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The Role of Adenylyl Sulfate Reductase to Abiotic Stress in Tomato

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ABSTRACT The full-length cDNA of LeAPR1 encoded a protein of 461 amino acid residues, which contained homology with phosphoadenosine phosphosulphate reductase (PAPS reductase) in N-terminal and an adenylylsulfate reductase in N-term and C-terminal. Analysis of the deduced amino acid sequence of LeAPR1 revealed that it shares high sequence identity with potato StAPR (96% identity) (Gene bank accession no. CDC44841). We found that multiple copies of LeAPR1 gene are present in the tomato genome through southern blot using genomic DNA was digested with 3 different restriction enzymes. The expression of LeAPR1 was also examined in various organs and its expression was also detected at high levels in roots and stems. Only high amounts of LeAPR1 transcripts were detected at high transcripts in the leaves at time 0, and then reduced as the plant stressed by the NaCl and abscisic acid (ABA). After 24h treatment of NaCl and ABA were showed increasing patterns of LeAPR1 gene. Time course of LeAPR1 gene expression was examined under oxidative stresses from metyl viologen (MV) and hydrogen peroxide (H_2O_2). In the presence of 10 mM H_2O_2 and 50 μM MV, the levels of LeAPR1 transcript in leaves decreased after 1 h, and then increased strongly, peaked at 24 h. Our results indicated that LeAPR1 may play a role function of circadian regulation involved in abiotic stresses signaling pathways.

Introduction

Plants or microbes synthesize the sulfur containing amino acid cysteine and important downstream metabolites such as methionine from sulfate or other inorganic sulfur sources. Five enzymes are required to synthesize cysteine from sulfate in plants. Although S research in vascular plants has a 30-year history, only recently has the process been studied at the molecular level. Molecular and biochemical evidence has demonstrated significant differences in the first enzyme-catalyzing sulfate reduction among vascular plants, bacteria, and fungi. In all of the organisms, sulfate assimilation begins with the enzyme ATP sulfurylase that catalyzes the adenylation of sulfate to 59-adenylylsulfate (APS). Adenylation is necessary because sulfate is relatively unreactive. APS is then reduced by APS reductase (APR) in plants and some bacteria (Bick and Leustek, 1997; Abola et al., 1999; Bick et al., 2000), whereas in other bacteria and fungi APS is further phosphorylated at the 39 position forming 39-phospho-59-adenylylsulfate (PAPS) before being reduced by PAPS reductase.

Sulfate assimilation is an energy intensive process that relies primarily on ATP and reductant generated by photosynthesis. Sulfide is assimilated in chloroplasts and also in the cytoplasm and perhaps mitochondria (Hell et al. 2002). The mechanism

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of sulfate assimilation was first reported in *Escherichia coli* and *Salmonella typhimurium* (Tones-Martimer 1968; Kredich 1971). Reduction of sulfate occur ATP sulfurylase forms adenosine 5_-phosphosulfate (APS), which is further phosphorylated by APS kinase to form adenosine 3_-phosphate 5_-phosphosulfate (PAPS). PAPS reduced in a thioredoxin-dependent reaction by PAPS reductase to sulfite. Sulfite is reduced by a NADPH-dependent sulfite reductase to sulfide, which is incorporated into the amino acid skeleton of O-acetyl-L-serine, thus forming cysteine (Brunold 1990). Plants and algae were shown to utilize APS as sulfonyl donor; the corresponding enzyme was originally called APS sulfotransferase (Schmidt 1972; Schmidt 1975). APS sulfotransferase was identified to play a key role and to be highly regulated by controlling sulfate reduction in plants (Brunold 1993). Nevertheless, sulfate reduction was included to PAPS-dependent pathway in purification of PAPS reductase isolated from spinach (Schwenn 1989). Plant APR is unique in that it is able to use reduced glutathione (GSH) as a source of electrons. By contrast, bacterial APR and PAPS reductase require the redox factors thioredoxin (Trx) or glutaredoxin (Grx) as a source of electrons. The GSH-dependency of plant APR is probably mediated through a carboxyl terminal domain that functions as a Grx, which is lacking in the bacterial and fungal enzymes. In flowering plants, APR is thought to be a primary regulation point for the sulfate assimilation pathway (Leustek and Saito 1999).

PAPS reductase, a small family of three cDNA clones was isolated from *Arabidopsis thaliana* (Gutierrez-Marcos et al. 1996; Setya et al. 1996). These clones encoded isoforms of an enzyme with a N-terminal organelle targeting peptide, a central part similar to *E. coli* PAPS reductase, and a C-terminal part similar to thioredoxin. The enzyme produced sulfite from APS, and the cDNAs complemented also *E. coli* mutants deficient in APS kinase. When both the PAPS reductase-like and the thioredoxin-like domains of APS reductase were expressed separately in *E. coli*, they were able to reconstitute the APS reductase activity (Bick et al. 1998, Prior et al. 1999). All the enzymes for synthesis of Cys are localized in the chloroplast, and APS reductase is exclusively localized in plastids (Leustek et al. 2000). APS reductase is significantly regulated by treatments such as sulfur starvation, heavy metal exposure, addition of Cys or other reduced sulfur sources to the irrigation medium, or oxidative stress (Leustek et al. 2000, Leustek and Saito 1999).

Massive changing of tomato genes reported by the overexpression of *CaPIF1* in transgenic tomato plants (Seong et al. 2007). LeAPR1 was induced in *CaPIF1* overexpressed tomato microarray data at 5.2 fold change (Seong et al. 2007). Therefore, we studied to investigate the role of LeAPR1 gene involved in response to abiotic stresses in tomato.

Materials and Methods

Plant Material

'MicroTom' (*Lycopersicum esculentum*) seeds were cultured in MS medium (MS salts including MS vitamins, 3% sucrose, 0.8% agar, pH 5.8). After 2 weeks laters, germinated plants were transferred to pots and kept in a growth chamber at 24℃ for 4 weeks. Several abiotic elicitors were applied to the leaves of the tomato plants. For the abscisic acid (ABA) and NaCl treatments, the leaves of tomato plants were detached in 100 μM ABA, 200 mM NaCl, 50 μM MV or 10mM H_2O_2. The treated leaves were frozen in liquid nitrogen and stored at -70℃, until used for RNA extraction.

DNA Sequencing

We searched a tomato cDNA clone (SGN-U155567), APR gene, from (http://www.sgn.cornell.edu/). And full-length primers designed to amplify APR gene of tomato. Detected band by PCR amplification cloned into pGEM T easy vector (Promega). We confirmed that sequences result match with one at Cornell University's homepage.

Southern Blot Analysis

Genomic DNA was prepared according to Dellaporta et al. Twenty micrograms of total DNA was digested with *Xba* I, *Eco*R I or *Hin*d III and the digested DNA was fractionated by

size on 0.8% (w/v) agarose gels. Membranes were hybridized overnight with a ^{32}P-labeled fragment of the PCR product of the *LeAPR* cDNA in a buffer consisting of 1% BSA /1 mM EDTA/0.5 M $NaHPO_4$, pH 7.2/7% SDS at 65℃ and washed in 0.5% BSA/1 mM EDTA/40 mM $NaHPO_4$, pH 7.2/5% SDS at room temperature for 5 min. The blot was then washed three times with high stringency wash buffer (1 mM EDTA/40 mM NaHPO4, pH 7.2/5% SDS) at 65℃, and the dried blots were placed on X-ray film at −80℃ for a week and developed.

Northern Blot Analysis

Total RNA was isolated from the various organs of tomato (Yi et al. 2004). Plant materials (1−1.5 g) that had been frozen in liquid nitrogen and ground to the powder were homogenized in 10 ml extraction buffer [4 M guanidine isothiocyanate, 25 mM sodium citrate at pH 7.0, 0.55% (w/v) N-laurylsarcosine and 0.1 M 2-mercaptoethanol]. A mixture of 2 M sodium acetate (pH 4.0), water saturated phenol and chloroform−isoamylalcohol (24:1) was added to the homogenate. Precipitation was performed with isopropanol at -20℃ for 2 h. After centrifugation, the pellet was suspended in 2 M LiCl solution and incubated at 4℃ for 18 h. The total RNA concentration and purity were determined by spectrophotometer and staining of the ribosomal RNA with ethium bromide, respectively. Equal quantities of total RNA (10 μg) were loaded into 1% agarose gel, containing 7.4% formaldehyde. After electrophoresis and visualization under UV light, the RNA was transferred onto nylon membranes (Hybond N^+, Amersham), followed by cross-linkage under irradiation with UV light. To generate *LeAPR1* gene-specific probe, each coding sequence was PCR-amplified with two primers: (5'-atggctttgactttcacttcttca-3' and 5'-gatgttgagggtgtatagagggag-3') for *LeAPR1*. The purified PCR products were ^{32}P-labeled. Hybridization was performed overnight at 65℃ in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS and 1 mM EDTA. After hybridization, the filter was washed twice with 2 X SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.1 X SSC and 0.1% SDS for 5 min each at 65℃.

RT-PCR

First-strand cDNA was synthesized from 1 μg samples of the DNase-treated total RNA (M-MLV RT, Invitrogen, USA). The primers used for reverse transcriptase-PCR were as follows: (5'-atggctttgactttcacttcttca-3' and 5'-gatgttgagggtgtatagagggag-3') for *LeAPR1* or (5'-gcagtgtttcccagtattgt-3' and 5'-accgctagcatac-agagaaa-3') for *LeActin*. PCR conditions were as follows: initial denaturation for 5 min at 94℃; followed by 25 cycles of 94℃, 1min; 55℃, 1min; and 72℃, 1min; and a final 7 min at 72℃. Twelve μl samples of the reaction products were separated on 1% agarose gels and visualized by staining with ethidium bromide. All experiments were preformed in triplicate.

Results and Discussion

Isolation of Tomato *APR1* Gene

To isolate tomato *APR1* gene up-regulated by *CaPIF1*-over-expressed transgenic tomato, the tomato EST cDNA database was searched to identify annotated APR cDNA clone (http:// www.sgn.cor nell. edu/) (Seong et al. 2007). The evolutionary relationships among five APR proteins, including LeAPR1, were analyzed (Figure 1). AtPRH26 of Arabidopsis was most similar to *LeAPR1* (76% identity). LeAPR1 shares high sequence identity with potato StAPR (96% identity). Popula PoAPR (78% identity) are other homologs of LeAPR1. Soybean GmAPR has 79% identity with LeAPR1. The LeAPR1 polypeptide is composed of 461 amino acids. The deduced amino acid of LeAPR1 cDNA is indicative of a typical APR protein comprising a conserved phosphoadenosine phosphosulphate (PAPS) reductase domain (115−289) and adenylylsulfate (APS) reductase domain (at positions 349−457) (Figure 1). Alignment of LeAPR1 with StAPR, PoAPR and GmAPR reveals that the N-terminal and C-terminal domains are highly conserved (Figure 1). The APS reductase from plants is an enzyme with a bipartite structure consisting of an amino terminal domain that may carry the APS reductase catalytic center and a C-terminal electron transfer domain that mediates the interaction with the natural electron donor, reduced glutathione (Bick et al. 1998,

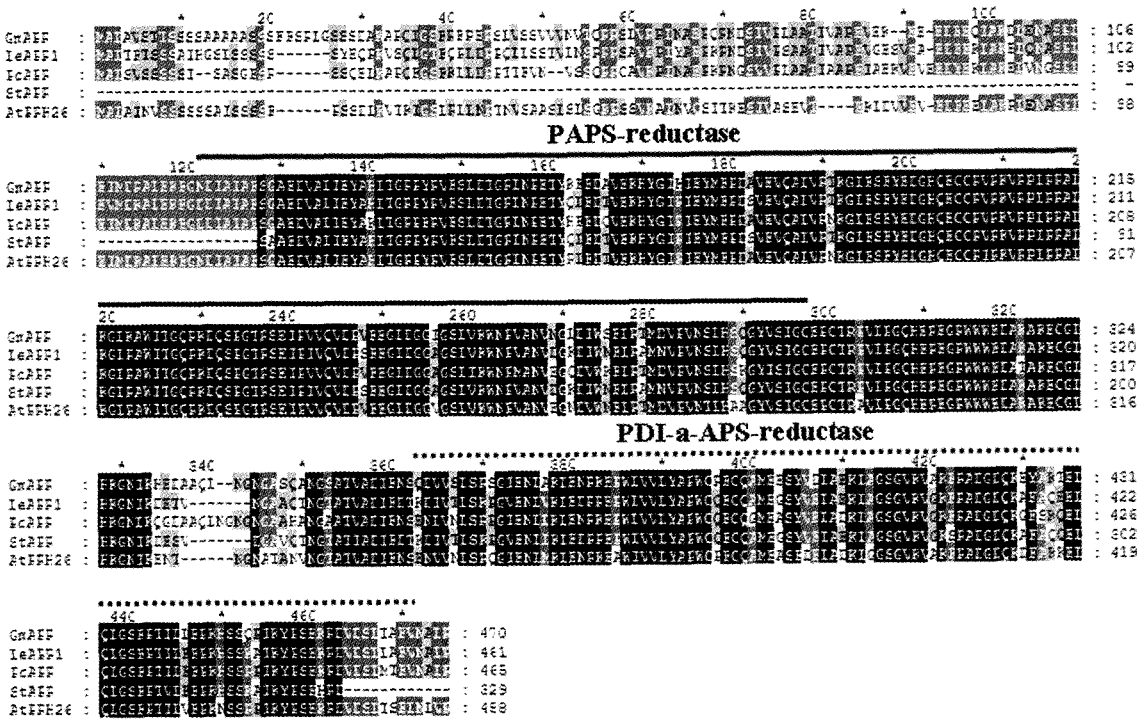

Figure 1. Characterization of the LeAPR1 gene. Comparison of derived amino acid sequences of tomato LeAPR1 with those of Arabidopsis PRH26, Patato APR, Soybean APR and Popular APR. Residues shaded in black are identical between the four proteins. The GenBank, DDBJ, EMBL, and NCBI accession numbers of nucleotide sequences are the following: tomato cDNA is AAU03359 (LeAPR1, isolated from this study), Arabidopsis cDNA is AAM65133 (AtPRH26): Potato cDNA is CAD44841 (StAPR): Popular cDNA is AAQ57202 (PoAPR): Soybean cDNA is AAL66290 (GmAPR)and AB013887 (AtRAV2).

Kopriva et al. 2001). Some species use an APS reductase that has homology with the N-terminal domain of plant APS reductase (Abola et al. 1999, Bick et al. 2000, Neumann et al. 2000, Kopriva et al. 2002, Williams et al. 2002). Other sulfate assimilating prokaryotes and eukaryotes employ a different sulfite-forming enzyme that uses 3'-phosphoadenylylsulfate (PAPS) as a substrate (Berendt et al. 1995). The plant APS reductases are the cysteine residues that serve as iron sulfur ligands, the sulfite ligand, and the glutaredoxin active site. Very similar enzymes have been detected in algae, *Enteromorpha intestinalis* and *Chlamydomonas reinhardtii* (Gao et al. 2000, Ravina et al. 2002), in *Catheranthus roseus* (Prior et al. 1999), *Brassica juncea* (Heiss et al. 1999), *Lemna minor* (Suter et al. 2000), *Oryza sativa and Zea mays* (Houston et al. 2005).

Gel Blot Analysis of Genomic DNA

Genomic DNA was digested with 3 different restriction enzymes (Figure 2), and the probe used was a 1383-bp PCR product of the LeAPR1 cDNA clone. All restriction enzyme digestions gave above two hybridizing bands, suggesting that multiple copies of LeAPR1 gene are present in the tomato genome. DNA blot analysis was used to examine the size of the *APR* gene family in *A. thaliana*. This result reported that there are at least three DNA fragments that hybridize with the *APR1* cDNA (Setya et al. 1996). *C. reinhardtii* contains single genes encoding APS reductase. It showed that DNA gel blots using the APR1 cDNA as probes detected simple banding pattern that indicated single gene (Ravina et al. 2002). APS reductase has the three conserved domains in *C. reinhardtii*. These include an amino terminal transit peptide responsible for targeting the protein to the chloroplast followed by a portion homologous to bacterial APS and PAPS reductases (Leustek et al. 2000).

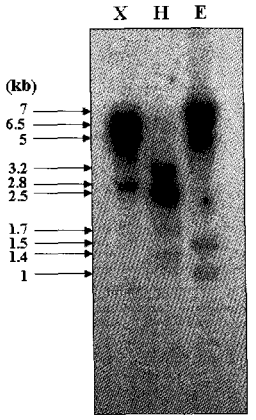

Figure 2. Genomic DNA-blot analysis of LeAPR1. Genomic DNA blot analysis of tomato LeAPR1. Each lane was loaded with 20 μg pepper genomic DNA digested with *Xba* I (X), *Hind* III (H), and *Eco* RI (E). The membrane was hybridized with 32P-dCTP-labeled full-length LeAPR1 cDNA. The sizes of the molecular weight markers are indicated in kilo-bases to the left.

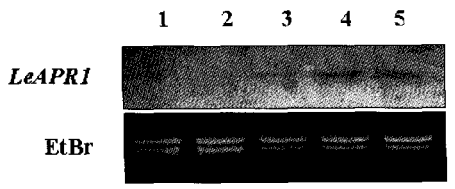

Figure 3. Organ-specific expression of LeAPR1. Organ-specific expression of the LeAPR1 gene in tomato plants. Total RNA was isolated from old leaves (1), young leaves (2), flowers (3), roots (4), and stems (5), and the RNA gel blot was hybridized with full-length LeAPR1 cDNA.

LeAPR1 Expression in Relation to Developmental Signals

The expression of LeAPR1 was also examined in various organs (Figure 3). Its expression was not restricted to old and young leaves or flower; it was also detected in roots, stems. Roots and stems had the highest level of LeAPR transcripts. APS-reductase were showed highest expression in the upper section of the root (Hopkins et al. 2004). In the leaves, the sulfate transporters, APS-reductase was induced by S-starvation with the most mature leaf showing increased mRNA abundance first (Hopkins et al. 2004). Sulfur assimilation is a highly regulated process in plants. The pathway is controlled in response to plant developmental hints, the demand for cysteine and methionine for growth, and the demand for glutathione to remediate against with broad environmental attacks.

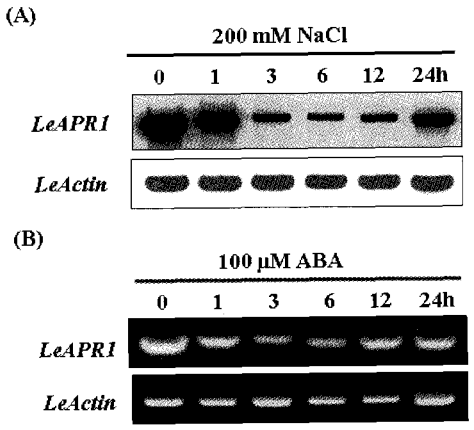

Figure 4. Expression of LeAPR1 mRNA in response to treatment with abiotic stresses. Detached chili pepper leaves submerged in NaCl (A) or ABA (B) solution for various time points. Treated leaves were harvested at the indicated times, and total RNAs were isolated. To carry out RT-PCR, cDNA was synthesized from total RNAs. RT-PCR analysis was performed as described in Materials and methods.

LeAPR1 gene expression responds to various environmental stresses in tomato

Both detached leaves of tomato were used to investigate the effect of salt on LeAPR expression. Only high amounts of LeAPR transcripts were detected at high levels in the leaves at time 0, and then reduced as the plant stressed by the NaCl (Figure 4A). To investigate the effect of ABA on *LeAPR1* transcript levels, young detached leaves of *tomato* were placed on Petri dish in 100 μM abscisic acid (ABA). Osmotic stress (100 μM ABA) did reduce *LeAPR1 after treated 1h*, while LeAPR1 expression was slightly induced at 12h-treatment (Figure 4B). The results show that salt or ABA regulated the amounts of LeAPR transcripts. Time course of LeAPR1 gene expression was examined under oxidative stresses from metyl viologen (MV) and hydrogen peroxide (H_2O_2). All two stresses induced a decreased and then increased LeAPR1 accumulation in leaves of tomato (Figure 5A and 5B). In particular, LeAPR1 gene expression was constitutive strongly in intact tomato leaf. But in the presence of 10 mM H_2O_2 and 50 μM MV, the levels of LeAPR1 transcript in leaves decreased after 1 h, and then increased strongly, peaked at 24 h (Figure 5A and 5B). All aerobic organisms are damaged by reactive oxygen species (ROS) (Hquptmann and Cadenas 1997). The life of plants is

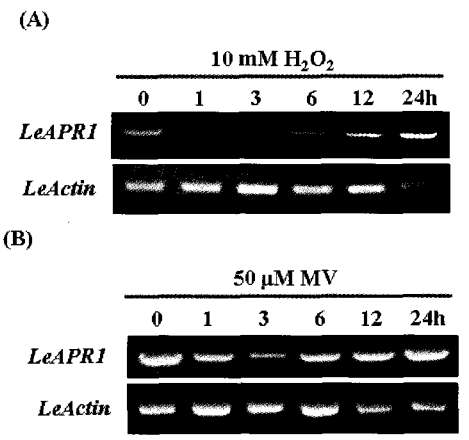

Figure 5. Expression patterns of LeAPR1 in tomato plants treated with chemicals related to oxidative stress. Expression patterns of LeAPR1 gene using RT-PCR analysis. Total RNA was extracted from tomato leaves treated by 10 mM H_2O_2 **(A)** and 50 μM MV **(B)** at the indicated time points after treatment. To carry out RT-PCR, cDNA was synthesized from total RNAs. Twelve μl of RT-PCR product was separated by electrophoresis. RT-PCR analysis was performed as described in Materials and methods.

especially precarious because ROS are generated as a by-product of oxygenic photosynthes is (Polle 1997). In their own defense plants have evolved an antioxidant system in which glutathione plays a pivotal role in the removal of hydrogen peroxide and in maintaining the level of another antioxidant (May et al. 1998, Alscher 1989). APS reductase is likely a control point of sulfate assimilation in plants. It is the only enzyme activity in the pathway that is significantly regulated by treatments such as sulfur starvation, heavy metal exposure, addition of Cys or other reduced sulfur sources to the irrigation medium (Leustek et al. 2000, Leustek and Saito 1999), or oxidative stress (Bick et al. 2001). Moreover, regulation by oxidative stress was not accompanied by changes in steady-state mRNA or protein level, whereas both mRNA and protein change in response to diurnal regulation (Bick et al. 2001). It was reported that *A. thaliana* APS reductase is diurnally regulated by a transcriptional or posttranscriptional mechanism (Kopriva et al. 1999). Some sulfur assimilation genes are regulated by circadian rhythm (Kopriva et al. 1999; Harmer et al. 2000). The expression of sulfate transporters, APS reductase, Ser acetyltransferase, and 3-phosphoglycerate dehydrogenase, the first enzyme of the plastidic Ser biosynthetic pathway, are at a peak just before the onset of the light period. The coordinate circadian control of a cluster of genes involved in sulfur assimilation and related pathways seems to ensure the efficient production of Cys by the preparation of sulfate and key enzymes just before the generation of reductants and substrates from photosynthesis. Abiotic stresses such as heavy metals and oxidative stress affect sulfur assimilation (Dominguez-Solis et al. 2001, Nocito et al. 2002). The general role of APS reductase in controling sulfur assimilation was attempted with *Z. mays* overexpressed bacterial APS reductase (Martin et al. 2005). APS reductase limits the production of reduced sulfur compounds in transgenic Z. mays (Martin et al. 2005).

In conclusion, we reported here suggest that the role of LeAPR1 to abiotic stress responses in tomato plant. LeAPR1 can be used in a genetic engineering strategy to increase cysteine production. Future experimentation must focus on whether the relationship about LeAPR1 expression and cystein production using transgenic tomato plants. And how impact of environmental conditions on sulfur assimilation is involved in the diverse role that glutathione plays in mitigating oxidative stress.

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