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Differential expression of the members of the *Asr* gene family in tomato (*Lycopersicon esculentum*)

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Abstract

In this work, we continued to dissect the *Asr* (ABA/water stress/ripening-induced) gene family originally described in tomato. A RT-PCR-based strategy was developed to assess the organ (leaf, root and fruit) and developmental (immature and ripe fruit) specificity of expression of the three known members of the *Asr* gene family under normal and stress conditions. Our results allow us to conclude that whereas *Asr*1 and *Asr*2 are the members of the family preferentially induced by desiccation in leaves, *Asr*2 is the only one activated in the roots from water-deficit-stressed plants. We also observed that expression of the three genes does not change significantly in fruit at different developmental stages, except for that of *Asr*2, which decreases after the breaker yellow stage. In addition, we identified a 72-amino acid polar peptide region, rich in His, Lys, Glu and Ala, which contains two internal imperfect repeats and is highly conserved in more recently discovered *Asr*-like proteins from other plant species exposed to different kinds of abiotic stress such as water deficit, salt, cold and/or limiting amount of light.

Keywords: *Asr* genes; *Lycopersicon esculentum*; ABA; Water stress; Fruit ripening

1. Introduction

Water availability is a major factor in life maintenance of plants. The response of plants to drought is very complex and involves physiological changes such as decreased rates of photosynthesis [1] and stomatal closure [2]. Drying soil conditions initially cause loss of turgor pressure eliciting signals moving from the roots to the shoots [3] which in turn induce a rapid mobilisation and oscillating fluxes of Ca^{2+} in at least stomatal guard cells [4,5] and ubiquitous expression of various target genes [6]. It is largely known that accumulated abscisic acid (ABA) is responsible for the expression of some of these genes (reviewed in [7] and [8]). The exact mechanisms by which plants translate stress environmental signals into changes in gene expression are not yet fully understood. Nevertheless, it has been clear for many years that ABA brings about the accumulation of different gene products during periods of water deficit [9].

Another interest of ours focuses on the genes expressed during fruit ripening [10,11] as well as in water stress. In this context, tomato *Asr*1 was the first reported member of the *Asr* (ABA/water stress/ripening-induced) family, which subsequently drew the attention of other groups at the level of gene expression [12]. *Asr*1 then led way to the discovery of other members of the same family [13–16]. To date there are three well studied members of this family, named *Asr*1, *Asr*2 and *Asr*3, that map to chromosome 4 of tomato [15]. Recently, a fourth member was reported [16], but is still poorly characterised.

Asr-like genes have also been found to be expressed in desiccated lily pollen [17], water-stressed loblolly pine [18,19], wild potato [20], cold-stressed commercial potato [21], common ice plant (GenBank Acc. # 7484607), water-stressed maize [22], salt-tolerant rice

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seedlings [23], grape (GenBank Acc # AAD53125) and non-acidic pummelo fruit [24]. The homologous gene in the five latter species was named *Asr*, thus following our nomenclature.

Expression of an endogenous *Asr* gene in tomato had been studied by Iusem et al. [10] and Amitai-Zeigerson et al. [25], unaware of the complexity of the *Asr* gene family [13]. Taking into account the considerable sequence homology between the family members [15], those earlier hybridisation-based studies suffer from the fact that the therewith single reported mRNA might be an unravelled combination of at least three transcripts of about the same size. Therefore, the aim of the present paper is to investigate whether the individual members of the family are differentially expressed in several organs under various physiological conditions.

Besides, in an attempt to identify consensus peptide motifs associated with function (i.e. protection upon stress), we thoroughly searched sequence data bases for homology to novel structurally related gene products from plant species other than tomato.

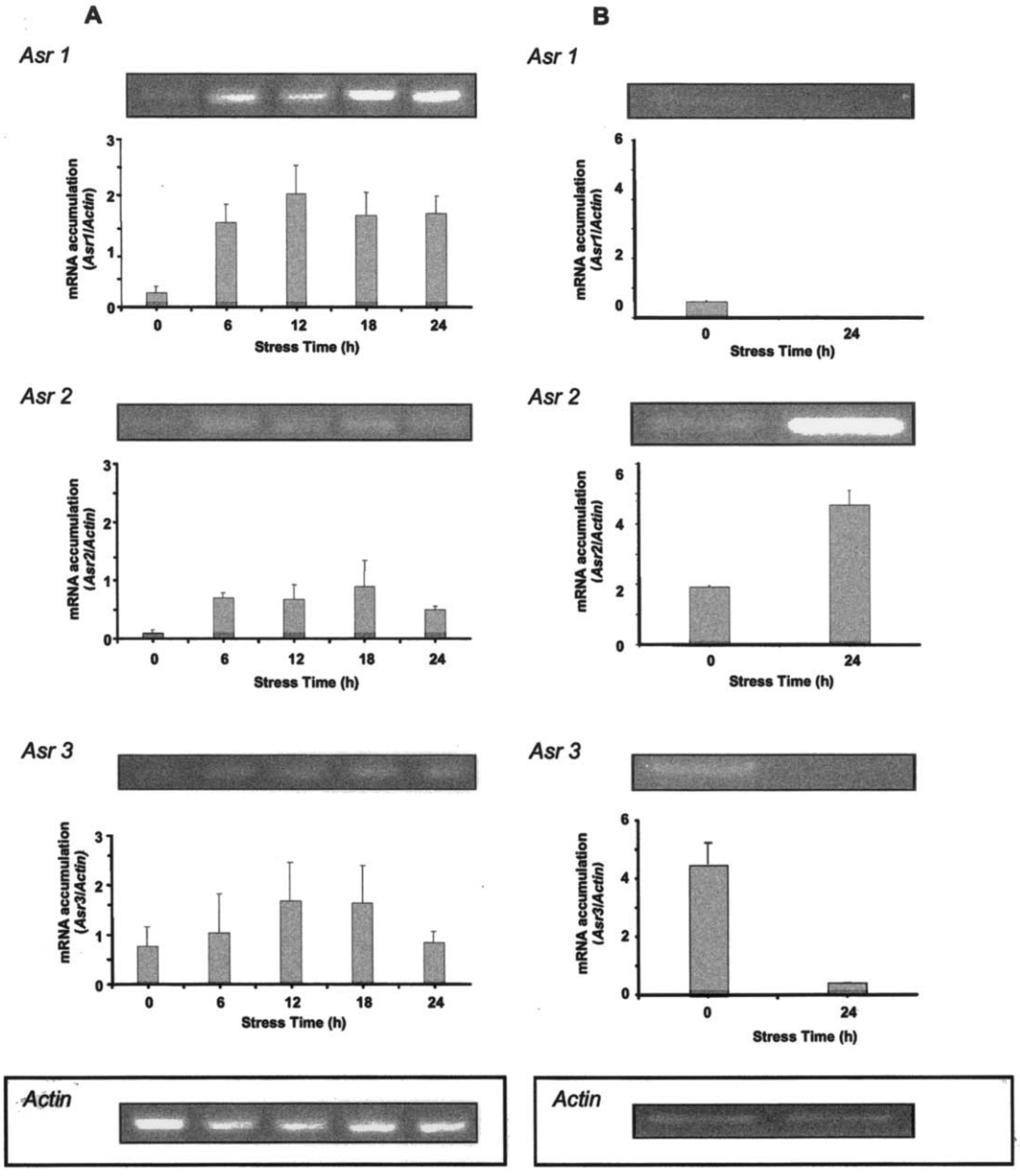

Fig. 1. Analysis of *Asr1*, *Asr2* and *Asr3* expression in leaves and roots in response to drought stress. mRNA accumulation was analysed by RT-PCR as described in Section 2 using total RNA from A) watered (0 h stress) or drought-stressed (after 6, 12, 18 or 24 h) tomato leaves and B) roots of normally irrigated (0 h) or drought-stressed (24 h) tomato plants. RT-PCR was also performed on actin mRNA as external control. mRNA levels normalised against actin transcript are shown below photographs of amplification products as seen after agarose gel electrophoresis. The whole experiment was repeated three times. Bars represent standard errors of the mean.

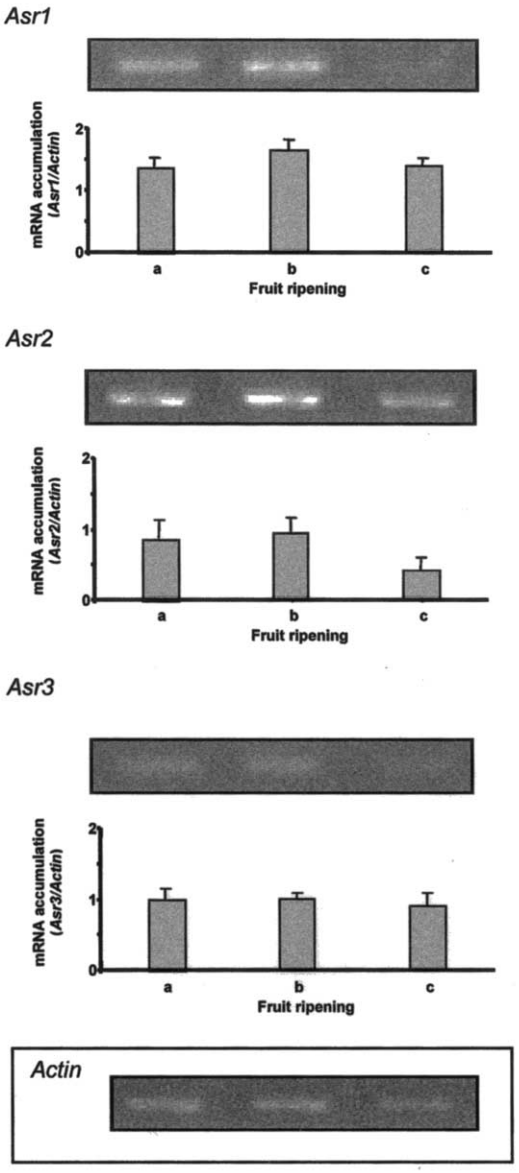

Fig. 2. Expression of *Asr*1, *Asr*2 and *Asr*3 genes during various stages of tomato fruit ripening. mRNA accumulation was analysed by RT-PCR as described in Section 2, using total RNA from (a) mature green, (b) breaker and (c) red fruit pericarp. RT-PCR was also performed on actin mRNA as external control. mRNA levels normalised against actin transcript are shown below photographs of amplification products as seen after agarose gel electrophoresis. The whole experiment was repeated three times. Bars represent standard errors of the mean.

2. Materials and methods

2.1. Plant material and treatments

Tomato (*Lycopersicon esculentum* M. Cv. UC82b) plants were grown in soil pots for 1 month in the greenhouse and watered every day to field capacity. For water stress experiments, plants were removed from pots, roots were quickly rinsed and the whole plants were let dry during different times (6, 12, 18 and 24 h) on the laboratory bench. Leaves and roots were then detached, immediately immersed in liquid nitrogen and stored at −80 °C until RNA extraction. Unstressed controls were obtained from plants analysed without being let dry. In our hands, this gave same results as control plants kept moist for the same periods of time as those chosen for the stress treatments. Three independent stress experiments were replicated.

Fruits were harvested at mature green stage, breaker stage (showing the first external signs of orange or yellow) or ripe red stage, from 4 month greenhouse grown tomato plants. Pericarp tissue was taken, frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA isolation and RT-PCR amplification

Total RNA was obtained from leaves, roots and fruits according to the Trizol procedure (Gibco BRL) followed by incubation with 1.5 U RQ1 RNAase-free DNAase (Promega, Madison, WI, USA) at 37 °C for 15 min. The reaction was stopped by 2.5 mM EDTA and by heating at 70 °C for 15 min. About 3.5 μg total RNA was used as template for M-MLV reverse transcriptase (RT) (300 U, Promega) in a 20 μl reaction containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 20 U Rnasin (Promega), 400 μM dNTPs and 3.3 mM dT_{15} (Pharmacia) at 35 °C for 1 h. The reaction was stopped at 95 °C for 5 min. PCR amplification of *Asr* 1, *Asr*2, *Asr*3 and actin cDNAs was performed in buffer mix with 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 U *Taq* DNA polymerase and the primers (265 nM) shown below, designed to anneal to non-homologous regions of the three *Asr* cDNAs,

Transcript	Upper primer	Lower primer
Asr1	5′ GAT AGA TTT ATT GTT TCA GAT GGA G 3′	5′ GAC ACA ACA CTT ATA CCA AAT ATG G 3′
Asr2	5′ TTA AGA GAA GCA ATA CAA TAT GGC T 3′	5′ TCC ACC TGC CCC AAC TGC AGC AAC A 3′
Asr3	5′ CAA AGC ATA AAT TGT CTA TCG ACG T 3′	5′ TCA ACT GGA CCA CCT TCT TCC TCT C 3′
Actin	5′ TGG CAT CAT ACC TTT TAC AA 3′	5′ TCC GGG CAT CTG AAC CTC TC 3′

The 5′ annealing nucleotide positions of the primers and corresponding amplicon sizes were as follows

	Upper	Lower	Amplicon size (pb)
*Asr*1	61	480	420
*Asr*2	41	314	274
*Asr*3	16	155	140
Actin			519

Controls without RT ruled out possible genomic DNA contamination in subsequent PCR reactions.

PCR cycles consisted of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. To detect changes in steady state levels of each type of cDNA present before amplification -ensuring a linear range in relation to template amounts-, the number of cycles was adjusted as follows: in leaf experiments, the number of cycles was set at 22, 26 and 30 for *Asr*1, *Asr*2 and *Asr*3, respectively. When examining expression in roots, the number of cycles was 23 for the three transcripts. In fruit experiments, the numbers were 23, 28 and 31, for *Asr*1, *Asr*2 and *Asr*3, respectively. For the control actin transcript, the cycle number was 23 in all cases. All RNA samples were subjected to RT-PCR analysis for at least five times. No quantitative comparison between different types of transcripts was intended.

RT-PCR products from each replicated reaction were electrophoresed in 2.5% agarose gels and detected by ethidium bromide staining. Results from gels showing the most uniform actin signals were chosen for the figures. Identity of amplicons was confirmed by sequencing.

2.3. DNA quantification

PCR was carefully performed using different numbers of cycles to ensure that the mass of reaction products reflects the amount of cDNA template originally present in samples. Bands were scanned with a UMAX Scanner and intensity was quantitated densitometrically using the NIH image software.

2.4. Protein sequence analysis

The GenBank database was searched for sequences homologous to tomato *Asr*1 protein using the BLASTP program [26]. Multiple alignment was performed with CLUSTALW version 1.8 [27]. Remarkable features were highlighted using functions provided by the ANTHEPROT version 4.9 freeware.

Prediction of secondary structure and accessibility to water molecules was estimated using the PHDsec [28] and PHDacc [29] computer programs.

3. Results

3.1. Differential expression of endogenous Asr genes

To investigate if the members of the *Asr* family are differentially expressed in tomato leaves, roots and fruit, we set up an experimental system that discriminates between the three transcripts. We chose RT-PCR as a valid alternative, under well-controlled experimental settings that allow estimation of relative changes in

Table 1
Reported *Asr*-like gene products. 'Id.' and 'Sim.' stand for level of amino acid identity and similarity of peptide regions ranking in the top BLASTP scores, expressed as percentage, respectively

Clone name	Species	Organ	Environmental or physiological signal	Id.	Sim.	Reference
*Asr*1	Tomato	Root, leaves, fruit	Water stress (leaves)	100	100	[10,25], this work
*Asr*2	Tomato	Root, leaves, fruit	Water stress (roots) Ripening (fruit)	83	91	[13], this work
*Asr*3	Tomato	Root, leaves, fruit	–	70	86	[16], this work
ci21A	Commercial potato	Tubers	Cold stress	95	95	[21]
ci21B	Commercial potato	Tubers	Cold stress	82	92	[21]
DS2	Wild potato	Leaves and stems	Water stress	79	94	[20]
GASR	Grape	–	–	76	95	Acc # AAD53125
PAPRI41	Apricot	Fruit	Ripening	75	88	Acc # AAB97140
Segment 1–3	Maize	Coleoptile	Limiting light	86	99	[34]
*Asr*1	Acidless pummelo	Fruit	Immaturity?	63	80	[24]
LP3-1	Loblolly pine	Root	Water stress	62	74	[18]
LLA-23	Lilly	Pollen	Desiccation	50	60	[17]
Asr	Common ice plant	–	Cold acclimation	53	63	Acc # 7484607
*Asr*1	Rice	–	Salt stress	52	60	[23]
Asr	Maize	Root	Water stress	50	55	Acc # AAA21866.1

Organ refers to the one in which major expression was found. The type of environmental stress or physiological condition indicates the ones eliciting induction. A hyphen indicates lack of information.

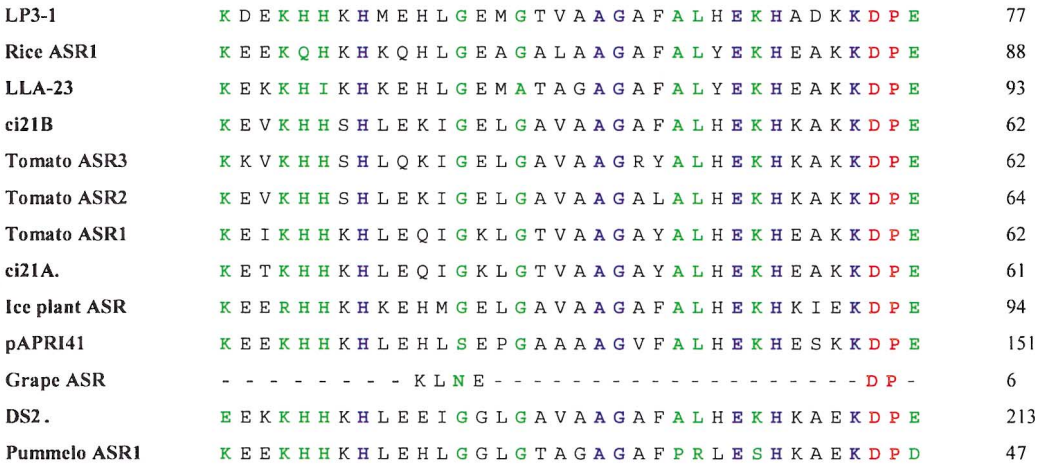

Fig. 3

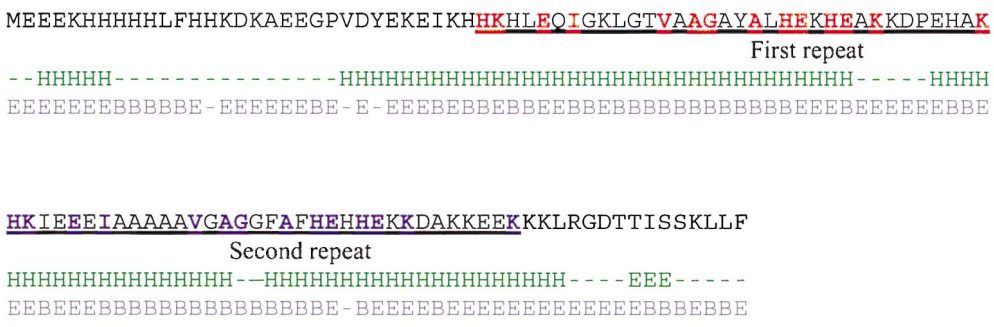

Fig. 4

Fig. 3. Multiple alignment of *Asr*-related peptide sequences from several plant species. Clone name abbreviations are the same as in Table 2. The numbers in the right-hand-side column indicate the position number of the last amino acid on each line. A highly conserved region consisting of 72-amino acids is shown. Red, blue, green and black columns indicate 13/13, 12/13, 11/13 and $<11/13$ conserved residues, respectively. Charge conservation is shown by an asterisk.

Fig. 4. Entire amino acidic sequence of *Asr*1 from *Lycopersicon esculentum*. Imperfect repeats are underlined. Red and blue colours are used to display conserved residues between each repeat. Green letters indicate second structure prediction: 'H' standing for alpha helix and 'E' for extended beta sheet. Grey letters show predicted relative solvent accessibility, 'E' (exposed) meaning high solvent accessibility and 'B' (buried), poor solvent accessibility.

mRNA steady-state levels. Comparison of their signal strength to that of actin allows to distinguish the response of each family member to stress or to varying physiological conditions.

It can be observed (Fig. 1A) that mRNAs of *Asr*1 and *Asr*2 are barely detected in watered leaves, becoming quite perceptible after 6 h since the beginning of stress (6- and 7-fold induction, respectively. On the contrary, *Asr*3 showed no significant induction upon water deficit stress despite different basal values found in replicated experiments. Besides, expression of *Asr*1 and *Asr*3 turned out to moderately increase further under prolonged stress, opposite to *Asr*2, which did not seem to change significantly after 6 h under the same conditions.

Following a similar analysis, expression of the individual mRNAs in tomato roots under drought conditions clearly showed (Fig. 1B) that *Asr*2 was the only member of this gene family that is induced, increasing its expression over 2-fold on the average, while *Asr*1 and *Asr*3 are down-regulated.

In addition, we examined the individual *Asr* expression patterns during fruit ripening. No significant change in accumulation of any of the mRNAs occurred, except for the *Asr*2 transcript, which showed a decreasing trend in ripe fruit (Fig. 2).

3.2. Comparison of structurally related coding sequences in other species

A search for elsewhere reported *Asr*-like coding sequences in the plant kingdom was done using the BLASTP program. The outcome with the most similar sequences is displayed in Table 1. A common feature of all of them is the relative abundance of Glu, Ala, His, Lys and Gly amino acid residues. In addition, they all have a high average hydrophilicity (data not shown). The sequences found belong to proteins from different plant species accumulated as a result of sensing miscellaneous types of abiotic stress, such as water deficit, salt, cold and limiting source of light, in addition to proteins abundant in non-stressed fruit.

We were able to identify a highly conserved 72-amino acid sequence shared by these proteins (Fig. 3). This region is rich in His, Lys, Ala and Glu and contains two imperfect repeats spanning residues 32–66 and 67–101 in tomato *Asr*1. The repeat closer to the N-terminus turned out to be less conserved among the examined proteins.

According to PHDsec and PHDacc softwares (See Section 2), the conserved peptide sequence taken from tomato *Asr*1 predicts a high proportion of alpha helix secondary structure (29 out of 34 residues for the first repeat and 32 out of 34 residues for the second repeat), as shown in Fig. 4. In addition, these repeats contain 14 and 15 residues, respectively, highly accessible to water (Fig. 4). Taken together, these features are compatible with a globular protein.

4. Discussion

Here, we report the expression patterns of *Asr* (ABA/water stress/ripening-induced) genes, initially described in tomato [10,13,25,16] and known to be organised in a family [15]. Analysis of the regulatory sequences of one of them (*Asr*2) revealed a promoter region [14] which possesses various consensus elements [30] involved in ABA responsiveness [31,32] but lacks the sequence TACCGACAT, thought to regulate some dehydration-inducible genes [33].

We have earlier relied on transgenic plants carrying a reporter gene driven by a particular *Asr* promoter to study the expression of one member of the *Asr* family [30]. Alternatively, in this paper we describe an easy procedure to study individual expression by means of RT-PCR, with the concomitant advantage of avoiding the artefacts associated with the random integration site of the transgene [34]. By using this approach, we are able to conclude that the earlier reported increase in expression of '*Asr*' in tomato developing leaves [16], stressed leaves [10,25] and stressed roots [25] may have been the result of specific induction of some but not all of these genes, for example *Asr*1 and *Asr*2 in stressed leaves and *Asr*2 in stressed roots.

Our survey on studies in other species indicates that *Asr*-structurally related genes are also stress-induced. Interestingly, similar effects on the expression of *Asr* genes were evoked not only by other types of stress involving water movement such as cold treatment and salinity exposure but also by restricted light [35].

It is worth mentioning the two recently found members belonging to an *Asr*-like family in potato [21], ci21A and ci21B. ci21A is more similar to tomato *Asr*1 than is to ci21B, which resembles tomato *Asr*2 more than ci21A. Therefore, these two genes seem to be the *Asr* counterparts in potato and suggest that duplication and divergence of *Asr* genes occurred early in plant evolution, prior to separation of genus in the *Solanaceae* family.

In contrast to earlier data found with *L. esculentum* cv. Ailsa Craig [10,16], our results with tomato cv. UC82b fruit show no significant changes in expression of any *Asr* member during ripening. Genotype-dependent polymorphism in *Asr* upstream sequences can account for that contrasting behaviour, as found in low- and high-acid pummelo (*Citrus maxima*) fruit [24].

Our search for *Asr* homologs revealed the existence of conserved protein molecules that accumulate in fruit and/or as a result of miscellaneous types of stress in various somatic and reproductive cells. Their high proportion of charged residues is striking and may be

relevant to function. In this regard, based on evidences supporting a nuclear localisation [10,16], a role for the *Asr*-like protein from wild potato was suggested by Silhavy et al. [20]. This author envisaged a sort of nucleoplasm protection from desiccation, exerted by an N-terminal hydrophilic repeated sequence resembling a class of LEA (late embryogenesis abundant) proteins, also known to accumulate under drought conditions [36]. Tomato *Asr* proteins lack those N-terminal repetitive sequences but still maintain a high degree of hydrophilicity.

Interestingly, the 72-amino acid domain reported in this work is highly conserved in the plant kingdom and may contain one or several consensus motifs functionally meaningful. In this context, its predicted helix secondary structure with considerable accessibility to water molecules is compatible with the potential protective role in restricting water loss from internal tissues during stress. In agreement with this idea, a recent report [37] groups most of seed-specific LEA proteins in a more widespread class called hydrophilins, defined by their high glycine content and high hydrophilicity. All *Asr*-like proteins fall into this large group, which portrays dissimilar proteins, even from prokaryotes, encoded by osmotic stress-induced genes, thus suggesting that they represent a common adaptation to water deficit stresses. We are currently exploring the question of whether *Asr* proteins are present at high levels during the last stage of seed formation.

It is tempting to suggest that these widespread proteins may act as downstream components of a common signal transduction pathway involved in responses triggered in plant cells by any of all the forementioned types of environmental signals thus leading to tolerance. It is clear that more research is needed to gain knowledge on the precise biochemical and/or biophysical function of the proteins from the *Asr* family, which seem to participate at a point of a pathway where different types of abiotic stress signals can merge.

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