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The E3 ubiquitin-ligase SEVEN IN ABSENTIA like 7 mono-ubiquitinates glyceraldehyde-3-phosphate dehydrogenase 1 isoform in vitro and is required for its nuclear localization in *Arabidopsis thaliana*

Diego A. Peralta [a], Alejandro Araya [b], Maria V. Busi [a,*], Diego F. Gomez-Casati [a,*]

[a] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI-CONICET), Universidad Nacional de Rosario, Suipacha 531, Rosario 2000, Argentina
[b] Centre National de la Recherche Scientifique & UMR 1332 – Biologie du Fruit et Pathologie, Institute National de la Recherche Agronomique (INRA) Bordeaux Aquitaine, 71 avenue Edouard Bourlaux, Villenave D'Ornon 33882, France

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ABSTRACT

The E3 ubiquitin-protein ligases are associated to various processes such as cell cycle control and diverse developmental pathways. *Arabidopsis thaliana* SEVEN IN ABSENTIA like 7, which has ubiquitin ligase activity, is located in the nucleus and cytosol and is expressed at several stages in almost all plant tissues suggesting an important role in plant functions. However, the mechanism underlying the regulation of this protein is unknown. Since we found that the SEVEN IN ABSENTIA like 7 gene expression is altered in plants with impaired mitochondria, and in plants deficient in the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase 1, we decided to study the possible interactions between both proteins as potential partners in plant signaling functions. We found that SEVEN IN ABSENTIA like 7 is able to interact in vitro with glyceraldehyde-3-phosphate dehydrogenase and that the Lys231 residue of the last is essential for this function. Following the interaction, a concomitant increase in the glyceraldehyde-3-phosphate dehydrogenase catalytic activity was observed. However, when SEVEN IN ABSENTIA like 7 was supplemented with E1 and E2 proteins to form a complete E1–E2–E3 modifier complex, we observed the mono-ubiquitination of glyceraldehyde-3-phosphate dehydrogenase 1 at the Lys76 residue and a dramatic decrease of its catalytic activity. Moreover, we found that localization of glyceraldehyde-3-phosphate dehydrogenase 1 in the nucleus is dependent on the expression SEVEN IN ABSENTIA like 7. These observations suggest that the association of both proteins might result in different biological consequences in plants either through affecting the glycolytic flux or via cytoplasm-nucleus relocation.

1. Introduction

The seven in absentia (*SINA*) gene was first identified in *Drosophila* regulating photoreceptor differentiation by targeting the transcription factor Tramtrack for proteasomal degradation (Carthew and Rubin, 1990; Li et al., 1997; Cooper, 2007). SINA proteins are E3 ligases composed by an N-terminal RING (Really Interesting New Gene) finger domain C3HC4-type (pfam00097), linked to a conserved C-terminal domain required for oligomerization and binding to target proteins (Hu and Fearon, 1999). The interaction of E2 ubiquitin-conjugating protein with E3 ubiquitin-ligases is carried out by the RING finger domain in the E3 ligases, leading to the transfer of ubiquitin to the target protein. E3 ligases are able to recognize diverse targets through adaptor proteins, which provides to these elements a remarkable functional specificity (Deshaies and Joazeiro, 2009; Lipkowitz and Weissman, 2011).

Protein turnover through the ubiquitin-mediated proteasome pathway plays an essential role in many regulatory pathways such as cell differentiation, cell growth, cell cycle control, stress response and apoptosis (de Bie and Ciechanover, 2011; Komander and Rape, 2012; Teixeira and Reed, 2013). Additionally to the proteolytic pathways, protein ubiquitination can also regulate protein functions (Sun and Chen, 2004; Behrends and Harper, 2011), making these enzymes key mediators of post-translational protein regulation.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDHs) enzymes are conserved in all living organisms, where they play a

* Corresponding authors.
E-mail addresses: peralta@cefobi-conicet.gov.ar (D.A. Peralta), alexandre.araya@reger.u-bordeaux2.fr (A. Araya), busi@cefobi-conicet.gov.ar (M.V. Busi), gomezcasati@cefobi-conicet.gov.ar (D.F. Gomez-Casati).

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central role in carbon metabolism in the cell. *Arabidopsis thaliana* possess four different isoforms of GAPDH (www.arabidopsis.org): a cytosolic, phosphorylating, NAD^+ dependent glyceraldehyde-3-phosphate dehydrogenase isoform (GAPC1, gene number At3g04120) which catalyzes the conversion of glyceraldehyde-3-phosphate (Ga3P) to 1,3-bisphosphoglycerate; the isoform GAPC2 (At1g13440) also involved in copper ion binding; and the plastidial isoforms GAPCP1 (At1g79530) and GAPCP2 (At1g16300). In particular, GAPC1, whose role in glycolysis is well-known (Anderson et al., 2004), undergoes several post-translational modifications (Zaffagnini et al., 2013b) conferring multifunctional (moonlighting) properties to this protein (Zaffagnini et al., 2013a). Interestingly, GAPDH isoforms are translocated to the nucleus (Holtgrefe et al., 2008; Vescovi et al., 2013). In addition, they participate in other important roles in the cell such as being associated both with the cytoskeleton and mitochondria, thus involving GAPC1 in the regulation of mitochondrial functions (Giegé et al., 2003). According with this hypothesis, the *gapc1* plants, lacking the GAPC1 isoform, present a mitochondrial dysfunction and male-sterile phenotype (Rius et al., 2008). Interestingly, it was found that the mRNA levels of the seven in absentia like 7 (*SINAL7*) gene were down-regulated in plants that showed mitochondrial dysfunction (Busi et al., 2011). It has been reported that the interaction of the murine E3 ligase Siah-1 with the S-nitrosylated form of the cognate GAPDH leads to its nuclear translocation initiating an apoptotic cell death cascade (Hara et al., 2005). Based on these observations, we hypothesize that the recently characterized E3 ubiquitin-ligase SINAL7 (Peralta et al., 2013) may play a role in some signaling pathways involving GAPC1 and particularly in mitochondrial retrograde signaling in *Arabidopsis*. To gain insight on this pathway in plants, we decided to verify if the *A. thaliana* E3 ubiquitin ligase SINAL7 interacts with the glycolytic enzyme GAPC1 and to study the biochemical consequences of this event.

In the present study, we report evidence that SINAL7 is able to interact with GAPC1 in vitro by pull down and far western blot analysis, and find that residue Lys231 of GAPC1 is involved in this process. We also study the catalytic properties of the complex formed by GAPC1 and SINAL7. Moreover, using an in vitro ubiquitination assay, we show that SINAL7 E3 ligase is able to mono-ubiquitinate GAPC1 on residue Lys76, affecting the glycolytic activity of this enzyme. The analysis of nuclear extracts indicates that GAPC1 can be found in the nucleus in wild type *A. thaliana* plants, but is absent in the nucleus of plants lacking SINAL7 (*sinal7*). Taken together, our data suggest a role for SINAL7 in the regulation of both, the activity and the localization of GAPC1 that could be relevant to the physiology of plant cells.

2. Materials and methods

2.1. Plant material and bacterial strains

Arabidopsis thaliana (var. Columbia Col-0), grown in a greenhouse under long day conditions (16 h day/8 h night), was used as wild type in these experiments. *sinal7* mutant plants that contain a T-DNA insertion in the second exon (*sinal7.1*, SALK_096989) or in the first exon (*sinal7.2*, CS833574) of the *SINAL7* gene At5g37890 (SALK_096989) were obtained from the T-DNA Express Collection at the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress?gene=AT5G37890). The *gapc1* mutant used in these experiments containing a T-DNA insertion in the ninth exon of the *GAPC1* gene At3g04120 (SALK_010839), has been described (Rius et al., 2008). *Escherichia coli* BL21-(DE3)-pLysS strain (*E. coli* B F–dcm ompT hsdS(rB–mB–) gal λ(DE3) [pLysS Camr]) was used as hosts for recombinant expression assays.

2.2. Identification of insertional sinal7 mutants

The position of the T-DNA insert in *sinal7.1* and *sinal7.2* mutants was determined by PCR using the primers LBb1 (GCGTGGACCGCTTGCTGCAACT; http://signal.salk.edu) and SINAL7up (see Table 1). Genomic DNA was extracted from leaves using the cetyltrimethyl-ammonium bromide method (Sambrook et al., 1989). The genotype and T-DNA insertion was evaluated by PCR on genomic DNA using primers flanking the insertion point of the T-DNA, SINAL75′/SINAL73′ and LBb1/SINAL75′ primer pairs (Table 1).

2.3. Isolation of RNA and RT-PCR analysis

Total RNA was isolated from 30 mg of fully expanded rosette leaves using SV Total RNA Isolation System (Promega, WI, USA) as described in the manufacturer's protocol. cDNA was synthesized using 5X M-MLV buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), random hexamers pd(N)6 (Amershan #27-2166-01) as primers, RNasin ribonuclease inhibitor (Catalog# N2518 Promega, WI, USA), and MMLV reverse transcriptase (USB Corp. Cleveland, OH, USA). The resultant cDNA was quantified spectrophotometrically and used as a template in PCR reaction with the appropriate primer pairs (Table 1). Semi-quantitative RT-PCR analyses were performed from, at least, three independent samples measured at 16, 20, 24 and 28 PCR cycles. Data obtained at the exponential phase of the PCR reaction were considered. *β-ACTIN* (At3g18780) was used as an internal control. PCR products were analyzed on agarose gels and visualized using ethidium bromide staining and/or transferred onto Hybond N^+ membranes (Amersham Biosciences). Probe labeling and membrane hybridization were performed according to the ECL Direct Nucleic Acid Labeling and Detection System protocol (Amersham Biosciences).

2.4. Cloning, expression and purification of recombinant proteins

AtSINAL7 was cloned, expressed and purified as described previously (Peralta et al., 2013). Total RNA extracted from *Arabidopsis* leaves was used as template for cDNA synthesis using random hexamers. The 1017 bp cDNA corresponding to *GAPC1* (At3g04120) was amplified by PCR using GoTaq Polymerase (Promega) and the primers: GAPC1up and GAPC1down (Table 1). The resulting PCR product was cloned into pGEMT-Easy (Catalog# A1360 Promega, WI, USA). The *GAPC1* ORF region was PCR amplified using the pGEMTGAPC1 vector as a template with primers GAPC1NcoIup and GAPC1XhoIdown (Table 1). The amplified DNA fragment was digested with NcoI (Promega) and XhoI (Promega) restriction enzymes, and ligated to the double digested (NcoI and XhoI) pETGB1a vector using T4 Ligase (Promega, WI, USA) (Unger et al., 2010). This construct, named pETGB1aGAPC1, encodes GAPC1 (*Arabidopsis thaliana* glyceraldehyde-3-phosphate dehydrogenase 1), aminoacids 1 to 337, containing a N-terminal His-tag sequence which can be removed with TEV protease when necessary.

2.5. Purification of GAPC1, GAPC1K231A and GAPC1K76A

E. coli BL21 (DE3) pLysS cells harboring plasmid pETGB1aGAPC1, pETGB1aGAPC1K231A or pETGB1aGAPC1K76A were grown at 37 °C in LB medium containing 100 mg ml^{-1} of ampicillin to an OD_{600} = 0.6. GAPC1, GAPC1K231A and GAPC1K76A production was induced by the addition of 1 mM IPTG and subsequent incubation for 8 h at 30 °C. Cells were harvested and re-suspended in 20 mM Tris–HCl pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), disrupted by sonication and centrifuged at 7000 × *g* for 15 min at 4 °C. The supernatant was loaded onto a HiTrap chelating column (GE Healthcare) and washed with 20 ml of 20 mM Tris–HCl pH 7.4, 20 mM imidazole. The recombinant proteins were eluted

Table 1
Primers used in this work.

Primer name	Primer sequence, forward/reverse	Gene
SINAL75′	AAAGGATCCAATGGGTGCCGCGATTTTG	At5g37890
SINAL73′	CTCGAGATTCTCTTTGTTCAACTTCTTGAC	At5g37890
GAPC1up	TCTCATATGGCTGACAAGAAGAT	At3g04120
GAPC1down	ACCGAATTCGGCCTTTGACATGTGGAC	At3g04120
GAPC1NcoIup	CCATGGATGGCTGACAAGAAGAT	At3g04120
GAPC1XhoIdown	CTCGAGAATTCGGCCTTTGACATGTGGAC	At3g04120
Act2 Fw	AATCTCCGGCGACTTGACAG	At3g18780
Act2 Rv	AAACCCTCGTAGATTGGCACAG	At3g18780
LBb1	GCGTGGACCGCTTGCTGCAACT	
GAPC1K231Aup	GCTTCCAGCTCTTAACGGAGCGTTGACTGGAATGTCTTTC	
GAPC1K231Adown	GAAAGACATTCCAGTCAACGCTCCGTTAAGAGCTGGAAGC	
GAPC1K76Aup	CCCTTCTCTTCGGTGAGGCGCCAGTCACTGTTTTCG	
GAPC1K76Adown	CGAAAACAGTGACTGGCGCCTCACCGAAGAGAAGGG	

with a 20–500 mM imidazole gradient in 20 mM Tris–HCl pH 7.4. The presence of GAPC1 was monitored by measuring the catalytic activity of the eluted fractions and by SDS-PAGE electrophoresis. Purified enzymes were pooled and concentrated to >1 mg ml^{-1} and used immediately after the purification process. The concentration of proteins was carried out using a Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Catalog# UFC901008 EMD Millipore) as described by the manufacturer.

2.6. Far-western blot analysis

Far western blotting was performed as described previously (Wu et al., 2007) with minor modifications. First, recombinant $SINAL7_{His}$ was subjected to electrophoresis (SDS-PAGE) and transferred to a poly(vinylidene fluoride) membrane. The membrane was blocked in 10 mM sodium phosphate pH 7.2, containing 0.9% (w/v) NaCl, 3% (w/v) BSA and 0.1% (v/v) Tween-20, and incubated overnight at 4 °C with 2 μg/mL of the bait protein (GAPC1). After this, the membrane was washed three times for 10 min with 10 mM sodium phosphate buffer pH 7.2, 0.9% (w/v) NaCl and 0.1% (v/v) Tween-20. GAPC1 protein bound to $SINAL7_{His}$ was detected by incubation with a polyclonal antibody raised against recombinant GAPC1 (α-GAPC). Controls were incubated with α-His-tag antibodies (dilution 1:200, Catalog# 34660 Qiagen) and α-GAPC antibodies (dilution 1:200) antibodies. The antigen-antibody complexes were visualized as described in Section 2.8.

2.7. Pull-down assays

Pull-down assays were carried out as described previously (Wayllace et al., 2010). Purified recombinant His-Tagged GAPC1 or GAPC1K231A were bound to a Ni^{2+}-Sepharose high-performance resin (GE Healthcare Bio-Sciences) previously equilibrated with binding buffer (20 mM NaH_2PO_4 pH 7.4, 20 mM Imidazole, 50 mM NaCl and 1 mM 2-mercaptoethanol). The recombinant AtSINAL7 lacking the His-tag, used as a prey, was added to the immobilized bait protein. In control experiments, BSA was used as a prey. Unbound proteins were eluted by washing twice with 5 ml of binding buffer. The washed resin was centrifuged at 500 × *g* for 3 min at 4 °C and the supernatant discarded. Bound proteins were eluted with a buffer containing 20 mM NaH_2PO_4 pH 7.4, 500 mM imidazole, 50 mM NaCl and 1 mM 2-mercaptoethanol. The recovered proteins were subjected to SDS-PAGE and further analyzed by Coomassie Blue staining and immunoblotting.

2.8. Protein analysis

SDS-PAGE was performed using 12% (w/v) gels as described (Laemmli, 1970). Gels were stained with Coomassie Blue or transferred to nitrocellulose membranes (BioRad). Electro-blotted membranes were incubated with either penta-His antibody (dilution 1:200, Catalog# 34660 Qiagen), α-Acetyl-Histone H3 antibody (α-AcH3) (dilution 1:200, catalog# 06-599, EMD Millipore) or polyclonal α-GAPC antibodies. The antigen–antibody complex was visualized using alkaline phosphatase-linked α-mouse IgG or α-rabbit IgG antibody, followed by staining with BCIP and NBT (Bollag et al., 1996). Total protein concentration was determined as described by Bradford (1976).

2.9. E3 ubiquitin ligase activity assay

In vitro ubiquitination assay was adapted from the protocol described by Wertz et al. (2004). Each reaction (25 μl final volume) contained the ubiquitination assay buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM DTT), 5 μg cMyc-ubiquitin (Boston Biochem), 150 nM yeast E1 (Boston Biochem), 200 nM human recombinant UbcH2 (Boston Biochem), 5 μg purified recombinant AtSINAL7 (as E3 ubiquitin ligase) and the purified recombinant GAPC1, GAPC1K231A or GAPC1K76A (as ubiquitination targets). The reaction mixtures were incubated at 30 °C for 2 h. The reaction was stopped by adding 5 μl of 5× SDS-PAGE Sample buffer (125 mM Tris–HCl pH 6.8, 20% Glycerol, 4% SDS and 10% β-mercaptoethanol), boiled at 100 °C for 5 min and then analyzed by SDS-PAGE electrophoresis. Gels were transferred into nitrocellulose membranes and probed using α-cMyc antibodies (Boston Biochem), followed with α-mouse IgG conjugated with alkaline phosphatase (Sigma) and NBT/BCIP staining.

2.10. Site-directed mutagenesis of GAPC1

Site directed mutagenesis of the codons encoding for Lys76 and Lys231 of GAPC1 were performed using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The pETGB1aGAPC1 vector was used as template for PCR amplification. The primers used for the conversion of Lys to Ala residues were: GAPC1K76Aup, CCCTTCTCTTCGGTGAGgcGCCAGTCACTGTTTTCG; GAPC1K231Afwd, GCTTCCAGCTCTTAACGGAgcGTTGACTGGAATGTCTTTC, and their respective complementary oligonucleotides. Base substitutions are indicated by lower-case letters. The resultant, pETGB1aGAPC1K76A and pETGB1aGAPC1K231A vectors were verified by DNA sequencing and used to transform *E. coli* BL21 (DE3) pLysS cells.

2.11. Circular dichroism (CD)

Far-UV CD spectra were obtained using a Jasco J-810 spectropolarimeter (Jasco International Co.) over a wavelength range from 200 to 250 nm, at 25 °C. Measurements were performed in a 0.2 cm quartz cuvette at rate of 100 nm min^{-1}, bandwidth of 1 nm, response time of 2 s, data pitch of 1 nm, and accumulation of 10. CD

data are shown as the mean residue ellipticity (deg cm^2 $dmol^{-1}$) obtained after substracting the baseline, smoothing, and data normalization. CD spectra for GAPC1, GAPC1K76A, GAPC1K231A (0.1–1 mg ml^{-1}) were recorded in 20 mM sodium phosphate buffer pH 7.4. Secondary structure analysis from CD spectra data was performed using the K2d algorithm (Andrade et al., 1993).

2.12. Enzymatic activity of GAPC

GAPC1 activity was measured by following the reduction of NAD^+ as described by Rius et al. (2008) using recombinant proteins. The buffer containing 50 mM triethanolamine-HCl pH 8.5, 10 mM Na_3AsO_4, 4 mM NAD^+, 2 mM D-Ga3P and 3 mM dithiothreitol. The reaction was initiated by the addition of Ga3P and was linear for at least 3 min. One enzyme unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol min^{-1} of NADH under specified assay condition. All the determinations were performed at least by triplicate and the average values ± SD are reported.

2.13. Nuclear protein isolation

Nuclear protein extracts were obtained from flowers of 40 days-old *Arabidopsis thaliana* wt and *sinal7* plants as described previously by Calikowski and Meier (2006) with minor modifications. Proteins from nuclear and cytosolic compartments were analyzed by SDS-PAGE electrophoresis and transferred into nitrocellulose membranes. α-Acetyl-Histone H3 antibody (α-AcH3) was used as a positive control for the detection of nuclear proteins (N) and α-ADPGlc PPase was used as a control for non-nuclear proteins (C) in the extracts. The antigen–antibody complexes were resolved as indicated above in Section 2.8.

2.14. Mass spectrometry analysis

Mass spectrometric analysis was performed in the proteomic facility CEQUIBIEM, at the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. The equipment used was a MALDI-TOF-TOF, Ultraflex II from Bruker. The bottom-up approach used was based on MS fingerprint and MSMS of representative peptides for each protein; the search was performed against NCBI database of *Arabidopsis thaliana* (for the GAPC proteins) and *Homo sapiens* (for ubiquitin); two miscleavages for trypsin were allowed; a constant modification of carbamidomethylation was used for cysteine, and a variable modification of GlyGly to detect ubiquitinated uncleaved Lys; peptide tolerance: 0.1 Da; fragment tolerance: 0.5 Da. The samples subjected to mass spectrometry analysis in Fig. 4A were the bands migrating between 130 and 200 kDa in lanes 6 and 8 (upper bands shown with an asterisk in the membrane in Fig. 4B), that were previously cut from the gel and frozen until mass spectrometry analysis.

2.15. Statistical analysis

The experiments were conducted at least three times (biological samples). The significance of differences was determined using Student's *t*-test. A One Way Analysis of Variance was performed to analyze the statistical significance for the determination of GAPC1 specific activity after the ubiquitination assay (see Fig. 5). The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$). The Values statistically different are denoted with an asterisk in the figure.

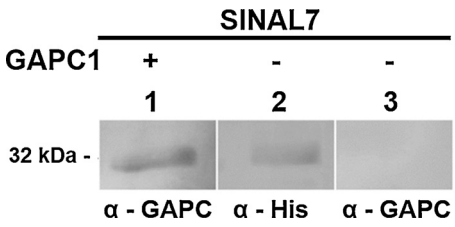


Fig. 1. Analysis of AtSINAL7 and GAPC1 interaction by far western blotting. Recombinant SINAL7-His was subjected to SDS-PAGE and immunoblotting. The membrane in lane 1 was incubated with GAPC1 and the protein detected using α-GAPC serum. In parallel, two other membranes containing electroblotted SINAL7-His were developed by incubation with α-His-tag (lane 2) or α-GAPC (lane 3) antibodies, respectively. The antigen–antibody complex was visualized with alkaline phosphatase-conjugated α-mouse IgG or α-rabbit IgG, followed by staining with BCIP and NBT.

3. Results

3.1. SINAL7 physically interacts with GAPC1

Siah1 (murine homologue of SINAL7) has been reported to specifically bind to GAPDH (homologue of GAPC) (Hara et al., 2005). To assess a possible interaction between SINAL7 and GAPC1, the *Arabidopsis* homologue of murine GAPDH, far western blot analysis was conducted (Fig. 1). For this purpose recombinant GAPC1 and His_6-tagged SINAL7 were used. In far-western experiments, His_6-SINAL7 was electrophoresed and then transferred to PVDF membranes as described in Section 2. After incubation of the membrane in lane 1 with GAPC1 and extensive washing, the SINAL7 containing membranes were probed with the α-GAPC antibody. A positive band was observed at 32 kDa position (Fig. 1, lane 1) as expected for the recombinant SINAL7. Lane 2 corresponds to SINAL7 revealed with α-His_6 antibodies, whereas no signal was observed when the membrane containing SINAL7 was exposed to α-GAPC antibodies (lane 3).

3.2. GAPC1 residue K231 is involved in the interaction with SINAL7

GAPDH lysine residue 225 is responsible for the interaction with Siah1 (Hara et al., 2005). A lysine residue in position 231 (K231) is conserved in GAPC1 which is equivalent to residue K225 from the rat homologous. To verify if the K231 residue from GAPC1 was responsible for the interaction with SINAL7, a pull down analysis was performed (Fig. 2). The recombinant SINAL7 (without any tags) was incubated with either the His-tagged GAPC1 or the GAPC1K231A mutant where the K231 residue was replaced by alanine (see Section 2). As shown in Fig. 2 (lane 5), SINAL7 was recovered in the fraction eluted with buffer containing 500 mM imidazole. However, the K231A GAPC1 mutant was unable to bind to the SINAL7 protein (lane 6) indicating that K231 in GAPC1 is involved in the interaction with SINAL7.

3.3. Effect of SINAL7 on GAPC1 kinetic parameters

To study the effect of SINAL7 binding to GAPC1, the kinetic parameters, K_m and V_{max} of GAPC1 were determined. The enzymatic activity was measured by following the reduction of NAD^+ at 340 nm as described previously (Rius et al., 2008) (see Section 2). GAPC1 activity was measured using different concentrations of Ga3P (Fig. 3A) and NAD^+ (Fig. 3B) in the absence or presence of purified recombinant SINAL7 (GAPC1:SINAL7 1:3 molar ratio). Results show that the presence of SINAL7 increases the catalytic activity of GAPC1 about 3-fold, while no significant effect were observed for the K_m values for both substrates (Fig. 3, Table C).

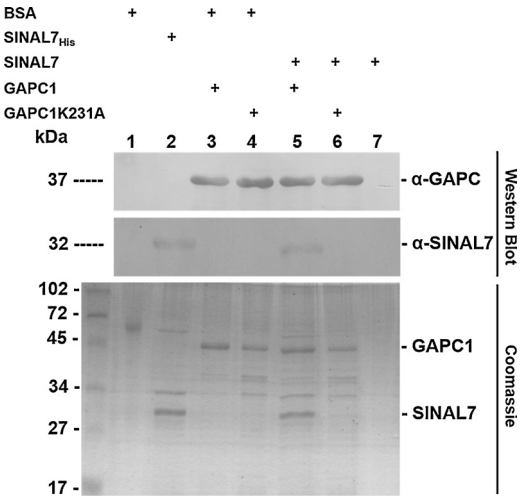
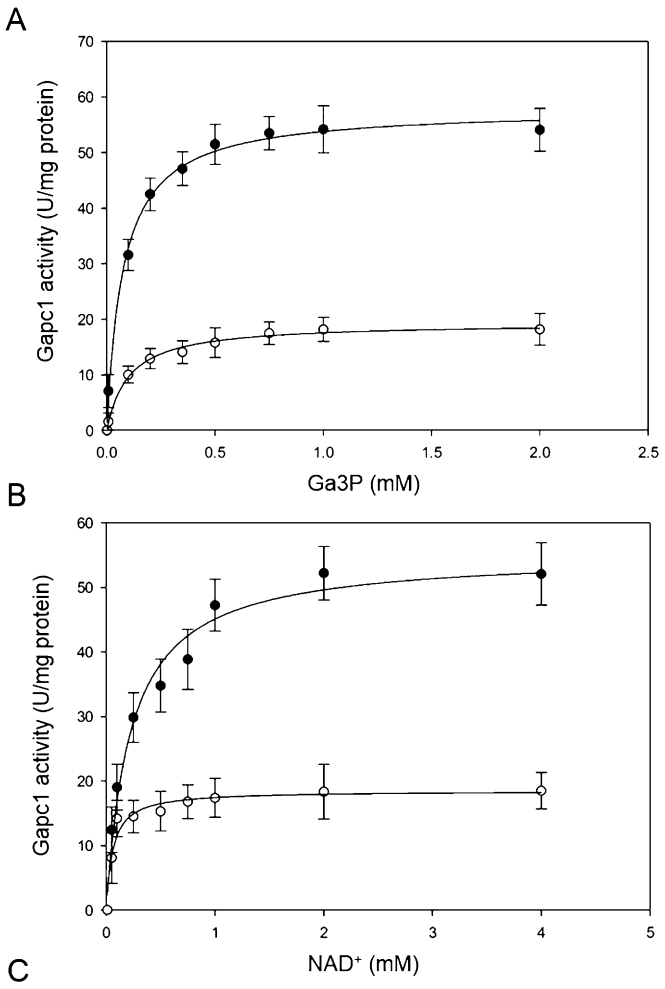


Fig. 2. Pull-down analysis of recombinant SINAL7 and GAPC1 or GAPC1K231A proteins. The physical interaction between SINAL7 without any tag and His-tagged GAPC1 and GAPC1K231A proteins was determined after pull-down assays and SDS-PAGE, followed by western blot analysis. Immunodetection of the respective proteins was performed using α-GAPC and α-SINAL7 antibodies (upper panel). Proteins eluted from the resin in pull-down assays were analyzed by Coomassie Blue staining (lower panel). Lane 1, BSA (control of no interaction); lane 2, $SINAL7_{His}$ (positive control for the identification of SINAL7); lane 3, BSA and GAPC1 bound to resin; lane 4, BSA and GAPC1K231A bound to resin; lane 5, SINAL7 and GAPC1 bound to resin; lane 6, SINAL7 and GAPC1K231A bound to resin; lane 7 shows the absence of nonspecifically bound proteins to the resin (negative control). Numerals on the left indicate molecular masses of the standards (low range Prestained SDS-PAGE Standars BioRad).



C

	GAPC1		GAPC1 + SINAL7	
Substrate	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)	K_m (μM)
Ga3P	18.3 ± 3.1	70 ± 5	53.1 ± 1.9	66 ± 8
NAD^+	18.6 ± 2.5	243 ± 39	53.4 ± 4.92	252 ± 52

Fig. 3. Glyceraldehyde-3-phosphate and NAD^+ saturation plots of purified recombinant GAPC1. Saturation plots of GAPC1 in the absence (empty circles) or in the presence (black circles) of purified recombinant SINAL7 (1:3 molar ratio) using variable concentrations of Ga3P and a saturating concentration of NAD^+ (4 mM), panel A; and variable concentrations of NAD^+ in the presence of saturating Ga3P levels (2 mM), panel B. Plots were fitted with a hyperbola, single rectangular, 2 parameters ($f = a^*x/(b+x)$ were $a = K_m$ and $b = V_{max}$) using SigmaPlot program. The K_m and V_{max} determined are shown in the table. Values are the mean ± SD of at least three independent replicates.

3.4. GAPC1 is mono-ubiquitinated by SINAL7

In previous work, we showed that SINAL7 has an E3 ubiquitin ligase activity (Peralta et al., 2013). As GAPC1 interacts with SINAL7 (Figs. 1 and 2) involving the Lys231 from GAPC1, we decided to investigate if GAPC1 acts as a potential ubiquitin acceptor. For this purpose, SINAL7 was incubated either with the recombinant GAPC1 or the non-interacting GAPC1K231A mutant. As shown in Fig. 4A (Coomasie staining) and B (western blot), it is possible to observe protein bands of high molecular mass (marked with an asterisk in B). These bands correspond to products of the ubiquitination reactions of GAPC1 (lane 6) and GAPC1K231A (lane 8) in the presence of SINAL7. These bands were not found in the reaction mixtures where SINAL7 was absent. However, signals of autoubiquitination of SINAL7 products were observed (lane 4). The high molecular mass bands, specific from the GAPC1 and the GAPC1 mutated protein, obtained after the ubiquitination reaction were subjected to mass spectrometry analysis (see Section 2). They were identified as including mono-ubiquitinated K76 on GAPC1 (lane 6) and GAPC1K231A (lane 8) recombinant proteins. No other ubiquitinated products were identified using mass spectrometry.

3.5. The residue K76 of GAPC1 is involved in ubiquitination

As described above, GAPC1 undergoes a mono-ubiquitination by SINAL7 in vitro. To define the potential residues involved in the ubiquitination of GAPC1, in silico analysis was conducted using the UbPred software "Predictor of ubiquitination sites" (Radivojac et al., 2009). Five out of 31 Lys residues, K76, K109, K111, K145 and K302, from GAPC1 were found as potential acceptors of the ubiquitin molecule according to the bio-computer analysis (http://www.ubpred.org/) (Table S1). This prediction was tested by mass spectrometry analysis which showed that the ubiquitin moiety was linked to the K76 residue either on GAPC1 or the correspondent modified protein (see Section 2). To confirm this result, and to evaluate whether another Lys residue different from K76 is able to link ubiquitin, we constructed the GAPC1K76A protein, where the K76 residue was replaced by alanine (see Section 2). When GAPC1K76A was used as substrate for in vitro ubiquitination experiments, we found no evidence of the occurrence of ubiquitination after SDS-PAGE and western blot analysis (Fig. 4C and D, lane 9), in contrast to what happens with the wild-type GAPC1 protein (Fig. 4C and D, lane 8). This result clearly shows that the K76 residue from GAPC1 is involved in mono-ubiquitination by SINAL7.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2015.11.007.

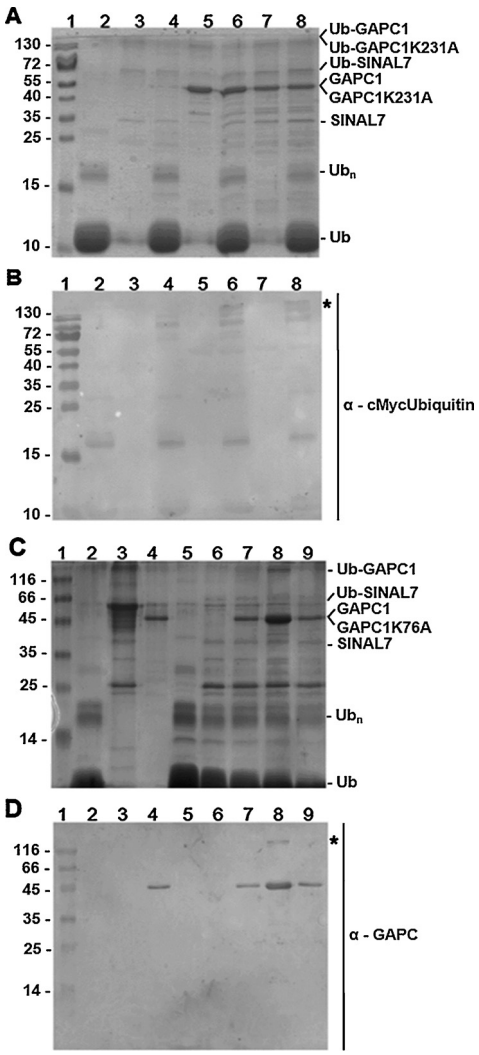
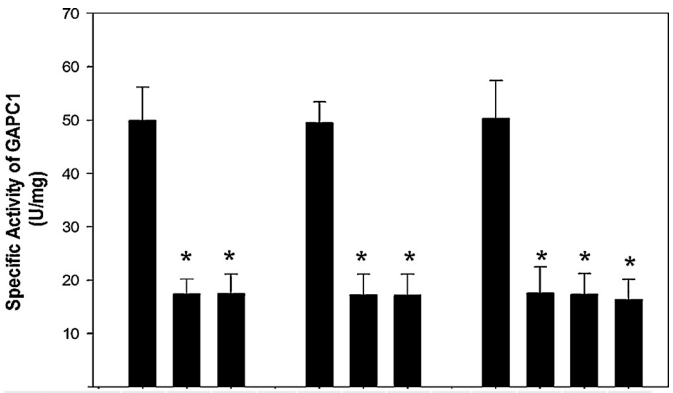


Fig. 4. *SDS-PAGE and western blot analysis of the E3 ligase activity of SINAL7 in the presence of GAPC1 or GAPC1K231A.* (A) Coomassie stain. (B) Immuno-detection of ubiquitinated proteins by western blot using α-cMyc antibodies. In vitro ubiquitination reactions were performed by incubating purified SINAL7 in the presence of GAPC1 (lane 6), GAPC1K231A (lane 8) with the ubiquitination assay buffer, cMyc-Ubiquitin, yeast E1, E2 UbcH2 at 30 °C for 2 h. Self-ubiquitinated SINAL7 control is in lane 4. Target protein/ubiquitin conjugates (*) were resolved by 12% (w/v) SDS-PAGE and detected by immunoblot analysis using α-cMyc antibody. Lane 2: buffer + ubiquitin as antibody cross-reaction control, lane 3: buffer + SINAL7, lane 5: buffer + GAPC1, lane 7: buffer + GAPC1K231A. Numbers and bands on the left indicate the molecular masses of the standard in kDa (PageRuler Prestained Protein Ladder, Fermentas, Lane 1). *SDS-PAGE and western blot analysis of the E3 ligase activity of SINAL7 in the presence of GAPC1 or GAPC1K76A.* (C) Coomassie stain. (D) Immuno-detection of ubiquitinated proteins by western blot. In vitro ubiquitination reactions were performed incubating purified SINAL7 in the presence of GAPC1 (lane 8), GAPC1K76A (lane 9) with the ubiquitination assay buffer, cMyc-Ubiquitin, yeast E1, E2 UbcH2 at 30 °C for 2 h. GAPC1/ubiquitin conjugates (*) were resolved by 12% (w/v) SDS-PAGE and detected by immunoblot analysis using α-GAPC antibodies. Lane 2: buffer + ubiquitin. Lane 3: buffer + SINAL7. Lane 4: buffer + GAPC1K76A. Lane 5: buffer + cMyc-ubiquitin, yeast E1, E2 UbcH2. Lane 6: SINAL7, buffer, cMyc-Ubiquitin, yeast E1, E2 UbcH2. Lane 7: GAPC1K76A, buffer, cMyc-Ubiquitin, yeast E1, E2 UbcH2. Numbers and bands on the left indicate the molecular masses of the molecular weight standard in kDa (Pierce Unstained Protein MW Marker, Lane 1).



Samples	1	2	3	4	5	6	7	8	9	10	11	12
GAPC1	X	X	X	X								
Buffer		X	X	X		X	X	X		X	X	X
2h a 30°C		X	X	X		X	X	X		X	X	X
E1+E2+Ub			X	X			X	X			X	X
SINAL7				X				X				X
GAPC1K231A					X	X	X	X				
GAPC1K76A									X	X	X	X
Activity (U/mg)	49.94 ± 6.23	17.41 ± 2.81	17.45 ± 3.71	N.D.	49.45 ± 3.97	17.24 ± 3.92	17.15 ± 4.01	N.D.	50.31 ± 7.11	17.53 ± 4.96	17.31 ± 3.92	16.37 ± 3.78
Ubiquitination				Yes				Yes				No

Fig. 5. Effect of ubiquitination on GAPC1 specific activity. GAPC1 specific activity was determined in different samples containing recombinant GAPC1 or GAPC1K231A alone or after ubiquitination by SINAL7, and non-ubiquitinated GAPC1K76A. Sample composition, occurrence of ubiquitination and specific activity values are indicated in the table. Lane 1: GAPC1 specific activity; lane 2: GAPC1 activity after 2 h incubation at 30 °C; GAPC1 activity after incubation with ubiquitination medium in the absence (lane 3) or presence (lane 4) of SINAL7; lane 5: GAPC1K231A specific activity; lane 6: GAPC1K231A activity after 2 h incubation at 30 °C; GAPC1K231A activity after incubation with ubiquitination medium in the absence (lane 7) or presence (lane 8) of SINAL7; lane 9: GAPC1K76AA specific activity; lane 10: GAPC1K76A activity after 2 h incubation at 30 °C; GAPC1K76A activity after incubation with ubiquitination medium in the absence (lane 11) or presence (lane 12) of SINAL7. N.D: "Not detected". Values are the mean ± SD of at least three independent replicates. The asterisk signals a statistically different result from the control value ($P < 0.05$).

3.6. GAPC1 mono-ubiquitination abolishes its catalytic activity.

To investigate whether ubiquitin modification of GAPC1 affects its catalytic activity, we performed in vitro ubiquitination assays using SINAL7 as E3 ligase in the presence of either, GAPC1, GAPC1K231A or GAPC1K76A, followed by the analysis of the GAPC1 activity (Fig. 5). After 2 h of incubation with the complete ubiquitination reaction medium, GAPC1 has no detectable catalytic activity (lane 4), compared to the sample where SINAL7 was absent (lane 3). Similar result was observed for the GAPC1K231A mutant (lane 8, compared to lane 7). Interestingly, the GAPC1K76A mutant, which does not undergo mono-ubiquitination, presents no inhibition of its enzymatic activity (lane 12). This result indicates that the loss of GAPC1 catalytic activity was due to the ubiquitination of the enzyme. We found no differences in the enzymatic activity regardless the enzyme used (lanes 1, 5 and 9). It should be mentioned that GAPC1 and the modified proteins retain about 40% of the catalytic activity after incubation for 2 h at 30 °C (see lines 1, 5 and 9 vs. lines 2, 6 and 10, respectively).

3.7. Mutations K76A and K231A do not affect GAPC1 protein folding

Since the mutation of lysine residues may result in structural changes in the protein, we decided to study the secondary structure of recombinant GAPC1 and the respective K- to A mutated proteins. The folding of pure recombinant GAPC1, GAPC1K76A and GAPC1K231A proteins was analyzed using circular dichroism (CD) (Supplementary Fig. 1). The percentages of the secondary structure were determined from CD spectra using the K2D algorithm (Andrade et al., 1993). Interestingly, both K76A and K231A mutants presented identical CD spectra respect to the wild type GAPC1, showing that no changes in secondary structure were induced in both mutants. Considering that the average error observed from CD

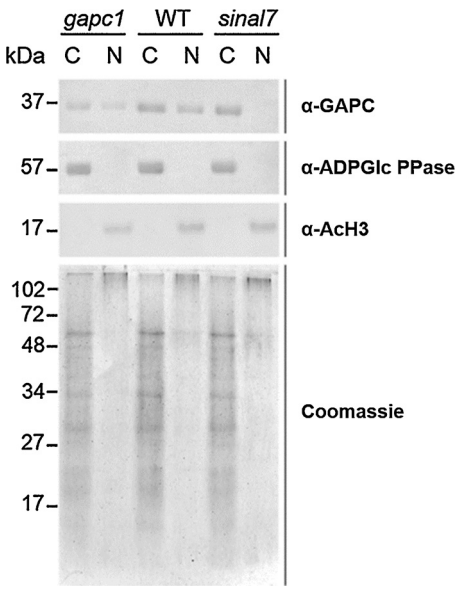


Fig. 6. Detection of GAPC1 in nuclear extracts of *Arabidopsis* flowers. GAPC1 was detected by western blot analysis using α-GAPC antibodies in nuclear protein extracts of *gapc1*, wt and *sinal7.1 Arabidopsis* flowers. (C) Cytosolic, mitochondrial, and plastidial protein fraction from flowers. (N) Nuclear protein fraction. α-ADPGlc PPase was used to identify cytosolic/organellar contamination and α-AcH3 (acetylated form of Histone H3) antibodies were used to identify nuclear proteins in extracts. Lower panel correspond to the Coomassie stained polyacrylamide gel. Numbers on the left indicate the molecular masses of the identified proteins and the molecular masses of molecular weight standards corresponding to Bio Rad Low Range Prestained SDS-PAGE Standards.

spectra was lower than the values obtained in secondary structure prediction using K2D, we conclude that the lack of ubiquitination in GAPC1K76A and the lack of interaction with SINAL7 in GAPC1K231A was not due to differences in the secondary structure.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2015.11.007.

3.8. *GAPC1 is absent in the nucleus of* sinal7 *mutant plants*

To evaluate the possible functions of seven in absentia *like* 7 (*SINAL7*) gene, we carried out the selection of SINAL7 knock-out (*sinal7*) *Arabidopsis* plants from T-DNA insertion mutants (see Section 2). *SINAL7* (At5g37890, 860 bp) is composed of two exons and one intron. In *sinal7.1* and *sinal7.2* plants the T-DNA was found inserted at the beginning of the second and first exon, respectively (Supplementary Fig. 2A and B). *sinal7* homozygous plants containing one copy of T-DNA were identified by using PCR screening, segregation analysis and DNA gel-blot hybridization. The expression of *SINAL7* was verified by RT-PCR analysis showing that *SINAL7* mRNA was absent in *sinal7* T-DNA insertion mutant plants (Supplementary Fig. 2C).

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2015.11.007.

Our working hypothesis is that SINAL7 interaction with GAPC1 is involved in the translocation and/or stabilization of GAPC1 protein in the nuclear compartment. To verify this possibility, nuclei from wt and *sinal7 Arabidopsis* plants were isolated and nuclear proteins were analyzed for the presence of GAPC1 by western blot analysis (see Section 2). Acetylated histone H3 was used as a marker of the nuclear fraction and ADPGlc PPase was used to identify possible contamination of the nuclear extracts. As shown in Fig. 6, no cross-contamination was detected either in nuclear (N) or cytosolic (C) fractions. GAPC1 was found in both nuclear and cytoplasmic compartments in extract from wild-type plants (Fig. 6, WT, lanes N and C). Interestingly, no traces of GAPC1 were found in the nucleus of *sinal7.1* plants (Fig. 6, *sinal7*, lane N), suggesting that SINAL7 is required for the nuclear localization of GAPC1. Similar results were obtained with the *sinal7.2* line (not shown). In the *gapc1* knock-out mutant (Rius et al., 2008), a nuclear signal band is observed with α-GAPC antibodies (Fig. 6, *gapc1*, lane N), suggesting that the GAPC2 isoform, which shares 98% identity with GAPC1, is also translocated to the nucleus in *Arabidopsis* plants.

4. Discussion

The RING finger protein SINA *like 7* from *A. thaliana*, which has ubiquitin E3 ligase activity, is postulated to participate in flowering functions (Lorick et al., 1999; Deshaies and Joazeiro, 2009; Peralta et al., 2013). We reported that in male sterile plants with impaired mitochondria, the transcript levels of *SINAL7* were down-regulated (Busi et al., 2006; Rius et al., 2008; Busi et al., 2011). Interestingly, plants lacking one of the cytosolic glyceraldehyde-3-phosphate dehydrogenase genes, *gapc1*, also present a male-sterile phenotype with alterations in flowering and fructification, suggesting that SINAL7 could play an important role in a retrograde signaling pathway associated with GAPC1 (Rius et al., 2008).

It was reported that the glycolytic enzymes are functionally associated to mitochondria in *Arabidopsis* (Giegé et al., 2003). Although, the role of GAPC1 in glycolysis is well known (Anderson et al., 2004), several reports also designate non-metabolic roles for GAPC1 (Giegé et al., 2003; Sirover, 2012). Moreover, reactive oxygen or nitrogen species could induce post-translational modifications, affecting the catalytic activity of GAPC1 and inducing changes in its subcellular localization and, probably, metabolic function (Zaffagnini et al., 2013a,b; Holtgrefe et al., 2008).

Several poly-ubiquitination targets of SIAH, the SINA homologue from *Arabidopsis*, have been described in the context of proteasome-mediated protein turnover. Since compartment-specific substrates have been postulated for human SIAH-1, different effects may be induced depending on the subcellular localization of the ubiquitinated targets (Brauckhoff et al., 2011). The interaction between murine SIAH-1 and S-nitrosylated GAPDH via nuclear translocation and its participation in an apoptotic cell death cascade have already been extendedly studied (Hara et al., 2005). In plants, SINAT5, a SINAL7 paralog, promotes ubiquitin-mediated degradation of NAC1 to down-regulate auxin signals (Xie et al., 2002).

Considering the important role of the ubiquitin-proteasome system in the regulation of plant physiology (Vierstra, 2012; Ling and Jarvis, 2013; Shabek and Zheng, 2014), the knowledge of the specific targets of the family of ubiquitin-protein ligases is crucial for understanding the mechanisms of signaling and gene expression in plants. The need to specifically target a broad array of substrates accounts for the great diversity among the estimated >1000 potential E3-ligases (Vierstra, 2012). Ubiquitination of proteins may lead to several outcomes depending on the type and extent of this modification. Ubiquitination either targets the substrate proteins for proteolysis by the 26S proteasome or alters their biochemical properties and subcellular localization.

We show that SINAL7 ubiquitin E3 protein ligase is able to interact with GAPC1 and that its binding ability was affected when the K231 was changed to alanine in GAPC1, suggesting that the K231 residue is involved in complex formation or is found at the protein–protein interface. It should be noted that the lack of interaction was not due to a loss of secondary structure of K231A as shown by the conservation of the kinetic parameters between GAPC1 and GAPC1K231A proteins and the CD analysis.

GAPC1 is a substrate for ubiquitination catalyzed by SINAL7 since when the complex was incubated in the presence

of the complementary E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and cMyc-Ubiquitin high molecular weight product was detected by gel electrophoresis. These products were identified as containing K76 mono-ubiquitinated GAPC1 by mass spectrometry analysis. Indeed, when K76 was changed by Ala, the ubiquitination of GAPC1 was abolished. It should be noted that the GAPC1K231A mutant, which does not interact with SINAL7 in the pull down assay, was also ubiquitinated by SINAL7, probably because the interaction is weaker compared to the wild-type and cannot be detected by this procedure.

An important fact is the increase we observe in the V_{max} of GAPC1 when interacting in vitro with SINAL7, which turns GAPC1 into a more effective enzyme, increasing its catalytic efficiency. This could probably affect the glycolytic pathway, increasing the glycolytic flux rate in response to cellular metabolic needs. In contrast, the recruitment of GAPC1 by SINAL7 (E3) in the complete E1–E2–E3 modifier complex leads to the mono-ubiquitination of GAPC1 and to a dramatic decrease in the glycolytic activity. The observation that the GAPC1K76A mutant, unable to undergo mono-ubiquitination, retains the GAPDH activity strongly supports the idea that the loss of the enzymatic activity is due to the ubiquitination on residue K76. Thus, we report two potential ways in which the association of SINAL7 with GAPC1 might result in different biological consequences, affecting the glycolytic flux in different ways.

GAPC may be translocated from the cytoplasm into the nucleus (Sirover, 2012; Zaffagnini et al., 2013a), and the nitrosylation of GAPC has been proposed as responsible for this event (Zaffagnini et al., 2013b). Animal GAPDH after NO-dependent apoptotic stimuli is found in the nucleus (Nakamura and Lipton, 2013). GAPDH relocation from the cytoplasm to the nucleus appears to be due to the S-nitrosylation of its catalytic cysteine acquiring the ability to bind Siah1 (seven in absentia homologue 1). In contrast, nuclear relocation of GAPC1 was stimulated by the substitution of the catalytic cysteine with a serine (Vescovi et al., 2013), suggesting that the redox modification of the cysteine is not required for nuclear translocation of GAPC1 in *Arabidopsis*. However the possibility that oxidative stress or other stimuli might modulate the translocation events cannot be discarded. We present evidence that GAPC1 can be mono-ubiquitinated by SINAL7. While post-translational modifications could also have an important role, it is clear that the presence of SINAL7 is required for the localization of GAPC1 into the nucleus, since the nuclear extracts from knock-out *sinal7* plants are depleted of GAPC1. Interestingly, in nuclear extracts of GAPC1 deficient plants, we observed the presence of a similar protein band that could probably be GAPC2 confirming the observations made by Holtgrefe et al. (2008). This result is not surprising considering that GAPC1 and GAPC2 present 98% identity and thus probably share common biochemical properties. Our results are consistent with the fact that GAPC1 is ubiquitinated by SINAL7 and, since mono-ubiquitination is known to cause translocation of affected proteins in the cell (Tanaka et al., 2008; Cai et al., 2012), the poly-ubiquitination is mainly associated with proteolytic degradation of substrates by the 26S proteasome (Hellmann and Estelle, 2002). Thus, we believe that the mono-ubiquitination of GAPC1 might modify this protein in order to change its localization. However, we cannot assume as a fact that this modification is responsible for the nuclear translocation of GAPC1.

5. Conclusions

In *Arabidopsis thaliana* the glycolytic enzyme GAPC1 is able to interact in vitro with the E3 ubiquitin ligase SINAL7. This interaction is dependent on the presence of residue Lys231 from GAPC1, leading to the increase in its catalytic efficiency which could increase the glycolytic flux within the cell. In addition, when supplementing the E3 ligase, SINAL7, with the E1 and E2 complements, it catalyze the mono-ubiquitination of GAPC1 at residue Lys76 and the glycolytic activity of GAPC1 is completely abolished. Interestingly, the presence of GAPC1 in the cellular nucleus is dependent of the presence of SINAL7 in *Arabidopsis*. Our data suggest that SINAL7 modulates GAPC1 activity and participates in the nucleus-cytoplasm traffic of the glycolytic enzyme. Thus, SINAL7 could be involved in conferring some moonlighting features to GAPC1 such as the participation in novel metabolic or signaling pathways inside the plant cell.

Authors' contribution

DAP, AA, MVB and DGC conceived and designed the experiments. DAP performed the experiments. DAP, AA, MVB and DGC analyzed the data. DAP, AA, MVB and DGC wrote the manuscript.

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