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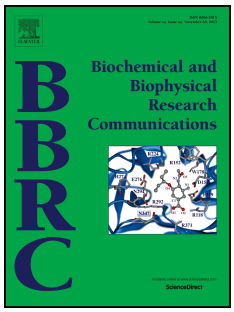

Hetero- and homodimerization of *Arabidopsis thaliana* arginine decarboxylase AtADC1 and AtADC2

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Hetero- and homodimerization of *Arabidopsis thaliana* Arginine Decarboxylase AtADC1 and AtADC2

Running tittle:

AtADC1 and AtADC2 interaction in plant cytosol and chloroplast

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Abstract

The arginine decarboxylase enzyme (ADC) carries out the production of agmatine from arginine, which is the precursor of the first polyamine (PA) known as putrescine; subsequently, putrescine is turned into the higher PAs, spermidine and spermine. In *Arabidopsis thaliana* PA production occurs only from arginine and this step is initiated by two ADC paralogous, AtADC1 and AtADC2. PA production is essential for *A. thaliana* life cycle. Here, we analyzed the sub-cellular localization of AtADC1 and AtADC2 enzymes through GFP translational fusions. Our data revealed that the *A. thaliana* arginine decarboxylase enzymes exhibit a dual sub-cellular localization both in the cytosol and chloroplast. Moreover, we examined the protein dimer assembly using a Fluorescence Complementation (BiFC) approach, which showed that AtADC1 and AtADC2 proteins were able to form homodimers in the cytosol and chloroplast. Interestingly, we found the formation of AtADC1/AtADC2 heterodimers with similar sub-cellular localization than homodimers. This study reveals that both ADC proteins are located in the same cell compartments, and they are able to form protein interaction complexes with each other.

Keywords: BiFC, heterodimer, homodimer, chloroplast localization, polyamines.

Introduction

Putrescine (Put), spermidine (Spd), and spermine (Spm) are broad distributed low-molecular weight and positively charged compounds known as polyamines (PAs) (Handa and Mattoo, 2010). In higher plants, the major pathway for Put production is through arginine. In *Arabidopsis thaliana*, Put is synthesized by arginine decarboxylase (ADC, EC 4.1.1.19), which exists as couple isoforms AtADC1 and AtADC2 (Hanfrey et al., 2001). These isoforms convert arginine to agmatine, which is subsequently turned into Put by an N-carbamoylputrescine transitional compound through two reactions, which involves agmatine deiminase/iminohydrolase (AIH, EC 3.5.3.12) and N-carbamoylputrescine amidase/amidohydrolase (NCPAH EC 3.5.1.53) (Janowitz et al., 2003). Put is the precursor of Spd and Spm, through reactions catalyzed by spermidine synthases (AtSPDS1 and 2; EC 2.5.1.16) and spermine synthase (AtSPMS; EC 2.5.1.22), respectively.

In *A. thaliana*, Put synthesis has been related to a wide range of physiological processes throughout its growth and development, such as stem length, flowering rate, and seed morphology (Sánchez-Rangel et al., 2016). *AtADC1* and *AtADC2* genes are essential for seed development. In *A. thaliana*, the double mutant *adc1*$^{-/-}$ *adc2*$^{-/-}$ is embryo lethal (Urano et al., 2005). *AtADC1* and *AtADC2* mRNAs accumulate during development and stress responses (Hummel et al., 2004a). The *A. thaliana adc1*$^{-/-}$ and *adc2*$^{-/-}$ single mutants exhibit an increased freezing sensitivity phenotype in contrast to the wild-type plants; which was alleviated by exogenous Put addition (Cuevas et al., 2008). Furthermore, in an *A. thaliana AtADC2* over-expressing line, drought tolerance was attributed to Put accumulation (Alcázar et al., 2010). In tobacco species and Solanaceae, Put also functions as a substrate for the synthesis of nicotine, tropane, and nortropane alkaloids, which are all thought to deter herbivores attack (Biastoff et al., 2009).It is well known that the *A. thaliana AtADC1* and *AtADC2* genes are differentially expressed. In fact, during early vegetative development, the *AtADC1* promoter activity is low but it is considerably increased in roots and leaves under chilling

treatment. In contrast, *AtADC2* promoter activity is more related to seed germination, root and leaf development, and also it responds to light, sucrose and ethylene treatments (Hummel et al., 2004). Previous reports about ADC protein sub-cellular localization revealed that the ADC protein from oat (*Avena sativa*) has been localized inside the chloroplast bound to thylakoid membranes (Borrell et al. 1995); whereas, the tobacco ADC protein showed dual chloroplast and nuclear localization (Bortolotti et al. 2004). An *in silico* analysis of *A. thaliana* AtADC1 and AtADC2 protein sequences revealed that they possess chloroplast-targeting peptides (Illingworth et al., 2003).

Since in *A. thaliana* Put synthesis only occurs via the ADC pathway, it is important to analyze the *in vivo* localization of the ADCs enzymes and their ability to interact with each other. Herein, we provide evidence on the *in vivo* sub-cellular localization of both AtADC1 and AtADC2 proteins. In addition, we study the assembly and localization of ADC complexes through the use of a Bimolecular Fluorescence Complementation (BiFC) approach. Our research discloses a dual cytoplasmic and chloroplast localization for AtADC1 and AtADC2 enzymes, and the assembly of homo and heterodimeric ADC complexes.

Material and Methods

Plant growth conditions

Nicotiana benthamiana seeds were dropped on a mix of vermiculite and soil (1:1) and grown for three to four weeks in a growth chamber under long-day conditions (16 h light/8 h dark) at 22ºC ± 2ºC.

Entry constructs

To generate the entry vectors, the *AtADC1* and *AtADC2* open reading frames were PCR amplified using the Phusion high-fidelity DNA polymerase (Thermo scientific, Carlsbad, CA, USA). After amplification, each PCR amplicon was introduced into the pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA). Selected clones were sequenced using the M13 forward primer.

Destination constructs

For sub-cellular localization analyses, each pCR8-AtADC1 and pCR8-AtADC2 entry construct were shifted to the pMDC43 binary vector in order to generate GFP translational fusion constructs (Curtis and Grossniklaus, 2003). To carry out the Bimolecular Fluorescence Complementation (BiFC) tests, the entry constructs were shifted to the pYFN43 and pYFC43 binary vectors (Belda-Palazón et al., 2012). The shifting was done through site-specific recombination using Gateway LR Clonase II Enzyme Mix (Invitrogen). Finally, *Agrobacterium tumefaciens* GV3101/pMP90 strains were transformed with all of the generated destination vectors.

Plant transient expression

N. benthamiana leaf abaxial cells were transiently transformed using *A. tumefaciens* GV3101/pMP90 cells harboring the generated constructs. To avoid gene silencing, an *A. tumefaciens* strain containing the tomato bushy stunt virus p19 protein was also co-infiltrated (Voinnet et al., 2003; Belda-Palazón et al.,

2012). Thus, *A. tumefaciens* cells at OD_{600} of 1.0 were collected and re-suspended in infiltration buffer containing 10 mM $MgCl_2$, 10 mM MES pH 5.6, 200 µM acetosyringone. The *A. tumefaciens* strains were shaken at room temperature for 3 h. Subsequently, the *N. benthamiana* leaf abaxial space was co-infiltrated with the translational fusion constructs and the p19 plasmid using a needleless syringe. Three days after infiltration, two leaves from three independent transformed plants were analyzed for fluorescence under confocal microscope. The analyses were repeated three times for all constructs, obtaining similar results.

Nuclei Staining

For nuclei staining, DAPI reagent (Sigma, St. Louis, MO) was used. Briefly, *N. benthamiana* transformed leaf segments were cut from the plant and then incubated in a water solution containing 5µg/ml DAPI for 5 min.

Fluorescence confocal microscopy

The abaxial sides of the agro-infiltrated *N. benthamiana* leaves were observed through an inverted confocal laser-scanning microscope (LSM 780, Carl Zeiss, Jena, Germany). To prevent photobleaching, each DAPI-incubated leaf sections were collocated on a microscope slide and covered with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). The laser excitation wavelength was 488 nm and the spectral detection was set between 497 and 537 nm for GFP and 684-758 nm for chlorophyll fluorescence, with a beam splitter MBS 488. For DAPI laser excitation the wavelength was set to 405 nm and detection was made at 410-492nm. The objective used was C-Apochromat 40x/1.20 W. Image analysis was carried out with ZEN imaging software (Carl Zeiss).

Results

Sub-cellular localization of *Arabidopsis thaliana* Arginine Decarboxylase 1 (AtADC1)

It is known that *A. thaliana* genome contain two ADC genes, *AtADC1* (At2G16500) and *AtADC2* (At4G34710), which codify two functional enzymes to initiate PA biosynthesis (Urano et al., 2005). To resolve where the *A. thaliana* arginine decarboxylase 1 (AtADC1) enzyme is *in vivo* localized, we generated a translational fusion between GFP and AtADC1. Briefly, the open reading frame (ORF) of *AtADC1* gene was PCR amplified and introduced into the pCR8 entry vector for its subsequent shifting into the pMDC43 gateway binary expression vector (Curtis and Grossniklaus, 2003) (Figure 1A). To assess the fluorescent signal, the 2x35S::GFP-AtADC1 construct was agro-infiltrated in *Nicotiana benthamiana* leaves. Afterward infection, leaf sections were analyzed by a laser-scanning confocal microscope. To visualize nuclei localization DAPI staining was used. As shown in Figure 1C, the green fluorescence of GFP-AtADC1 fusion was slightly present in the cytoplasm and it co-localized with the red auto-fluorescence of chlorophyll channel in *N. benthamiana* leaf epidermal cells (Figure 1C). The localization of the control GFP (expressed from 2x35S::GFP construct) was localized in both cytoplasm and nuclei of tobacco cells (Supplementary Figure S1). In the case of the GFP-AtADC1 fusion, signal in tobacco nuclei was absent.

Homodimer formation of Arginine Decarboxylase 1 (AtADC1)

In order to know whether the AtADC1 enzyme is able to homodimerize *in vivo*, we carried out a Bimolecular Fluorescence Complementation (BiFC) test. Briefly, the pCR8-AtADC1 entry clone was shifted in both pYFN43 and pYFC43 BiFC gateway binary vectors (Belda-Palazón et al., 2012) (Figure 1B). Subsequently, the generated pYFN43-AtADC1 and pYFC43-AtADC1 vectors were transformed together into *N. benthamiana* leaves. After infiltration, the GFP reconstitution was

monitored using a confocal microscope. Our BiFC analyses revealed the assembly of homodimers for AtADC1 (Figure 1D). In accordance with its sub-cellular localization, the AtADC1-AtADC1 homodimer was situated in cytoplasm and chloroplasts (Figure 1D). The positive interaction control of a pair subunits of the Arabidopsis SnRK kinase AKIN10 and AKINβ2 was included (Ferrando et al., 2001), and there was no auto-fluorescence when the pYFN43-AtADC1 construct was agro-infiltrated (Supplementary Fig. S2). In the same way, the interaction between AtADC1 and AKINβ2 did not show fluorescence under confocal microscopy (Supplementary Figure S2).

Sub-cellular localization of *A. thaliana* Arginine Decarboxylase 2 (AtADC2)

As mentioned above, the AtADC1 protein presents a dual cytoplasm and chloroplast localization in *N. benthamiana* epidermal cells (Figure 1C). To investigate the sub-cellular localization of the AtADC2 paralog, we generated an N-terminal translational fusion between GFP and AtADC2. Thus, the ORF from *A. thaliana AtADC2* gene was introduced and then shifted into pCR8 and pMDC43 gateway vectors, respectively (Figure 2A). The 2x35S::GFP-AtADC2 vector was tested through transitory expression in agro-infiltrated *N. benthamiana* leaf abaxial cells (Figure 2C). Our confocal analysis revealed that similar to AtADC1, the GFP-AtADC2 fluorescence occurred in the cytoplasm, and it co-localized with the red auto-fluorescence of chlorophyll of tobacco leaf epidermal cells (Figure 2C).

Homodimer formation of Arginine Decarboxylase 2 (AtADC2)

To assess if the AtADC2 protein is able assembled into homodimers, we performed a BiFC test in *N. benthamiana* leaves. The pCR8-AtADC2 vector was shifted into the BiFC gateway binary vectors (Figure 2B). As a positive BiFC interaction control, we included the Arabidopsis SnRK kinase heterodimer, AKIN10 and AKINβ2; which was able to reconstitute GFP signal. In contrast, the interaction between AtADC2 and AKINβ2 did not show fluorescence under

confocal microscopy (Supplementary Figure S2). As shown in Figure 2D, the AtADC2-AtADC2 protein interaction was detected into *N. benthamiana* cytoplasm and chloroplast. These results are in accordance to the sub-cellular localization of AtADC1 dimers.

Heterodimer formation between the AtADC1 and AtADC2 enzymes

In order to determine whether the *A. thaliana* AtADC1 and AtADC2 enzymes are able to heterodimerize *in vivo*, we carried out a BiFC analysis in agro-infiltrated *N. benthamiana* leaves using the following vector combinations: pYFN43-AtADC1/pYFC43-AtADC2 and pYFN43-AtADC2/pYFC43-AtADC1 (Figure 3A and B). After infiltration, the fluorescence reconstitution was monitored using a laser-scanning confocal microscope. Our BiFC analyses revealed the assembly of heterodimers for AtADC1 and AtADC2 to all vector combinations (Figure 3C and D). In accordance to the homodimer localization, the AtADC1/AtADC2 and AtADC2/AtADC1 interactions occurred in both plant chloroplast and cytoplasm (Figure 3).

Discussion

Interestingly, *Arabidopsis thaliana* is an ideal plant model for studying ADCs, since it only has this route to produce Put compared to other plants that also use ornithine decarboxylase (ODC) enzyme to synthetize Put from ornithine (Hanfrey et al., 2001). Therefore, the study of *A. thaliana* ADC pathway is an interesting field to explore PA role during plant life span. Herein, we reported the *in vivo* sub-cellular localization for the *A. thaliana* AtADC1 and AtADC2 enzymes using N-terminal GFP translational fusions. Our results demonstrated that both enzymes are localized in the cytosol and chloroplast of *Nicotiana benthamiana* leaf epidermal cells. Borrell et al. (1995) used cell fractionation and immunoblotting approaches to show that oat ADC can be detected in leaf chloroplast and is bound to thylakoid membranes. The authors suggested that the thylakoid association could be through putative amphiphilic α-helix. Afterward, Bortolotti et al. (2004) performed an *in situ* immunological technique to determine the tissue distribution and sub-cellular localization of *N. tabacum* ADC; they found that tobacco ADC is present in flowers, leaves, stems, and roots. At the sub-cellular level, they found that the tobacco ADC protein is localized inside the chloroplast and nucleus, in photosynthetic tissues (leaves and stems), tobacco ADC presents a chloroplast localization; whereas in non-photosynthetic tissues (flowers and roots), the tobacco ADC was located in nuclei.

It has been reported that the *A. thaliana adc1* T-DNA mutant insertion line did not show any differences in its morphology and PA levels in contrast to wild type; however, the *adc2* mutant line displayed a reduction in Put and Spd levels (Urano et al., 2004). In addition, the simultaneous silencing of both *A. thaliana ADCs* genes reduced PA levels and altered its development (Sánchez-Rangel et al., 2016). This silencing *adc1* and *adc2* line exhibited root architecture changes, stunted growth, delayed flowering time, reduced seed number and size, and abortive seeds (Sánchez-Rangel et al., 2016). Moreover, the authors reported that *adc2* mutant line showed a diminished seed size compared to *adc1* mutant and wild-type seeds (Sánchez-Rangel et al., 2016). The *AtADC1* and *AtADC2*

double-knockout mutant was lethal at the embryo stage (Urano et al., 2005). Although both ADC paralogous are differentially expressed, our data showed that these enzymes were located in the same sub-cellular places.

Arginine decarboxylase dimerization process has not been completely studied. Herein, through a BiFC, approach we demonstrated that *in planta* the AtADC1 and AtADC2 proteins are able to assemble into homodimer with a dual cytoplasmic and chloroplast localization. Previously, Hanfrey et al. (2001) proposed that the AtADC1 enzyme might function as homodimer complex similar to ODC. In fact, the authors observed that the residues lysine 136 and cysteine 524 could function in the same way that the lysine 69 and cysteine 360 from mouse ODC, which are related to Schiff base formation with the co-factor PLP and substrate binding, respectively (Poulin et al., 1992). The point mutation of these residues in AtADC1 protein reduced drastically its enzyme activity (Hanfrey et al. 2001). In another study, a recombinant ADC from *Dianthus caryophyllus* expressed *in vitro* was shown to be a tetramer, which is probably a dimer of dimers (Ha et al., 2004).

In addition, we observed that AtADC1 and AtADC2 are able to form a heterodimer with cytoplasmic and chloroplast localization. In this sense, it has been reported that *A. thaliana* spermidine synthases AtSPDS1 and AtSPDS2, enzymes involved in spermidine synthesis, can interact with itself and also form heterodimers in yeast and tobacco epidermal cells (Panicot et al., 2002, Belda-Palazón et al., 2012). The *A. thaliana* genome has been through a whole-genome duplication, gene loss and local gene duplications (Arabidopsis Genome Initiative, 2000). As *AtSPD1* and *AtSPDS2* genes, there is evidence suggesting that *AtADC1* and *AtADC2* genes are result from gene duplication (Minguet et al., 2008; Hummel et al., 2004). Ispolatov et al. (2005) proposed that duplicated proteins are more probably to interact among them than other proteins; in addition it is suggested that paralogous interactions are heritable from ancient homodimeric proteins, rather than established de novo after gene duplication. Further, it has been proposed that AtSPDS1 and AtSPDS2 heterodimer might be

enzymatically active, which might provide a biological advantage that could either facilitate the synthesis of products or exhibit regulatory functions on the activity of a partner enzyme domain (Panicot et al., 2002).

Early studies performed in spinach and oat revealed that PAs are localized in membranes of thylakoid associated with photosystem II (Kotzabasis et al. 1993; Legocka and Zajchert 1999). It was reported that high concentrations of CO_2 augmented the Put binding to the thylakoid membranes (Logothetis et al., 2004). Moreover, PA addition to oat leaves inhibited chlorophyll loss and stabilizes thylakoid membranes during osmotic stress (Besford et al. 1993, Borrell et al. 1996). In salt stressed cucumber seedlings, exogenously Put enhances the level of the photochemical efficiency of photosystem II (Zhang et al., 2009). In this regard, the *N. benthamiana* chloroplastic ADC could be involved in photosynthesis, whereas the nuclear ADC form could play a role in cellular signaling (Bortolotti et al., 2004). Another association about the ADC cytosol and chloroplast localization is related to that the arginine metabolism is distributed into three sub-cellular compartments: cytosol, plastids, and mitochondria (Winter et al., 2015).

In *A. thaliana*, the arginine-derived metabolites, such as PAs and nitric oxide (NO) are essential for many cellular and developmental processes (Kusano et al., 2008; Mur et al., 2012). In this sense, the PA roles during plant lifetime have been studied; however, the molecular mechanism how they act is still a mystery. The study of spatial localization of PA biosynthetic enzymes, substrates, and final products absolutely will help to resolve its cellular function. Thus, know where PA synthesis occurs through the ADC sub-cellular localization analysis, and knowing that Arabidopsis ADCs can form homo and heterodimers, are important clues to understand the mechanisms of activity of the ADC enzymes.

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Legend of figures

Figure 1. *in vivo* AtADC1 sub-cellular localization and self-protein interaction. (A) Schematic representation of pMDC43-AtADC1 construct. (B) BiFC pYFN43-AtADC1 and pYFC43-AtADC1 vectors. (C) Cytosol and chloroplast localization of GFP-AtADC1 construct. The white and grey arrowheads indicate cytosolic and chloroplast signal, respectively. (D) AtADC1/AtADC1 interaction is shown in green by reconstitution of GFP. Chlorophyll auto-fluorescence is shown in red. From left to right: the GFP and Chlorophyll fluorescence spectrum, DAPI, bright field, and overlay signals. Regions that appear orange in the overlap pictures confirm that protein interaction takes place inside the chloroplast. The scale bar corresponds to 40 µm.

Figure 2. *in vivo* AtADC2 sub-cellular localization and itself protein interaction. (A) Schematic representation of pMDC43-AtADC2 construct. (B) BiFC pYFN43-AtADC2 and pYFC43-AtADC2 vectors. (C) Cytosol and chloroplast localization of GFP-AtADC2 construct. The white and grey arrowheads indicate cytosolic and chloroplast signal, respectively. (D) AtADC2/AtADC2 interaction is shown in green by reconstitution of GFP. Chlorophyll auto-fluorescence is shown in red. From left to right: the GFP and Chlorophyll fluorescence spectrum, DAPI, bright field, and overlay signals. Regions that appear orange in the overlap pictures confirm that protein interaction takes place inside the chloroplast. The scale bar corresponds to 40 µm.

Figure 3. Visualization of AtADC1/AtADC2 heterodimer formation. (A, B) Schematic representation of *AtADC1* and *AtADC2* genes cloned into pYFN43 and pYFC43 vectors. (C, D) BiFC analysis of pYFN43-AtADC1/pYFC43-AtADC2 vectors and its swapped constructs. White and grey arrowheads indicate cytosolic and chloroplast signal, respectively. The fluorescence was assessed by laser-scanning confocal microscopy. From left to right: the GFP, chlorophyll, and

DAPI fluorescence spectrum, bright field, and overlay channels. The scale bar corresponds to 40 µm.

Supplementary legend of figures

Supplementary Figure S1. Control GFP expressed from pMDC43 and BiFC positive interaction control. *N. benthamiana* leaves were agro-infiltrated with pMDC43 plant expression vector (A) and with the pYFN43-AKIN10 and pYFC43-AKINβ2 BiFC vectors (B). The fluorescence was analyzed by laser-scanning confocal microscopy. From left to right: the GFP, Chlorophyll, and DAPI fluorescence spectrum, bright field, and overlay signals. The scale bar corresponds to 40 µm.

Supplementary Figure S2. Auto-fluorescence and negative interaction tests. The pYFN43-AtADC1 and pYFN-AtADC2 constructs were expressed alone (A, B) and with pYFC43-AKINβ2 (C, D) vector in *N. benthamiana* leaves; clearly, not fluorescence signal was observed. From left to right: the GFP, Chlorophyll, and DAPI fluorescence spectrum, bright field, and overlay signals. The scale bar corresponds to 40 µm.

Fig. 1

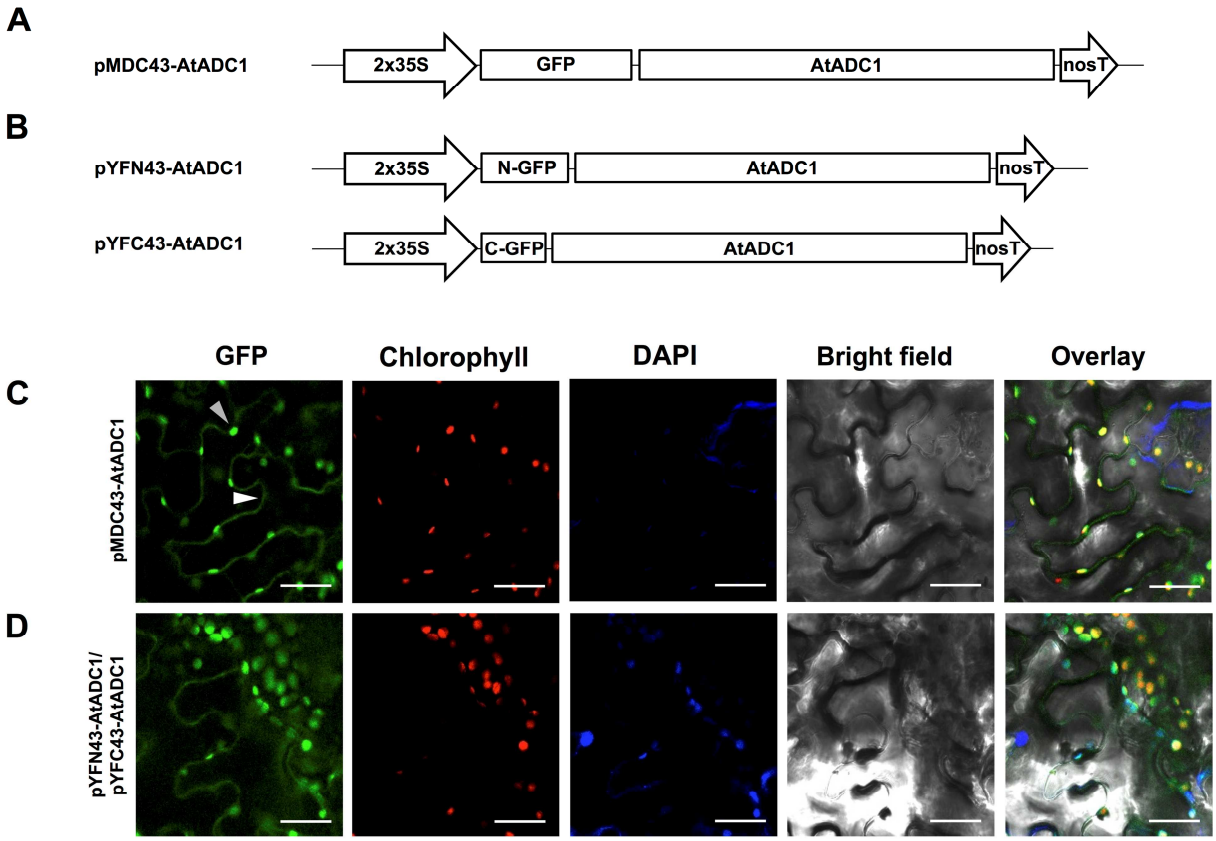


Fig. 2

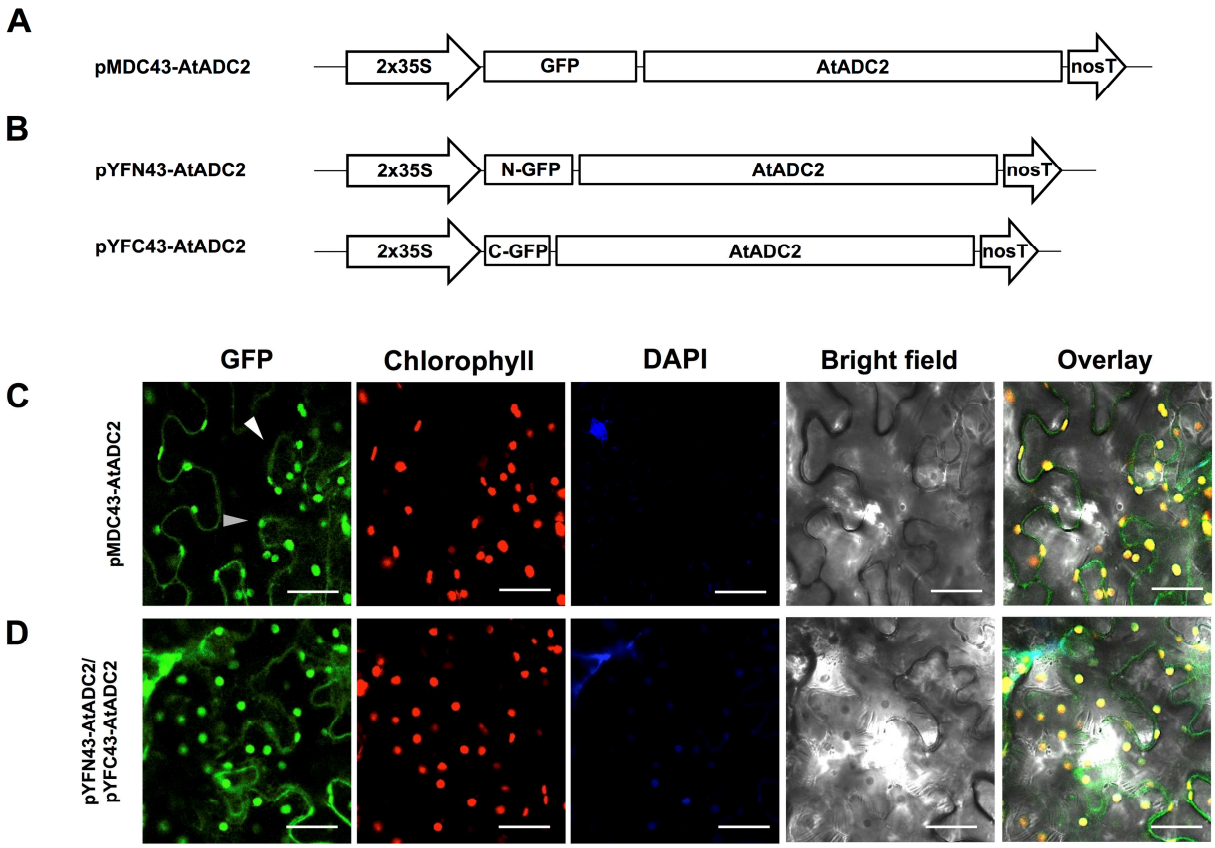
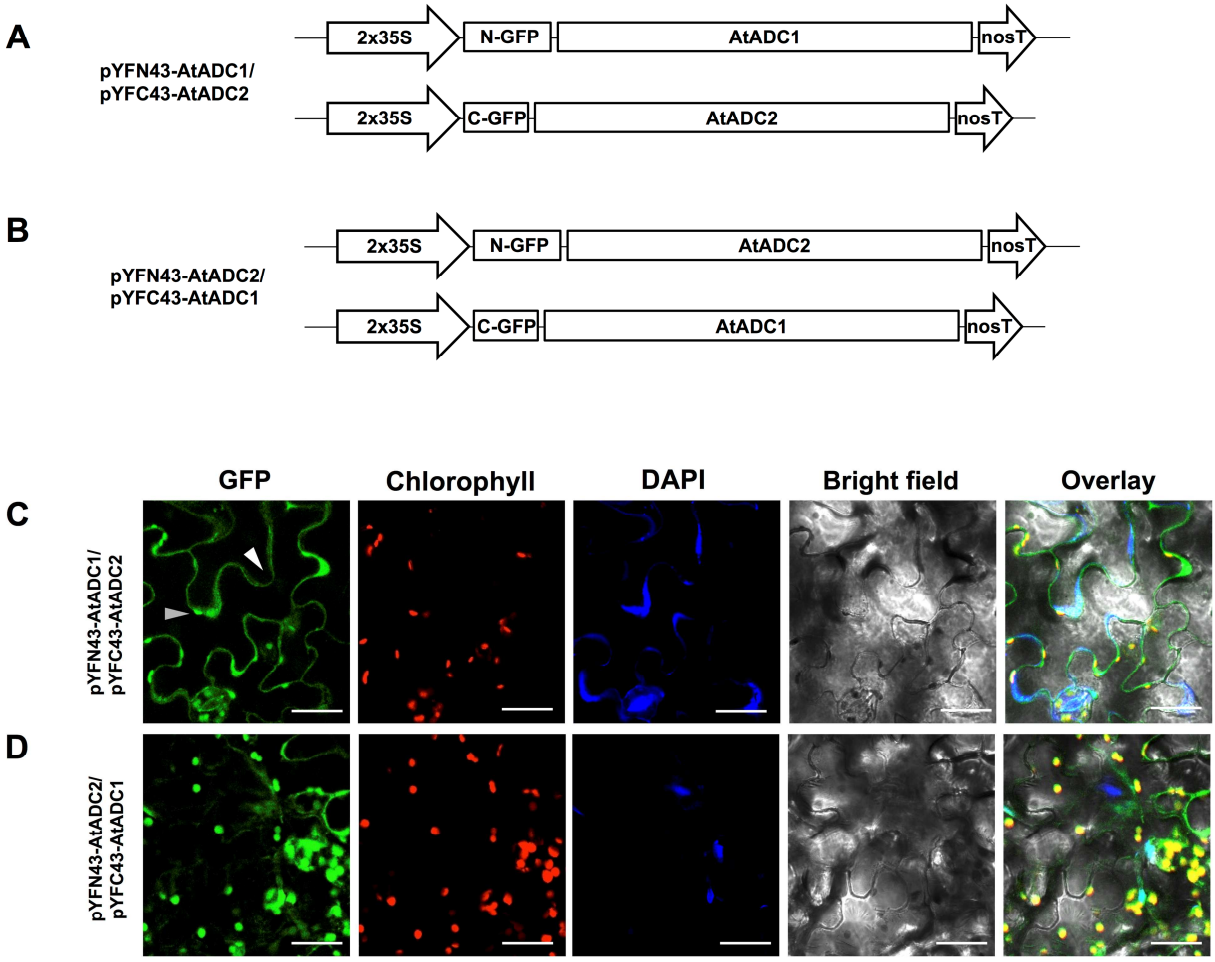


Fig. 3



- The *Arabidopsis thaliana* arginine decarboxylases AtADC1 and AtADC2 exhibit a dual sub-cellular localization both in the cytosol and chloroplast.
- The AtADC1 and AtADC2 proteins are able to assemble into homodimers, which are localized in the cytosol and chloroplast.
- This study reveals that both ADC proteins form heterodimers in the same cell compartments as the homodimers.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.