Tomato *ABSCISIC ACID STRESS RIPENING (ASR)* Gene Family Revisited

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**Abstract**

Tomato *ABSCISIC ACID STRESS RIPENING 1 (ASR1)* was the first cloned plant ASR gene. ASR orthologs were then cloned from a large number of monocot, dicot and gymnosperm plants, where they are mostly involved in response to abiotic (drought and salinity) stress and fruit ripening. The tomato genome encodes five ASR genes: *ASR1, 2, 3 and 5* encode low-molecular-weight proteins (ca. 110 amino acid residues each), whereas *ASR4* encodes a 297-residue polypeptide. Information on the expression of the tomato ASR gene family is scarce. We used quantitative RT-PCR to assay the expression of this gene family in plant development and in response to salt and osmotic stresses. *ASR1* and *ASR4* were the main expressed genes in all tested organs and conditions, whereas *ASR2* and *ASR3*/*5* expression was two to three orders of magnitude lower (with the exception of cotyledons). *ASR1* is expressed in all plant tissues tested whereas *ASR4* expression is limited to photosynthetic organs and stamens. Essentially, *ASR1* accounted for most of *ASR* gene expression in roots, stems and fruits at all developmental stages, whereas *ASR4* was the major gene expressed in cotyledons and young and fully developed leaves. Both *ASR1* and *ASR4* were expressed in flower organs, with *ASR1* expression dominating in stamens and pistils, *ASR4* in sepal and petals. Steady-state levels of *ASR1* and *ASR4* were upregulated in plant vegetative organs following exposure to salt stress, osmotic stress or the plant abiotic stress hormone abscisic acid (ABA). Tomato plants overexpressing *ASR1* displayed enhanced survival rates under conditions of water stress, whereas *ASR1*-antisense plants displayed marginal hypersensitivity to water withholding.

**Introduction**

The first member of the tomato *ABSCISIC ACID STRESS RIPENING* (*ASR*) gene family, *ASR1*, was identified by screening a tomato fruit cDNA library with cDNA from stressed leaves [1], hence its name. Since then, a large number of *ASR* orthologs have been cloned from many plant species, including gymnosperms and angiosperms (reviewed by [2]). *ASR* gene families are found in the model plant *Arabidopsis* [2]. Interestingly, no *ASR* orthologs have been found in organisms outside the plant kingdom. *ASR* genes have been shown to be induced by abscisic acid (ABA) and abiotic stress, mainly salinity and drought [1,3–18]. They are also highly expressed in ripening fruit [1,4,14,19–23], and during potato-tuber development [24,25].

The tomato *ASR* gene family consists of five genes localized in one cluster on chromosome 4. Four members of the *ASR* gene family have been cloned from tomato [4,26–30]. These genes encode highly homologous proteins and possess a single intron of different size, but conserved location [4,27,28]. Upon completion of the tomato genome sequence [31], a fifth *ASR* gene was annotated, whose exon nucleotide sequence is highly similar to that of *ASR3* (88% identity in coding sequences, see also [32]). The loci of *ASR1–ASR5* genes in the tomato genome are Solyc04g071610, Solyc04g071580, Solyc04g071590, Solyc04g071620 and Solyc04g071600, respectively. Four of the genes (*ASR1–ASR3, ASR5*) encode low-molecular-weight proteins, whereas the polypeptide encoded by *ASR4* is approximately double the size of the other proteins [30]. Wild tomato species also encode this five member *ASR* gene family, suggesting that this family was not lost during tomato domestication and breeding [30,33]. Furthermore, *ASR1, 2 and 4* genes from wild tomato species were also induced by drought and cold. The majority of plant *ASR* genes encode low molecular weight proteins. In addition, genomes of a number of plant species contain in addition a gene encoding higher molecular *ASR* polypeptides [25,34,35]. *ASR* proteins have been proposed to belong to the hydrophylin group of proteins [36]. Tomato *ASR1* was shown to be a natively unordered protein [37] that possesses chaperone-like activity [38], and was localized to both the cytosol and nucleus [39]. Dual subcellular localization was also shown for the lily pollen *ASR* proteins [39].
protein [40]. Rice ASR1 was also shown to possess chaperone-like activity [41,42]. ASR1 has Zn$^{2+}$-dependent DNA-binding activity [39,43]. Upon binding of Zn$^{2+}$, ASR1 becomes structured and dimerizes [26,37], and is translocated to the nucleus [37,44,45]. Zn$^{2+}$ and Fe$^{3+}$ ions affected the structure of a soybean ASR [46]. Nuclear ASR proteins modulate gene expression via binding to specific promoter sequences [14,39,47,48].

ASR genes have been shown to play a central role in drought and salinity stress. Overexpression of ASR genes resulted in increased tolerance of the transgenic plants to water/osmotic [42,48–51], salinity [48,50,52,53] and cold [45,54] stresses. However, until now these responses have been seen only in heterologous systems. Transgenic Arabidopsis plants expressing ASR proteins from other plant species [42,54] demonstrated increased tolerance to abiotic stresses. Arabidopsis plants do not encode ASR proteins. Ectopic expression of tomato ASR1 in Arabidopsis was shown to affect the plant’s response to ABA, glucose and tolerance to abiotic stress via competition for DNA binding with the transcription factor ABI4 [55]. Thus, expression studies in heterologous organisms that do not naturally have the studied gene(s) should be analyzed with caution, especially for regulatory proteins, as results may not directly reflect the biological role of the analyzed gene. In addition, ASR gene involvement in carbohydrate signaling, sugar trafficking and metabolism has been shown [14,24,56,57], as has its influence on the biogenesis of branched-chain amino acids [58].

In tomato, the best-studied member of the ASR family is ASR1, followed by ASR2. Information on ASR3, ASR4 and ASR5 is scarce. Although a few studies have compared the expression of some members of the tomato ASR gene family [59,60], results are from northern blot, semi-quantitative PCR, or histological staining studies analyzing two or three of the family’s genes, under rather restricted conditions. In this work, we revisited the tomato ASR gene family using the highly accurate quantitative RT-PCR technology to determine the expression patterns of its members in vegetative and reproductive organs. Because of their high sequence homology, we could not design gene-specific primers for ASR3 and ASR5, we determined the summed expression of these genes (ASR3/5). We found that ASR1 and ASR4 are highly expressed in vegetative tissues of nonstressed plants, whereas the steady-state levels of ASR2 and ASR3/5 transcripts are two to three orders of magnitude lower. ASR1 was the major member expressed in roots, stems, stamens, pistils and fruits. ASR4 accounted for more than two-thirds of total ASR transcripts in leaves, shoot vegetative meristem, sepals and petals. Both of these genes were induced by ABA, osmotic stress and salt stress. Tomato plants overexpressing ASR1 (ASR1-OE) survived better under water stress than wild-type (WT) plants, where ASR1-antisense (ASR1-AS) plants had slightly lower survival rates than the WT.

### Materials and Methods

#### Plant material and growth conditions

**Generation and selection of transgenic lines.** The 348-bp coding region of the tomato ASR1 gene (GenBank U86130.1) was cloned in sense or antisense orientation into the multiple cloning site of the vector [61] between the Cauliflower Mosaic Virus (CaMV) 35S promoter and the octopine synthase (ocs) terminator. Tomato plants (Solanum esculentum cv. Moneymaker) were transformed by Agrobacterium as previously described [62]. Emerging shoots were excised and selected on Murashige and Skoog (MS) medium containing kanamycin (100 mg/l), and then transferred to the greenhouse for selection by qPCR in T1 plants. Transgenic plants were numbered XX-YY, where XX represents an independent transformation event, and YY represents a subline of the founder of T2 generation seeds. Plants were self-pollinated and seeds were collected. T3 generation plants were used in this study. Western blot analyses showed that the ASR1-OE lines have higher levels of ASR1 than WT plants (Figure S1 in File S1).

**Plant growth.** Plants were grown in pots in the greenhouse or hydroponically in the growth room in aerated half-strength Hoagland mineral solution at 28°C and 70% relative humidity, under a diurnal cycle of 18 h light, or in the greenhouse at an average temperature of 28°C, and >50% relative humidity. Seeds were germinated in water-soaked vermiculite. Ten-day-old seedlings were transferred to aerated containers with half-strength Hoagland mineral solution [63], or to pots containing equal volumes of planting mix and vermiculite. Hydroponic growth medium was replaced 1 week after transfer and every 3–4 days thereafter.

**Water-stress tolerance and survival assays.** Seedlings were grown in pots under optimal conditions for 3 weeks. Water was then withheld for 22 days, followed by rewatereing. Plant survival was scored 17 days later. At least 20 plants from each line were used. Plants were grown in random order and pot location was changed every few days.

**NaCl, polyethylene glycol (PEG) and ABA treatment.** WT seedlings were germinated on vermiculite and transferred to aerated 0.5X Hoagland’s solution as described above. After 1 week acclimation, plants were transferred to fresh mineral solution containing, where indicated, 40 μM ABA, 0.2 M NaCl, or 8% (w/v) PEG 8000. Plants were harvested 24 h after treatments.

**Figure 1. Relative expression of the tomato ASR genes.** Steady-state levels of the indicated genes were determined in leaves and roots of 10-day-old hydroponically grown tomato seedlings. Expression of ASR1 in each tissue was defined as 1000. Data shown are average ± SE. Bars with different letters represent statistically different values by Tukey’s HSD post-hoc test (P≤0.05).

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mRNA level assay

Total RNA was extracted from the indicated plant tissues using EZ-RNA (Biological Industries, Israel) according to the manufacturer's protocol. This protocol uses improved RNA-extraction methods as described by Chomczynski and Sacchi [64]. RNA quality integrity was checked spectrally (at 230 nm, 260 nm and 280 nm) and by running samples on denaturing agarose gels electrophoresis. Relative steady-state transcript levels were assayed by RT-qPCR as described previously [50,55,65,66]. cDNA was synthesized from DNase-treated RNA using high-capacity cDNA reverse transcription kit (Applied Biosystems). Primers were designed by Primer-Express software Vers. 2.0 (Applied Biosystems). When possible, one of the primers in each set was anchored at an exon–exon border to reduce possible amplification from contaminating genomic DNA. All amplicon lengths were between 75 and 90 bp. Primer sequences are presented in Table S1 in File S1. Transcript levels were assayed using the 7300 Real-Time PCR System (Applied Biosystems), with 18S rRNA as the internal standard. PCR efficiency was close to 100%. RNA relative quantification analyses were performed using 7300 System SDS software (Applied Biosystems). The list of primers used is shown in Table S1 in File S1. The data represent the mean ± SE of n = 3 independent experiments. Each data point was determined in triplicates in each of the three biological replicates and presented as mean ± SE. Data presented in a single graph were carried in a single run. Differences between groups were analyzed by Tukey's HSD post-hoc test (P<0.05).

Results and Discussion

Relative expression levels of the tomato ASR genes

Relative steady-state levels of the members of the ASR gene family were determined in leaves and roots of hydroponically grown seedlings. In general, ASR1 and ASR4 were the most highly expressed genes in this family (Figure 1). In young leaves, ASR4 levels were 2.6 times higher than those of ASR1, whereas transcript levels of ASR2 and ASR3/5 were more than two orders of magnitude lower. In tomato roots, ASR1 was the most abundantly expressed gene, whereas transcript levels of ASR3/5 and ASR4 were approximately one order of magnitude lower than that of ASR1, and that of ASR2 two orders of magnitude lower than the steady-state levels of ASR1. These results are in agreement with Frankel et al. [30] who reported that ASR2 and ASR3/5 transcripts could not be detected in tomato leaves by northern blot analysis. On the other hand, previous studies from the same laboratory reported that ASR2 transcripts are highly abundant in roots of stressed tomato plants [60] and that the ASR2 promoter can drive transcription in both tomato and other Solanaceae plant cells [60,67]. RNA Seq also suggest that ASR1 and ASR4 transcripts in tomato leaves and fruits are relatively
abundant, whereas transcripts from ASR2, ASR3 and ASR5 are hardly found [31]. (data presented at the tomato Sol Genomic Network database (http://solgenomics.net)).

Expression of tomato ASR genes in vegetative tissues

Expression of tomato ASR genes was determined in vegetative tissues of hydroponically grown tomato (Figure 2). Transcript levels of each of the genes were normalized to the expression of the same gene in developing leaves. This reference tissue was selected since it is present in all plant ages used in the study. Highest levels of ASR1 transcript were found in roots and stems (ca. 4.5 and 2 times that in the leaves, respectively). ASR1 transcript levels decreased with leaf development (Figure 2A), in agreement with Amitai-Zeigerson et al. [68]. ASR1 expression in stems and roots suggests its role in the plant’s vascular system [59]. ASR2 transcript levels were only slightly different in vegetative organs (Figure 2B), and its steady-state levels in all vegetative organs were marginal (see Figure 1). ASR3/5 levels were highest in the cotyledons (Figure 2C), reaching transcript levels in the same order of magnitude of that of ASR1 in cotyledons, suggesting that ASR3 and/or ASR5 may play a role in advanced stages of seed development. ASR proteins were detected immunologically in tomato seeds using anti-ASR1 antiserum [37]. Since there is high amino acid conservation between the different members of the tomato ASR proteins, it is likely that this antibody crossreacts with other ASR proteins such as ASR3 and ASR5. ASR4 was expressed mainly in leaves, cotyledons and meristem, with relatively low expression rates in roots and stems (Figure 2D). After normalization of the relative expression of each gene in young leaf tissues (Figure 2A), ASR1 seemed to be the main expressed gene in the roots and stems. On the other hand, ASR4 was the highest expressed ASR gene in cotyledons, and young and fully expanded leaves. ASR1 accounted for up to one quarter of of ASR gene family expression in these tissues. Expression of ASR2 and ASR3/5 in leaves was negligible. Interestingly, and Our results indicate that ASR1 is the primarily expressed gene in tomato roots, stems and fruits, whereas both ASR1 and ASR4 are both expressed in cotyledons, leaves and meristems, where ASR4 levels exceed that of ASR1. Transcript levels of ASR2 and ASR3/5 were marginal in vegetative tissues, with the exception of ASR3/5 in cotyledons. Our analyzes determine averaged transcript levels in the entire organ, rather than cell specific expression. Thus, it will be interesting to find out if in these organs, ASR1 and ASR4 coexpress in the same cells, or in different cell types.

Expression of the tomato ASR genes in reproductive tissues

The ASR gene family showed differential expression in flower organs. Although ASR2 and ASR3/5 expression varied in the different flower organs (Figure 3B, C), their expression levels can be estimated to be two order of magnitude order lower than that of ASR1 and ASR4 of ASR1 and ASR4 were highly expressed in the sepals and stamens, and to a lower extent in petals and pistils (Figure 3A, D), where ASR4 estimated transcript levels were higher than ASR1 in the sepals and petals. One the other hand,
ASR1 is the most highly expressed ASR gene in the stamen and pistils. Although ASR1 expression increased during fruit development (Figure 4A), it was already higher than that in all other tissues in young immature green fruits. ASR1 was essentially the main fruit-expressed ASR gene at all fruit developmental (Figure 4). Steady-state levels of ASR2 and ASR3/5 transcripts in fruit tissues were also the highest measured in any plant tissue. Nevertheless, their levels in fruits are approximately two order of magnitude lower of that of ASR1. An increase in ASR1 transcript levels in tomato fruit development and ripening is in agreement with Gilad et al. [4] and Iusem et al. [1], but not with Maskin et al. [60]. Increase in ASR1 during tomato fruit ripening also correlates with increase in its protein levels [20]. Increased steady state transcript levels during fruit ripening of SlASR1 orthologous genes were reported in a number of plant species [21–23,69,70].

Tomato ASR genes are differentially responsive to ABA and abiotic stress

Steady-state levels of ASR1 and ASR4 transcript increased following plant exposure to salt stress, osmotic stress (PEG) or to the hormone ABA (Figure 5). The relative induction levels by these treatments were rather similar for these two highly expressed ASR genes. On the other hand, the less expressed genes ASR2 and ASR3/5 showed different responses: ASR2 responded most strongly to ABA treatment and to a lesser extent to salt or osmotic stress, whereas ASR3/5 was not affected by ABA and was relatively highly expressed after NaCl treatment, suggesting that they are induced by an ABA-independent pathway. A large
number of ASR genes have been identified in different plant species based on their response to abiotic stresses such as water, salinity and osmotic stress [9,10,14,71,72]. Expression results for the tomato ASR1 gene are in agreement with previous studies [4,59,60,68]. In contrast, activity of the tomato ASR2 promoter was enhanced by ABA when expressed in papaya and tobacco, but not in tomato or potato [67].

Genetic manipulation of ASR1 in tomato affects water-stress tolerance

Tomato plants overexpressing CaMV 35S:ASR1 (ASR1-OE) or CaMV 35S:Reverse ASR1 (ASR1-AS) were tested for tolerance to water and salt stresses. The modified plants did not perform significantly differently from WT plants when treated with NaCl (not shown). However, ASR1-OE plants survived water stress better than WT plants (Figure 6A and 6B). The response of ASR1-AS plants was highly variable: many lines showed hypersensitivity to water stress, whereas others showed no difference, or even better tolerance (Figure S2 in File S1). Averaging the recovery rates of all lines showed that ASR1-OE plants were significantly more tolerant to water stress than WT controls and ASR1-AS plants, whereas the latter were slightly less sensitive to water than WT plants (Figure 6C). Transgenic plants overexpressing ASR genes have been reported to be more tolerant to abiotic stresses such as water/osmotic stress [42,48–51], salinity stress [48,50,52,53] and cold stress [44,54]. However, most of those studies expressed the studied gene in a heterologous system [42,48–50,52–54]. In some of those studies, the biological species of the gene of origin and the transgenic plant belonged to the same botanical genus or family [24,53,56], but only a few studies have been performed within a single species [45,51]. The biological relevance of studying the role of a regulatory protein on a genetic background that naturally lacks it has been questioned [55]: the phenotype of Arabidopsis plants expressing tomato ASR1 was shown to result from competition for DNA binding between the ectopically expressed tomato gene and the Arabidopsis transcription factor AB4, essentially resulting in an ab4-1-mutant-like phenotype [55]. Thus, expressing the studied gene on the same genetic background ([45,51] and this study), or in closely related species [24,53,56], is more likely to shed light on the actual role of the studied gene and its protein product. The increased survival of transgenic tomato plants overexpressing tomato ASR1 following water stress (Figure 6) is in agreement with reports on the expression of plant ASTR genes on similar or different genetic backgrounds in response to stress [42,48–51]. Results obtained using the ASR1-AS lines were more variable: lines ASR1-AS5-4 and ASR1-AS18-4 were significantly hypersensitive to water stress as compared to WT plants (Figure S2 in File S1), whereas other lines were not significantly different. On the other hand, no significant differences were found in the sensitivity of ASR1-OE and ASR1-AS plants to NaCl stress (not shown), most probably due to the relative tolerance of the tomato WT line. ASR proteins are localized in the cytosol and nucleus. In the latter organelle, they are associated with DNA [14,39,40,48,73]. Tomato and grape ASR proteins have been shown to bind specific DNA sequences [14,53], suggesting that they regulate the expression of genes involved in abiotic stress responses or sugar metabolism [4,14,24,34,57,74]. In addition, ASR proteins have been shown to possess protein-chaperone-like activity [38,41], increasing protein stability. Thus, the increased water-stress tolerance of ASR1-OE plants most likely results from an increase in the transcription of ASR1-regulated genes and from ASR1 chaperon activity.

Conclusions

Two of the five genes in the ASR gene family (ASR1 and ASR4) are significantly expressed in tomato plants, whereas the expression levels of the other three member of the gene family are less pronounced. ASR1 and ASR4 encode 115- and 297-amino acid polypeptides, respectively, thus most likely encoding proteins whose activity may not be fully redundant. ASR1 is expressed in all plant tissues tested: it is most highly expressed in the stem, roots and reproductive organs–stamen, pistils and fruit at all developmental stages. ASR4 is mainly expressed in photosynthetic organs, in in sepal and petals. The steady-state levels of ASR1 and ASR4 increased following salt stress, osmotic stress and treatment with ABA. ASR2 expression is negligible in all tested tissues. ASR3 and ASR5, being highly similar genes, were assayed together, with significant expression detected only in the cotyledons. These results suggest that ASR2/3/5 may be very low expressing genes, or that their expression is limited to specific low abundant cells, thus resulting in low transcript activity when assayed in the whole tissue. Tomato plants overexpressing ASR1 show increased tolerance to water stress, whereas ASR1-AS plants show a certain degree of hypersensitivity to water withholding. Our data suggest that ASR1 and ASR4 may be expressed in different cell types. The differential expression patterns of the tomato ASR gene family under non-stress and stress conditions may be used for genetic manipulation of tomato (as well as other crop plants) to affect vegetative and fruit parameters under non-stress and stress conditions.
Supporting Information

File S1 Western-blot analysis of ASR1-overexpressing tomato, response of individual transgenic lines to water stress, and list of primers used for RT-qPCR. (DOC)

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Author Contributions

Conceived and designed the experiments: IG DSI DBZ. Analyzed the data: IG ZK DSI DBZ. Contributed to the writing of the manuscript: IG PGD DSI FC DBZ. Constructed the transgenic tomato plants: PGD FC.
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