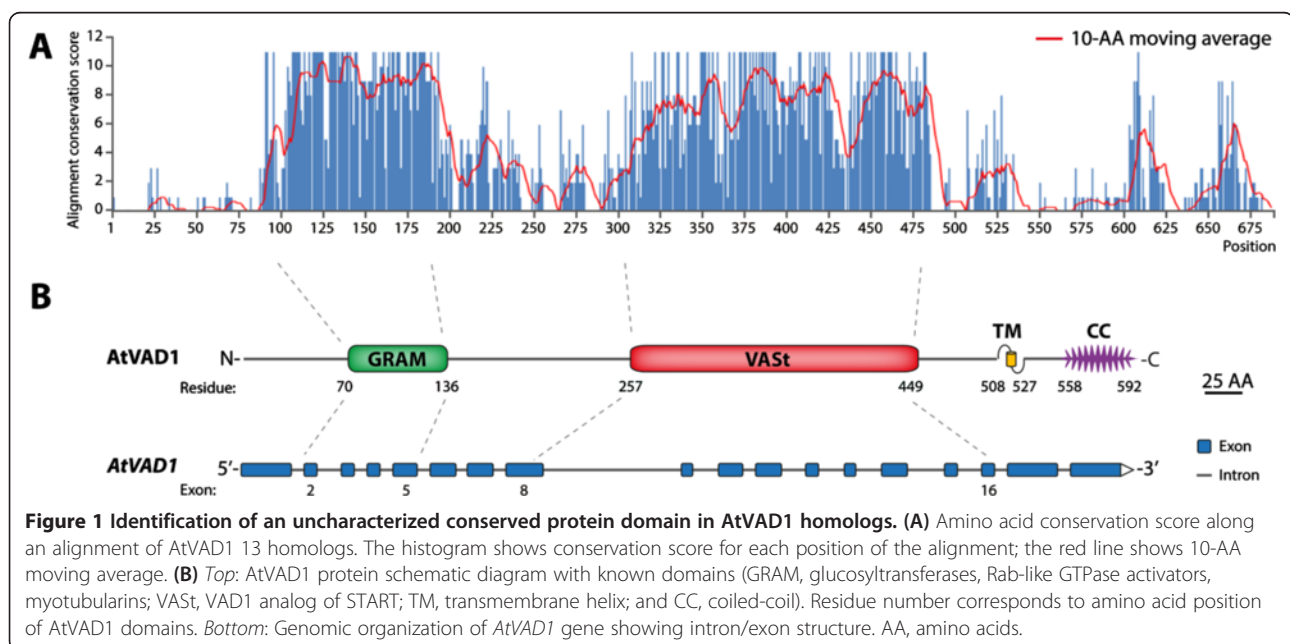
Here, we analyzed sequence conservation in the VAD1 family and identified an uncharacterized conserved domain we designated as VAS_t (VAD1 Analog of *St*AR-related lipid transfer). Using sequence- and structure-based phylogenetic analyses we demonstrate that this domain is present in all major eukaryotic lineages but no molecular function has been assigned to it. VAS_t is related to Bet v1-like, a superfamily including lipid- and hormone-binding domains. The VAS_t domain will be referred to with accession number PF16016 in release 28.0 of the Pfam database [20]. These findings open new perspectives for the functional analysis of VAS_t-domain containing proteins such as *A.*

thaliana VAD1, yeast YSP2 and human GRAM1A, B and C.

Results

Proteins in the VAD1 family contain an uncharacterized conserved domain

To get insights into VAD1 putative biochemical function, we analyzed protein sequence conservation among VAD1 homologs. First, to identify VAD1 homologs, we used the full length sequence of VAD1 protein in stringent phmmer searches against the Uniprot database. We identified 13 AtVAD1 homologs with e-value below $1e^{-100}$ across twelve angiosperm plant species, including monocots (*Brachypodium distachyon*, *Musa acuminata*, *Oryza brachyantha*, *Oryza sativa*, *Setaria italica*, *Sorghum bicolor*) and eudicots (*Arabidopsis lyrata*, *Glycine max*, *Ricinus communis*, *Solanum lycopersicum*, *Vitis vinifera*). In this stringent approach, all species showed a single VAD1 copy except *G. max* that had three copies. The retrieved homologs showed at least 55% identity and all contained a clearly identified GRAM domain. To identify conserved regions in these 14 sequences, we aligned them using the Multiple Sequence Alignment (MSA) tool MAFFT [21] and we plotted the consensus conservation score along the alignment using a ten Amino-Acids (AA) sliding window (Figure 1A). Two major conserved regions were clearly apparent. The first one spanning positions 80 to 200 in the alignment, and the second spanning positions 300 to 480. To characterize and precisely delimit VAD1 conserved regions we mapped VAD1 gene and protein annotations onto the conservation plot (Figure 1B).



The N-terminal conserved region (position 80–200) overlapped largely with the predicted GRAM domain (position 116–182). The second conserved region (position 300–480) corresponded to an uncharacterized domain of approximately 190 AA. This domain is encoded by a region spanning from *AtVAD1* exon 8 to exon 16. We therefore set the limits of this uncharacterized domain at positions 257 and 449, for a total length of 193 AA (Figure 1B). Based on further characterization described hereafter, we designated this domain as the VAS_t (VAD1 Analog of *START*) domain. A close up view on the MSA of the VAS_t domain revealed a high degree of conservation among plant homologs, with an average 70.5% identity over the VAS_t domain (Additional file 1: Figure S1, Additional file 2). Since no annotation could be mapped onto the VAS_t domain, it represents a yet uncharacterized protein domain highly conserved in plants.

The VAS_t domain is conserved among Eukaryotes

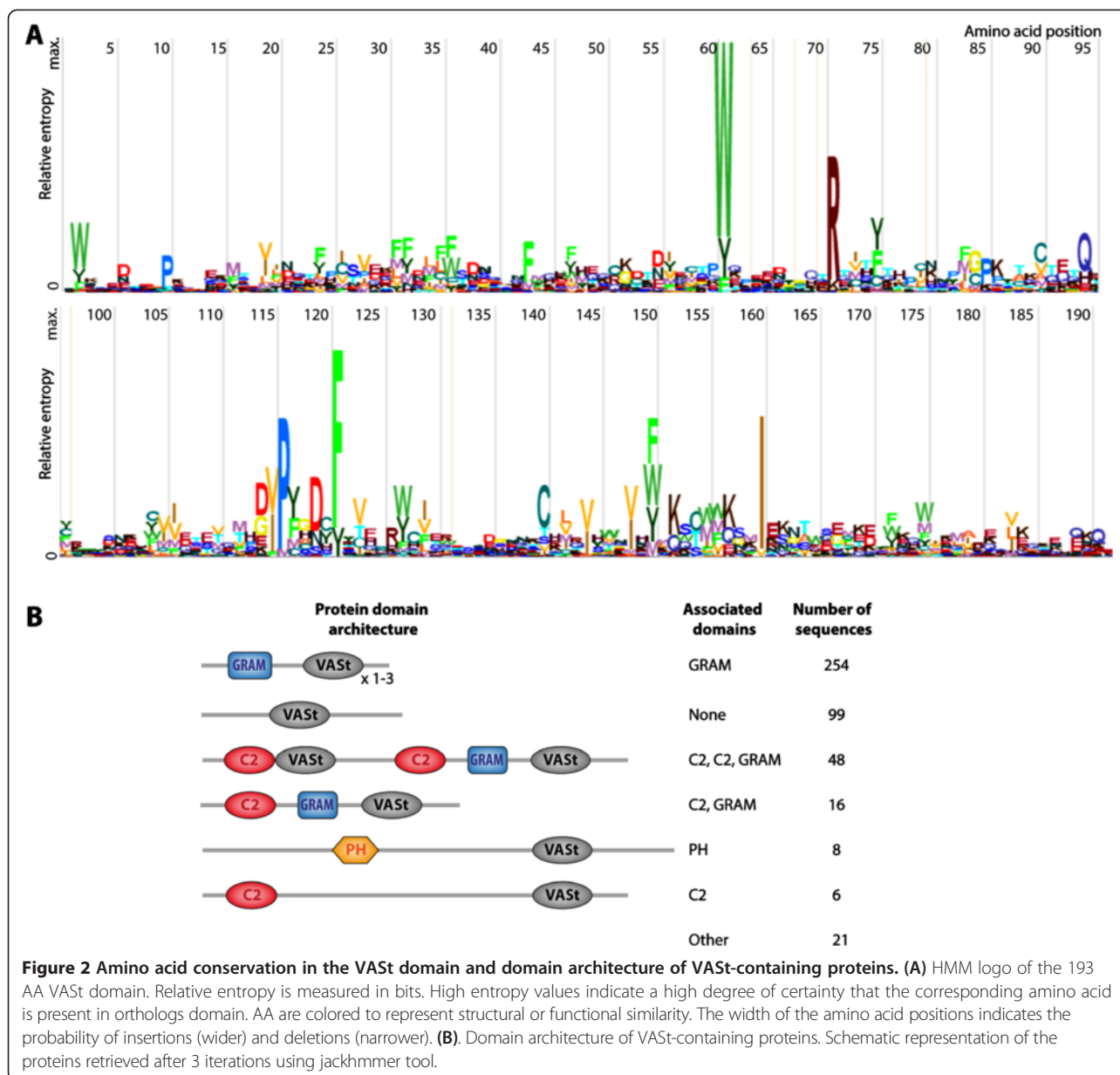
Since none of *AtVAD1* homologs had characterized biochemical functions, we extended our search of related protein domains using Hidden Markov Models (HMM) to get insights into the VAS_t domain putative function and evolution. For this, we built a HMM using the 13 plant homologs of *AtVAD1* VAS_t domain. To highlight important sequence motifs in this HMM, we examined the corresponding sequence logo (Figure 2A). Amino acids W61, R71, P116, F121 and I160 were strongly conserved in the model, suggesting an important contribution of these residues to the protein function. To identify protein domains related to the VAS_t domain, we searched the Uniprot database with the VAS_t HMM model. After three iterations of jackhammer search, we retrieved 452 hits distributed exclusively in eukaryotic proteins, including yeasts and other Fungi, Oomycetes, Mammals, and Plants (Additional file 3). Most of the identified proteins contained one copy of the VAS_t domain (~85.8%), with a few containing two or more VAS_t domains (Figure 2B). As little as ~21.9% of the protein hits contained VAS_t as the only domain identified. The VAS_t domain is frequently associated with lipid binding domains such as GRAM (in 70.3% of the hits), C2 and Pex24p domains, suggesting a functional link between VAS_t and lipid-binding domains. Among proteins related to *VAD1* retrieved by our HMM search was *YSP2*, a major cell death regulator in Yeast [22]. However, there was no protein for which a biochemical function has been described.

Multiple domain combinations contributed to the diversification of VAS_t-containing proteins

To document the evolution of the VAS_t domain, we examined the phylogenetic relations of VAS_t domains in 17 fully sequenced species representing all major Eukaryotic lineages (see Methods), corresponding to a

total of 85 protein sequences (after redundancy and incomplete sequence filtering). (Figure 3, Additional file 4, Additional file 5, Additional file 6). Sequences clustered into nine groups defined by their taxonomic range and domain organization. To highlight the phylogenetic relationship between copies of the VAS_t domain present in a single protein (clades 1, 2, 4 and 5), we have connected together VAS_t copies present in the same protein (Figure 3A). Group 1 gathered sequences from mammals and other metazoans. In this group, the VAS_t domain is found either (i) alone, (ii) associated with a GRAM domain or (iii) associated with another VAS_t domain. The two copies of VAS_t in GRAM-VAS_t-VAS_t proteins clustered in the same clade indicating that the duplication of VAS_t domain is recent. This group included human *GRAMD1A*, *GRAMD1B* and *GRAMD1C* proteins. Group 2 exclusively consisted in sequences from fungi, with the same domain structures as found in Group 1. Group 2 contained the yeast *YSP2* protein. Group 3 and 8 contained N-terminal and C-terminal VAS_t domains respectively of proteins with a C2-VAS_t-(C2)-GRAM-VAS_t domain architecture. Group 3 and 8 are restricted to plants, including the moss *Physcomitrella patens*, and phylogenetically distant, suggesting that duplication of the VAS_t domain early in land plant evolution allowed the divergence of two VAS_t copies in these proteins. Group 4 contained proteins from Stramenopiles, Ciliates and Amoebozoa, with either a VAS_t domain alone or a GRAM-VAS_t architecture. Group 5 contained exclusively plant sequences with a GRAM-VAS_t structure, include *A. thaliana* *VAD1*. Group 6 contained exclusively Stramenopile sequences with either a VAS_t domain alone or a FCH-GRAM-VAS_t architecture. Group 7 contained exclusively Ciliate sequences comprising either a VAS_t domain alone, or GRAM domain with one to three copies of VAS_t. Finally, Group 9 contained sequences from Amoebozoa, Tracheophyta (vascular plants) and Stramenopiles with diverse domain architectures: (i) VAS_t alone, (ii) GRAM-VAS_t, (iii) C2-GRAM-VAS_t or (iv) VAS_t-VAS_t-Pex24p. Groups 3, 5, 8 and 9 show support value of 1.0 suggesting that the function of VAS_t domains from these groups could have diverged, and that this divergence could be essentially driven by the association with the C2 and the GRAM domains.

Next, we attempted to reconstruct the evolutionary history of domain combinations in VAS_t-containing proteins (Figure 3B). Similar to GRAM [13], the VAS_t domain would originate from the last common eukaryote ancestor (LCEA). Since GRAM and VAS_t domains are associated in nearly all eukaryotic lineages the GRAM-VAS_t combination probably dates back from the LCEA. Sequences harboring a VAS_t domain alone were also found in all lineages except Plants and Fungi, suggesting that a copy of the ancestral VAS_t domain gene has



been maintained in most phyla. Alternatively, the GRAM domain could have been lost from a putative GRAM-VASt ancestor in several lineages. Adjacent VASt duplications within a single protein are observed in Ciliates, Stramenopiles, Fungi and Mammals that probably arose recently, judging from high sequence similarity between adjacent copies. Consistent with [13], VASt association with both GRAM and C2 appeared Plant-specific. A parsimonious scenario for the emergence of the complex C2-VASt-C2-GRAM-VASt domain architecture specific to Plants could be the combination of a C2 domain with ancestral VASt alone and GRAM-VASt proteins, followed by the fusion of a C2-VASt and a

C2-GRAM-VASt module early in the evolution of Plants (Figure 3B). The C2-VASt and C2-GRAM-VASt modules could have been maintained in vascular plants but not in mosses. Alternatively, a VASt-GRAM-VASt fusion could have emerged in an ancestral Eukaryotic lineage (it has been maintained in Mammals), combined with two C2 domains in plants, then C2-VASt and C2-GRAM-VASt could have emerged from the split of a C2-VASt-C2-GRAM-VASt plant ancestor in vascular plants but not in mosses. The association of VASt with Pex24p or FCH domains seems to be innovations from the Oomycete lineage. In addition to data presented in Figure 3, proteins with a PH-VASt architecture were found in some fungal species.

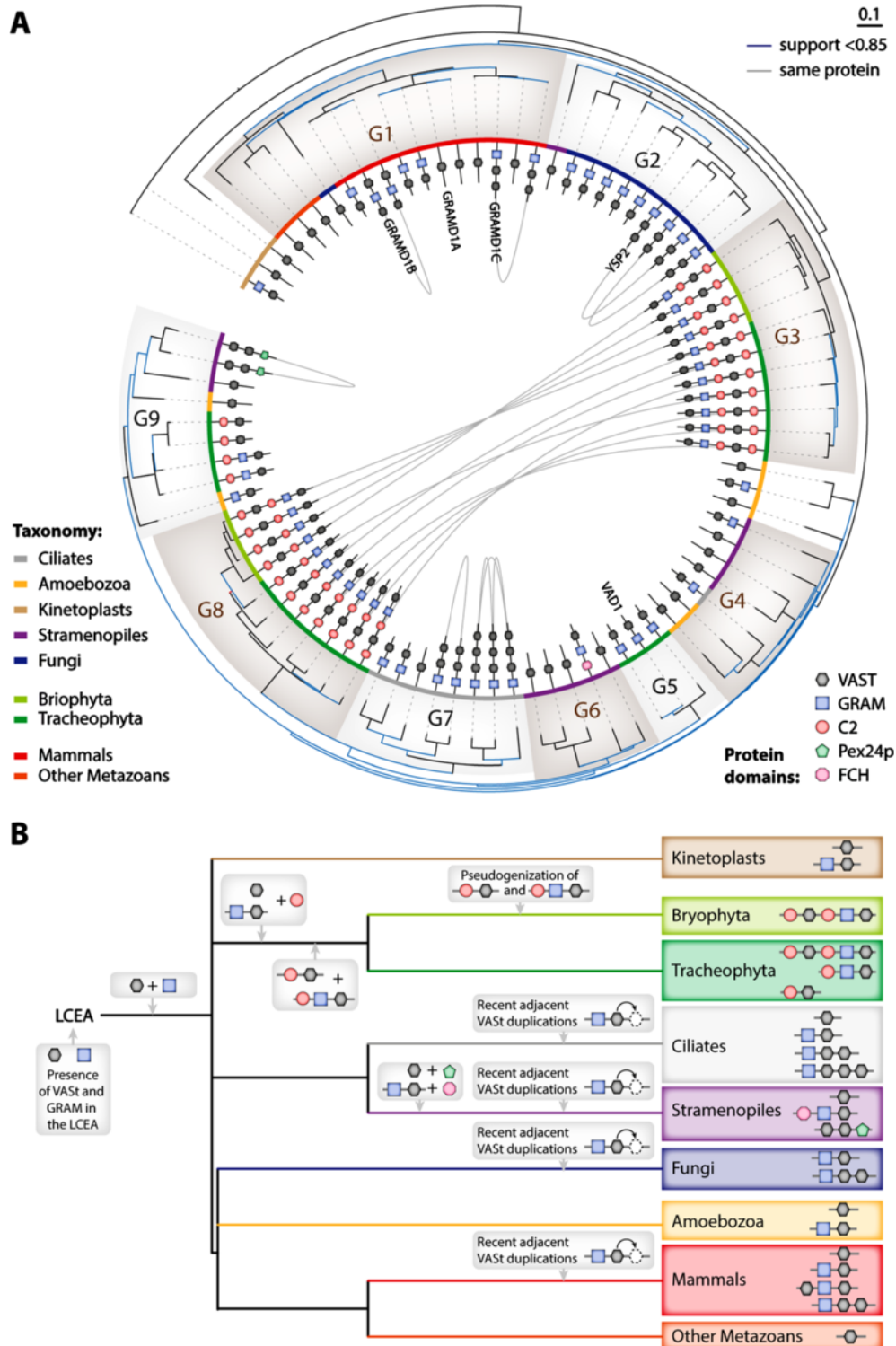


Figure 3 Phylogeny and evolutionary history of the VASt domain. (A) Maximum likelihood phylogenetic tree of 85 VASt protein domains from 17 fully-sequenced species representative of major Eukaryotic lineages. Multiple copies of VASt found in a single protein are linked with central connectors. Protein Domain architecture and taxonomy are shown along branches. Nine phylogenetic groups (G1 to G9) are highlighted. Proteins cited in the main text are labeled along branches. **(B)** Proposed scenario for the evolution of VASt-containing proteins. Domain symbols are as in **A**. LCEA, last common Eukaryote ancestor.

Homology modeling of the VAS_t domain 3D structure

Classical sequence-based phylogeny did not allow identifying protein domains of known function related to VAS_t. Structure-based phylogenetic network inference may be used to improve the resolution of deep evolutionary relationships and assist in inference of protein function [23]. To analyze relationships between VAS_t and protein domains of known three-dimensional structure, we conducted a structure-based clustering of AtVAD1 VAS_t domain and its closest analogs. First, to obtain atomic coordinates of a 3D model for AtVAD1 VAS_t domain, we submitted its 193 AA sequence to the homology and threading structure prediction server I-TASSER [24]. The best model (Additional file 7) showed a two-layer sandwich alpha beta fold (CATH 3.30, also called “helix grip fold”, [25]) containing three alpha helices (α_1 to 3), six beta-sheets (β_1 to 6) and two loops (Ω_1 and 2) numbered from N to C terminus (Figure 4A). This model had a C-score of -1.41 indicating that quality predictions can be estimated with more than 90% confidence [24], and expected TM-score of 0.54 suggesting a correct topology. Eight of the top 10 threading alignments had normalized Z-score higher than 1, thus accuracy of the model is expected to be high [26]. The predicted VAS_t model encompasses a large hydrophobic cavity delimited by sheets β_2 , 3 and 4, loop Ω_1 and helices α_2 and 3 (Figure 4B).

Structure-based phylogeny reveals relationships between the VAS_t domain and Bet v1-like domains

To investigate structural relationships between AtVAD1 VAS_t domain and its closest analogs, we conducted a structure-based tree inference analysis including AtVAD1 VAS_t domain, predicted three-dimensional structure

for 15 VAS_t homologs, and the top structural analogs retrieved by fold recognition searches. The best structural analogs retrieved by I-TASSER and NCBI Vector Alignment Search Tool fold recognition searches were human MLN64 (Metastatic axillary lymph node protein 64) STAR-related lipid transport domain [PDB:1EM2] and *Streptomyces* ZhuI polyketide aromatase/cyclase [PDB:3TFZ]. Close analogs also included human CERT ceramide trafficking protein [PDB:2E3M] and *Arabidopsis thaliana* PYL2 ABA receptor [PDB:3KDI]. Gene Ontology terms associated with AtVAD1 VAS_t domain based on the 3D model included hormone binding [GO:0042562], isoprenoid binding [GO:0019840] and monocarboxylic acid binding [GO:0033293]. To build a structure-based phylogenetic tree, we modeled the three-dimensional structure of 15 VAS_t domains using AtVAD1 VAS_t as a template, and searched for AtVAD1 VAS_t closest structural analogs in the medium redundancy subset of the Molecular Modeling database (Additional file 8). We next performed a multiple structure alignment, calculated normalized pairwise RMSD distances for aligned C α atoms (Additional file 9), and used this distance matrix to produce a neighbor-joining tree (Additional file 10). Structures clustered into five major groups (Figure 5). Group I contained plant Bet v1 phytohormone-binding proteins and pathogenesis-related (PR) 10-like proteins. Group II contained *Arabidopsis thaliana* PYL2 abscisic acid (ABA) receptor, belonging to the Pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/Regulatory components of ABA receptors (RCAR) family and uncharacterized bacterial proteins. Group III contained mammalian START proteins binding sterols and sphingolipids, and *Streptomyces* aromatase-cyclases. Group IV contained VAS_t-domains. Finally Group V contained mammalian

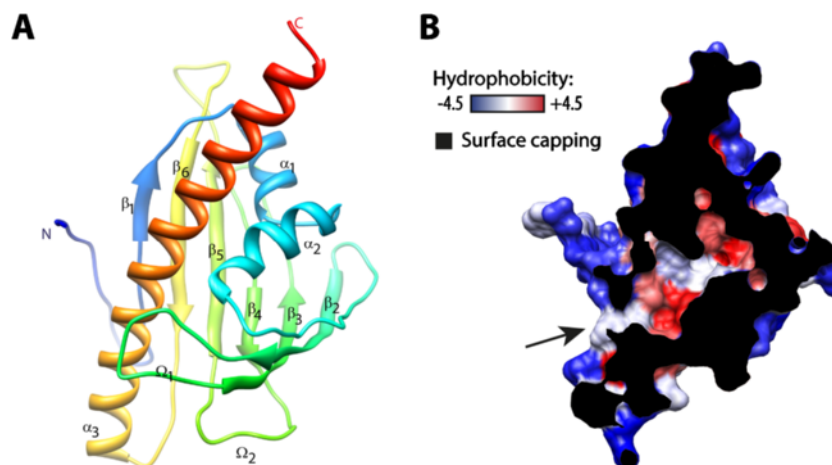
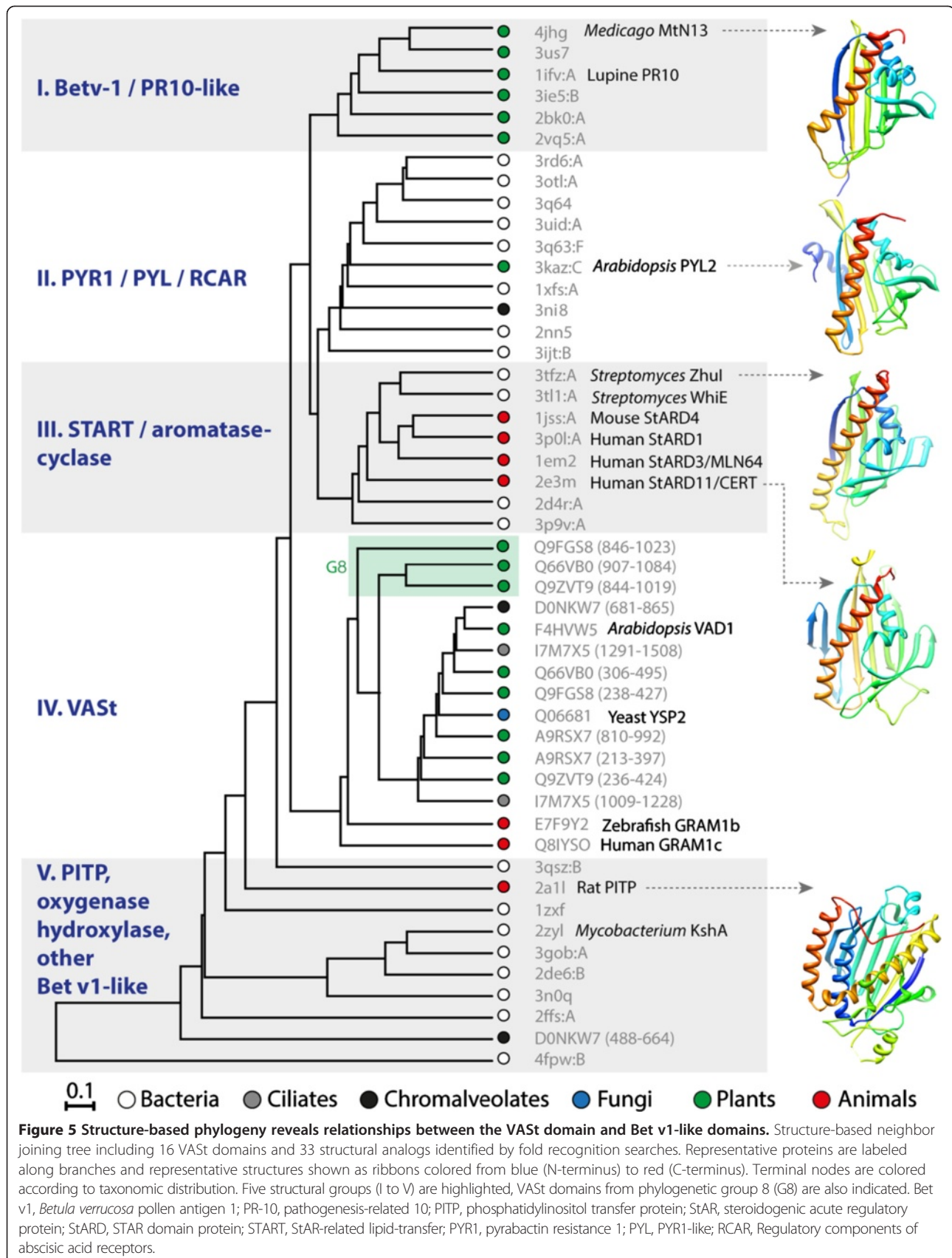


Figure 4 Homology modeling of the VAS_t domain 3D structure. (A) Ribbon diagram representation of the predicted 3D structure of AtVAD1 VAS_t domain, colored from blue (N-terminus) to red (C-terminus). Secondary structure elements are numbered from N to C terminus. (B) The VAS_t domain harbors a large hydrophobic cavity. Surface of AtVAD1 VAS_t model colored according to residue hydrophobicity. Capping surface is shown in black, the entrance of the hydrophobic cavity is marked by an arrow.



phosphatidylinositol transfer protein (PITP), bacterial oxygenase and hydrolases, and other Bet v1-like domains. With the exception of the N-terminal VAS_t domain of *Phytophthora infestans* predicted protein PITG_12663 [Uniprot:DONKW7], all VAS_t domains clustered together into structural group IV. A separate sub-group within structural group IV was formed by VAS_t domains from sequence-based phylogenetic group 8, supporting a possible functional divergence. This analysis revealed that VAS_t domains are structurally related to Bet v1-like domains [Pfam:CL0209] known to bind bulky hydrophobic ligands such as phytohormones, lipids and polyketides. Association with lipid-binding domains in large multidomain proteins is typical for START and VAS_t domains.

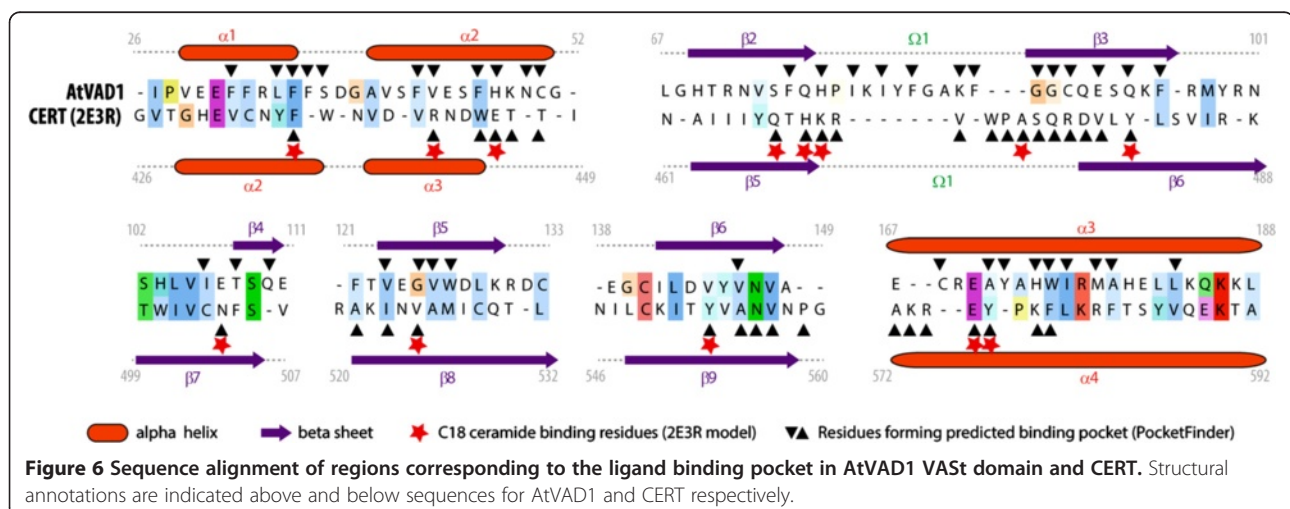
To further test whether AtVAD1 VAS_t domain and the well-characterized START domain of the CERT protein could be evolutionary related, we closely examined the ligand-binding pockets of these two proteins. For this, we performed a structural alignment of AtVAD1 VAS_t and CERT START domain models. The structural alignment of AtVAD1 VAS_t domain and CERT START domain highlighted a good conservation of secondary structure elements lining the substrate binding pocket (Figure 6). Although the overall sequence conservation between the two proteins is limited, residues binding to C18 ceramide in CERT showed conservation or similar environments in AtVAD1 VAS_t domain. These observations are consistent with hidden homology between VAS_t and Bet v1-like domains. Alternatively, the analogy between VAS_t and Bet v1-like domains could result from convergent evolution. Testing the importance of the predicted ligand-binding pocket residues for VAS_t function could help discriminate between these hypotheses.

Discussion

We report the identification of the VAS_t protein domain in the VAD1 plant cell death regulator. This domain is

conserved across eukaryotes and is structurally related to Bet v1-like domains, including START lipid-binding domains. The predicted structure of VAD1 VAS_t domain is consistent with a function in binding large hydrophobic ligands. Our findings open new perspectives for the analysis of functions of the VAS_t domain associated with the GRAM, C2 and PH lipid-binding domains and the characterization of novel mechanisms regulating PCD in plants.

What is the physiological role of VAS_t-containing proteins? Most of the proteins containing VAS_t domain have no characterized function to date. The *A. thaliana* VAD1 is the only exception in plants. In the context of pathogen attack, controlled programmed cell death (PCD) is one of the prevailing plant defense responses, allowing confinement of the pathogen locally in dead cells. The *vad1* mutant exhibits spontaneous PCD lesions initiated in cells surrounding vascular tissue progressively expand to the whole leaf, hence its classification as “propagation lesion mimic mutant” [7]. This phenotype suggests that *vad1* is impaired in the control of cell-to-cell propagation of PCD, involving a yet unknown mechanism. Amiodarone is a Ca²⁺ channel-targeted drug inducing apoptosis mediated by reactive oxygen species (ROS), *via* the same pathway as natural pheromones [27]. Genetic screens have revealed the function of the YSP2 (Yeast Suicide Protein 2) in enhancing survival after amiodarone treatment. YSP2 is a mitochondrial membrane protein involved in mitochondrial fragmentation, probably acting downstream of ROS production triggered by intracellular acidification [22]. YSP2 harbors a GRAM and a VAS_t domain but its molecular function is unknown. In human, a polymorphism in the GRAMD1B gene has been associated with susceptibility to chronic lymphocytic leukemia [28]. Recently, a whole genome modified-siRNA screen identified GRAMD1B as a protein associated with chemoresistance in epithelial ovarian cancer (OvCa) cells. Consistent with



the view that acquired chemoresistance is a major contributor to patient mortality from OvCa, reducing GRAMD1B expression increased overall survival in OvCa patients and decreased tumour burden in mouse models [29]. GRAMD1C has been identified as part of a quantitative trait locus associated with hepatic iron overload in mice, but its function has not been validated [30]. The molecular function of GRAMD1 proteins has not been investigated but their association with several disorders supports the relevance of VASSt-containing proteins for cell integrity. What may be the signal(s) associated to VAD1 that mediate propagation of PCD? The VASSt domain is related to domains from the Bet v1-like superfamily [Pfam:CL0209] that bind large hydrophobic ligands such as lipids, hormones and antibiotics [31]. In the Bet v1-like superfamily, PR-10, Bet v1 and PYR/PYL/RCAR domains (Figure 5, groups I and II) typically bind phytohormones such as brassinosteroids, cytokinins and abscisic acid [32-34]. Some other Bet v1-like domains (Figure 5, groups III and V) bind secondary metabolites such as flavonoids, polyketides and various antibiotics [31,35]. These ligands are diffusible molecules that could act as intercellular signals regulated by VAD1. Domains belonging to the START subfamily of Bet v1-like domains bind lipids such as sterols and sphingolipids [31,36]. In animal cells, intercellular transport of sterols and sphingolipids is mainly mediated by non-vesicular transport *via* the action of dedicated lipid transport proteins (LTPs) or *via* spontaneous lipid exchange [37]. Phytohormone- and secondary metabolite-binding proteins in the Bet v1-like superfamily often function as single-domain proteins, or multimers of single domain-proteins, whereas START and VASSt-containing proteins are generally large multidomain proteins. Notably, the mammalian CERT and *Arabidopsis* VAD1 proteins share a common domain structure involving a PH superfamily domain (PH and GRAM respectively) and a Bet v1-like superfamily domain (START and VASSt respectively). Cooperation between the PH and START domains in CERT is critical for its function as a ceramide transport protein [38]. Ceramides and other sphingolipids are important regulators of cell death programs in animals and plants [39,40]. VAD1 may therefore sense or transport lipids to modulate cell death signals intercellularly.

What is the evolutionary history of the VASSt domain? Our sequence- and structure-based phylogenetic analyses suggest that the VASSt domain evolved from a primordial Bet v1-related protein that existed in the last universal common ancestor, and emerged with the divergence of Eukaryotes. Alternatively, the analogous structure of VASSt and Bet v1-like domains could result from convergent evolution. By contrast to the PR10-like subfamily of Bet v1 domains, the VASSt domain is conserved across all major Eukaryotic lineages, and therefore probably serves

a function relatively conserved across all Eukaryotes. Radauer *et al.* proposed that the primordial Bet v1 protein would bind lipids, and would have evolved by addition of secondary structural elements or fusion to other domains into multi-domain proteins [31]. Our results suggest that the VASSt domain has been associated with the GRAM domain very early in the history of Eukaryotes, and was later combined with C2 domains in Plants and with Pex24p domains in Oomycetes. The 3D model we obtained for VAD1 VASSt domain features a long loop connecting helix $\alpha 2$ and sheet $\beta 2$, instead of a beta-sheet in typical Bet v1 domains, leading to a β - $\alpha 2$ - $\beta 5$ - α instead of β - $\alpha 2$ - $\beta 6$ - α secondary structure arrangement. VASSt domains of Plants span across four phylogenetic groups (Group 3, 5, 8 and 9, Figure 3), suggesting the emergence of novel adaptations in the Plant kingdom, that may either reflect the evolution of new catalytic activities or the adaptation to plant-specific ligand(s).

Conclusions

Local variations in membrane protein and lipid composition create subcellular compartments with diverse physico-chemical properties. Such local variations in lipid and protein content may be critical for defining the specific structure membrane compartments and flagging them for the addressing of proteins and other signals [41-43]. The trafficking routes between membrane compartments, and the proteins implicated, are just starting to be uncovered. Our analyses revealed the VASSt domain as a member of the Bet v1-like superfamily predominantly associated with lipid binding domains such as GRAM, C2 and PH domains. This finding opens new perspectives for molecular and genetics studies of the function and regulation of VASSt domain containing proteins.

Methods

Identification of VAD1 homologs and conservation analysis in plants

The VAD1 protein sequence [Uniprot: F4HVW5] was used as a query for a profile Hidden Markov Model (HMM) search with phmmer [44] against the Uniprot database using Blosum62 matrix. Hits with e-value $1e^{-100}$ or less were selected and manually curated resulting in the identification of 13 homologs, exclusively from plants. Sequences were aligned using MAFFT version 7 [21] with default parameters. Amino acids conservation score, calculated according to [45], was plotted as a moving average using a sliding window approach with window size 10 and steps of size 1. Protein sequence features retrieved through the phmmer search were manually mapped along the alignment. AtVAD1 gene model (At1g02120.1) was retrieved from TAIR (www.arabidopsis.org) and mapped on the protein alignment using GeneWise (<http://www.ebi.ac.uk/Tools/psa/genewise/>) with default parameters.

Conservation and phylogenetic analyses of VAD1 VASt domain

The precise boundaries of the newly identified VASt domain were set based on a conservation score >4 among plant AtVAD1 homologs and to include well-conserved N-terminal residues F1, D7 and P11, delimiting a 193 amino acids domain. The ungapped alignment of the 193 amino acids domain of VAD1 VASt domain with its 13 homologs was used as entry for HMM searches with jackhammer [44] using Blosum90 matrix against NR database, with cut off e-value of $1e^{-10}$. The final list of proteins containing VASt domains was obtained after 3 iterations of jackhammer search using all hits from previous iteration as a seed. Sequence logo of the 452 VASt domains alignment was done with LOGOMAT-M [46]. To built the VASt domains phylogenetic tree, we selected all hits from 17 fully sequenced species representative of all major Eukaryotic lineages as follows: *Homo sapiens* and *Mus musculus* (Mammals), *Caenorhabditis elegans* and *Drosophila melanogaster* (Other Metazoans), *Arabidopsis thaliana* and *Oryza sativa* (Tracheophyta), *Physcomitrella patens* (Briophyta), *Saccharomyces cerevisiae*, *Aspergillus nidulans*, *Neurospora crassa* and *Ustilago maydis* (Fungi), *Phytophthora infestans* and *Thalassiosira pseudonana* (Stramenopiles), *Entamoeba histolytica* and *Dictyostelium discoideum* (Amoebozoa), *Leishmania major* (Kinetoplasts), *Tetrahymena thermophila* (Ciliates). After removing incomplete sequences and redundant sequences with CD-HIT [47], 85 sequences were aligned using MAFFT version 7 [21]. The alignment was automatically curated using TrimAI [48] to keep 125 positions out of 341. Selection of LG + I + G + F as best-fit models with alpha value 2.108 for omega distribution was performed in ProtTest2 [49]. A phylogenetic tree showing branch support values as aLRT SH-like test was generated using PhyML 3.0 [50] and visualized using iTOL [51].

AtVAD1 VASt domain 3D structure modeling and structure-based clustering

The 3D structure of AtVAD1 (At1g02120) VASt domain (residues 257 to 449) was predicted with I-TASSER web server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). For structural tree inference, we first searched for AtVAD1 VASt domain structural analogs using the Vector Alignment Search Tool server [52]. Forty six analogs aligned over 100 amino-acids or more were retrieved. Second, we predicted the three dimensional of 15 VASt domains with Modeller v9.12 [53] using AtVAD1 VASt domain as a template. A structural alignment of AtVAD1 VASt domain, 15 other VASt domain structures and 46 structural analogs was generated with Mustang-MR [54]. Pairwise root-mean-square deviation (RMSD) between C α atoms were normalized as $100 \times \text{RMSD}/\text{number}$

of aligned residues, and used to build the distance matrix for tree inference. The tree was constructed using Fitch-Margoliash method in Phylip [55] with power 2.0, and a number of terminal branches connecting uncharacterized proteins were manually removed for clarity, resulting in a distance tree with 49 structures. All protein structures were rendered with UCSF Chimera [56].

Availability of supporting data

The original version of the tree shown in Figure 3A can be accessed at http://itol.embl.de/shared/lipm_bioinfo under the project 'VASt'. All other supporting data associated with this manuscript are included as additional files.

Additional files

Additional file 1: Figure S1. Multiple sequence alignment of VASt domains from AtVAD1 and its 12 closest plant homologs. Positions showing > 70% identity are highlighted in blue.

Additional file 2: Multiple alignment file in .FASTA format of AtVAD1 and its 12 closest homologs.

Additional file 3: Table of the 452 proteins retrieved from Uniprot after HMM search using the model built from 14 VAD1 homologs as bait.

Additional file 4: Multiple alignment file in .FASTA format of 85 VASt domains.

Additional file 5: Multiple alignment file in .FASTA format of 85 VASt domains after clean-up by trimAI, used to generate the tree shown in Figure 3A.

Additional file 6: Phylogenetic tree of 85 VASt-containing proteins from 17 fully-sequenced genomes in newick format.

Additional file 7: Atomic coordinates of AtVAD1 VASt domain best model obtained via modeling by I-TASSER in .pdb format.

Additional file 8: List of AtVAD1 VASt domain closest structural analogs identified in the medium redundancy subset of the Molecular Modeling database using NCBI Vector Alignment Search Tool. Only structures that aligned over 100 residues or more were included in further analyses.

Additional file 9: Pairwise RMSD matrix used for the generation of the structure-based neighbor-joining tree of VASt domain analogs in phylip format.

Additional file 10: Structure based neighbor-joining tree of VASt domain analogs in newick format.

Competing interests

The authors declare no conflict of interests.

Authors' contributions

Conceived and designed the analyses: MK, LC, SR. Performed and interpreted the analyses: MK, LC, CB, SR. Wrote the paper: MK, CB, SR. All authors read and approved the manuscript.

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References

1. Forslund K, Sonnhammer EL: **Predicting protein function from domain content.** *Bioinformatics* 2008, **24**(15):1681–1687.
2. Söding J, Lupas AN: **More than the sum of their parts: on the evolution of proteins from peptides.** *Bioessays* 2003, **25**(9):837–846.
3. Chothia C, Gough J, Vogel C, Teichmann SA: **Evolution of the protein repertoire.** *Science* 2003, **300**(5626):1701–1703.
4. Vogel C, Berzuini C, Bashton M, Gough J, Teichmann SA: **Supra-domains: evolutionary units larger than single protein domains.** *J Mol Biol* 2004, **336**(3):809–823.
5. Lorrain S, Vaillieu F, Balagué C, Roby D: **Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants?** *Trends Plant Sci* 2003, **8**(6):263–271.
6. Moeder W, Yoshioka K: **Lesion mimic mutants: a classical, yet still fundamental approach to study programmed cell death.** *Plant Signal Behav* 2008, **3**(10):764–767.
7. Lorrain S, Lin B, Ariaci MC, Kroj T, Saindrenan P, Nicole M, Balagué C, Roby D: **vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues.** *Plant Cell* 2004, **16**(8):2217–2232.
8. Bouchez O, Huard C, Lorrain S, Roby D, Balagué C: **Ethylene is one of the key elements for cell death and defense response control in the *Arabidopsis* lesion mimic mutant vad1.** *Plant Physiol* 2007, **145**(2):465–477.
9. Doerks T, Strauss M, Brendel M, Bork P: **GRAM, a novel domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins.** *Trends Biochem Sci* 2000, **25**(10):483–485.
10. Berger P, Schaffitzel C, Berger I, Ban N, Suter U: **Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module.** *Proc Natl Acad Sci USA* 2003, **100**(21):12177–12182.
11. Tsujita K, Itoh T, Ijuin T, Yamamoto A, Shisheva A, Laporte J, Takenawa T: **Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3, 5-bisphosphate interaction.** *J Biol Chem* 2004, **279**(14):13817–13824.
12. S-i Y, Oku M, Wasada Y, Ano Y, Sakai Y: **PI4P-signaling pathway for the synthesis of a nascent membrane structure in selective autophagy.** *J Cell Biol* 2006, **173**(5):709–717.
13. Jiang S-Y, Ramamoorthy R, Ramachandran S: **Comparative transcriptional profiling and evolutionary analysis of the GRAM domain family in eukaryotes.** *Dev Biol* 2008, **314**(2):418–432.
14. Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF: **A fatty acid desaturase modulates the activation of defense signaling pathways in plants.** *Proc Natl Acad Sci USA* 2001, **98**(16):9448–9453.
15. Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Ødum N, Jørgensen LB, Brown RE, Mundy J: **Knockout of *Arabidopsis* accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense.** *Genes Dev* 2002, **16**(4):490–502.
16. Liang H, Yao N, Song JT, Luo S, Lu H, Greenberg JT: **Ceramides modulate programmed cell death in plants.** *Genes Dev* 2003, **17**(21):2636–2641.
17. Tang D, Ade J, Frye CA, Innes RW: **Regulation of plant defense responses in *Arabidopsis* by EDR2, a PH and START domain-containing protein.** *Plant J* 2005, **44**(2):245–257.
18. Wang W, Yang X, Tangchaiburana S, Ndeh R, Markham JE, Tsegaye Y, Dunn TM, Wang G-L, Bellizzi M, Parsons JF: **An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*.** *Plant Cell* 2008, **20**(11):3163–3179.
19. Vorwerk S, Schiff C, Santamaria M, Koh S, Nishimura M, Vogel J, Somerville C, Somerville S: **EDR2 negatively regulates salicylic acid-based defenses and cell death during powdery mildew infections of *Arabidopsis thaliana*.** *BMC Plant Biol* 2007, **7**(1):35.
20. Punta M, Coghill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements JF, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD: **The Pfam protein families database.** *Nucleic Acids Res* 2012, **40**(D1):D290–D301.
21. Katoh K, Misawa K, Kuma K-i, Miyata T: **MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform.** *Nucleic Acids Res* 2002, **30**(14):3059–3066.
22. Sokolov S, Knorre D, Smirnova E, Markova O, Pozniakovskiy A, Skulachev V, Severin F: **Ysp2 mediates death of yeast induced by amiodarone or intracellular acidification.** *Biochim Biophys Acta Bioenerg* 2006, **1757**(9):1366–1370.
23. Lundin D, Poole AM, Sjöberg B-M, Högbom M: **Use of structural phylogenetic networks for classification of the ferritin-like superfamily.** *J Biol Chem* 2012, **287**(24):20565–20575.
24. Zhang Y: **I-TASSER server for protein 3D structure prediction.** *BMC Bioinformatics* 2008, **9**(1):40.
25. Iyer LM, Koonin EV, Aravind L: **Adaptations of the helix-grip fold for ligand binding and catalysis in the START domain superfamily.** *Protein Struct Funct Genet* 2001, **43**(2):134–144.
26. Roy A, Kucukural A, Zhang Y: **I-TASSER: a unified platform for automated protein structure and function prediction.** *Nat Protoc* 2010, **5**(4):725–738.
27. Pozniakovskiy AI, Knorre DA, Markova OV, Hyman AA, Skulachev VP, Severin FF: **Role of mitochondria in the pheromone-and amiodarone-induced programmed death of yeast.** *J Cell Biol* 2005, **168**(2):257–269.
28. Di Bernardo MC, Crowther-Swanepoel D, Broderick P, Webb E, Sellick G, Wild R, Sullivan K, Vijayakrishnan J, Wang Y, Pittman AM, Sunter NJ, Hall AG, Dyer MJS, Matutes E, Dearden C, Mainou-Fowler T, Jackson GH, Summerfield G, Harris RJ, Pettitt AR, Hillmen P, Allsup DJ, Bailey JR, Pratt G, Pepper C, Fegan C, Allan JM, Catovsky D, Houlston RS: **A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia.** *Nat Genet* 2008, **40**(10):1204–1210.
29. Wu SY, Yang X, Gharpure KM, Hatakeyama H, Egli M, McGuire MH, Nagaraja AS, Miyake TM, Rupaimoole R, Pecot CV, Taylor M, Pradeep S, Sierant M, Rodriguez-Aguayo C, Choi HJ, Previs RA, Armaiz-Pena GN, Huang L, Martinez C, Hassell T, Ivan C, Sehgal V, Singhanian R, Han HD, Su C, Kim JH, Dalton JC, Kovvali C, Keyomarsi K, McMillan NAJ, Overwijk WW, Liu J, Lee JS, Baggerly KA, Lopez-Berestein G, Ram PT, Nawrot B, Sood AK: **2'-OME-phosphorodithioate-modified siRNAs show increased loading into the RISC complex and enhanced anti-tumour activity.** *Nat Commun* 2014, **5**:1–12.
30. Guo X, Zhang Z, Zhang F, Tao Y, An P, Wu Q, Wang C-Y, Knutson MD, Wang F: **Fine-mapping and genetic analysis of the loci affecting hepatic iron overload in mice.** *PLoS One* 2013, **8**(5):e63280.
31. Radauer C, Lackner P, Breiteneder H: **The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands.** *BMC Evol Biol* 2008, **8**(1):286.
32. Marković-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H: **Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier.** *J Mol Biol* 2003, **325**(1):123–133.
33. Fernandes H, Michalska K, Sikorski M, Jaskolski M: **Structural and functional aspects of PR-10 proteins.** *FEBS J* 2013, **280**(5):1169–1199.
34. Miyakawa T, Fujita Y, Yamaguchi-Shinozaki K, Tanokura M: **Structure and function of abscisic acid receptors.** *Trends Plant Sci* 2012.
35. Lee E-J, Facchini P: **Noroclaurine synthase is a member of the pathogenesis-related 10/Bet v1 protein family.** *Plant Cell* 2010, **22**(10):3489–3503.
36. Alpy F, Tomasetto C: **Give lipids a START: the StAR-related lipid transfer (START) domain in mammals.** *J Cell Sci* 2005, **118**(13):2791–2801.
37. Lev S: **Non-vesicular lipid transport by lipid-transfer proteins and beyond.** *Nat Rev Mol Cell Biol* 2010, **11**(10):739–750.
38. Kumagai K, Kawano M, Shinkai-Ouchi F, Nishijima M, Hanada K: **Interorganelle trafficking of ceramide is regulated by phosphorylation-dependent cooperativity between the PH and START domains of CERT.** *J Biol Chem* 2007, **282**(24):17758.
39. Young MM, Kester M, Wang H-G: **Sphingolipids: regulators of crosstalk between apoptosis and autophagy.** *J Lipid Res* 2013, **54**(1):5–19.
40. Berkey R, Bendigeri D, Xiao S: **Sphingolipids and plant defense/disease: the “death” connection and beyond.** *Front Plant Sci* 2012, **3**:68.
41. Tilsner J, Amari K, Torrance L: **Plasmodesmata viewed as specialised membrane adhesion sites.** *Protoplasma* 2011, **248**(1):39–60.
42. Ehlers K, Westerloh MG: **Developmental Control of Plasmodesmata Frequency, Structure, and Function.** In *Symplasmic Transport in Vascular Plants*. Science+Business Media New York: Springer; 2013:41–82.
43. Perraki A, Cacas JL, Crowet JM, Lins L, Castroviejo M, German-Retana S, Mongrand S, Raffaele S: **Plasma Membrane Localization of *Solanum tuberosum* Remorin from Group 1, Homolog 3 Is Mediated by Conformational Changes in a Novel C-Terminal Anchor and Required for the Restriction of Potato Virus X Movement.** *Plant Physiol* 2012, **160**(2):624–637.
44. Finn RD, Clements J, Eddy SR: **HMMER web server: interactive sequence similarity searching.** *Nucleic Acids Res* 2011, **39**(suppl 2):W29–W37.

45. Livingstone CD, Barton GJ: **Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation.** *Comput Appl Biosci* 1993, **9**(6):745–756.
46. Schuster-Böckler B, Schultz J, Rahmann S: **HMM Logos for visualization of protein families.** *Bioinformatics* 2004, **5**(1):7.
47. Li W, Godzik A: **Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences.** *Bioinformatics* 2006, **22**(13):1658–1659.
48. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T: **TrimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses.** *Bioinformatics* 2009, **25**(15):1972–1973.
49. Abascal F, Zardoya R, Posada D: **ProtTest: selection of best-fit models of protein evolution.** *Bioinformatics* 2005, **21**(9):2104–2105.
50. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O: **New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0.** *Syst Biol* 2010, **59**(3):307–321.
51. Letunic I, Bork P: **Interactive tree of life v2: online annotation and display of phylogenetic trees made easy.** *Nucleic Acids Res* 2011, **39**(suppl 2):W475–W478.
52. Gibrat J-F, Madej T, Bryant SH: **Surprising similarities in structure comparison.** *Curr Opin Struct Biol* 1996, **6**(3):377–385.
53. Sali A, Blundell TL: **Comparative protein modelling by satisfaction of spatial restraints.** *J. Mol. Biol.* 1993, **234**:779–815.
54. Konagurthu AS, Reboul CF, Schmidberger JW, Irving JA, Lesk AM, Stuckey PJ, Whisstock JC, Buckle AM: **MUSTANG-MR structural sieving server: applications in protein structural analysis and crystallography.** *PLoS One* 2010, **5**(4):e10048.
55. Felsenstein J: **PHYMLIP - phylogeny inference package (version 3.2).** *Cladistics* 1989, **5**:164–166.
56. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE: **UCSF chimera—a visualization system for exploratory research and analysis.** *J Comput Chem* 2004, **25**(13):1605–1612.

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