Changes in ascorbate peroxidase activities during fruit ripening in *Capsicum annuum*

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Received 12 October 1994; revised version received 9 December 1994

Abstract The ascorbate peroxidase (APX) system was studied in *Capsicum annuum* during fruit ripening. A large increase in organelle APX activity was found during chloroplast–chromoplast transition whereas only a slight difference was detected in total fruit extracts. On native gels, four different isoforms were found in total fruit extracts but the patterns for red and green fruit were quite different. In isolated organelles, six isozymes were found and a comparison of the patterns showed significant differences. A cDNA encoding a cytosolic APX was cloned and sequenced. The corresponding transcript was shown to increase 3–4-fold during fruit ripening.

Key words: Ascorbate peroxidase; Fruit-ripening; Isozyme; Organelle; cDNA Sequence; *Capsicum annuum*

1. Introduction

Whereas in animals scavenging of hydrogen peroxide is performed using glutathione, cytochrome *c* or pyridine nucleotide as electron donor, in plants and algae ascorbate peroxidase represents the most important hydrogen peroxide-scavenging enzyme. Ascorbate peroxidase has been found in all angiosperms so far surveyed and also been detected in the eukaryotic algae *Euglena*, *Chlamydomonas* and even in the cyanobacteria [1].

In chloroplasts, immediate scavenging of hydrogen peroxide is indispensable to the maintainance of photosynthetic activity. In leaf cells the electron donor for the peroxidase reaction has been identified as ascorbate [2]. Ascorbate peroxidase has been found to occur as at least three distinct isozymes: the cytosolic isoform, and two chloroplastic isoforms, one in the stroma and the other associated with the thylakoid membranes [3]. The differentiation of chloroplast to chromoplast, for example in fruits, is characterized by a breakdown of chlorophyll, considerable structural modifications and the accumulation of carotenoids and other polyunsaturated lipids. During this process, the synthesis of several antioxidants is induced to prevent the formation of harmful lipid hydroperoxides. It has been shown that the formation of superoxide radicals is increased during fruit ripening as indicated by the accumulation of lipid peroxidation products [4]. In addition, high levels of plastidial superoxide dismutase (SOD), which functions as detoxifier, accumulate during chromoplast formation [5]. In an attempt to characterize the antioxidant systems stimulated during fruit-ripening, we have investigated the ascorbate peroxidase (APX) activities during ripening of bell pepper fruits. In this paper, we present the evolution of the ascorbate peroxidase system in whole tissues and in organelles during fruit ripening. We have cloned a cDNA encoding the cytosolic enzyme and analyzed its expression during fruit ripening.

2. Materials and methods

2.1. Materials

Bell pepper (*Capsicum annuum*, cv. Yolo Wonder) seeds were germinated and plants were grown under greenhouse conditions.

2.2. Plant fractionation

All purification steps were carried out at 4°C. 1 g of pericarp of green or red bell pepper fruit was homogenized in a chilled mortar with 0.5 ml of buffer [6] modified as follows: 150 mM potassium phosphate (pH 7.6), 3 mM ascorbic acid (AsA), 3 mM EDTA, 20% sorbitol (w/v), 2% soluble polyvinylpyrrolidone (PVP) (w/v). The concentrations of the buffer components were increased to take into account the relatively high water content of the fruit tissues. After filtration through 4 layers of cheesecloth, the homogenate was centrifuged for 15 min at $15{,}000 \times g$.

Aliquots of total proteins (10–20 μg) of the supernatant determined by Bradford reaction [7] were used immediately in all the experiments for measuring APX activity.

Chloroplasts and chromoplasts were purified according to [8] except that AsA was added to all buffers to give a final concentration of 1 mM.

Purified chloroplasts or chromoplasts were resuspended in washing buffer and aliquots were plated on a Malassez counting chamber, covered with a slide and counted with a microscope. Organelle suspensions were adjusted to the same density and centrifuged at $5{,}000 \times g$ for 10 min. Organelle pellets were immediately resuspended in 0.4 ml medium containing 50 mM potassium phosphate (pH 7.6), 1 mM AsA, 1 mM EDTA, 20% sorbitol (w/v), 2% PVP [6] and frozen at −80°C, then slowly thawed to 4°C. This treatment was repeated once.

After lysis, the suspension was centrifuged at $20{,}000 \times g$ for 15 min and a given volume of the chloroplast and chromoplast supernatants (corresponding to $6\text{–}25 \cdot 10^4$ organelles) was used to test APX activity.

2.3 Enzyme assay

APX activity was determined as described by [9] in 0.4 ml reaction mixture. Oxidation of AsA was followed by monitoring the decrease in the absorbance at 298 nm. One unit of APX activity is defined as the amount of AsA which has been oxidized per mg of protein per min at room temperature under the above conditions.

2.4. Electrophoresis and activity staining

Native PAGE was performed on 8% polyacrylamide gels (8×8 cm) without stacking gels. The gel buffer was 0.45 M Tris-HCl (pH 8.9). The running buffer was 5 mM Tris, 38.4 mM glycine, 2 mM AsA. After pre-electrophoresis for 15 min at 20 mA, the samples were loaded and separated by electrophoresis at 4°C for 1.5 h with constant current of 20 mA.

APX activity was localized as an achromatic band on a blue background according to Mittler and Zilinskas [10].

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The sequence data reported in this paper will appear in the EMBL database under Accession Number X81376.

SSDI 0014-5793(94)01413-2

2.5. DNA/RNA techniques

Differential screening of the cDNA library, subcloning and sequencing were performed as described [11]. RNA extracted from fruits at different stages of fruit development were separated by formamide/formaldehyde agarose gel electrophoresis (1.2%, w/v) and blotted onto Hybond N membranes (Amersham) as previously described [12]. Isolated APX cDNA insert was labelled with [α-^{32}P]dCTP by random priming and hybridized with the RNA blots for 24 h at 37°C in a reaction mixture containing 50% formamide, 5 × SSC (3M sodium chloride, 0.3 M sodium citrate), 1 × Denhardt's solution, 200 μg sonicated salmon sperm DNA and 0.2% sodium dodecyl sulfate (SDS). Filters were washed with 0.1 × SSC, 0.1% SDS at 65°C for 10 min.

3. Results

3.1. Changes of ascorbate peroxidase activity during fruit ripening

Guaiacol peroxidase (GPX), usually present in total extracts but not in chloroplasts can use ascorbate as an electron donor. To ascertain that the measured activity can be attributed to APX, we have tested the occurrence of GPX in pepper fruit extracts following the procedure described by Amako et al. [13]. Surprisingly, GPX activity was barely detectable, in contrast to other tissues like bean root extracts in which we found an activity of nearly 20% of the total peroxidase activity in the presence of ascorbate.

As shown in Table 1, APX activity is slightly higher in red fruits as compared with mature green fruits when determined in total fruit extracts. In contrast, when using isolated organelles for APX activity determination, a large increase is found during the chloroplast-chromoplast transition (the activity is around 3 times higher in chromoplasts as compared with chloroplasts). Taking into account the difference in protein content between the two types of organelles it is more accurate to express the activity on the basis of an organelle number rather than on a protein basis.

3.2. Characterization of APX activity in gels

The solution assay used for the determination of APX activity in soluble protein extracts does not allow the separation of different APX isozymes. Therefore we took advantage of the method allowing direct detection of APX activity in native gels.

Table 1
Ascorbate peroxidase activities in *Capsicum annuum*

	Green	Red	Ratio
Total fruit extracts μmol/mg protein/min	0.817	1.117	1.4
Organelle extracts μmol/10^6 plastids/min	$2.02 \cdot 10^{-2}$	$5.62 \cdot 10^{-2}$	2.8

Each value represents the mean of 6 and 3 measurements for total fruit extract and organelles, respectively.

As shown in Fig. 1a, four different bands can be distinguished in total fruit extracts. The patterns in green and red fruits are significantly different, corresponding either to an increase of a given isoform or to the appearance of a new isoform. In addition the band b seems to decrease or to disappear in red fruits whereas the band d shows a marked increase. It seems that during fruit ripening, the various isoforms behave differently, such that some increase and others decrease, leading to only a slight overall rise (1.4) of activity in total fruit extracts during fruit ripening.

The plastidial isoforms are very labile, especially in the absence of ascorbate [1,10,14]. In order to reveal these forms it was essential to improve the organelle purification procedure by adding sorbitol and ascorbate to the isolation buffers and to shorten the extraction method as much as possible.

In isolated organelles the number of isoforms detected is very high, at least six different forms being found (Fig. 1b). The significance of this high number of isoforms remains unknown. By enzyme purification it could be shown that in isolated chloroplasts, APX exists in both the stromal fraction and in the thylakoid fraction [3] whereas, using the techniques of native gels separation, Mittler and Zilinskas [10] were able to detect 5–6 bands. Following exactly the same procedure, we were also able to detect 4–5 bands in isolated chloroplasts of *Picea abies* L. cv. Karsten (Guillemaut and Schreiber, unpublished results). The comparison of the patterns obtained from chloroplasts and chromoplasts shows significant differences, especially the band 3 which exhibits a marked increase. These differences can be correlated to the overall increase (3-fold) of APX activity during the chloroplast to chromoplast transition (Table 1). It is difficult to attempt to identify the plastidial isoforms in the

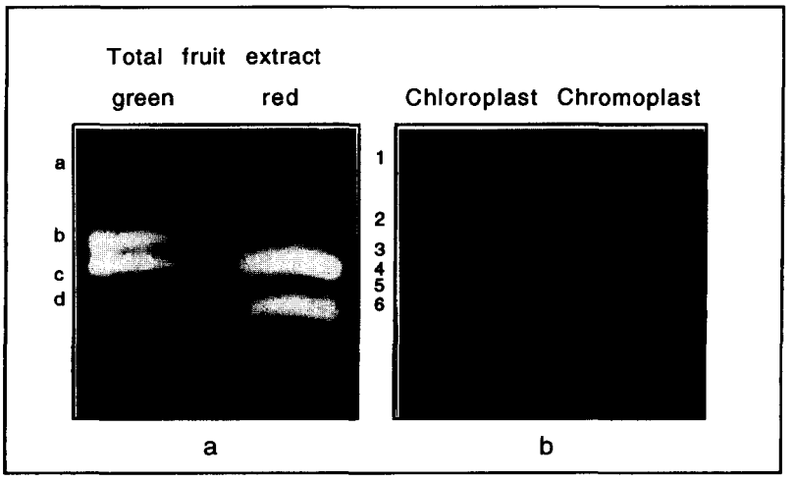

Fig. 1. Detection of APX activities on native PAGE. (a) in total fruit extracts. (b) in isolated organelles. For total extracts, 80 μg proteins per lane were loaded onto the gel. For organelles, the supernatant corresponding to $2 \cdot 10^6$ organelles was loaded.

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                1                                                    50
Capsicum        MGKcYPTVSE EYlKAVDKCK RKLRGLIAEK NCAPLMLRLA WHSAGTyDvc
Arabidopsis     MtKnYPTVSE DYkKAVEKCr RKLRGLIAEK NCAPiMvRLA WHSAGTFD.c
Pisum           MGKsYPTVSp DYqKAiEKaK RKLRGfIAEK kCAPLiLRLA WHSAGTFDsk
Consensus       MgK-YPTVSe dY-KAveKck RKLRGlIAEK nCAPlmlRLA WHSAGTfD-c

                51                                                   100
Capsicum        .SKTGGPFGT MRFktEQsHG ANNGIDIALR LLEPlgEQFP ivSYADFYQL
Arabidopsis     qSrTGGPFGT MRFdAEQAHG ANsGIhIALR LLdPIrEQFP tiSfADFhQL
Pisum           .tKTGGPFGT ikhqAElAHG ANNGlDIAvR LLEPIkEQFP ivSYADFYQL
Consensus       -skTGGPFGT mrf-aEqaHG ANnGIdIAlR LLePi-EQFP ivSyADFyQL

                101                                                  150
Capsicum        AGVVAVEVTG GPDVPFHPGR EDKPEPPvEG RLPDATKGSD HLRDVFvKQM
Arabidopsis     AGVVAVEVTG GPDiPFHPGR EDKPqPPPEG RLPDATKGcD HLRDVFaKQM
Pisum           AGVVAVEiTG GPeVPFHPGR EDKPEPPPEG RLPDATKGSD HLRDVFgKaM
Consensus       AGVVAVEvTG GPdvPFHPGR EDKPePPPEG RLPDATKGsD HLRDVF-KqM

                151                                                  200
Capsicum        GLSD.QDIVA LSGGHTLGRC HKERSGFEGP WTaNPLIFDN SYFKELLgGE
Arabidopsis     GLSD.kDIVA LSGaHTLGRC HKdRSGFEGa WTSNPLIFDN SYFKELLsGE
Pisum           GLSvvQDIVA LSGGHTiGaa HKERSGFEGP WTSNPLIFDN SYFtELLtGE
Consensus       GLSd-qDIVA LSGgHTlGrc HKeRSGFEGp WTsNPLIFDN SYFkELL-GE

                201                                                  250
Capsicum        KEGLLQLPSD KALLsDPaFR PLVEKYAADE DAFFADYAEA HLKLSELGFA
Arabidopsis     KEGlLQLvSD KALLdDPVFR PLVEKYAADE DAFFADYAEA HmKLSELGFA
Pisum           KdGLLQLPSD KALLtDsVFR PLVEKYAADE DvFFADYAEA HLKLSELGFA
Consensus       KeGLLQLpSD KALL-DpvFR PLVEKYAADE DaFFADYAEA HlKLSELGFA

                251
Capsicum        EA*
Arabidopsis     dA*
Pisum           EA*
Consensus       eA*
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Fig. 2. Comparison of the derived bell pepper protein sequence with those from pea [15] and *Arabidopsis* [16]. Upper case letters in the consensus sequence indicate conservation across all three species.

whole fruit extracts, even by running all the extracts on the same gel (data not shown). Nevertheless, we can tentatively assume that band a and c in total fruit extracts correspond to band 1 and 4 in the plastidial extracts, respectively. We should point out that two comigrating bands do not necessarily contain identical proteins.

3.3. Isolation of a pepper cDNA encoding APX

In order to isolate clones corresponding to genes preferentially expressed during fruit ripening, we have screened by differential hybridization a cDNA library constructed with poly(A)$^+$ RNA from pepper fruit at an early ripening stage. Among several clones identified as ripening-specific, one was found to be homologous to recently described sequences encoding cytosolic APX [15,16].

The pepper cDNA sequence 953 nt long encodes a protein of theoretical molecular weight of 27.3 kDa with an isoelectric point of 5.3. As shown in Fig. 2, the sequences of APX are highly conserved. The pepper APX shares 84% and 83% identities and 92% and 89% similarities with the *Arabidopsis* and pea counterparts, respectively. The homology found in the N-terminal region indicates the cytosolic location of the encoded protein, moreover upstream from the ATG, the presence of a stop codon excludes the possibility of a sequence corresponding to a transit peptide. Screening of different cDNA libraries constructed from ripening or fully ripe fruits did not allow the isolation of clones encoding different APX proteins, even on using low stringency hybridization conditions (30% formamide, 5 × SSC, 42°C and washings with 2 × SSC, 0.1% SDS, 50°C). Experiments designed to isolate the cDNAs encoding the plastidial isoforms following a different strategy with specific antibodies are in progress.

3.4. Expression of APX during fruit ripening

The steady state level of the corresponding transcripts was studied by Northern blot analysis during fruit ripening. A single transcript of 1.1 kb was found in all RNA samples (Fig. 3). Great differences were found during fruit ripening. The transcript level was lowest in young green fruits, barely detectable only on overexposed autoradiograms. The signal remained almost constant during fruit development in mature green and orange fruits and increased about 3–4-fold up to the fully ripe stage. This change in transcript level is much higher than the differences of activities (1.4 times) measured in total fruit extracts (Table 1). This result suggests either that APX expression is mainly regulated at the level of protein synthesis or enzymatic activity or that other isozymes not detected by our cDNA probe are differentially regulated during fruit ripening. Interestingly, the pea cytosolic APX appeared also to be regulated post-transcriptionally after recovery from drought stress [17].

4. Conclusion

In conclusion, we determined a rise of APX activity during fruit ripening and especially within the chromoplast of the bell pepper. Similarily, we have shown previously during the chloroplast-chromoplast transition increased activities of cysteine synthase, glutathione synthase and glutathione reductase [18]. On the other hand, in tomato the plastidial SOD accumulates during the chromoplast formation [5]. Taken together, these

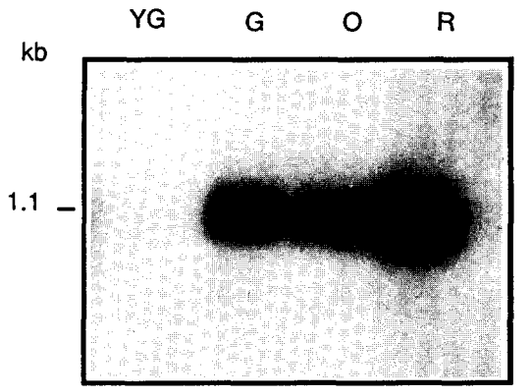

Fig. 3. Northern blot analysis of the cytosolic ascorbate peroxidase transcripts during fruit ripening. Total RNA samples (8 μg) from young green fruits (YG), mature green fruits (G), orange ripening fruits (O) and fully ripe red fruits (R), were tested using the cDNA as a probe.

data indicate that in the process of chloroplast to chromoplast differentiation, a general program of antioxidant synthesis and enhanced activity of detoxifying enzymes occur. Our data point out that different expression of the isoforms occurs in the cytosol as well as in the plastidial compartment, indicating a very complex regulatory system. Molecular analysis of APX gene structure and expression may help to unravel the mechanisms regulating the expression of the individual gene(s) at the appropriate location and time in plants. It will be of special interest to investigate the behaviour of the different APX isoforms detected within the organelles. Isolation of cDNAs encoding the plastidial isozymes in addition to the cytosolic cDNA described in this work will be a useful tool for investigating APX gene expression during fruit ripening.

Acknowledgements: We thank Dr. M. Kuntz (I.B.M.P.) for the help with the cDNA isolation.

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