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SHORT COMMUNICATION



# Moonlighting proteins: putting the spotlight on enzymes

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**ABSTRACT**

AROGENATE DEHAYDRATASE2 (ADT2) is a member of the *Arabidopsis thaliana* ADT family. All members of this family act as arogenate dehydratases in phenylalanine biosynthesis, decarboxylating/dehydrating arogenate to phenylalanine. ADT2 is detected in stromules, and as a ring around the equatorial plane of dividing chloroplasts, indicating it has a second, non-enzymatic function in chloroplast division. Here, we provide further evidence for this alternative role of ADT2. First, we demonstrate that ADT2 and FtsZ co-localize around the equatorial plane at the same time. Second, FtsZ expression in an *adt2* mutant was analyzed, as well as ADT2 expression in three *Arabidopsis* chloroplast division mutants, ACCUMULATION AND REPLICATION OF CHLOROPLASTS3 (ARC3), ARC5 and ARC6. In *arc3* and *arc6* mutants, ADT2 is misexpressed and resembles the expression of FtsZ in the same mutants. However, in the *arc5* mutant, ADT2 ring positioning is observed at constriction points indicating proper relative timing. ADT2 expression in the *arc* mutants shows that the role of ADT2 in chloroplast division occurs prior to ARC5, but is dependent on ARC3 and ARC6.

**Abbreviations** used: ADT: arogenate dehydratase, ARC: accumulation and replication of chloroplasts, CFP: cyan fluorescent protein, dpi: days post infiltration, FtsZ: filamentous temperature sensitive Z, PD: plastid division, Phe: phenylalanine, YFP: yellow fluorescent protein.

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## Multifunctional and moonlighting proteins

In recent years, our understanding of proteins has changed as it has been more frequently reported that proteins can have multiple functions. Multifunctional proteins are thought to contribute to cellular complexity and can be important for the regulation of key enzymatic reactions.[1] The different functions can be achieved through a variety of ways, such as gene sharing, alternative splicing, or promiscuous enzymes.[1,2] The different roles of multifunctional proteins can be related or distinct. For instance, plant annexins have been demonstrated to have multiple functions, such as phosphodiesterase activity, $Ca^{2+}$ binding and peroxidase activity,[3] with each function correlated to a different domain of the protein.[3–5]

Moonlighting proteins have emerged as a new theme in protein evolution and they are considered a subclass of multifunctional proteins where the same protein has two distinct unrelated functions. These proteins perform both functions independently from each other using essentially the same whole protein entity, without requiring any sequence modifications.[6–8] In contrast to other multifunctional proteins, moonlighting proteins are not the result of splicing events, nor are their different functions split into separate domains.[9,10] Despite their differences, both moonlighting and multifunctional proteins open up evolutionary avenues to increase cellular complexity and flexibility in protein function without increasing genome size. In addition, a protein with two distinct functions might allow a more dynamic control over cellular processes, and a fine-tuned response to environmental stimuli.[11]

As the multiple functions of moonlighting proteins are usually unrelated, proteins can not only have two distinct enzymatic functions, but they often combine an enzymatic with a non-enzymatic function or have two non-enzymatic functions. Such non-enzymatic functions include proteins with structural roles, transcriptional repressors, and transcription factors. For instance, RACK1 (receptor for activated C kinase 1) from *Saccharomyces cerevisiae* acts as a scaffolding protein for various receptors, but is also a component of the ribosome to recruit additional proteins to this multimeric protein complex.[12] Moonlighting proteins can not only acquire new functions, but these new functions can act in unrelated cellular processes which may even occur in different tissues.[8,10] Almost 400 proteins have been listed in the MoonProt database with reported moonlighting functions[13] and we believe this is just the beginning.

Our interest was piqued when we discovered that select members of the arogenate dehydratase (ADT) enzyme family from *Arabidopsis thaliana*, typically targeted to chloroplasts, had additional subcellular localization patterns consistent with non-enzymatic roles.[14] Specifically, we show that one member, ADT2, also has a role in chloroplast division aside from its enzymatic role in phenylalanine (Phe) biosynthesis,-[15,16] suggesting it is a moonlighting protein.

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## Spotlight on ADT2

ADT2 is one of six nuclear encoded ADTs in *Arabidopsis* that catalyze the final reaction in Phe biosynthesis, converting arogenate to Phe.[15–17] All six members of this enzyme family catalyze this reaction, and all six localize to the stromules of chloroplasts.[14,15,18] This localization was expected as Phe biosynthesis has been shown to occur in chloroplasts.[19–21] However, ADT2 has a surprising additional subcellular localization pattern. We have shown that ADT2 localizes as a ring around the equatorial plane during chloroplast division (Figures 1A and 2A[14]). This localization pattern is strikingly similar to that of FtsZ1 and FtsZ2, the dimerizing components of the Z-ring, which is one of the constricting rings in chloroplast division.[22,23] Consistent with a role in chloroplast division, *adt2* mutants have large, and at times, misshapen chloroplasts (Figure 1B).[14] Rather than having the classical wild-type ellipsoid shape, *adt2-1D* chloroplasts are abnormal in shape with distorted and irregular outlines. To test if ADT2 and the Z-ring occur at the same time, we co-expressed ADT2-CFP and FtsZ2-YFP (Figure 2B). The merged images of the fluorescent proteins unambiguously show that both proteins are localized to the chloroplast division plane at the same time, forming a ring with matching curvature and positioning. We then determined what happens to FtsZ2 expression in an *adt2-1D* mutant. While expressing ADT2-CFP in an *adt2-1D* mutant leads to the formation of almost normal ring structures, essentially complementing the mutant phenotype, FtsZ2-YFP expression in the same mutant background is abnormal and spiral-forming (Figure 2A, bottom images), suggesting that the presence of ADT2 is required for proper Z-ring formation.

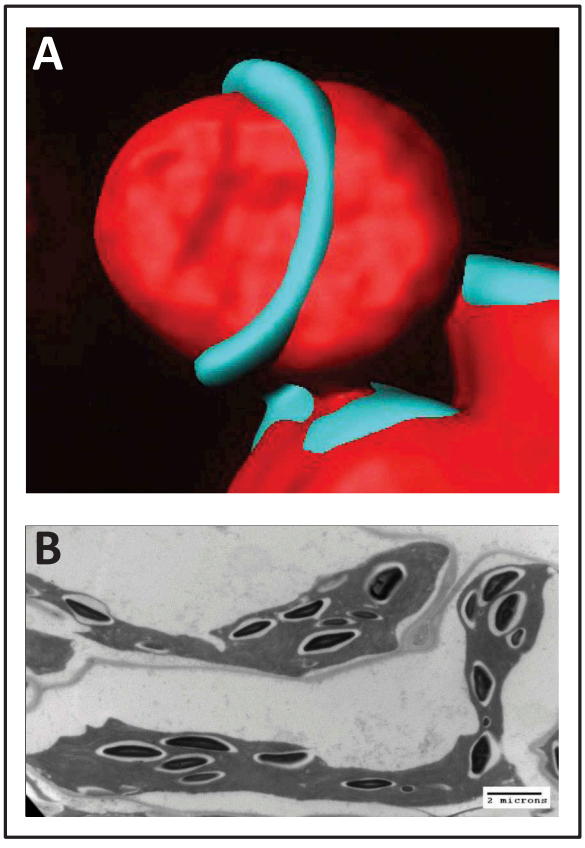


**Figure 1.** ADT2 and chloroplast morphology.
A. Confocal 3D image of a WT chloroplast expressing ADT2-CFP. ADT2-CFP forms a ring at the equatorial plane of dividing chloroplasts.
B. TEM image of a chloroplast in *adt2-1D*. Chloroplasts in the *Arabidopsis adt2* mutant line are often larger and misshapen.

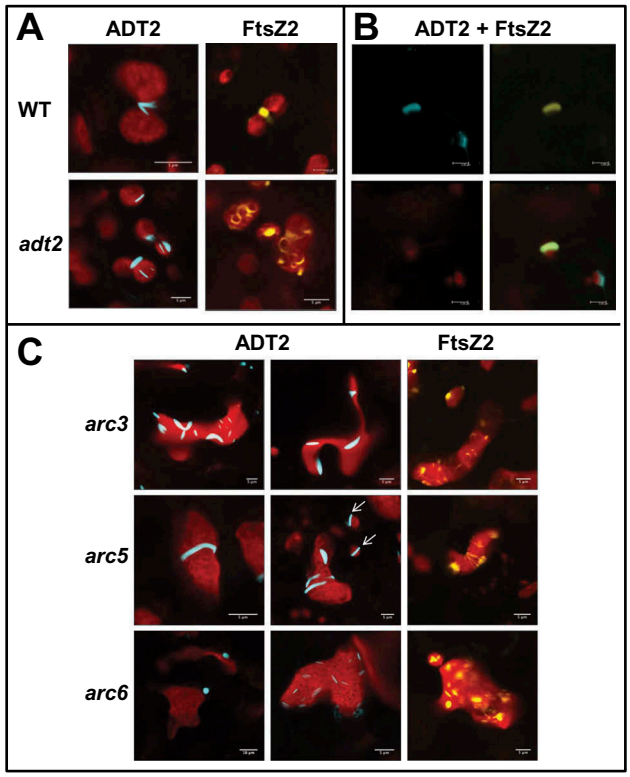


**Figure 2.** Expression of ADT2 and FtsZ2 in wildtype and *arc* mutants.
A. ADT2 and FtsZ2 were transiently expressed as ADT2-CFP or FtsZ2-YFP fusion proteins in *A. thaliana* wildtype and *adt2-1D* mutant leaves. Expression of wild type ADT2 in the *adt2-1D* background essentially complements the mutant while FtsZ2 is misexpressed. Shown are merges of chlorophyll fluorescence and ADT2-CFPs (left) and FtsZ2-YFP (right).
B. Co-expression of ADT2-CFP and FtsZ2-YFP in *N. benthamiana* leaves. Both proteins co-localize as rings at the equatorial plane of dividing chloroplasts. Shown are all individual channels image (CFP: top left, YFP: top right, chlorophyll: bottom left) and the merged (bottom right) where co-localization of YFP and CFP is seen as green.
C. ADT2 and FtsZ2 expression patterns in three *A. thaliana* chloroplast division mutants (*arc3-1*, *arc5-1* and *arc6-1*). Two samples of ADT2 expression are shown (left and middle column) and compared to FtsZ2 expression (right column). In *arc3* and *arc6* mutants, ADT2 is misexpressed, and expression patterns resemble that of FtsZ2 in the same mutants. In *arc5*, ADT2 is often seen as a single ring in both enlarged and chloroplasts that are wild type in size (arrows). Occasionally multiple rings are observed coinciding with constriction points. Merged images of chlorophyll autofluorescence and ADT2-CFPs (left and middle) and FtsZ2-YFP (right).

Genetic screens identified many *Arabidopsis* mutants with altered chloroplast size, shape, and number,[24–26] which have been used to identify many genes encoding chloroplast division proteins.[27–29] In addition, the effects of these mutations on chloroplast appearance and Z-ring positioning have allowed functional characterization of several chloroplast division proteins. In *arc3-1* plants, multiple Z-rings are able to form within a single chloroplast,[30] resulting in chloroplasts being heterogeneous in size and shape,[24] consistent with the role of ARC3 in regulating Z-ring placement.[31] In *arc5-1*, Z-ring formation is unaffected,[32] however, chloroplast division is

stalled during constriction, causing chloroplasts to be enlarged and dumbbell shaped,[25] in agreement with ARC5 (dynamin) providing the force required to constrict chloroplasts.[27,33] In *arc6-1* mutant chloroplasts, the Z-ring does not form,[29] causing a drastic phenotype of approximately two large chloroplasts per mesophyll cell.[26] In such chloroplasts, FtsZ filaments do not assemble into rings and are found scattered throughout the stroma.

We hypothesized that ADT2 chloroplast ring localization will be affected by mutations of known chloroplast division components. To test this we transiently expressed ADT2-CFP in three *Arabidopsis arc* mutants. Expression of ADT2-CFP in *arc3-1* mutants revealed differences in localization patterns compared to wild-type *Arabidopsis*. ADT2-CFP was observed as multiple bands within chloroplasts that varied in both linearity and thickness, consistent in appearance with the multiple Z-rings that form in *arc3-1* chloroplasts (Figure 2C).[30] Typically *arc3-1* chloroplasts are large and irregular in shape, however, some chloroplasts were observed that were indistinguishable from those in wild-type plants. ADT2-CFP localization in these smaller chloroplasts resembled observations from wild-type, localizing to a pole or the equatorial plane (data not shown).

In *arc5-1* chloroplasts, ADT2-CFP predominantly localized to the central constriction, once again consistent with Z-ring location[32] and a role in chloroplast division prior to ARC5. As in *arc3-1* chloroplasts, the appearance of ADT2-CFP bands varied, and could be linear or curved. Multiple bands were occasionally observed, although consistently appeared centered at constricted areas of chloroplasts (Figure 2C). Some chloroplasts in *arc5-1* leaf cells were indistinguishable from wild-type, and ADT2-CFP localization in these was comparable to that in wild-type (Figure 2C, white arrows).

In *arc6-1* plants, ADT2-CFP was visualized as short rod-shaped bands scattered throughout the stroma of chloroplasts, strikingly similar in appearance to FtsZ filaments (Figure 2C).[29] In addition, ADT2-CFP was observed as round structures commonly seen at the periphery of chlorophyll autofluorescence (Figure 2C).

Based on the ADT2 localization patterns observed in wild-type chloroplasts and chloroplasts of division mutants, we believe ADT2 has a role in chloroplast division aside from its catalytic enzymatic role (Figures 1 and 2).[14] Co-localization cannot distinguish between an ADT2-FtsZ interaction or two distinct rings being formed. Therefore, it is possible that ADT2 is a stabilizing component of the Z-ring or is involved in positioning the ring but a direct interaction between ADT2 and FtsZ1 or FtsZ2 has not yet been shown. Alternatively, the composition of the inner plastid division (PD) ring is still unidentified,[22,34] and we propose ADT2 as a candidate.

We propose that ADT2 is a moonlighting protein, with an enzymatic function in Phe biosynthesis and a non-enzymatic role in chloroplast division. We present a simple model of ADT2 involvement in chloroplast division. As ADT2 is required for proper Z-ring formation and/or placement (Figure 2A), ADT2 either acts simultaneously with (Figure 3A) or before (Figure 3B) FtsZ. While current models place the formation of the inner PD ring after Z-ring formation,[22,35] if ADT2 is the inner PD ring, or contributes to its formation, the formation of these two rings might occur in quick succession.

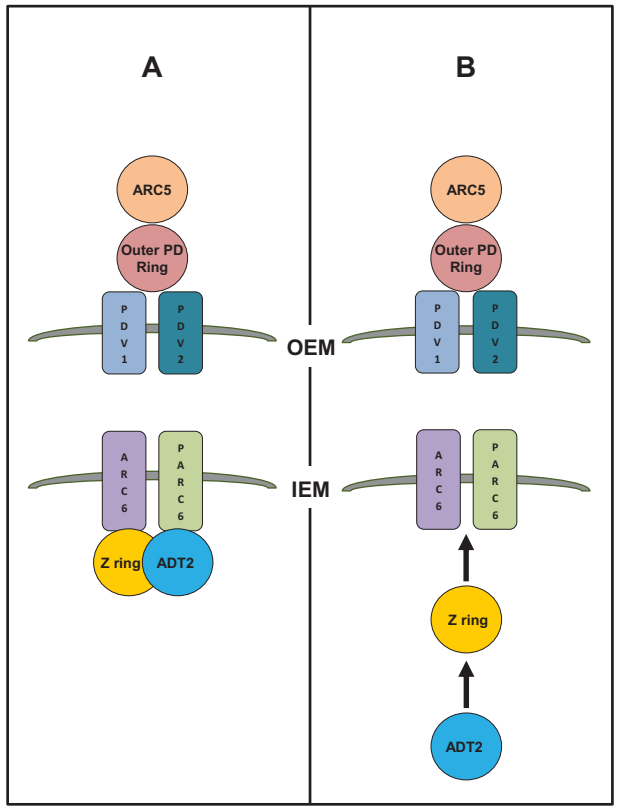


**Figure 3.** Proposed role of ADT2 in chloroplast division.
The placement of chloroplast division rings is a highly co-ordinated process where the inner two rings are anchored first inside of the inner membrane with the help of ARC6 and PARC6. This is followed by the anchoring of the outer PD ring and then the ARC5 (dynamin) ring on the outside of the outer membrane. Anchoring of the outer rings requires the function of PDV1 and PDV2. Based on our data we propose that ADT2 is forming one of the inner rings either (A) coinciding with establishing the Z-ring, formed by the FtsZ1 and FtsZ2 heterodimer or (B) preceding and required for the formation of the Z-ring.

## Why do proteins moonlight?

The evolution of multifunctional and moonlighting proteins is not well understood. The development of additional protein functions might be easier than originally anticipated, and can probably occur over a relatively short evolutionary time, as it has been shown that only a few amino acid changes can be sufficient to implement a new function.[36–39] In fact, even a single amino acid change can be sufficient to alter protein function. For example, a single substitution is enough to alter the substrate specificity of a β-glucosidase,[40] or to introduce new substrate recognition to a cyclic nucleotide phosphodiesterase.[41] That small sequence changes can have drastic effects on function has been documented for a number of proteins found in organisms ranging from microorganisms to plants and animals.[42–46] Once a sequence change is established, the change may affect existing protein interaction partners, or may introduce a new interaction site. The cytoplasm of a cell is crowded with macromolecules and proteins at any given time. A study in *E. coli* determined that a

cytosolic protein at any given time encounters over 100 different molecules within microseconds[11,47] leaving ample opportunities for new variations of proteins to form new interactions that may give rise to novel functions. This phenomenon, in combination with mutations, could be the driving force in the evolution of alternative or secondary functions for certain members of protein families.

Organelles are centers of metabolic activities such as photosynthesis, cellular respiration, and primary and specialized metabolism. Hence, chloroplasts and mitochondria are highly sensitive to internal and external cues; they are linked closely with metabolism, a highly dynamic, integral process that requires large networks of proteins at any given time.[48–51] Given that organelles are hubs of metabolic activity, we anticipate that mitochondrial and chloroplast proteins are more likely to have moonlighting functions. A well described example is STAT3, a transcription factor in mice which has a role in response to inflammation in the nucleus and cytosol, and a second function in mitochondria regulating the electron transport chain.[52] In angiosperms, WHIRLY1 (or PBF-2) is a transcription factor that regulates pathogen response genes[53] and plays a role in the chloroplast where it ensures plastid genome stability.[54] As part of these roles, WHIRLY1 has been shown to exit from the chloroplast and migrate to the nucleus,[55] suggesting that WHIRLY1 is involved in retrograde signalling acting as a sensor of internal and external stimuli in the chloroplast and communicator of these changes to the nucleus to elicit a transcriptional response.[51,55] Similar to WHIRLY1, ADT5, another member of the *Arabidopsis* ADT family localizes to both the chloroplast and the nucleus, where it is proposed to moonlight in transcriptional regulation.[14] Although the MoonProt database[13] contains information on almost 400 documented moonlighting proteins, only 8 of these entries are for proteins found in plants, suggesting that many plant moonlighting proteins have yet to be characterized. However, of these eight proteins, two have moonlighting functions in chloroplasts. For this reason, we believe that only the tip of the iceberg of plant moonlighting enzymes has been uncovered.

## Materials and methods

### Growth conditions for bacteria and plants

The *Agrobacterium tumefaciens* strain LBA4404 carrying the Ti helper plasmid pAL4404 NCCB accession PC2760,[56,57] was used for transient transformations of *Nicotiana benthamiana* and *A. thaliana*. *A. tumefaciens* were grown at 28°C in YEB media[58] supplemented with appropriate antibiotics.

Three to five-week old *N. benthamiana* plants were used to determine the subcellular localizations of fluorescent fusion proteins. Tobacco plants were grown in incubators (Conviron) under a 16 h light (80–100 µmol $m^{-2}$ $s^{-1}$) and 8 h dark cycle, with temperatures set to 24°C and 22°C, respectively. *A. thaliana* plants (accession Columbia-0, Col-0, and the mutants described below) were grown 3 to 4 weeks and watered with a 20 mM L-ascorbic acid solution using the same photo-period and a light intensity of 150 µmol $m^{-2}$ $s^{-1}$.[14]

Several different *Arabidopsis* mutant lines were used in this study. The homozygous *adt2-1D* (Col) line has an ethyl methanesulfonate (EMS)-induced point mutation resulting in a serine to alanine substitution in the ACT regulatory domain. This mutant was identified as it exhibits a change in the phenylalanine-mediated allosteric regulation.[59] The *Arabidopsis* lines carrying mutations in various organelle division genes, *ACCUMULATION AND REPLICATION OF CHLOROPLASTS* (*arc3-1*: CS264; *arc5-1*: CS1633; and *arc6-1*: CS286), were obtained from ABRC. *arc3-1* and *arc5-1* are EMS-induced point mutations resulting in a premature stop codon in the 13th exon[24,28] and 5th exon[25,27], respectively. *arc6-1* carries a cytosine to thymine point mutation in exon 3 also causing a premature stop codon[26,29]. *arc3-1* and *arc5-1* are in the Landsberg *erecta* (L*er*) background and *arc6-1*in Wassilewskija (Ws).

### Cloning of ADT2-CFP and FtsZ2 fusion constructs

*ADT2 (At3g07630)* and *FtsZ2* were cloned as full-length coding sequences fused 5′ to a CFP or YFP coding sequence, respectively, as described by Bross et al.[14] *ADT2* was expressed from pEZT-NL[60] and *FtsZ2* was integrated into pEarleygate101.[61] Both fusion genes are under the control of the cauliflower mosaic virus (CaMV) *35S* promoter and are translated as C-terminally tagged fusions to avoid masking of the transit peptide sequences.[14]

### Bacterial and plant transformations

*A. tumefaciens* were transformed as described.[14,62] Transformed *Agrobacterium* strains were used to pressure-infiltrate *N. benthamiana* and *Arabidopsis* leaves.[14,60,63,64] For co-infiltrations, equal volumes of *A. tumefaciens* cultures containing different vectors were combined to maintain a final $OD_{600}$ of 1.0. The p19 vector encodes a 19 kDa protein from tomato bushy stunt virus, which has been shown to enhance transgene expression through suppression of post-transcriptional gene silencing (PTGS;[65] and was added to all transient transformations.

*Agrobacterium*-infiltrated leaves were assayed at 5 dpi using a Leica SP2 confocal laser scanning microscope equipped with a 63X water immersion objective. Leaf tissues were viewed from the abaxial side to identify fluorescent proteins in mesophyll cells. To excite chlorophyll and CFP, a blue diode laser (405 nm) was used, and emission was collected from 630 to 690 nm and 440 to 485 nm, respectively. YFP was excited with a 514 nm argon laser, and the emission was collected from 540 to 550 nm. In co-localization experiments, YFP and CFP emissions were collected separately to avoid emission cross-talk of the fluorophores.[60,66] Images were analyzed using Leica Confocal Software (Leica, V2.61) or ImageJ 1.45s.[67] Chlorophyll, CFP, and YFP fluorescence was false coloured red, cyan, and yellow, respectively.

### Transmission electron microscopy (TEM)

Leaf sections of 2 × 2 mm from 6-week-old *Arabidopsis adt2-1D* (Col) were cut and fixed overnight in 2% (v/v) glutaraldehyde at 4°C, rinsed once with 70 mM Na phosphate buffer (pH 7.2) and then post-fixed in 1% $OsO_4$ for 2 hrs at room temperature, in the dark. Samples were dehydrated in an increasing series of

ethanol dilutions (50, 70, 90, 2 × 100%) and embedded in Spurr low viscosity resin (Sigma EM0300). Thin sections (60 µm) were prepared with a Reichert Jung Ultra E microtome (Reichert-Jung AG, Vienna, Austria), placed on copper grids and stained with 2% Reynold's lead citrate solution. The sections were viewed with a Philips CM10 TEM microscope.

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