Remarkable Evolutionary Conservation of Antiobesity ADIPOSE/WDTC1 Homologs in Animals and Plants

Eric Ducos,¹ Valentin Vergès, Thomas Dugé de Bernonville, Nathalie Blanc, Nathalie Giglioli-Guivarc'h, and Christelle Dutilleul

EA2106 "Biomolécules et Biotechnologies Végétales," UFR de Pharmacie, Université François Rabelais de Tours, Parc de Grandmont, F-37200, France

ABSTRACT ASG2 (Altered Seed Germination 2) is a prenylated protein in *Arabidopsis thaliana* that participates to abscisic acid signaling and is proposed to act as a substrate adaptor for the DDB1 (DNA damage-binding protein 1)-CUL4 (Cullin 4) E3 ubiquitin ligase complex. ASG2 harbors WD40 and Tetratricopeptide Repeat (TPR) domains, and resembles the well-conserved animal gene called ADP (antiobesity factor ADIPOSE) in fly and WDTC1 (WD40 and TPR 1) in humans. Loss of function of WDTC1 results in an increase in adipocytes, fat accumulation, and obesity. Antiadipogenic functions of WDTC1 involve regulation of fat-related gene transcription, notably through its binding to histone deacetylases (HDACs). Our sequence and phylogenetic analysis reveals that ASG2 belongs to the ADP/WDTC1 cluster. ASG2 and WDTC1 share a highly conserved organization that encompasses structural and functional motifs: seven WD40 domains and WD40 hotspot-related residues, three TPR protein–protein interaction domains, DDB1-binding elements [H-box and DWD (DDB1-binding WD40 protein)-box], and a prenylatable C-terminus. Furthermore, ASG2 involvement in fat metabolism was confirmed by reverse genetic approaches using *asg2* knockout *Arabidopsis* plants. Under limited irradiance, *asg2* mutants produce "obese" seeds characterized by increased weight, oil body density, and higher fatty acid contents. In addition, considering some ASG2- and WDTC1-peculiar properties, we show that the WDTC1 C-terminus is prenylated *in vitro* and HDAC-binding capability is conserved in ASG2, suggesting that the regulation mechanism and targets of ADP/WDTC1-like proteins may be conserved features. Our findings reveal the remarkable evolutionary conservation of the structure and the physiological role of ADIPOSE homologs in animals and plants.

KEYWORDS fatty acids; histone deacetylase; protein isoprenylation; WD40 protein; obesity

LIPIDS are essential to animal and plant cells, being involved in cellular and organellar structure, or in signaling pathways, as well as in carbon and energy storage (Okazaki and Saito 2014). Among lipids, triacylglycerols (TAGs) represent the majority of lipids stored in adipocytes (fat cells) in mammals (Voet and Voet 1990; Ali *et al.* 2013) or in seeds of plants (Murphy 1993; Baud and Lepiniec 2010).

In *Arabidopsis thaliana* and even more so in oleaginous seeds, stored TAGs are carbon and energy reserves to initiate the germination process by promoting embryo development

until photosynthesis takes over (Baud and Lepiniec 2010). On the other hand, seed development involves two major phases initiated by embryo morphogenesis followed by seed maturation. The latest phase leads to embryo accumulation of storage compounds (Baud *et al.* 2008). Reserve accumulation is tightly controlled by environmental and genetic factors. For instance, irradiance strongly impacts seed metabolite distribution as shown by decrease of total oil content under light deficiency (Li *et al.* 2006; Baud and Lepiniec 2010). Moreover, seed reserve composition is also the outcome of hormonal cross talk mainly governed through abscisic acid (ABA) signaling (Finkelstein *et al.* 2002; Shu *et al.* 2016). In *Arabidopsis*, recent advances highlight the role of the ubiquitin–proteasome systems in the modulation of ABA perception. Up to now, > 15 proteins related to E3 ubiquitin ligases have been described as ABA negative regulators promoting the degradation of ABA signaling factors such as kinases, phosphatases, or transcription factors (Yu *et al.* 2016).

Copyright © 2017 by the Genetics Society of America

doi: <https://doi.org/10.1534/genetics.116.198382>

Manuscript received November 16, 2016; accepted for publication June 25, 2017; published Early Online June 28, 2017.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.198382/-/DC1.

¹Corresponding author: EA2106 "Biomolécules et Biotechnologies Végétales," UFR de Pharmacie, Université François Rabelais de Tours, Parc de Grandmont, F-37200 Tours, France. E-mail: eric.ducos@univ-tours.fr

ABA perception is therefore modulated through an intricate network, which highlights its crucial physiological functions in ensuring the plant's adaptive responses to environmental stress, seed development, and embryo filling (Nakashima and Yamaguchi-Shinozaki 2013; Yu *et al.* 2016).

In mammals, TAG accumulation and adipocyte differentiation is notably controlled by PPAR γ (Peroxisome Proliferator-Activated Receptor γ), a ligand-regulated transcription factor (Rosen *et al.* 2000). In humans, the transcriptional PPAR γ activity is, in part, regulated by the DDB1 (DNA damage-binding protein 1)-CUL4 (Cullin 4) E3 ubiquitin ligase adaptor DCAF9 (*DDB1-CUL4 Associated Factor 9*, Rosen *et al.* 2000; Angers *et al.* 2006; Jin *et al.* 2006; Suh *et al.* 2007). DCAF9, also named *WDTC1* (*WD and Tetratricopeptide Repeats 1*) in humans and *ADIPOSE* (*ADP*) in flies, is a conserved single-copy gene encoding a singular WD40 protein that harbors additional Tetratricopeptide Repeats (TPR). *ADP* was first identified in *Drosophila* (Doane 1960), in which the dysfunction is accompanied by an increase in the number of adipocytes and an obese phenotype (Häder *et al.* 2003; Suh *et al.* 2007). Antiobesity functions of ADP have then been extended to several ADP homologs from worms to mammals (Suh *et al.* 2007; Galgani *et al.* 2013). Moreover, in humans, population genetic studies have highlighted a significant correlation between *WDTC1* SNP variations and obesity in two US populations (Lai *et al.* 2009), and therefore *WDTC1* variants have been defined as obesity markers.

In *Arabidopsis*, *ASG2* (Altered Seed Germination 2) harbors WD40 and TPRs (Lee *et al.* 2008), and its domain organization resembles that of animal ADP/*WDTC1*. *ASG2* acts as an ABA negative regulator of seed germination since the transcription factor ABI5 (ABA INSENSITIVE 5) shows increased stability in *ASG2*-deficient plants during this process (Bassel *et al.* 2011). We recently retrieved *ASG2* in a proteomics approach to capture prenylated proteins in *Arabidopsis* cell cultures (Dutilleul *et al.* 2016). Indeed, *ASG2* is equipped with a C-terminal CaaX-box that is a target for isoprenylation (Dutilleul *et al.* 2016). Protein isoprenylation is mediated either by the protein farnesyl-transferase (PFT) or the protein geranylgeranyl-transferase type I (PGGT-I), adding a farnesyl or a geranylgeranyl to the Cys of the CaaX-box, respectively (Crowell and Huizinga 2009). The CaaX-box of *ASG2* is a specific target for farnesylation mediated by PFT (Dutilleul *et al.* 2016). Moreover, because *ASG2* displays DDB1-binding capacities, it was proposed to function as a substrate adaptor for the DDB1-CUL4 E3 ubiquitin ligase (Lee *et al.* 2008; Dutilleul *et al.* 2016). Interestingly, the farnesylation of *ASG2* does not prevent *ASG2*-DDB1 interaction but modifies the subcellular localization of the complex (Dutilleul *et al.* 2016). Furthermore, *asg2* knockout plants mimic some of the PFT knockout plant phenotypes, which suggests that *ASG2* fully operates when it is farnesylated (Dutilleul *et al.* 2016). In the present work, by considering phylogenetic relationships, functional and physiological properties of *ASG2*, and animal ADP/*WDTC1*-related proteins, we aimed to determine whether *WDTC1* homologs have similar features that have been conserved through kingdom evolution.

Materials and Methods

Plant material

The *A. thaliana* wild-type (WT) and *asg2* mutant lines used for this study are described in Dutilleul *et al.* (2016): *asg2-1* in plants bears a T-DNA insertion in the first intron (SALK_040151) whereas *asg2-2* in plants bears a T-DNA insertion in the 3'-UTR (SALK_113565). Plants were grown in a growth chamber (22° at 70% humidity) under a 16 hr light/8 hr dark cycle, and were exposed to optimal or low irradiance (*i.e.*, 300 or 25 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). Once harvested, seeds were maintained in the dark at 4°.

Bioinformatics and phylogenetic analysis

Protein sequences were recovered using Blast and reciprocal Blast with NCBI Databases (see text and Supplemental Material, Tables S1 and S2 in File S1) with blastp default parameters (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was performed using Seaview multiplatform (<http://doua.prabi.fr/software/seaview>). Protein sequences were aligned using the Clustal Ω v1.2 program. The PhyML v3.1 program was used to compute maximum likelihood phylogenetic trees. Phylogenetic trees were colored using Adobe Illustrator.

A comparison of *ASG2* and *WDTC1* gene organization was drawn using PowerPoint software. Exon-intron location was determined by combining protein sequence alignments with coding and genomic sequence alignments using ExPASy Bioinformatics Resources Portal (www.expasy.org). The topology of the *ASG2* and *WDTC1* N-termini were predicted with PSIPRED v3.3 server (<http://bioinf.cs.ucl.ac.uk/psipred>).

Expression data were retrieved from the Bio-Analytic Resource (BAR) Expression browser (<http://bbc.botany.utoronto.ca>) by querying the database with the Seed and Silique Development filter without the “Imbibed Seed” values (corresponding to the dormancy break rather than the seed development process). Raw expression data were centered and scaled per gene and visualized with the heatmap.2 function of the “gplots” R package. Clustering of the genes was obtained by a hierarchical classification based on Euclidian distances.

Seed analysis

For each genotype and condition, 400 seeds were weighed, in five independent replicates. The lengths and widths of 50 seeds were determined using ImageJ software (<https://imagej.nih.gov/ij/>) using microscopy pictures. The seed shape was likened to a spheroid and the seed volume calculation was done as described in Riefler *et al.* (2006).

Fatty acid (FA) analysis was performed as described in Focks and Benning (1998) with modifications. First, 5 mg of seeds were ground using a Mixer Mill MM 400 (Restsch), for 2 min at 30 Hz. They were then incubated in 0.5 ml of 1 N methanolic-HCl containing C_{17:0} used as an internal standard, at 80° for 2 hr. Fatty acyl methyl esters (FAMES) were

Figure 1 Phylogenetic analysis of ASG2 and WDTC1-like proteins. Phylogenetic tree of ASG2- and WDTC1-related amino acid sequences. Human proteins are red, *Arabidopsis* proteins are green, and proteins of other organisms are black (Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Os, *Oryza sativa* and Pp, *Physcomitrella patens*). WDTCs are in the gray box. A cluster of TPR proteins is indicated. The dotted frame indicates ADIPOSE and neighboring clusters with their corresponding protein domain organization. The inset corresponds to phylogeny of the seven-WD40 blocks of the proteins in the dotted frame. ADP, antiobesity factor ADIPOSE; ASG2, Altered Seed Germination 2; DWD, DDB1-binding WD40 protein; TPR, Tetratricopeptide Repeat; WDTC1, WD40 and TPR 1.

extracted in 0.5 ml of hexane following the addition of 0.5 ml of 0.9% NaCl. Next, 0.3 ml of the upper phase was dried under N_2 before analysis by gas chromatography with flame ionization detector (GC-FID) (AlphaMOS PR2100). FAMES were separated on a BPX-70 SGE capillary column (Perichrom).

For oil body observation, embryos were incubated in a $2 \mu\text{g}\cdot\text{ml}^{-1}$ aqueous solution of Nile red (Sigma [Sigma Chemical], St. Louis, MO) to visualize neutral lipids. Images were acquired using confocal laser scanning microscopy based on

the Leica SP8 gSTED system. Fluorescence labeling from Nile red was observed with a 488-nm light wavelength generated by a white light laser and an emission band of 550–650 nm.

In vitro isoprenylation assay

For the *in vitro* isoprenylation assay, the last 30 nucleotides of the WDTC1 cDNA (encoding the last 10 aa of the C-terminus) were added at the 3'-end of the glutathione *S*-transferase in the pGEX-5x-1 plasmid (GE Healthcare Life Sciences) by PCR using specific primers (Table S3 in File S1). A WDTC1 variant

Figure 2 Sequence alignment and representation of ASG2 and WDTC1 proteins. (A) Alignment of ASG2 and WDTC1 amino acid sequences. WD40 blades are framed and numbered, sequential strands a-b-c-d are indicated and highlighted in yellow, and “H” indicates hotspots (according to the WD40 Prediction Server: wu.scbb.pkusz.edu.cn). TPRs are highlighted in gray (according to the TPR Prediction Server: <https://toolkit.tuebingen.mpg.de/tprpred>). The ASG2-specific acidic domain is highlighted in green with the corresponding exons, and in pink in WDTC1. ASG2 and WDTC1 DWD-boxes are underlined in the fourth WD40. (B) Schematic representation of ASG2 and WDTC1 proteins deduced from (A). The different elements are colored and numbered as in (A). Arrows with black edge correspond to ASG2 and WDTC1 DWD-boxes (strand b-c of WD4). Black stars are ASG2 and WDTC1 common hotspots. Red and green stars indicate WDTC1- and ASG2-specific hotspots, respectively. ASG2, Altered Seed Germination 2; DWD, DDB1-binding WD40 protein; TPR, Tetratricopeptide Repeat; WDTC1, WD40 and TPR 1.

with the CaaX to SaaX mutation was also produced. Protein expression, purification, and isoprenylation assays were performed as described in Dutilleul *et al.* (2016).

Bimolecular Fluorescence Complementation (BiFC) experiments and HDAC9 subcellular localization

Y_C -ASG2_{CTQS} and Y_C -ASG2_{STQS} constructs are described in Dutilleul *et al.* (2016). The full-length *AtHDAC6* and *AtHDAC9* cDNAs were obtained by RT-PCR with total

RNA extracted from an *Arabidopsis* cell culture. cDNAs were cloned in the *NcoI* and *NotI* sites of the pSY728 plasmid (Bracha-Drori *et al.* 2008) to generate the Y_N -HDAC6/9 constructs. *AtHDAC9* cDNA was cloned in the *BglII* and *SpeI* sites of the pSCA-cYFP vector (Guirimand *et al.* 2009) to generate the *AtHDAC9*-YFP construct (Table S3 in File S1 for primers). Constructs were used for protoplast transfection and YFP (yellow fluorescent protein) fluorescence was detected as described in Dutilleul *et al.* (2016).

Figure 3 Comparison of *ASG2* and *WDT C1* genomic organization. Exons are numbered and their location in the different domains indicated (black, TPRs and gray, WD40s). + indicates *ASG2* additional introns. Light gray strips indicate conserved intron locations. *ASG2* sequence and *WDT C1* exons are represented at scale, size of *WDT C1* introns are indicated above (base pair). *ASG2*, Altered Seed Germination 2; TPR, Tetratricopeptide Repeat; WD, WD40; *WDT C1*, WD40 and TPR 1.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Molecular constructs and seeds of mutant lines are available upon request.

Results and Discussion

ASG2 is the human *WDT C1* ortholog in *Arabidopsis thaliana*

To clarify phylogenetic relationships between plant and animal *WDT C1*-like sequences, *ASG2* and *WDT C1* protein sequences were used as queries for BLAST and reciprocal BLAST searches in *Arabidopsis* and human NCBI databases. For both organisms the 10 best hits of both approaches, including *WDT C1*-like, WD40, and TPR proteins (Tables S1 and S2 in File S1), were submitted to phylogenetic analysis. The phylogenetic tree (Figure 1) shows that *ASG2* belongs to the ADP/*WDT C1* cluster, which consists of *WDT C1*, DmADP (*D. melanogaster*), and CeADP (*C. elegans*). *WDT C1* homologs are also present in rice (Os*WDT C1*) and moss (Pp*WDT C1*) (Figure 1), suggesting that *WDT C1*-like proteins are widely distributed in plant and animal kingdoms. Querying the fungal database (<http://fungidb.org/>) identified *WDT C1*-like proteins in only two oomycete geni, *Albugo candida* and *Plasmopara halstedii*, revealing that *WDT C1* homologs are not as well-distributed in the fungal kingdom (Figure S1 in File S1). The ADP/*WDT C1* cluster is close to the DCAF6/DCAF8 WD40 protein cluster, which includes three *Arabidopsis* WD40 proteins that also harbor putative DWD (DDB1-binding WD40 protein)-boxes (Figure 1 and Figure S2 in File S1). To monitor the impact of TPR sequences on ADP/*WDT C1* homologs and DCAF6/8 sequence alignment and phylogeny, additional phylogenetic analysis focused on WD40 sequences was conducted and returned similar results (Figure 1, inset).

Plant and animal *WDT C1*-like proteins share a similar domain organization: three TPRs surrounded by five N-terminal WD40s

and two C-terminal WD40s (Figure 1). Alignment of *ASG2* and *WDT C1* shows 29.4% identity (42.3% similarity) of the overall sequence, 29.5% identity (42.6% similarity) in the N-terminal WD40s, 22.6% identity (41.4% similarity) in TPRs, and 39.6% identity (57.4% similarity) in the C-terminal WD40s (Figure 2A). Plant *WDT C1*-like proteins contain an additional ~100-aa acidic region (24.1% Asp/Glu vs. 12.6% overall) located between the TPRs and C-terminal WD40 domains (Figure 1 and Figure 2A), suggesting an additional plant-specific function. Nevertheless, the conservation of WD40 sequential strand a-b-c-d locations in *ASG2* and *WDT C1* (Figure 2) suggests that the seven WD40s of *ASG2* adopt the typical β -propeller (Li *et al.* 2006; Wu *et al.* 2012). According to the WD40 Prediction Server, *ASG2* and *WDT C1* harbor 13 and 15 putative hotspots, respectively (Figure 2), which correspond to WD40 protein–protein-contacting residues (Wu *et al.* 2012). Among these hotspots, 12 display conserved locations (Figure 2), suggesting that *Arabidopsis* and human *WDT C1* protein partners may bear similarities.

ASG2 and *WDT C1* are 19- and 15-exon genes, respectively. Three additional introns are present in *ASG2* within TPR, WD7, and the C-terminus coding sequences (Figure 3). The plant-specific acidic rich region described above is encoded by a 332-bp additional exon in the *ASG2* gene (14th exon, Figure 3). Finally, *ASG2* and *WDT C1* share seven intron locations distributed throughout the TPR and WD40 coding regions (Figure 3). This suggests that the specific domain organization of *WDT C1*-like sequences results from a singular assembly of WD40s and TPRs that occurred in the genome of a plant–animal common ancestor rather than kingdom-specific events. It is likely that *ASG2* and ADP/*WDT C1* originate from a common primordial *ASG2*/ADP/*WDT C1* sequence.

asg2 mutants accumulate higher FA content in seed

Considering the physiological functions of ADP/*WDT C1* in fat metabolism, we then wondered whether *ASG2* might also behave as an *Arabidopsis* “antiobesity” factor. To check

Figure 4 Characteristics of FA contents and oil bodies of *Arabidopsis* WT and *asg2* seeds. (A) *Arabidopsis* WT and *asg2* seed measurements. Seeds were harvested from plants grown under optimal or low irradiance (i.e., 300 or 25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Values are means $\pm$ SD of 50 independent measures. (B) Total FA contents of seeds harvested from plants grown at 25 or 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light, as indicated. Values are means $\pm$ SD of

whether ASG2 is engaged in fat metabolism, we examined shape and FA contents of mature seeds produced by *Arabidopsis asg2* knockout plants.

Because sucrose supply, produced through photosynthesis, is an important factor that influences fat storage in seeds (Li *et al.* 2006; Weselake *et al.* 2009; Baud and Lepiniec 2010), *Arabidopsis* plants were grown under 300 or 25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ irradiance, which corresponds with optimal and low irradiance, respectively (Li *et al.* 2006; Mishra *et al.* 2012). Under both irradiances, seed and embryo morphologies of WT and *asg2* did not display obvious morphological differences (Figure S3, A and B in File S1). This suggests that ASG2 is not essential for seed and embryo morphogenesis. However, under optimal irradiance, seed weight and size measurements revealed that *asg2* seeds were slightly wider than WT, while weight and length were similar (Figure 4A). In addition, under low irradiance, seed size and weight were both altered in *asg2*. *asg2* seeds were 18.3% heavier than WT (i.e., 14.2 and 16.8 μg for WT and *asg2-1*, respectively), were significantly longer, and therefore displayed a larger volume (Figure 4A). Under optimal irradiance, *asg2* and WT accumulated ~ 7 μg FA.seed $^{-1}$ (Figure 4B). Light limitation reduced the total FA accumulation in all genotypes (Figure 4B); however, *asg2* maintained up to 6 μg FA.seed $^{-1}$, whereas in WT seeds it decreased to 4.5 μg (which corresponds to 357 (*asg2-1*) and 316 (WT) μg FA per mg of seeds). Nile red staining of embryo cells showed that, under low irradiance, a denser network of oil bodies was observed in *asg2* compared to WT (Figure 4C). This observation substantiates the higher content of FA in *asg2* seeds in this condition. Furthermore, no qualitative changes in storage protein and oil body-associated protein patterns were observed (Figure S4, A and B in File S1), suggesting that ASG2 acts on FA biosynthesis regulation rather than embryo subcellular organization. Detailed analysis of FA contents revealed that under optimal irradiance, *asg2* and WT seeds shared a similar overall composition (Figure 5A). Interestingly, seeds from *asg2* plants grown under low irradiance displayed an increased monounsaturated FA (muFA) content (i.e., C18:1 and C20:1) compared to WT and a reduced C18:3 content, while saturated FA (C16:0, C18:0, and C20:0) and C18:2 distributions were unchanged (Figure 5B). In *Arabidopsis* seed, C18:3 synthesis proceeds by sequential desaturation of C18:1, which involves ω -6-fatty acid desaturase (FAD2) and ω -3-fatty acid desaturase (FAD3) (Baud *et al.* 2008). Because the distribution of C18s is altered in *asg2*, we can hypothesize that ASG2 modulates the gene expression or the activity of FAD2 and/or FAD3. Moreover, since C20:1 results from the addition of two-carbon moieties on preexisting

seven independent replicates (each composed of ~ 300 seeds). Student's *t*-test was performed to compare the data and asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). (C) Confocal sections of living mature *Arabidopsis* embryos stained with Nile red prepared from the seeds described in (A). Bar, 5 μm . FA, fatty acid; WT, wild-type.

Figure 5 FA composition of *Arabidopsis* WT and *asg2* seeds. FA content of seeds harvested from plants grown at 300 (A) or 25 (B) $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ light, as indicated. See *Materials and Methods* for technical details. Values are means $\pm$ SD of seven independent replicates (each composed of ~ 300 seeds). Student's *t*-test was performed to compare the data and asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). FA, fatty acid; WT, wild-type.

C18:1 (Baud *et al.* 2008), an increase in C20:1 in *asg2* seeds may be linked to a higher C18:1 content. This would explain the differential distribution of unsaturated FAs in *asg2* seeds.

As observed in animal cells, *ASG2* disruption in *Arabidopsis* plants is associated with an increased FA content that may be likened to an obese seed phenotype. Moreover, *WDTC1*-related obesity is associated with an increase in muFA intake in human populations (Lai *et al.* 2009). This reveals that human and *Arabidopsis* *WDTC1*-like proteins control FA content in adipose tissue and seeds, respectively, and specifically modulate muFA distribution. These results highlight a remarkable conservation of *ASG2*/*WDTC1* functions in the regulation of fat metabolism in plants and animals.

Recently, Groh *et al.* (2016) highlighted the critical role of a peculiar H-box in the antiobesity properties of *WDTC1*. H-boxes are poorly conserved 13-aa sequences that fold into an α -helix (Li *et al.* 2010). The *WDTC1* H-box has been fully characterized and its molecular modeling has revealed that it is required for DDB1-binding (Li *et al.* 2010). Up to now, no H-box has been described in *ASG2*; however, prediction of *ASG2* N-terminus topology reveals an α -helix structure that may correspond to the *ASG2* H-box (Figure 6, A and B). Indeed, despite a low amino acid conservation, four of the *WDTC1* H-box key DDB1-contacting residues are conserved in the putative *ASG2* H-box (Figure 6, B and C). The presence of a DWD box and a putative H-box in *ASG2* suggests that *ASG2*-related fat regulations may occur in a

Figure 6 Comparison of *ASG2* and *WDTC1* H-boxes. (A) Sequence alignment of *ASG2* and *WDTC1* N-termini. Putative H-boxes are framed in pink. Cylinders indicate the predicted α -helix (PSIpred). (B) Sequence alignment of *ASG2* and human DCAFs H-boxes [adapted from Gérard *et al.* (2014)]. Residues of the H-boxes previously reported to be involved in DDB1-DCAF interactions are highlighted and numbered. (C) Helical diagrams of *WDTC1* and putative *ASG2* H-boxes. Numbers are the same as in (B). Green and red circles indicate conserved and different putative DDB1-interacting residues, respectively. *ASG2*, Altered Seed Germination 2; DCAF, DDB1-CUL4 Associated Factor; *WDTC1*, WD40 and TPR 1.

DDB1-dependent manner similarly to that of *WDTC1*. Involvement of the DDB1-CUL4 E3 ubiquitin ligase in fat metabolism in seeds has not been described so far. However, loss of *CUL4* or *DDB1* affects early steps in embryo development that may lead to seed abortion (Bernhardt *et al.* 2006, 2010). According to the *Arabidopsis* BioArray Expression Browser, *ASG2*, *DDB1a*, and *CUL4* expression increase during seed development with similar kinetics (Figure S5A in File S1). Interestingly, heat map and cluster analysis including TAG biosynthesis genes and seed development regulatory genes (Figure S5B in File S1) revealed that *ASG2*, *DDB1a*, and *CUL4* expression patterns were close to that of the transcription factor *ABI5* and the diacylglycerol O-acyltransferase (Figure 7), involved in the last steps of seed development and accumulation of storage compounds (Baud *et al.* 2008). These findings suggest an involvement of *ASG2* in the last steps of seed maturation in relation to the DDB1-CUL4 E3 ubiquitin ligase complex.

Farnesylated *ASG2* interacts with *Arabidopsis* HDAC9

In characterizing *WDTC1* molecular functions, Suh *et al.* (2007) reported that coexpressed *WDTC1* and HDAC #3 (HsHDAC3) interact in human HEK293 cells. HsHDAC3 and HsHDAC1/2/8 are homologous to the yeast HDAC reduced potassium dependency 3 (RPD3), which defines the RPD3-like cluster (Emiliani *et al.* 1998 and Figure 8A).

Figure 7 Heat map and cluster analysis of *ASG2*, *DDB1a*, and *CUL4* during seed development. Expression data of *ASG2*, *DDB1a*, *CUL4*, and other genes related to TAG biosynthesis and seed development (see Figure S5 in File S1 for details) were retrieved from the Seed and Silique Development Data Source of *Arabidopsis* BioArray Resource. Dashed box indicates *ASG2* cluster. *ASG2*, Altered Seed Germination 2; TAG, triacylglycerols.

HDACs are responsible for the deacetylation of histones and give a tag for epigenetic repression (Kadonaga 1998). In mammals, HDAC3 participates in hepatic lipid homeostasis through the control of glucose metabolism (Gao *et al.* 2006; Sun *et al.* 2011) and is associated with obesity and type 2 diabetes [reviewed by McGee-Lawrence *et al.* (2015)]. HDAC3-driven HDAC mediates PPAR γ inhibition (Suh *et al.* 2007). It is thus hypothesized that WDTC1 functions in an antiobesity transcriptional PPAR γ corepressor complex with HDAC3 (Suh *et al.* 2007). No PPAR γ ortholog has been identified in flies (Suh *et al.* 2007) nor in plants (Figure S6 in File S1), suggesting that WDTC1-like proteins likely belong to a more primordial complex that functioned to control fat accumulation before PPAR γ existed. However, consistent with the conservation of WDTC1 WD40 hotspot locations in *ASG2* (Figure 2B), it is attractive to speculate that *ASG2* and WDTC1 protein interactions engage similar partners involved in fat metabolism. Thus, we set out to determine whether *ASG2* interacts with HDAC proteins and searched for RPD3-like homologs in *Arabidopsis*. Among the 12 proteins of the *Arabidopsis* HDAC family (Pandey *et al.* 2002), AtHDAC6/7/9/19 belong to the RPD3-like cluster (Alinsug *et al.* 2009 and Figure 8A). We investigated the interaction of *ASG2* with two RPD3-like HDACs: AtHDAC6 and AtHDAC9. AtHDAC6 is involved in ABA signaling, the salt stress response, seed development, and seed performance (Wang *et al.* 2014). AtHDAC9 has been characterized as regulating flowering time (Kim *et al.* 2013; Kang *et al.* 2015), salt/drought responsiveness (Zheng *et al.* 2016), and controlling seedling traits through the regulation of photosynthesis-related genes and glucose homeostasis (van Zanten *et al.* 2014). Consistent with the presence of an N-terminal nuclear localization sequence (NLS) (Alinsug *et al.* 2009), AtHDAC6-GFP fusion protein has been shown to localize in the nucleus (Wu *et al.* 2008). No NLS is predicted for AtHDAC9 (Alinsug *et al.* 2009) and the AtHDAC9-YFP fusion protein displays a nucleo-cytosolic subcellular localization (Figure 8B), as

Figure 8 *ASG2* interaction with AtHDAC9 and *in vitro* prenylation of WDTC1. (A) Phylogenetic tree of *Arabidopsis* (green) and human (red) HDAC proteins. *Arabidopsis* truncated AtHDAC10 and AtHDAC17 were removed from the analysis. The gray box indicates the RPD3-like cluster. Asterisks indicate AtHDAC6, AtHDAC9, and HsHDAC3. (B) *Arabidopsis* protoplast transiently expressing AtHDAC9-YFP fusion protein. (C) BiFC experiments: *Arabidopsis* protoplasts transiently coexpressing YFP_N- and YFP_C- fusion proteins, as indicated on the top. See Figure S7A in File S1 for BiFC controls. DAPI, nucleus stained with DAPI; YFP, YFP fluorescence; YFP + DAPI, overlay. (D) Prenylation scores for *ASG2* and putative WDTC1 CaaX-boxes (Prenylation Prediction Server). Green and red scores indicate, respectively, positive and negative predictions of isoprenylation. Putative prenylated Cys are underlined. (E) *In vitro* farnesylation assay for WDTC1 putative CaaX-box. *ASG2*_{CTQS} is a positive control corresponding to *ASG2* 10-aa C-terminus fused to the GST (Duttilleul *et al.* 2016). WDTC1_{SRPS}, GST fused to WDTC1 with a Cys-to-Ser mutated CaaX-box. Up, fluorescence. Down, Coomassie blue staining. *ASG2*, Altered Seed Germination 2; AtHDAC, *A. thaliana* HDAC; BiFC, ; HDAC, histone deacetylase; HsHDAC, human HDAC; PFT, protein farnesyl-transferase; PGGT-I, protein geranylgeranyl-transferase type I; RPD3, Reduced potassium dependency 3; WDTC1, WD40 and TPR 1; YFP, yellow fluorescent protein.

reported for several other plant HDAC proteins (Alinsug *et al.* 2012; Zhao *et al.* 2015), as well as HsHDAC3 (Gao *et al.* 2006). Because *ASG2* protein is subject to post-translational modification by farnesylation (Duttilleul *et al.* 2016), we monitored *ASG2* interaction with HDACs for both farnesylatable (*ASG2*_{CTQS}) and nonfarnesylatable (*ASG2*_{STQS}, farnesylated

Cys mutated to Ser) forms. No interaction was detected between ASG2 and AtHDAC6 through BiFC experiments (Figure S7A in File S1). However, BiFC interactions are observed in the cytosol with the farnesylatable ASG2_{CTQS} and AtHDAC9 (Figure 8C and Figure S7A in File S1), showing that HDAC-binding capacity is conserved in ASG2. No interaction is observed with the nonfarnesylatable ASG2_{STQS} (Figure 8C), revealing that ASG2 farnesylation is required for ASG2-AtHDAC9 interaction. Although AtHDAC6 belongs to the RPD3-like cluster, AtHDAC9 is more closely related to HsHDAC3 (Figure 8A). Interaction of ASG2 with AtHDAC9 probably depends on specific residues absent in AtHDAC6. Moreover, because ASG2-AtHDAC9 interaction occurs in the cytosol but not in the nucleus, we can suspect that the cytosolic localization of AtHDAC9 facilitates its interaction with the farnesylated form of ASG2, which is also localized in the cytosol (Dutilleul *et al.* 2016).

CaaX-boxes are found in ASG2 orthologs in plants (Dutilleul *et al.* 2016); however, up to now, no isoprenylation event has been reported in animal ADP/WDTC1 proteins. We identified a putative CaaX-box at the WDTC1 C-terminus: CRPS. According to the Prenylation Prediction Server (<http://mendel.imp.ac.at/sat/PrePS/index.html>), WDTC1 is predicted to be farnesylated with a similar score as ASG2 (Figure 8D). Actually, a GST fusion protein containing the C-terminal 10-aa peptide of WDTC1 can be farnesylated by PFT *in vitro* (Figure 8E). No signal was detected when PGGT-I was used in the assays (Figure S7B in File S1). Additionally, we found that all proteins in the ADP/WDTC1 cluster harbor a putative CaaX-box (*i.e.*, *Dm*, CFNN; *Ce*, CVQS; *Os*, CAQS; and *Pp*, CTQS), which strongly suggests that protein isoprenylation may be a common feature of ADP/WDTC1/ASG2 orthologs and could be required to fully achieve protein functions. Interestingly, farnesylation is required for the ASG2-HDAC9 binding but not for the ASG2-DDB1 interaction. Nevertheless, farnesylation modifies the subcellular localization of the ASG2-DDB1 complex (Dutilleul *et al.* 2016). Because the WDTC1-HsHDAC3 interaction was identified through immunoprecipitation and yeast two-hybrid assays using WDTC1 farnesylatable protein (*i.e.*, WT) (Suh *et al.* 2007), it is difficult to assess whether isoprenylation also influences WDTC1-HsHDAC3 binding or WDTC1 subcellular localization as observed for ASG2.

Conclusions

Our findings reveal a remarkable conservation of structural, functional, and physiological features of plant and animal ASG2/ADP/WDTC1 homologs, and extend the evolutionarily conserved role of ADP/WDTC1 proteins in FA metabolism to the plant kingdom. Furthermore, severe human diseases, such as progeria, cardiac hypertrophy, and cancers, have been associated with isoprenylated proteins (Xu *et al.* 2015). Because WDTC1 harbors a CaaX-box that is farnesylated *in vitro*, investigating its *in vivo* farnesylation with regard to its antiadipogenesis properties might be an attractive anti-obesity therapeutic prospect. Finally, in plants, seeds are of

great interest for food and biodiesel industries, and there are extensive efforts aimed at improving oil-producing crops. Because ASG2-deficient plants display a significant obese seed phenotype, engineering ASG2 orthologs through genome editing approaches may provide valuable tools to further improve oleaginous crop production.

Acknowledgments

The authors gratefully thank Nina Hautekeete (University of Lille, France), Johan-Owen de Craene, and Benoit St-Pierre for critical comments on manuscript, and Marie-Antoinette Marquet (EA2106, University of Tours) for her technical expertise in plant and cell culture. This research was financially supported by the “Enseignement Supérieur et de la Recherche” French Ministry and the Région Centre.

Literature Cited

- Ali, A. T., W. E. Hochfeld, R. Myburgh, and M. S. Pepper, 2013 Adipocyte and adipogenesis. *Eur. J. Cell Biol.* 92: 229–236.
- Alinsug, M. V., C. W. Yu, and K. Wu, 2009 Phylogenetic analysis, subcellular localization, and expression patterns of RPD3/HDA1 family histone deacetylases in plants. *BMC Plant Biol.* 28: 9–37.
- Alinsug, M. V., F. F. Chen, M. Luo, R. Tai, L. Jiang *et al.*, 2012 Subcellular localization of class II HDAs in *Arabidopsis thaliana*: nucleocytoplasmic shuttling of HDA15 is driven by light. *PLoS One* 7: e30846.
- Angers, S., T. Li, X. Yi, M. J. MacCoss, R. T. Moon *et al.*, 2006 Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. *Nature* 443: 590–593.
- Bassel, G. W., H. Lan, E. Glaab, D. J. Gibbs, T. Gerjets *et al.*, 2011 Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. *Proc. Natl. Acad. Sci. USA* 108: 9709–9714.
- Baud, S., and L. Lepiniec, 2010 Physiological and developmental regulation of seed oil production. *Prog. Lipid Res.* 49: 235–249.
- Baud, S., B. Dubreucq, M. Miquel, C. Rochat, and L. Lepiniec, 2008 Storage reserve accumulation in *Arabidopsis*: metabolic and developmental control of seed filling. *Arabidopsis Book* 6: e0113.
- Bernhardt, A., E. Lechner, P. Hano, V. Schade *et al.*, 2006 CUL4 associates with DDB1 and DET1 and its downregulation affects diverse aspects of development in *Arabidopsis thaliana*. *Plant J.* 47: 591–603.
- Bernhardt, A., S. Mooney, and H. Hellmann, 2010 *Arabidopsis* DDB1a and DDB1b are critical for embryo development. *Planta* 232: 555–566.
- Bracha-Drori, K., K. Shichrur, T. C. Lubetzky, and S. Yalovsky, 2008 Functional analysis of *Arabidopsis* postprenylation CaaX processing enzymes and their function in subcellular protein targeting. *Plant Physiol.* 148: 119–131.
- Crowell, D. N., and D. H. Huizinga, 2009 Protein isoprenylation: the fat of the matter. *Trends Plant Sci.* 14: 163–170.
- Doane, W. W., 1960 Developmental physiology of the mutant female sterile adipose of *Drosophila melanogaster*. II. Effects of altered environment and residual genome on its expression. *J. Exp. Zool.* 145: 23–41.
- Dutilleul, C., I. Ribeiro, N. Blanc, C. D. Nezames, X. W. Deng *et al.*, 2016 ASG2 is a farnesylated DWD protein that acts as ABA negative regulator in *Arabidopsis*. *Plant Cell Environ.* 39: 185–198.

- Emiliani, C., W. Fischle, C. Van Lint, Y. Al-Abed, and E. Verdin, 1998 Characterization of a human RPD3 ortholog, HDAC3. *Proc. Natl. Acad. Sci. USA* 95: 2795–2800.
- Finkelstein, R. R., S. S. Gampala, and C. D. Rock, 2002 Absciscic acid signalling in seeds and seedlings. *Plant Cell* 146: S15–S45.
- Focks, N., and C. Benning, 1998 Wrinkled1: a novel, low-seed-oil mutant of *Arabidopsis* with a deficiency in the seed-specific regulation of carbohydrate metabolism. *Plant Physiol.* 118: 91–101.
- Galgani, J. E., D. E. Kelley, J. B. Albu, J. Krakoff *et al.*, 2013 Adipose tissue expression of adipose (WDT1) gene is associated with lower fat mass and enhanced insulin sensitivity in humans. *Obesity* 21: 2244–2248.
- Gao, Z., Q. He, B. Peng, P. J. Chiao, and J. Ye, 2006 Regulation of nuclear translocation of HDAC3 by I κ B α is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. *J. Biol. Chem.* 281: 4540–4547.
- Gérard, F. C., R. Yang, B. Romani, A. Poisson, J. P. Belzile *et al.*, 2014 Defining the interactions and role of DCAF1/VRBP in the DDB1-cullin4A E3 ubiquitin ligase complex engaged by HIV-1 Vpr to induce a G2 cell cycle arrest. *PLoS One* 9: e89195.
- Groh, B. S., F. Yan, M. D. Smith, Y. Yu, X. Chen *et al.*, 2016 The anti-obesity factor WDT1 suppresses adipogenesis via the CRL4WDT1 E3 ligase. *EMBO Rep.* 17: 638–647.
- Guirimand, G., V. Burlat, A. Oudin, A. Lanoue, B. St-Pierre *et al.*, 2009 Optimization of the transient transformation of *Catharanthus roseus* cells by particle bombardment and its application to the subcellular localization of hydroxymethylbutenyl 4-diphosphate synthase and geraniol 10-hydroxylase. *Plant Cell Rep.* 28: 1215–1234.
- Häder, T., S. Müller, M. Aguilera, K. G. Eulenberg, A. Steuernagel *et al.*, 2003 Control of triglyceride storage by a WD40/TPR-domain protein. *EMBO Rep.* 4: 511–516.
- Jin, J., E. E. Arias, J. Chen, J. W. Harper, and J. C. Walter, 2006 A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol. Cell* 23: 709–721.
- Kadonaga, J. T., 1998 Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* 92: 307–313.
- Kang, M. J., H. S. Jin, Y. S. Noh, and B. Noh, 2015 Repression of flowering under a non-inductive photoperiod by the HDA9–AGL19-FT module in *Arabidopsis*. *New Phytol.* 206: 281–294.
- Kim, W., D. Latrasse, C. Servet, and D. X. Zhou, 2013 *Arabidopsis* histone deacetylase HDA9 regulates flowering time through repression of AGL19. *Biochem. Biophys. Res. Commun.* 432: 394–438.
- Lai, C. Q., L. D. Parnell, D. K. Arnett, B. García-Bailo, M. Y. Tsai *et al.*, 2009 WDT1, the ortholog of *Drosophila* adipose gene, associates with human obesity, modulated by MUFA intake. *Obesity (Silver Spring)* 17: 593–600.
- Lee, J.-H., W. Terzaghi, G. Gusmaroli, J.-B. F. Charron, H.-J. Yoon *et al.*, 2008 Characterization of *Arabidopsis* and rice DWD proteins and their roles as substrate receptors for CUL4-RING E3 Ubiquitin ligases. *Plant Cell* 20: 152–167.
- Li, T., E. I. Robert, P. C. van Breugel, M. Strubin, and N. Zheng, 2010 A promiscuous alpha-helical motif anchors viral hijackers and substrate receptors to the CUL4–DDB1 ubiquitin ligase machinery. *Nat. Struct. Mol. Biol.* 17: 105–111.
- Li, Y., F. Beisson, M. Pollard, and J. Ohlrogge, 2006 Oil content of *Arabidopsis* seeds: the influence of seed anatomy, light and plant-to-plant variation. *Phytochemistry* 67: 904–915.
- McGee-Lawrence, M. E., T. A. White, N. K. LeBrasseur, and J. J. Westendorf, 2015 Conditional deletion of Hdac3 in osteoprogenitor cells attenuates diet-induced systemic metabolic dysfunction. *Mol. Cell. Endocrinol.* 410: 42–51.
- Mishra, Y., H. J. Jänkänpää, A. Z. Kiss, C. Funk, W. P. Schröder *et al.*, 2012 *Arabidopsis* plants grown in the field and climate chambers significantly differ in leaf morphology and photosystem components. *BMC Plant Biol.* 12: 6.
- Murphy, D. J., 1993 Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Prog. Lipid Res.* 32: 247–280.
- Nakashima, K., and K. Yamaguchi-Shinozaki, 2013 ABA signaling in stress-response and seed development. *Plant Cell Rep.* 32: 959–970.
- Okazaki, Y., and K. Saito, 2014 Roles of lipids as signaling molecules and mitigators during stress response in plants. *Plant J.* 79: 584–596.
- Pandey, R., A. Müller, C. A. Napoli, D. A. Selinger, C. S. Pikaard *et al.*, 2002 Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res.* 30: 5036–5055.
- Riefler, M., O. Novak, M. Strnad, and T. Schmülling, 2006 *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18: 40–54.
- Rosen, E. D., C. J. Walkey, P. Puigserver, and B. M. Spiegelman, 2000 Transcriptional regulation of adipogenesis. *Genes Dev.* 14: 1293–1307.
- Shu, K., X. D. Liu, Q. Xie, and Z. H. He, 2016 Two faces of one seed: hormonal regulation of dormancy and germination. *Mol. Plant* 9: 34–45.
- Suh, J. M., D. Zeve, R. McKay, J. Seo, Z. Salo *et al.*, 2007 Adipose is a conserved dosage-sensitive antiobesity gene. *Cell Metab.* 6: 195–207.
- Sun, Z., D. Feng, L. J. Everett, A. Bugge, and M. A. Lazar, 2011 Circadian epigenomic remodeling and hepatic lipogenesis: lessons from HDAC3. *Cold Spring Harb. Symp. Quant. Biol.* 76: 49–55.
- van Zanten, M., C. Zöll, Z. Wang, C. Philipp, A. Carles *et al.*, 2014 HISTONE DEACETYLASE 9 represses seedling traits in *Arabidopsis thaliana* dry seeds. *Plant J.* 80: 475–488.
- Voet, D., and J. G. Voet, 1990 Lipids and membranes, pp. 271–314 in *Biochemistry*, edited by D. Voet, and J. G. Voet. Wiley, New York.
- Wang, Z., H. Cao, F. Chen, and Y. Liu, 2014 The roles of histone acetylation in seed performance and plant development. *Plant Physiol. Biochem.* 84: 125–133.
- Weselake, R. J., D. C. Taylor, M. H. Rahman, S. Shah, A. Laroche *et al.*, 2009 Increasing the flow of carbon into seed oil. *Bio-technol. Adv.* 27: 866–878.
- Wu, K., L. Zhang, C. Zhou, C.-W. Yu, and V. Chaikam, 2008 HDA6 is required for jasmonate response, senescence and flowering in *Arabidopsis*. *J. Exp. Bot.* 59: 225–234.
- Wu, X. H., Y. Wang, Z. Zhuo, F. Jiang, and Y. D. Wu, 2012 Identifying the hotspots on the top faces of WD40-repeat proteins from their primary sequences by β -bulges and DHSW tetrads. *PLoS One* 7: e43005.
- Xu, N., N. Shen, X. Wang, S. Jiang, B. Xue *et al.*, 2015 Protein prenylation and human diseases: a balance of protein farnesylation and geranylgeranylation. *Sci. China Life Sci.* 58: 328–335.
- Yu, F., Y. Wu, and Q. Xie, 2016 Ubiquitin–proteasome system in ABA signaling: from perception to action. *Mol. Plant* 9: 21–33.
- Zhao, L. J., J. Lu, P. Y. Zhang, S. Wu, S. Yang *et al.*, 2015 Identification and characterization of histone deacetylases in tomato (*Solanum lycopersicum*). *Front. Plant Sci.* 5: 760.
- Zheng, Y., Y. Ding, X. Sun, S. Xie, D. Wang *et al.*, 2016 Histone deacetylase HDA9 negatively regulates salt and drought stress responsiveness in *Arabidopsis*. *J. Exp. Bot.* 67: 1703–1713.

Communicating editor: S. Poethig