Research Article

Phenotypic Characterization of the Arabidopsis ufm1 (Ubiquitin Fold Modifier) Gene Involved in Seed Development

Cornejo Paula and Masuelli Ricardo Williams

Laboratorio de Biología Molecular, Instituto de Biología Agrícola de Mendoza (IBAM), Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, A. Brown 500 (M5528AHB) Chacras de Coria, Mendoza, Argentina

Abstract

Background and Objective: With the completion of the Arabidopsis genome sequencing, the next challenge is the determination of gene function. Post-translational modifications of proteins by small polypeptide are implicated in plant growth and development. Ubiquitin fold modifier 1 is a member of the ubiquitin like protein family. While, the enzymatic conjugation cascade of ubiquitin fold modifier 1 has been elucidated in recent years, its biological role is still unknown. The present study focuses in elucidate the role of the ufm1 in plants development. Materials and Methods: The researchers performed analyses of the development of wild-type Columbia plants and mutants of the ubiquitin fold modifier 1 gene to identify and interpret phenotypic changes in plants and seeds. Data were statistically analyzed with the Info Stat software. Results: In this study, evidence suggesting that ubiquitin fold modifier 1 is involved in the normal development of the seeds. Conclusion: The ufm1 gene would affect the normal development of the seed, particularly of the embryo, causing high percentage of seed abortion.

Key words: UBLs, embryo, endosperm, seed abortion, development, cell cycle, post translational modifications, embryogenesis

Received: Accepted: Published:

Citation: Cornejo Paula and Masuelli Ricardo Williams, 2016. Phenotypic characterization of the Arabidopsis ufm1 (Ubiquitin fold modifier) gene involved in seed development. Am. J. Plant Physiol., CC: CC-CC.

Corresponding Author: P. Cornejo, Laboratorio de Biología Molecular, Instituto de Biología Agrícola de Mendoza (IBAM), Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, A. Brown 500 (M5528AHB) Chacras de Coria, Mendoza, Argentina Tel: 0549-0261-4135000-1232

Copyright: © 2016 Cornejo Paula and Masuelli Ricardo Williams. This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.
INTRODUCTION

Post-translational modifications to proteins are one of the principal components of the organization and regulation of function and cell structure as well as the development of organism, refer to covalent and generally enzymatic modification of proteins during or after its biosynthesis. The protein modification systems produce a functional change in target proteins. The 1st protein modifier system discovered was ubiquitin (Ub). Ubiquitin is a highly conserved 76 amino acid polypeptide ubiquitously present in eukaryotes, from yeast to human. It is covalently ligated to a wide variety of target proteins through the action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein isopeptide ligase (E3). This addition can be reversed by the action of deubiquitylating enzymes (DUBs). Ubiquitin can participate in a wide variety of cellular processes like protein degradation pathways, histone regulation, transcriptional regulation, RNA metabolism, chromatin remodeling and cell cycle control. Besides ubiquitin, a number of other ubiquitin like proteins (UBLs) have been identified. These proteins do not necessarily share a high similarity to ubiquitin, but are structurally related and can be conjugated to target proteins in a similar manner than ubiquitin.

The most recently identified ubiquitin like protein, ufml (ubiquitin fold modifier 1), shares only 14% sequence identity with the ubiquitin proteins family but adopts the typical β-grasp fold. The ufml is synthesized as a precursor protein and is activated by an E1-like protein, Uba5, while conjugation is conducted by an E2-Like protein, ufcl. Recently, an E3-Like ligase protein termed ufll has been identified. Finally specific ufsp can also cleave ufml from its target proteins. Cascade of ufml is conserved among nearly all the eukaryotic organisms except yeast. Studies on mammalian cells showed that the only target of ufml identified, until now is the ubp1 protein and suggested a limited number of substrates. Mutant mice of Uba5 -/- are embryonic lethal and cultured human cells silenced by ufml and ufll showed accelerated apoptosis. Until now, there are no studies on the role of ufml in plant development. The aim of this study is conduct a functional analysis to understanding the role of ufml gene in plants. The phenotypic analysis described here, in conjunction with gene expression profiling, suggest that ufml protein has a fundamental role in development of plant primordial in seed, particularly embryo development.

MATERIALS AND METHODS

Plant material and growth conditions: Plants of Arabidopsis thaliana were derived from the Columbia-0 (Col-0) mutant lines ufml (SALK-040955C), locus AT1G77710, obtained from the Arabidopsis Biological Resource Center (Columbus, OH), from de SALK T-DNA insertion collection24. Surface-sterilized seeds were placed in petri dishes containing 15 mL of agar 0.8% w/v. Petri dishes were placed in a cold room for 3 days at 4°C to synchronize germination. Plates with cold stratified seeds were transferred into a room with day and night temperatures of 22 and 20°C, respectively. The average light intensity at the level of the rosette was ~65 μmol m−2 sec−1 supplied by fluorescent tubes. Seedlings were transferred to soil when 2 rosette leaves were greater than 1 mm in length. Plants were watered by sub irrigation every 2-3 days, depending on the growth stage. The plants, wild type and mutant were sown in 2 subgroups of 12 individuals each.

Phenotypic characterization: All plants were analyzed for the following phenotypic characteristics: Leaf morphology, rosette radius, flower morphology, length of major axis of silique and size of embryos. The phenotype of the mutant plants were compared to that of control plants during the different stages of growth.

All measurements were performed by analysis of digital images using AxioVision Rel. 4.8 software (Carl Zeiss).

Histology: For whole-mount preparations of embryos, silique were dissected with hypodermic needles. Dissected siliques were fixed on ice with FAA (10:7:2:1 ethanol:distilled water:acetic acid:formaldehyde 37%) for 30 min, hydrated in a graded ethanol series to 50 mM NaPOH, buffer, pH 7.2. Embryos were excised using fine forceps and surgical blades under a magnifying scope and mounted on a microscope slide in a clearing solution 8:2:1 chloral hydrate:distilled water:glycerol. Samples were cleared for 1 h at 4°C before inspection. Light microscope preparations were examined using a Zeiss Axioplan microscope (Jena, Germany). Pictures were taken with a Cannon PowerShot G11 camera and edited with Adobe Photoshop.

Tetrazolium staining: Seeds were imbibed for 2 h and embryos were excised using fine forceps and surgical blades
under a magnifying scope. Embryos were subjected to staining in 0.5% w/v 2, 3, 5-triphenyl tetrazolium chloride solution.

**Germination assay:** Seed lots were harvested from individual plants grown under identical conditions. Each plant population was sown in 5 petri dishes (20 seeds from each individual plant per petri dish) on agar and incubated in a climate-controlled room. Germination was measured 5 days after imbibition and was expressed as the percentage of plants with emerged roots in each plant population (germination percentage).

**Quantification of DNA:** Excised embryos were stained in the dark with DAPI dihydrochloride (Invitrogen, San Diego, Calif) 300 nM. Images were taken in a fluorescence Zeiss Axioplan microscope (Jena, Germany) with 358 nm excitation and 461 nm emission wavelengths, recorded with Cannon Power Shot G11 camera and processed using Image J.

**RNA extraction:** Siliques of mutant **ufm1** and wild type plants were dissected to isolate the seeds. Total RNA was extracted from a pool of seeds per plant using the TRIZOL reagent (Invitrogen GibCO/Life Technologies, Grand Island, NY) according to the protocol by Meng and Feldman.

**Semiquantitative reverse transcription (RT)-PCR analysis:** Equal amounts of RNA from seeds were treated with DNase and reverse transcribed using reverse transcriptase M-MLV (Invitrogen, San Diego, Calif) as indicated by the manufacturer. The cDNA was amplified with specific primers designed based on the sequence of **ufm1** transcript of *Arabidopsis thaliana* (forward primer, CGTTGGTATTATCTGTGGTTTCAC, reverse primer, CAACTCAGTCAATTGTGACGA) and specific primers of the constitutive control gene actin (forward primer, CATCAGGAAGCATTGTACGG, reverse primer, GATGAGCTGACTCGTCTAC). As template, 40 ng of cDNA were used in a 15 μL reaction volume that contained 20 mmol L⁻¹ tris-HCl, pH 8.4, 50 mmol L⁻¹ KCl, 1.5 mmol L⁻¹ MgCl₂, 300 μmol L⁻¹ of each dNTP, 0.4 μmol L⁻¹ of each primer and 0.4 U of Taq polymerase (Invitrogen, San Diego, Calif.). The thermocycler (PTC-100 MJ Research, Watertown, Mass.) was programmed for an initial denaturation step of 94°C for 1 min and then 35 cycles of 94°C for 30 sec, 51°C for 1 min and 72°C for 2 min. The PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. The stained gel was photographed with the Molecular Analyst Gel Photo documentation System (Bio-Rad, Calif.) and analyzed with the Image J software. The quantity of each PCR product was expressed as the ratio of the sample densitometry measurement to the corresponding internal standard (action). To evaluate the changes in relative mRNA levels, densitometry values for the target genes were normalized against those of the housekeeping action mRNA. Data were statistically analyzed with the Info Stat software (Córdoba, Argentina).

**RESULTS**

**Phenotypic characterization of a **ufm1** mutant:** To analyze **ufm1** function, a T-DNA insertion homozygous mutant of *Arabidopsis thaliana* (SALK 040955C) and wild-type plants were used. Analyzed growth stages included the development of the plant from seed through the completion of flowering and seed maturation. Figure 1 and 2 shows the vegetative growth of mutant and wild-type plants was not statistically different. Flower morphology of the mutant was normal. However, silique of the homozygous mutant plants contained approximately 75% of aborted seeds (Fig. 3).

Based on these findings, embryo development at different stages was investigated. In the mutants, the aborted seeds had embryos arrested at globular or torpedo stages. The rest of the mutant seeds presented embryos with delayed development respect to the wild type. At 6 Days After Pollination (DAP), mutant embryos were at globular, heart or torpedo stage, while wild type embryos were at torpedo stage. Between 8-12 DAP, the growth of the mutant embryos was retarded respect to the wild type. Mutant embryos at 8 DAP were arrested at heart or torpedo stage while the wild type was at cotyledon stage. At 12 DAP viable mutant seeds presented embryos at cotyledonal stage with several abnormalities, like enlarged cells and nuclei, disorganized cells in the tissue and flatten and smaller cotyledons. The mature embryos were significantly smaller than the wild type (Fig. 4a, b).

**Germination:** Tetrazolium staining showed that the mutant embryos had reduced germination potential, showing a reduce coloration and only morphologically mature embryos exhibited full staining by tetrazolium in wild type plants. Consistent with the viability of the embryos
Fig. 1(a-l): Representative mutant growth stages, (a) Imbibition, (b) Radicle emergence, (c) Hypocotyl and cotyledons emerged from seed coat, (d, e) Fully opened cotyledons, (f) Two rosette leaves of 1 mm in length, (g) Four rosette leaves of 1 mm in length, (h) Ten rosette leaves of 1 mm in length, (i) First visible flower buds (indicated by arrow), (j) First open flowers, (k) Flowering complete and (l) Mature silique using the tetrazolium staining method the seed germination percentage was significantly lower in the mutant compared to the wild type (Fig. 5).

**Embryo DNA content:** The DNA content in nuclei of wild type and mutant embryos was estimated by considering intensity of the DAPI staining. The signal was 1.7 times stronger in the
mutant embryos (45929 U per nucleus ± 1312.8, n = 60) compared with wild type embryos (25883 U per nucleus ± 1468.7, n = 60) (Fig. 6a, b).

**Gene expression analysis:** To verify the expression patterns, specific primers were designed to amplify the gene *ufm1*. The RT-PCR expression analysis was carried out on mature seeds of *ufm1* mutants and wild type plants. Figure 7 shows the results indicate that *ufm1* was expressed in seed of the wild type plants, but its expression was null in seeds of the mutant plants, which were characterized by the embryo retarded growth and seed abortion.

**DISCUSSION**

Post-translational modification by ubiquitin-like proteins (UBLs) plays a critical role in controlling stress responses, signaling, plant growth, cell cycle, transcription,
Fig. 4(a-i): Comparative analysis of embryos, (a) Comparative wild type and mutant embryo size. Significant differences were observed (p<0.01). Error bars indicate ±SE, (b-i) Image of cleared embryos of wild type and mutant seeds, (b) Embryos of the mutant to 6 DAP arrested at globular, heart and torpedo stage, (c) Wild type embryos to 6 DAP are uniformly developed at torpedo stage, (d) Mutant embryos to 8 DAP, (e) Wild type embryos to 8 DAP, (f) Mutant embryos to 12 DAP, (g) Wild type embryos to 12 DAP are mature, (h) Mutant mature embryos to 18 DAP present abnormalities and (i) Wild type embryos to 18 DAP are normal and mature
abnormalities and show reduced viability. A possible embryogenesis, circadian rhythms and other pathways\textsuperscript{3,4,16-29}. Recently, the ubiquitin fold modifier 1 (ufm1) pathway described in mammalian cells, involves E1 activating (Uba5), E2 conjugating (ufc1) and E3 ligating (ufl1) enzymes, which will conjugate ufm1 to targets both in vitro and in vivo\textsuperscript{38,39}. The abnormal ufm1 cascade is associated with many human diseases including cancer, ischemic heart diseases, diabetes, atherosclerosis, hip dysplasia and schizophrenia\textsuperscript{10}. Studies on mutant mice of the enzyme uba5 resulted lethal for the embryos. It was demonstrated that Uba5 was indispensable for erythroid differentiation\textsuperscript{23}. Also, cultured human cells silenced for ufm1 and ufl1 showed accelerated apoptosis\textsuperscript{22}.

Several genes have an essential role in plants life cycle, the absence of a functional gene product becomes critical or lethal. The distinction between embryo and gametophyte mutants is subjective. Some mutants with defects in embryos may also have defects in the gametophyte development\textsuperscript{31}. Plant embryogenesis involves a strict synchronization of cell division, elongation and differentiation\textsuperscript{32}.

An important objective in Arabidopsis research is to identify minimal number of genes necessary to have a viable plant under normal growth conditions\textsuperscript{33}. To understand the molecular bases of the embryonic development in plants, in the present study, an evidence was provided on the ufm1 gene function and seed phenotypes. The RT-PCR analysis showed that ufm1 is expressed in the seeds of the wild type plants and the expression is suppressed in the mutants. The use of null mutants revealed that ufm1 is involved in the normal development of the Arabidopsis embryo.

To understand the role of ufm1 in plants, reverse genetics to search for changes in phenotype of plants at different growth stages, morphology and DNA content in the nucleus of embryos in wild type (ecotype Col-0) and homozygous mutant (SALK 040955C) lines was used. Interestingly, phenotypic analysis reveal no significant differences in the vegetative parts but significant differences were observed in the number of viable seeds and size of the embryos, the mutant embryos are smaller and the number of seeds per
Fig. 7(a-b): RT-PCR analysis of mutant and wild type seeds of *Arabidopsis thaliana*. (a) Relative expression level of *ufm1* gene and (b) The wild type seeds amplified the expected product of 506 bp. The actin gene was used as a positive control.

Silique is lower than the wild type. Also was observed that mutant embryos have delayed development, present abnormalities and show reduced viability. A possible explanation for how some embryo defective mutant survive and develop normally, to complete their life cycle, involve the presence of genes with redundant function.

The results showed an increment in size and DNA content of embryo nuclei in *ufm1* mutants, denoting an alteration in the cell cycle and cellularization possibly by alteration of the ubiquitin pathway. Similar results, although more severe, were obtained in TTN6 seed mutants in *Arabidopsis* and in seeds of incompatible crosses in potato. This observation establishes an essential role for *ufm1* in plant development, demonstrating that *ufm1* is primordial for plant embryo genesis as observed in mammals. Further studies of *ufm1* interactions and pathway should clarify the molecular basis for the phenotypes resulting in mutant seeds.

CONCLUSION

It is observed that mutant embryos, which do not express *ufm1* have delayed development, present abnormalities and show reduced viability, relative to control. This is the 1st report to establish the role of *ufm1* in plant embryogenesis. These results are important because they contribute to the understanding of the mechanisms involved in seed development.

SIGNIFICANCE STATEMENT

Little is known about the role of the *ufm1* gene in plants. This study sets the role played by the *ufm1* gene in plant development, remains essential in embryogenesis of plants as well as in mammalian.

ACKNOWLEDGMENTS

This study was funded by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (ANPCyT) [BID 1201-OC-PICT-2007-01294]. We are greatful with Carina González member of the laboratory of deceased H. Boccalandro for helpful device and for plant material.

REFERENCES


40. Cai, Y., W. Pi, S. Sivaprakasam, X. Zhu and M. Zhang et al., 2015. UFBP1, a key component of the Ufm1 conjugation system, is essential for ufmylation-mediated regulation of erythroid development. PLoS Genet. 10.1371/journal.pgen.1005643.