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Dimerization and DNA-binding of ASR1, a small hydrophilic protein abundant in plant tissues suffering from water loss

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Abstract

The *Asr* gene family is present in Spermatophyta. Its members are generally activated under water stress. We present evidence that tomato ASR1, one of the proteins of the family, accumulates in seed during late stages of embryogenesis, a physiological process characterized by water loss. *In vitro*, electrophoretic assays show a homo-dimeric structure for ASR1 and highlight strong non-covalent interactions between monomers prone to self-assemble. Direct visualization of single molecules by atomic force microscopy (AFM) confirms that ASR1 forms homodimers and that uncovers both monomers and dimers bind double stranded DNA.

Keywords: *Asr* genes; *Lycopersicon esculentum*; Seed; Dimerization; Atomic force microscopy

Several types of environmental abiotic stress affect plant behavior by means of changes in gene expression [1]. We have focused on one of the many stress- and ABA-induced genes in plants, namely the genes of the *Asr* family, induced by ABA, stress, and fruit ripening. The first *Asr* gene was reported in *Lycopersicon esculentum* [2,3]. Despite the fact that the model plant Arabidopsis lacks *Asr* genes, the *Asr* gene family appears widespread in the plant kingdom [4]. In tomato, *Asr* is a family composed of at least four genes, *Asr1*, *Asr2*, *Asr3*, and *Asr4* [5], which are expressed differentially in leaf and root under water stress [4]. Biochemical experiments showed that ASR1 protein from tomato binds to a short DNA consensus sequence [6], which is in agreement with its localization in the nucleus [2]. Likewise, ASR protein from grape binds DNA [7] and those from pine and lily also have a nuclear localization [8,9]. Cakir et al. [7] have suggested that grape ASR forms dimers that might function as a part of a transcription-regulating complex involved in the control of sugar transport during grape ripening [10].

High hydrophilicity and water-stress induction in vegetative tissues are common features shared by ASRs and most LEA (late embryogenesis-abundant) proteins [4], which are expressed at high levels during late embryo maturation [11]. On the other hand, ASR proteins lack any of the known structural motifs typical of LEA proteins and their expression in seed development has not yet been evaluated. Therefore, to explore if physiological desiccation correlates with an accumulation of ASR proteins, we investigated its presence in developing seeds.

In addition, we wanted to focus on molecular aspects that might give us a clue about the mechanism underlying ASR1 involvement in the response to dryness. To that purpose, we looked at its quaternary structure and also took advantage of Atomic Force Microscopy (AFM) [12], a high-resolution technique which allows visualization of

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nucleoprotein complexes [13]. This is the first report of an ASR protein–DNA complex at the single molecule level by means of AFM. We show that, at least *in vitro*, ASR1 forms homodimers, which bind double strand DNA.

Materials and methods

Plant material and stress treatments..Tomato (*Lycopersicon esculentum* M. Cv. UC82b) plants were grown in soil pots for 1 month under standard greenhouse conditions. Fruit pericarp was collected from tomato fruit at a mature red stage. Seeds were harvested from tomato fruits (25–70 days post anthesis) grown under standard greenhouse conditions. The whole seed was used for protein extraction. For water-stress experiments, whole plants were removed from their pots, dried for 24 h on the laboratory bench and leaves, stems, and roots were then detached. Unstressed controls were obtained from plants that were not subjected to drying conditions. In all cases, the collected material was immediately immersed in liquid nitrogen and stored at −80 °C until protein extraction.

Antibody production. To develop an ASR1-specific antiserum, antibodies were raised against either the whole pure recombinant protein (see below) or a synthetic peptide corresponding to the C-terminus of ASR1, divergent from other members of the ASR family: GCKKKLRGDTT ISSKLLF-COOH (Bio-synthesis Inc., Lewisville, TX, USA). The purified peptide was intramuscularly injected into rabbits. The resulting antiserum was diluted 1/2500.

Protein extraction and Western blotting. Soluble proteins were extracted using Extraction Buffer (50 mM $NaHPO_4$, pH 7; 10 mM β-mercaptoethanol; 10 mM Na_2EDTA; 0.1% *N*-lauril-sarcosine; 0.1% Triton X-100). Western blots were performed according to [14], loading 50 μg of protein. After 0.1% SDS–16.5% PAGE, proteins were electroblotted onto a Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were stained with Ponceau Red to ensure equal loading and then incubated with ASR1-specific antiserum (1/2500). Bound antibodies were detected using an anti-rabbit immunoglobulin G-peroxidase conjugate (Bio-Rad) diluted (1/4000). For visualization of enzymatic activity, the ECL chemiluminescence detection system (Amersham Pharmacia Biotech) was used.

Urea treatment. Tomato pericarp proteins were incubated overnight with gentle stirring in cracking buffer containing 8 M urea. Samples were then loaded in 8 M urea–PAGE for Western blot assays under the same conditions as indicated below.

ASR1 purification. The coding region of the *Asr1* cDNA was subcloned into the PRSET B vector (Invitrogen). The recombinant plasmid was introduced into the *Escherichia coli* BL21 strain. ASR1 protein was purified by affinity chromatography (HisTrap Kit, Pharmacia Biotech) taking advantage of its histidine-rich tract. Purification was monitored by loading 5 μg ASR1 in PAGE stained with Coomassie blue.

Protein elution. Pure ASR1 was subjected to 0.1% SDS–16.5% PAGE. Gel regions corresponding to 15, 30, and 45 kDa were excised from the gel and left overnight in a Tris–glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The eluted proteins were concentrated using the methanol–chloroform–water method [15] and resuspended in extraction buffer. The two forms of pure eluted proteins were analyzed by a second 0.1% SDS–16.5% PAGE and Western blotting.

Atomic Force Microscopy (AFM). ASR1 samples (20 pg/μl) were pre-incubated for 20 min with CsCl-purified pBluescript SK+ plasmid (60 pg/μl) at a molar ASR1 monomer/DNA ratio of 10:1 in 4 mM Hepes, pH 7.4, in the presence of 1 mM $ZnCl_2$. The mix (10 μl) was then deposited onto freshly cleaved muscovite mica, incubated for 2–5 min, washed with 0.5 ml MilliQ water (Millipore) and blown dry briefly in a gentle stream of nitrogen. Tapping-mode AFM was performed using a Nanoscope III Multimode-AFM with a J-type piezoelectric scanner and microfabricated silicon cantilevers of 125 μm in length and a force constant of 40 N/m (NanoDevices, Veeco Metrology, Santa Barbara, CA, USA). The images (512 by 512 pixels) were captured with a scan size of between 0.5 and 3 μm at a scan rate of 1–2 Hz. Images were processed by flattening using Nanoscope software.

Results

ASR1 accumulation during embryogenesis

We explored if ASR proteins are present in programmed physiological events associated with desiccation. We concentrated on ASR1, for which we have a good immunodetection system and investigated its accumulation in developing seeds by Western blot analysis (Fig. 1). The protein was readily detected in seed only at developmental stages corresponding to late embryogenesis (55–70 days post anthesis).

ASR1 protein forms dimers in vitro

Immunodetection revealed ASR1 to accumulate in several tomato organs (leaf, root, stem, and fruit). Its electrophoretic mobility (15 kDa) was coherent considering the calculated molecular weight (115 amino acid residues, 13 kDa) (Fig. 2). To our surprise, we detected a band corresponding to 30 kDa, which suggested the existence of a

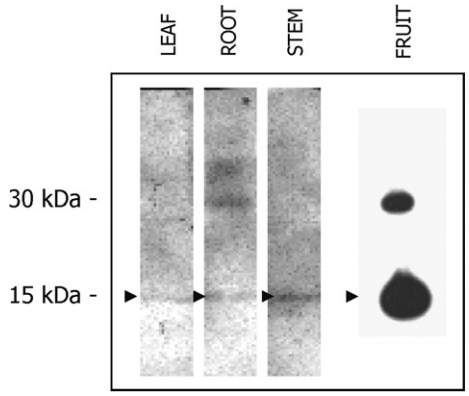

Fig. 2. ASR1 accumulates in different tomato organs. Western blot analysis was performed to test for the presence of ASR1 in tomato leaf, root, stem and fruit pericarp. The expected migration position for ASR1 (15 kDa) is indicated by arrowheads. Immunodetection was achieved by means of a specific anti-ASR1 antibody as described in Materials and methods.

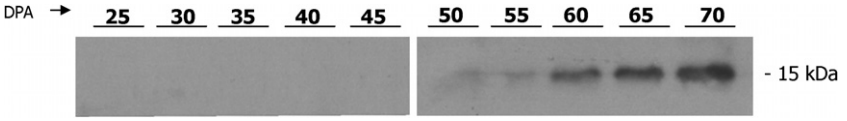

Fig. 1. ASR1 protein accumulation at different stages of seed development. Total protein was extracted from seed at different stages of ontogeny. Proteins were analyzed by Western blot as described in Materials and methods. The numbers over the lanes indicate the days post-anthesis. The migration position of the 15-kDa molecular weight marker is indicated on the right.

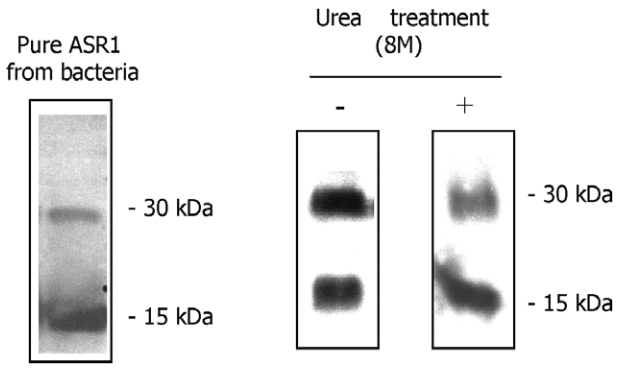

Fig. 3. ASR1 forms stable dimers. (Left panel) Western blot for ASR1 purified from transformed *E. coli*. (Right panel) Total fruit protein was subjected (+) or not (−) to urea treatment and analyzed by Western blot. Immunodetection was as indicated for Fig. 2.

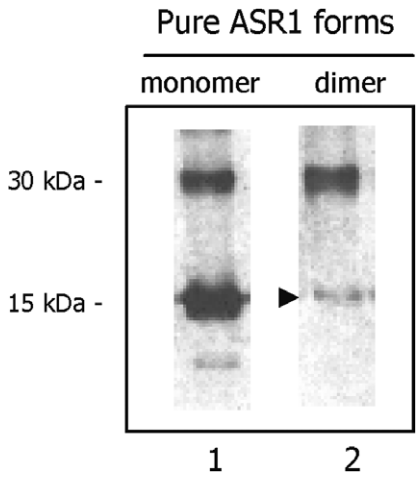

Fig. 4. ASR1 monomer self-assembles *in vitro*. Recombinant pure ASR1 monomer and dimer were eluted off a gel similar to that in Fig. 3 (left) and reloaded separately onto a new gel for Western blot using the same antibody.

dimer at least in some organs such as fruit and root. In order to discriminate between a heterodimer and a homodimer and rule out possible post-translational modifications, we analyzed the quaternary structure of recombinant ASR1 purified from transformed *E. coli*. Again, we detected the same two major forms (Fig. 3, left panel). The molecular mass corresponding to one of the bands from pure ASR1 turned out to be twice as much as the observed for the ASR1 monomer, thus confirming that it forms homodimers.

As the presence of SDS during electrophoresis had no effect on dimer disaggregation, we tested a caotropic agent such as urea for its ability to dissociate dimers. Fig. 3 (right panel) shows that urea had only a partial effect, if any, in disaggregation of the abundant dimer obtained from fruit. Given that ASR1 proteins cannot form disulfide bonds (as they lack cysteine residues) we concluded that ASR1 dimers result from strong non-covalent bonds between identical subunits.

To test the possibility that ASR1 forms can aggregate or disaggregate spontaneously, we isolated the pure monomeric and dimeric forms by eluting them directly off the gel. When re-subjected to electrophoresis separately, the resulting immunoblot shows that the pure monomer was able to dimerize even in the presence of detergent (Fig. 4, lane 1) and that the dimer dissociated into monomers (Fig. 4, lane 2). We can thus conclude that *in vitro*, self-association of ASR1 polypeptides occurs.

ASR1 homodimers bind DNA

Next, we took advantage of AFM to confirm and characterize the dimerization tendency of ASR1 and at the same time to test, in a direct way, its previously claimed DNA-binding activity (Kalifa et al. 2004). After pre-incubating ASR1 together with circular 3-kb dsDNA at the molar ratio of 10:1, ASR1 was observed to bind DNA as monomers and homodimers (Fig. 5, marked with an arrow). ASR1 monomers were visually identified as small globular particles of 0.43 ± 0.01 nm in height and 12 ± 0.27 nm in diameter on the average. These dimensions were identical when incubated without DNA (data not shown).

Discussion

The presence of ASR1 in tomato seeds, particularly during late embryogenesis, is worthy of note because this developmental stage is characterized by an extremely low water content [16]. A similar situation of physiological dryness occurs in pollen, where an ASR-like protein builds up as well, as reported in lily plants [9]. Although tomato ASR1 does not have any of the classic LEA sequence motifs, it possesses several short peptide sequences, like EEKK, which have been demonstrated to be over-represented in LEA group 2a [17].

Our findings provide the first evidence, by means of two independent methodological approaches, of *in vitro* dimerization of an ASR-like protein. In this context, there are other examples of dimeric stress-responsive non-ASR proteins in plants functioning as transcription factors [18]. Similarly, a group of known LEA proteins can form oligomers [19]. The unusual feature of SDS- and urea-resistant bonds observed for ASR1 polypeptides (which contain no cysteine residues) is likely to be the result of a particular packed oligomer structure with a close interface contact between subunits [20] generated by highly connected amino acid side chains [21]. Strong non-covalent bonds have also been reported for other small proteins, namely the SP1 protein from aspen [22] and the human amyloid peptide involved in Alzheimer disease [23].

The sequence specificity of the observed DNA-binding activity remains to be defined. Nevertheless, such an activity is consistent with the proposed role of Asr-like proteins as transcription factors [7]. However, based on its abun-

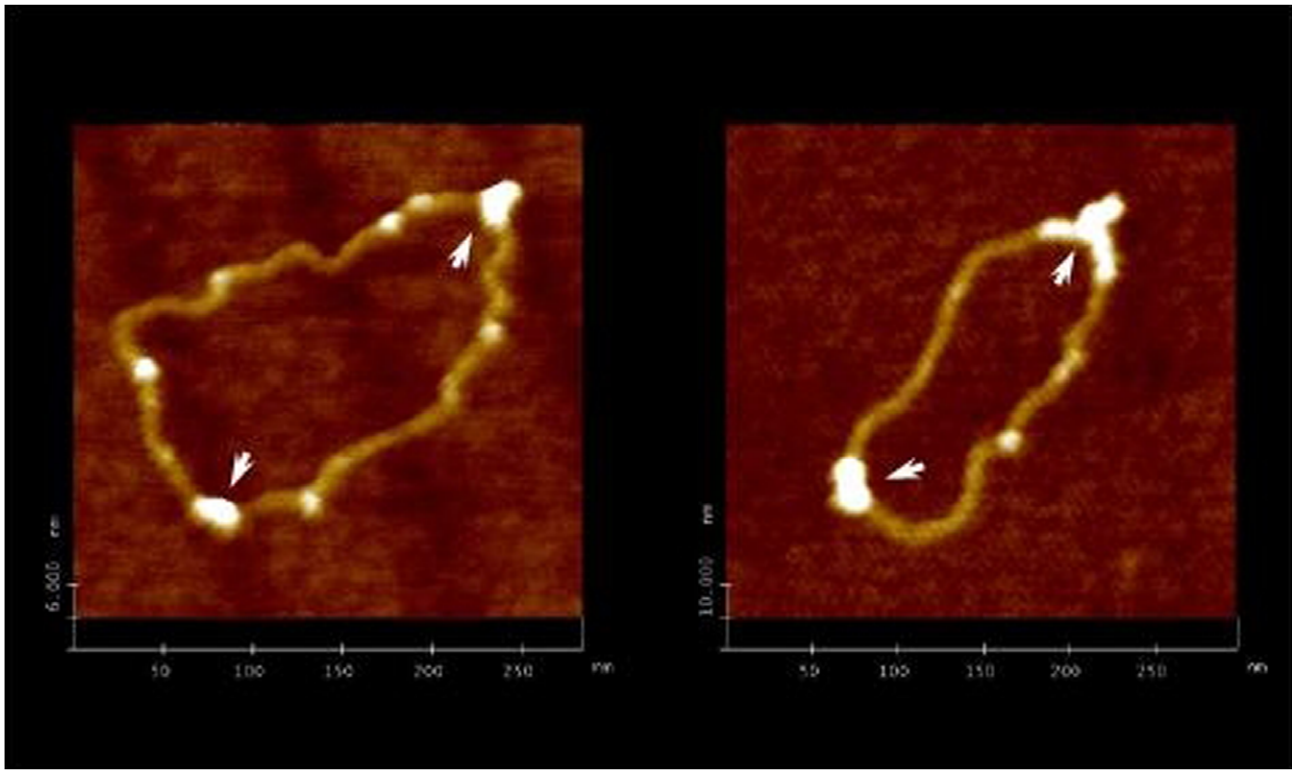

Fig. 5. AFM images of ASR1 bound to DNA. The images are presented as surface plots at a 62° viewing angle to emphasize topography. The small arrows point to ASR1 dimers bound to DNA.

dant expression in several tissues, at least for *Asr1* [5], and its high degree of hydrophilicity [4], an alternative and not mutually exclusive structural function such as direct protection from water loss cannot be ruled out, as is the case for a mitochondrial LEA protein able to protect enzymes from drying [24].

Whatever the molecular mechanism is, different approaches point in the same direction towards defining a physiological function for ASR proteins. Tolerance to osmotic stress achieved by ASR1 in the heterologous yeast model [25], positive selection on *Asr2* in a tomato wild species that inhabits a desertic region [26] and results from ASR-overexpressing transgenic plants [27] together strongly suggest that conferring drought and salt resistance is a main function of this family of proteins. Further research is needed to decipher the role of the demonstrated dimerization on this proposed physiological function.

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