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Iron-superoxide dismutase and monodehydroascorbate reductase transcripts accumulate in response to internode rubbing in tomato

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Abstract

A cDNA encoding an iron-superoxide dismutase (Fe-SOD) was isolated by RACE-PCR from a *Lycopersicon esculentum* cDNA library. The Fe-SOD cDNA consists of a 746-bp open reading frame and is predicted to encode a protein of 249 amino acids with a calculated molecular mass of 27.9 kDa. The deduced amino acid sequence was very similar to other plant Fe-SODs and a potential chloroplastic targeting was found. To study the induction of oxidative burst in response to mechanical stimulation, the accumulation of Fe-SOD and monodehydroascorbate reductase (MDHAR) mRNAs was analysed in response to young growing internode rubbing in tomato plants. Northern analyses show that Fe-SOD mRNA and MDHAR mRNA accumulated in tomato internodes 10 min after the mechanical stimulation. These results suggest that reactive oxygen species are early involved in the response of a plant to a mechanical stimulation, such as rubbing. The nucleotide sequence data reported in this paper will appear in the NCBI Nucleotide Sequence Databases under the accession number AY262025. ***To cite this article: I. Ben Rejeb et al., C. R. Biologies 327 (2004).***

Résumé

Accumulation des transcrits d'une superoxyde dismutase à fer et d'une monodéhydroascorbate réductase en réponse au frottement d'un entre-nœud chez la tomate. Une stratégie de RACE-PCR a été utilisée pour cloner l'ADNc complet d'une superoxyde dismutase à Fer (Fe-SOD) à partir d'une banque d'ADNc de tomate. L'analyse de cette séquence met en évidence un cadre de lecture ouvert de 746 pb. La séquence en acides aminés déduite de l'ADNc correspond à un polypeptide de 249 acides aminés, présentant une masse moléculaire de 27,9 kDa. L'analyse protéique montre que la séquence présente une forte similarité avec les autres SOD à fer identifiées chez les plantes et un peptide d'adressage dans le chloroplaste. Pour étudier le stress oxydatif induit en réponse à une stimulation mécanique, l'accumulation des transcrits d'une Fe-SOD et d'une monodéhydroascorbate réductase (MDHAR) a été analysée chez la tomate en réponse au frottement d'un jeune entre-nœud en croissance. L'étude par Northern montre que l'accumulation des transcrits Fe-SOD et MDHAR débute de manière significative

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aussi tôt que 10 min après l'application de la stimulation mécanique. Ces résultats semblent indiquer l'intervention précoce des espèces oxygénées réactives dans la réponse d'une plante à une stimulation mécanique de type frottement. ***Pour citer cet article : I. Ben Rejeb et al., C. R. Biologies 327 (2004).***

Keywords: iron superoxide dismutase; monodehydroascorbate reductase; oxidative stress; mechanical stimulation; *Lycopersicon esculentum*

Mots-clés : superoxyde dismutase ; monodehydroascorbate reductase ; stress oxydatif ; stimulus mécanique ; *Lycopersicon esculentum*

1. Introduction

In plant cells, an inevitable result of aerobic metabolism is the leaking of electrons onto molecular oxygen with the resultant production of reactive oxygen species (ROS) [1]. Environmental stresses can give rise to further increases in ROS levels [2,3]. The ROS can cause oxidative damage to many cellular components including membrane lipids, proteins and nucleic acids. The oxidative damage may result from the alteration of the balance between the production of ROS and their detoxification by the antioxidative system [4,5]. In plants, both enzymatic and non-enzymatic processes participate in ROS detoxification [6–8].

Superoxide dismutase (SOD, EC 1.15.1.1) is a key enzyme involved in the first steps of the ROS-scavenging system. The SODs are metalloenzymes that use Mn, Fe or Cu and Zn as prosthetic metals to catalyse the dismutation of the superoxide anion O_2^- to oxygen and hydrogen peroxide H_2O_2 [9]. H_2O_2 could be then scavenged by catalase and different classes of peroxidases [10]. Ascorbate peroxidase (APX) plays a key role in the ascorbate–glutathione cycle by reducing H_2O_2 to water using the reducing power of ascorbate and producing monodehydroascorbate (MDHA) [11]. In turn MDHA is reduced and recycled to ascorbate by the action of monodehydroascorbate reductase (MDHAR, EC 1.6.5.4). The cellular redox state is highly dependent with ascorbate pool, the major antioxidant buffer in plant tissues, and the cellular and apoplastic ascorbate pool is directly bound to antioxidant enzymatic capacities [12].

In plants, previous studies demonstrated that SOD can participate in early signalling pathways in responses to both biotic and abiotic stresses [10,13]. Based on the metal present at the catalytic site, SODs are classified into three types, Cu/Zn-, Mn- and Fe-SOD. These SODs are located in different compartments of the cell: Fe-SODs have been found in the chloroplasts of several plant species, Mn-SOD in the mitochondria and the peroxisome and Cu/Zn-SOD in the cytosol, the chloroplast and probably in the extracellular space [14]. Previous studies have focused on the role of Cu/Zn SOD in stress tolerance and only a few Fe-SODs have been described in higher plants [15]. In particular, there is little information on SOD involvement in oxidative damage generated by mechanical stress. But, several studies suggested the involvement of ROS-scavenging system in response to mechanical stress. In tomato, northern analyses demonstrated an increase in mRNA accumulation of two phospholipid hydroperoxide glutathione peroxidase (PHGPX) genes 1H after rubbing of tomato internode [16]. Furthermore, leaf wounding induces mRNA accumulation of MDHAR in tomato plants [17]. These results suggest the involvement of oxidative stress in the signalling pathway in response to a mechanical stimulus.

In order to understand the implication of oxidative stress in the growth response of tomato to mechanical stimulation, we have focused on two enzymes among the different antioxidant systems, the Fe-SOD involved in early steps, and the MDHAR as a witness of tissue redox state. We have first cloned a complete Fe-SOD cDNA in tomato and its nucleotide sequence was analysed. Then, we compared the mRNA accumulation of Fe-SOD and MDHAR genes in control and mechanically stressed plants.

2. Material and methods

2.1. Plant material and growth conditions

Tomato plants (*Lycopersicon esculentum* Mill. cv. VFN8) were raised from seeds in moist vermiculite in a controlled environment: 16 h daylight at 60 µmol m^{-2} s^{-1}, photosynthetically active radiation

provided by 40-W white daylight tubes (Mazda LDL, TF 40), 25 ± 1 °C (day) and 19 ± 1 °C (night), 70 ± 10% relative humidity. At the cotyledon stage, plants were transferred to a mineral solution [18].

2.2. Mechanical treatment

Rubbing was applied to 4-week-old plants, with seven developed internodes. The young growing internode was held between the thumb and forefinger and rubbed back and forth [19].

2.3. RNA isolation and northern blotting

Total RNA was extracted from internodes 4 of rubbed and unrubbed (control) tomato plants by the method of Bogorad et al. [20]. Extractions were carried out from control and rubbed plants at different times after stimulation (10 min, 30 min, 1 h, 2 h, 4 h, 6 h and 18 h). Total RNA (10 µg) was separated on 1.5% (w/v) formaldehyde/agarose gels, blotted onto nylon filters (Biodyne B, Pall) and probed with ^{32}P-labelled cDNA encoding tomato Fe-SOD or encoding tomato MDHAR (graciously gift by A.B. Bennet, California University, Davis, USA). The filters were washed at high stringency and exposed to X-ray film. Blots hybridisations were normalized with reference to 18S ribosomal RNA hybridisation.

2.4. Cloning and sequencing

To obtain Fe-SOD cDNA, RT-PCR experiments were performed using total RNA from rubbed tomato seedlings. First-strand cDNA synthesis was carried out from 5 µg of total RNA using T-primed First-Strand Kit from Amersham (Orsay, France). PCR experiments were performed using degenerated primers (SOD1: 5′-garttycaytggggnaarca-3′ and SOD2: 5′-tangcnarccangccca-3′) based on regions highly conserved from known Fe-SOD sequences as shown in Fig. 1. The PCR product of 330 bp was cloned in pGEM-T easy vector (Promega, Charbonnières, France) and sequenced by automated dye terminator sequence analysis using the CEQ sequencer (Beckman-Coulter, Roissy-Charles-de-Gaulle, France).

To obtain full-length cDNA, RACE-PCR amplifications were performed using primers: F-SOD (5′-ctccccttccagcattcaacaatg-3′) and R-SOD (5′-ggagccaa attgtgttgctgcagc-3′) shown in Fig. 1 and designed in the tomato Fe-SOD partial cDNA (330 bp) previously cloned. The 3′ and 5′ ends were amplified separately. The PCR products were cloned within the pGEM-T easy plasmid according to the manufacturer's instructions (Promega) and sequenced by automated terminator sequence analysis using the CEQ 2000 sequence (Beckman-Coulter).

2.5. Sequence analysis

Similarity scores between *Lycopersicon esculentum* Fe-SOD and other Fe-SOD were calculated using the BLAST software [21]. Sequence alignment, similarity scores and cladogram were calculated and performed according to the method of Higgins and Sharp using CLUSTAL program [22]. Signal peptide was predicted according to Chloro 1.1 software (Prediction results, CBS Danemark) and binding properties of the active site by the Scan Prosite program [23].

3. Results

3.1. Nucleotide and deduced amino acids sequence of Fe-SOD protein

To isolate cDNA-encoding Fe-SOD, total RNA from tomato plants was reverse-transcribed and first-strand cDNA were amplified using one set of degenerated primers designed in highly conserved regions of known plant Fe-SOD. A 330-bp partial Fe-SOD cDNA was isolated and subsequently used to design a new set of primers to obtain full-length cDNA by RACE-PCR (see materials and methods). The full length Fe-SOD cDNA (948 bp) contained an open reading frame of 746 bp with an initiation codon Methionin at nucleotide position 39 and a stop at nucleotide position 785 (Fig. 1).

The encoded protein would be 249 amino acids in length and have a calculated molecular mass of 27.9 kDa. As shown in Fig. 2, the amino acid sequence deduced from the cDNA contained conserved metal binding domain. Binding properties of the active site analysed by Scan PROSITE program display that glutamine involved in catalytic site of Fe-SOD [24] was conserved in tomato FeSOD (residue 121). Moreover, protein sequence contained some of characteris-

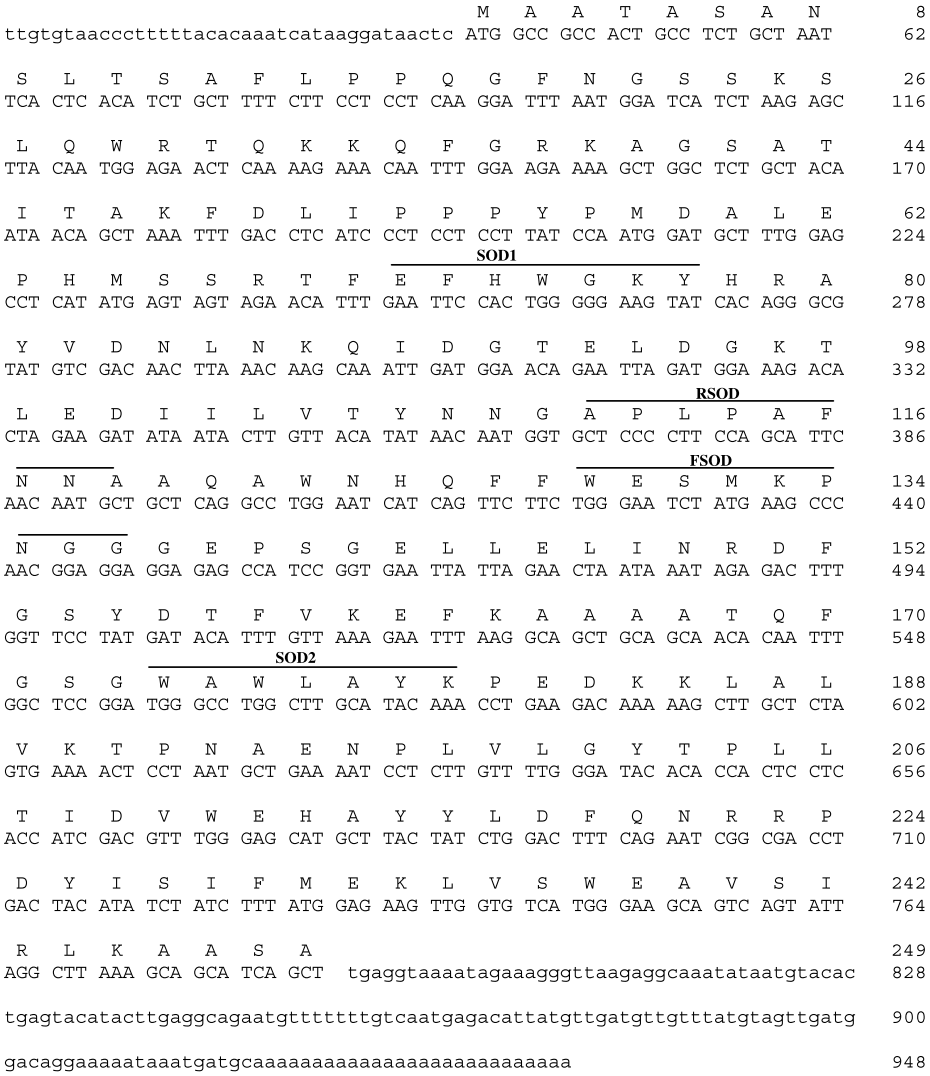

Fig. 1. Nucleotide sequence of Le-FeSOD and its deduced amino acid sequence. Oligonucleotides used for isolating partial cDNA fragment (SOD1 and SOD2) and for RACE-PCR approach (RSOD and FSOD) are underlined.

tic amino acids of Fe-SOD such as tryptophan 123 described as important amino acid for H_2O_2 sensibility of Fe-SOD [25]. The predicted protein seems to contain at its N-terminus a 58-amino acid signal peptide that would target it to the chloroplast. After peptide cleavage, the calculated molecular mass of the protein would be 21.7 kDa, as previously described for other Fe-SOD in higher plants [26].

The alignment of amino acids sequences (Fig. 2) indicated that tomato Fe-SOD shows high similarity with other Fe-SODs previously described in higher plants. The higher identity is obtained with the *Nicotiana plumbaginifolia* Fe-SOD protein (90%) and lower but significant identity (68%) has been found with the first *Arabidopsis thaliana* Fe-SOD protein identified [26].

The phylogenetic tree derived from the percentage of identity between Fe-SOD proteins from higher plants, chlorophytes and cyanobacteria was plotted. As shown in Fig. 3, the tomato Fe-SOD clusters

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RsFeSOD          ---------------------------------------MAASAAVTANYVLKPPPYPLDA  22
AtFeSOD          ----------------RFHWRVLSSLHSSLKLELQLQRMAASSAVTANYVLKPPPFALDA  44
LeFeSOD          MAATASANSLTSAFLPPQGFNGSSKSLQWRTQKKQFGRKAGSATITAKFDLIPPPYPMDA  60
NpFeSOD          -----------------------------------------------KFELQPPPYPMDA  13
                                                                 :: * ***:.:**

                          ▼
RsFeSOD          LEPHMSKQTLEFHWGKHHRAYVDNLKKQVLGSELEGKALEHIIQNTYNNGDLLPPFNNAA  82
AtFeSOD          LEPHMSKQTLEFHWGKHHRAYVDNLKKQVLGTELEGKPLEHIIHSTYNNGDLLPAFNNAA 104
LeFeSOD          LEPHMSSRTFEFHWGKYHRAYVDNLNKQIDGTELDGKTLEDIILVTYNNGAPLPAFNNAA 120
NpFeSOD          LEPHMSSRTFEFHWGKHHRAYVDNLNKQIDGTELDGKTLEDIILVTYNKGAPLPAFNNAA  73
                 ******.:*:******:********:**: *:**:**.**.**  ***:*  **.*****

                           ▼
RsFeSOD          QAWNHEFFWESMKPGGGGKPSGELLALLERDFTSYEKFYDEFNAAAATQFGAGWAWLAYA 142
AtFeSOD          QAWNHEFFWESMKPGGGGKPSGELLALLERDFTSYEKFYEEFNAAAATQFGAGWAWLAYS 164
LeFeSOD          QAWNHQFFWESMKPNGGGEPSGELLELINRDFGSYDTFVKEFKAAAATQFGSGWAWLAYK 180
NpFeSOD          QAWNHQFFWESMKPNGGGEPSGELLELINRDFGSYDAFVKEFKAAAATQFGSGWAWLAYK 133
                 *****:********.***:****** *::*** **: * .**:********:*******

                                                  ▼  ▼
RsFeSOD          --DNKLKVVKTPNAVNPLVLGSFPLLTIDVWEHAYYLDFQNRRPDYIKTFMNNLVSWEAV 200
AtFeSOD          --NEKLKVVKTPNAVNPLVLGSFPLLTIDVWEHAYYLDFQNRRPDYIKT-MTNLVSWEAV 221
LeFeSOD          PEDKKLALVKTPNAENPLVLGYTPLLTIDVWEHAYYLDFQNRRPDYISIFMEKLVSWEAV 240
NpFeSOD          PEEKKLALVKTPNAENPLVLGYTPLLTIDVWEHAYYLDFQNRRPDYISIFMEKLVSWEAV 193
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RsFeSOD          SSRLEAAKAASA 212
AtFeSOD          SARLEAAKAASA 233
LeFeSOD          SIRLKAASA--- 249
NpFeSOD          SSRLKAATA--- 202
                 * **:**.*
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Fig. 2. Comparison between the FeSOD sequences of *N. plumbaginifolia* (NpFeSOD, P22302), *Raphanus Sativus* (RsFeSOD, AAC15842), *Arabidopsis thaliana* (AtFeSOD, P21276) and *Lycopersicum esculentum* (LeFeSOD). Amino acids that are conserved in all sequences are shown by an asterisk. The metal ligands conserved between MnSOD and FeSOD are marked by an arrowhead (according to Van Camp et al. [24]). Amino acids important for distinguishing FeSOD from MnSOD are boxed.

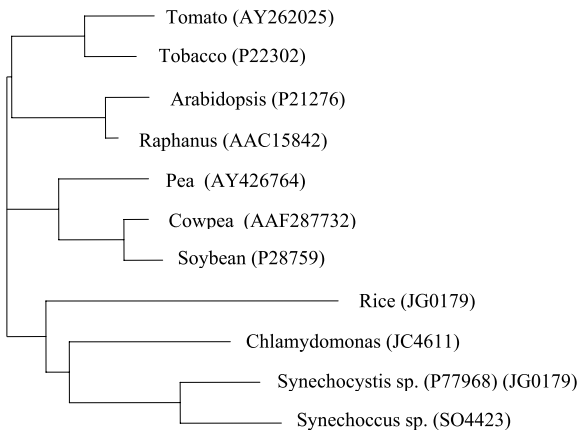

Fig. 3. Phylogenetic tree derived from Fe-SOD amino acids sequences from procaryotic and eucaryotic photosynthetic organisms. Accession numbers of the sequences are indicated on the figure. Protein sequences were compared by CLUSTAL software [20].

with the other higher plant Fe-SOD and in particular with the other solanacae sequence of *Nicotiana plumbaginifolia*. However, relative high similarity (59%) is observed with the sequence of the green algae *Chlamydomonas reinharditii*. Furthermore, tomato Fe-SOD presents 53% of identity with the prokaryotic cyanobacteria sequences.

3.2. Time-course accumulation of Fe-SOD and MDHAR mRNAs after mechanical treatment

To determine the effects of mechanical treatment on the expression of Fe-SOD gene and MDHAR gene, we realized a northern blot analysis with our tomato Fe-SOD cDNA or the tomato MDHAR cDNA as probes, in control plants (time 0) and in rubbed plants 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 18 h after rubbing. The 18S rRNA probe was used as loading control. To ensure consistency within each experiment, filters were stripped and reprobed with each of the cloned cDNAs.

Results first showed that the two genes expression is constitutive because a significant amount of Fe-SOD transcripts (Fig. 4) or MDHAR transcripts (Fig. 5) was observed in control internodes (times 0). The Fe-SOD

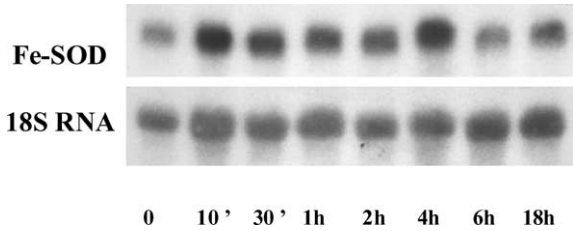

Fig. 4. Time-course induction of Fe-SOD mRNA accumulation following mechanical treatment (internode rubbing). Total RNA was extracted from unrubbed tomato internode (0) and from rubbed internodes at different times (10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 18 h). 10 µg total RNA were separated on a formaldehyde gel, blotted and probed with ^{32}P-labeled Fe-SOD cDNA. To estimate loading homogeneity, blots were subsequently hybridised to a cDNA probe for the 18S ribosomal RNA (18S RNA). Each experiment was repeated two times with different samples.

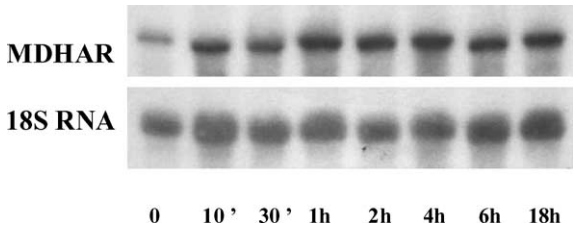

Fig. 5. Kinetic accumulation of MDHAR transcripts following mechanical treatment (internode rubbing). Total RNA was extracted from unrubbed (0) tomato internode and from rubbed internodes at different times (10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 18 h). 10 µg total RNA were separated on a formaldehyde gel, blotted and probed with ^{32}P-labeled MDHAR cDNA. To estimate loading homogeneity, blots were subsequently hybridised to a cDNA probe for the 18S ribosomal RNA (18S RNA). Each experiment was done two times.

mRNAs level increased very early, as soon as 10 min after the mechanical treatment. The transcripts accumulation was maintained during the four following hours and then decreased to the control level 6 h after the stimulation (Fig. 4). The accumulation of MDHAR mRNAs was detected as early as 10 min after the stimulation. The highest level of MDHAR transcripts was reached 1 h after the stimulation. Then, the transcripts level decreased but was still higher than the control 18 h after the mechanical stimulation (Fig. 5).

4. Discussion

For the first time, we report in this paper the cloning and characterisation of a complete cDNA encoding a tomato iron SOD. Sequence analysis from the cloned cDNA indicates that all amino acids residues used to distinguish Fe-SOD from Mn-SOD [26] are present in tomato sequence. The iron superoxide dismutases are found both in prokaryotes and in eukaryotes. In eukaryotes, Fe-SOD sequences have been isolated from *Nicotiana plumbaginifolia*, *Arabidopsis Thaliana* [26], *Glycine max* [27], *Pisum sativum* [28] and rice [29]. Three Fe-SODs were reported in *Arabidopsis Thaliana* [30]. Previously, it was thought that Fe-SODs were not present in all plants. The characterisation of a tomato Fe-SOD in this paper makes wider the group of higher plants having such particular SOD.

Alignment of the tomato deduced amino acids sequence with the Fe-SODs previously described indicates that tomato Fe-SOD shows higher identity with proteins from higher plants (90% of identity within the solanacae group). However, a relative high degree of identity (53%) is found with the prokaryotic sequence from cyanobacteria. These results confirm the hypothesis that chloroplast genome originates from the genome of an ancestral cyanobacterial endosymbiont and that Fe-SODs were probably originally encoded by the chloroplastic genome and were transferred later to the nucleus, explaining the absence of Fe-SOD in animals [26].

Analysis of tomato Fe-SOD amino acid sequence predicted a signal peptide responsible for targeting in the chloroplast. In all plant species examined to date, it is inferred that Fe-SODs are located in the chloroplast. When polyclonal antibodies raised against water lily (*Nuphar Luteum*), purified Fe-SOD protein were incubated with protoplasts, these antibodies predominantly associated with the chloroplasts [31]. A potential chloroplastic targeting sequence to the chloroplast was also found in the soybean Fe-SOD. Furthermore, the higher retention of the Fe-SOD in chloroplast fractions suggested that it was associated with thylakoid membranes [32] and finally an immunodetection of Fe-SOD proteins in *Arabidopsis* chloroplasts was shown [30]. Taken together, these data suggest that tomato Fe-SOD is probably also located within the chloroplast.

Before the *Arabidopsis* DNA-sequencing projects, Southern analyses suggested the existence of a single genomic gene for Fe-SOD. Based on the data of Kliebenstein et al. [30], who identified at least three different Fe-SOD genes, one may speculate the

existence of multiple Fe-SOD genes in the tomato genome.

Previous data in tomato have suggested that rubbing of a young growing internode initiated an oxidative stress [16]. Grantz et al. [17] reported that MDHAR gene expression was regulated by total ascorbate content in response to mechanical wounding. The increase of MDHAR mRNAs after rubbing presented in Fig. 5 argues for an increase in ascorbic acid content, as it was already reported in response to various plant stresses: strong illumination in wheat leaves [33], SO_2 and O_3 in conifer needles [34], drought in grasses [35]. Even if we cannot rule out the possibility that our MDHAR probe could cross-hybridise with other MDHAR mRNAs than the cytosolics, our first interpretation of the data showed in Fig. 5 is that the rubbing treatment induced a cytosolic oxidative stress.

Previous studies have demonstrated the regulation of Mn, Fe and cytosolic Cu/Zn SODs in response to environmental stresses [10]. In particular, Fe-SOD activity is modified by methyl viologen in different treated plants [36], rice Fe-SOD by light induction [31], and *Lingulodinium polyedrum* Fe-SOD mRNA modulated by metal stress [37]. Salt stress also induced in tomato antioxidant enzymes genes such as SOD, catalase and ascorbate peroxidases [38]. Recently, in tomato, SOD and GPX expression was demonstrated to be modulated also by various biotic stresses [39]. The present work shows for the first time the regulation of Fe-SOD mRNA by mechanical stimulation.

Based on the plastidic localization of Fe-SOD in plants [15,30], it may be questioned whether the accumulation of tomato Fe-SOD mRNA is due to the rapid generation of an oxidative stress within the chloroplast or to the binding of known transcription factors to the upstream region of the Fe-SODs genes, as proposed by Alsher et al. [15].

5. Conclusion and perspectives

In the present study, we have cloned and characterized a tomato cDNA encoding one of the major enzymes of the detoxification system of the ROS: the Fe-SOD. In addition, we have investigated the accumulation of Fe-SOD mRNA after internode rubbing, in parallel with MDHAR expression, another antioxidant enzyme. We confirm here that rubbing of tomato internodes initiates, in a very fast way, an oxidative stress and then an antioxidant response. However, it is now planned to estimate, using Southern, how many genes are expressed and to confirm the expression patterns of Fe-SOD and MDHAR by semi-quantitative RT-PCR strategy. Furthermore, it would be interesting to confirm these results by analysing the Fe-SOD regulation at the translational level.

It is still questioning why antioxidant enzymes such as Fe-SOD, probably located in the chloroplast, are involved in response to mechanical stress. It would be interesting to study the regulation of the other plastid-located SOD (Cu/Zn SOD) in such stress conditions. The measurement of the enzymatic activity and getting the protein would allow to explain why both plastid-located SOD (Cu/Zn SOD and Fe-SOD) are needed, particularly under stress conditions.

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