ISOLATION AND SEQUENCING OF A GENOMIC DNA ENCODING FOR ASCORBATE OXIDASE, A KEY ENZYME INVOLVED IN THE BIODEGRADATION OF ASCORBIC ACID IN MELON

Evangelos ALATSATIANOS[1], Marin ARDELEAN[2] *, Constantin BOTEZ[3], Mircea SAVATTI[3], Radu SESTRAŞ[2], Mirela CORDEA[2]

ABSTRACT

A melon genomic library was used to isolate and characterize a clone of genomic DNA coding for ascorbate oxidase (AO) which is considered a key enzyme in the biodegradation of ascorbic acid (AA).

The screening of genomic library was performed by using two probes: AO-PCR product and αE/CMAO3. Seven clones of recombinant phage, with positive signal, were isolated. The extracted DNA was digested with *EcoRI*, *Sal I* and *Bam HI* and the combinations among these restriction enzymes. Four clones of AO gene were isolated: AO_1; AO_2; AO_3 and AO_4. Since only clone AO_4 had not been characterized so far, the sequencing of this clone was performed. The isolated sequence of DNA has a length of 3072 bp being bordered at both ends by EcoRI enzymes.

The comparison between genomic DNA for AO4 in melon and DNA for AO in cucumber revealed a high similarity (94.45%).

KEYWORDS: isolation, sequencing, genomic DNA, ascorbate oxidaze, ascorbic acid, melon

AO_4 – A GENE FOR ASCORBATE OXIDASE SYNTHESIS

DETAILED ABSTRACT

At least four genes are considered by DIALINAS et al. (1997) responsible for AO biosynthesis and three of them (AO_1 ; AO_2 and AO_3) have already been isolated and sequenced by the above cited scientists. The purpose of the present paper was the isolation and characterization of AO_4 gene which is also involved in AO biosynthesis.

A melon genomic library, built up by CLONTECH, was used to isolate and characterize the genomic DNA clones encoding AO synthesis. Melon fruits were used as DNA source and the λ-EMBL-3 phage, with a cloning site in Bam HI, was employed as a vector. The digestion of λ-EMBL-3 with Bam HI resulted in melon DNA incorporation into the vector's DNA. Sal I enzyme was used to separate the melon DNA from the vector's DNA. The genomic library comprised 2.1 x 10^6 genes and the phage density was 0.5 x 10^9 pfu/ml. The bacterial strain K 802 of E.coli was used as host cell donnor for λ-EMBL-3 multiplication.

By means of two known AO, one from cucumber (BALAGUE et al., 1989) and the other from pumpkin [5], two primers were constructed: AO-PCR and $\alpha E/CMAO_3$. The screening of genomic library was performed four times, by using both probes in order to cover the whole length of the DNA phragment and to make the results more reliable.

At the end of three screening cycles, seven clones of recombinant phage, with positive signal, were isolated. They were assigned as 8_1 ; 6_γ ; 8_2 ; 10_1 ; 2_γ and 10_2. The extracted DNA from these clones was digested with Eco RI, Sal I and Bam HI and the combinations among these restriction enzymes. The utilization of these enzymes was imposed by the fact that Sal I sets free the genomic DNA from λ-EMBL-3 phage while the other enzymes do not cut the phage but only the free genomic DNA. The digested DNA was run on a 1% TAE gelagarose.

Four clones of AO gene were isolated: AO_1; AO_2; AO_3 and AO_4. Since only clone AO_4 had not been characterized so far, the sequencing of this clone was performed. The isolated sequence of DNA has a length of 3072 bp being bordered at both ends by EcoRI enzymes.

The comparison between genomic DNA for AO_4 in melon and DNA for AO in cucumber revealed a high similarity (94.45%).

KEYWORDS: isolation, sequencing, genomic DNA, ascorbate oxidaze, ascorbic acid, melon

INTRODUCTION

Ascorbic acid (AA) is an important compound of melon fruit conferring it a well-balanced taste and making this common vegetable a very serious competitor of oranges as a source of C vitamin for human needs.

Synthesis of AA in melon, as in most of the cultivated species, is carried out through the so called *non-inversed* pathway in which the biosynthesis starts from D-galactose and, in the last step, by menas of L-galacton-γ-lacton-dehidrogenase (GLDH), the C vitamin is formed [8]. The isolation and partial characterization of GLDH gene was published by [7] and completed by [1] and [2]. The *non-inversed* biosynthetic pathway of AA is rarely met in plant species, being put into evidence so far only in spinach and beans [9]. Figure1 presents the two biosynthetic pathways of AA discussed above.

Several strategies of achieving higher accumulation of biosynthetic products in plants, by means of altering the genetic information governing the anabolism and catabolism of these products, have been thoroughly discussed by [3, 4]. An attentive analysis of Figure1 shows that a sure way to increase the content of AA in melon would be that of intensifying its biosynthesis by controlling the gene for GLDH. On the other hand, if one takes a look at the lower part of Figure1, it appears clearly that the biodegradation of AA is controlled by a single enzyme: ascorbate oxidase (AO). It is obvious that by controlling the gene for AO, a lower rate of biodegradation and, consequently, a higher rate of AA accumulation in melon fruits could be achieved.

At least four genes are considered by [6] responsible for AO biosynthesis and three of them (AO_1 ; AO_2 and AO_3) have already been isolated and sequenced by the above cited scientists. The purpose of the present paper was the isolation and characterization of AO_4 gene which is also involved in AO biosynthesis.

MATERIAL AND METHODS

A melon genomic library, built up by CLONTECH, was used to isolate and characterize the genomic DNA clones encoding AO synthesis. Melon fruits were used as DNA source and the λ-EMBL-3 phage, with a cloning site in *Bam HI*, was employed as a vector. The digestion of λ-EMBL-3 with *Bam HI* resulted in melon DNA incorporation into the vector's DNA. *Sal I* enzyme was used to separate the melon DNA from the vector's DNA. The genomic library comprised 2.1 x 10^6 genes and the phage density was 0.5 x 10^9 pfu/ml. The bacterial strain K 802 of *E. coli* was used as host cell donor for λ-EMBL-3 multiplication.

By means of two known AO, one from cucumber (BALAGUE et al., 1989) and the other from pumpkin [5], two primers were constructed: AO-PCR and $\alpha E/CMAO_3$. The screening of genomic library was performed four times, by using both probes in order to cover the whole length of the DNA fragment and to make the results more reliable.

At the end of three screening cycles, seven clones of recombinant phage, with positive signal, were isolated. They were assigned as 8_1 ; 6_γ ; 8_2 ; 10_1 ; 2_γ and 10_2. The extracted DNA from these clones was digested with *Eco RI, Sal I* and *Bam HI* and the combinations among these restriction enzymes. The utilization of these enzymes was imposed by the fact that *Sal I* sets free the genomic DNA from λ-EMBL-3 phage while the other enzymes do not cut the phage but only the free genomic DNA. The digested DNA was run on a 1% TAE gelagarose.

RESULTS AND DISCUSSION

Fig. 2, 3, 4 and 5 present the results of genomic DNA migration through gelagarose. They are analysed only for 8_1 ; 2_γ; 8_2 ; 9_γ clones in which the differences are obvious. In the other three clones of recombinant phage the results are identical to those obtained in at least one of the four clones previously mentioned, as table 1 shows.

Tab. 1.: Similarity of DNA clones for AO isolated from melon

Clones	AO1	AO2	AO3	AO4
2γ	X			
6γ		X		
81				X
82		X		
9γ			X	
101		X		
102			X	

We can conclude that DNA obtained from the four analyzed clones was really differing from clone to clone. In each case, it is evident that the restriction enzymes completely cut the genomic DNA, no instances of incomplete digestion being encountered.

To identify the strip which hybridizes with the two probes used by us (AO-PCR-product and $\alpha E/CMAO_3$) DNA was transferred to a Hybom-TM membrane where each clone was hybridized with the probes. The results show that in each recombinant phage, there were complementary DNA zones with the probes. By comparing these results with those obtained in gel-electrophoresis of digested DNA, it was possible to attain a mapping of restriction sites of the analyzed DNA fragment. The results of this mapping are presented in Figure 6.

It is evident, from Figure 6, that clones 6_γ, 10_1 and 10_2 are identical to at least one of the other four clones, as it was clearly stated in table 1.

A comparison of these results with those obtained by mapping of cDNA in pumpkin and cucumber, reveals that the fragments of melon genomic DNA from clones 2γ (AO_1) and 8_1 (AO_4) are not of complete length. From the first clone there is missing an exon while from the second one three exons are missing. The other clones, 6_γ, 8_2 and 10_1 (representing AO_2), 9_γ and 10_2 (representing AO_3) had genomic DNA for AO of complete length.

Six restriction sites of *Eco RI* enzyme were identified in clone 8_1 while for *Bam HI* only one site was found. For clone 9_γ there were noted two restriction sites of *Eci RI* and one site for *Bam HI* and *Sal I*, respectively. In clones 8_2, 10_1 and 6_γ, *Eco RI* had two restriction sites, *Bam HI* four sites while *Sal I* only one site. Finally, in clone 2_γ, *Eco RI* had two restriction sites and *Sal I* only one site.

Since only clone 8_1 (AO_4) had not been characterized so far, the sequencing of this clone was performed. The DNA fragment is bordered at both ends by *Eco RI-Eco RI* and has a length of 2.9 kb. Figures 7a and 7b show the results of subcloning AO_4 gene and its approximate length (2.9 kb).

A comparison of genomic DNA for AO_4 in melon with cDNA for AO in cucumber and pumpkin was performed by means of CBLASTIN 1.4.11 computer programme. Results are presented in Figure 8.

From data shown in Figure 8 it can be concluded that the genomic DNA for AO_4, in melon, has a length of 3072 bp. In comparison with cDNA from cucumber, the genomic DNA for AO_4 in melon presents a high level of similarity (94.459%). At the 3' end, AO_4-DNA has a sequence of 802 bp non-homologous to that of cucumber cDNA. Also, the genomic DNA for AO_4 in melon does not comprise the whole length of the similar fragment of cDNA in cucumber, missing a sequence of 1163 bp.

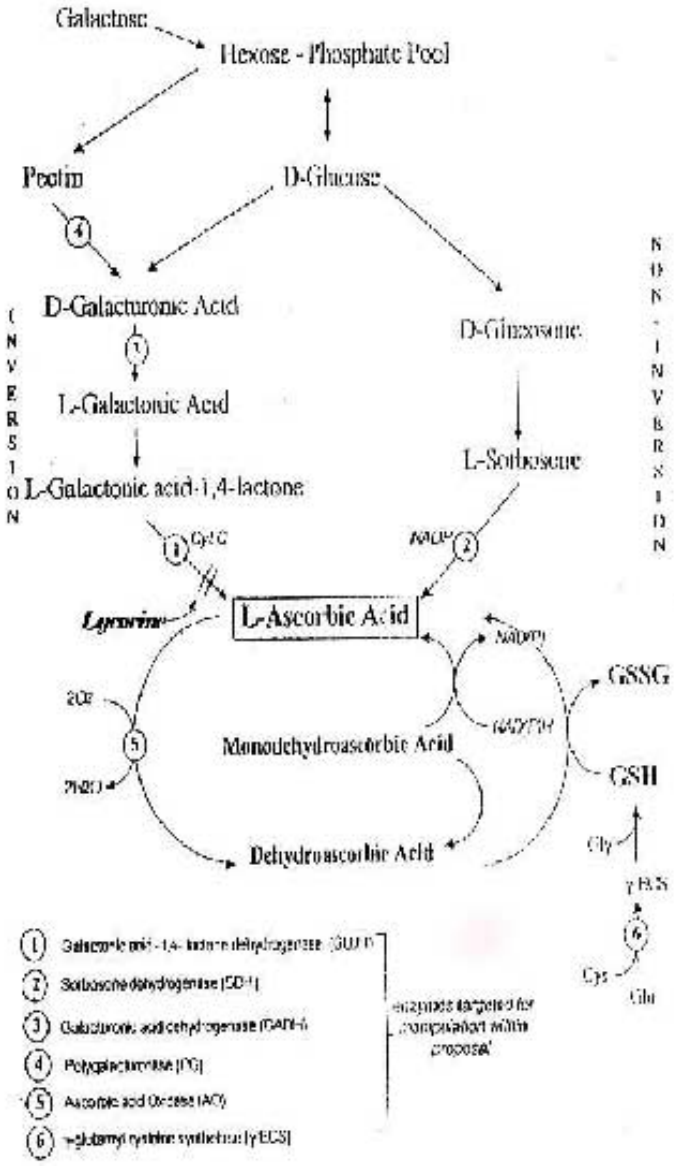

Fig. 1 Biosynthetic pathways of ascorbic acid

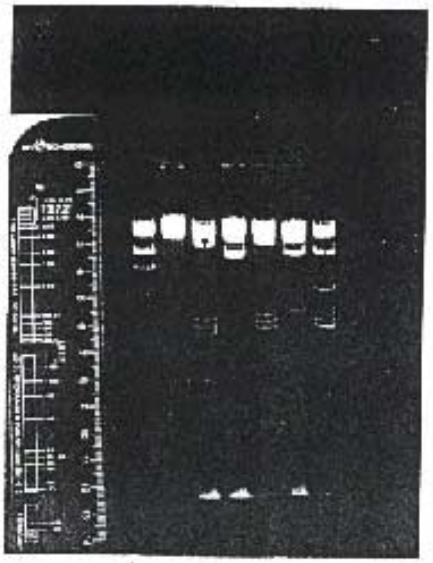

Fig.2 Electrophoresis gel of digested DNA from rocmbinant clone no.8_1

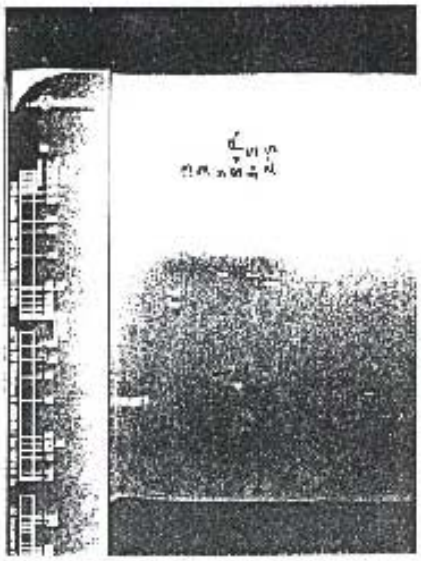

Fig.3 Electrophoresis gel of digested DNA from recombinant clone no. 2γ

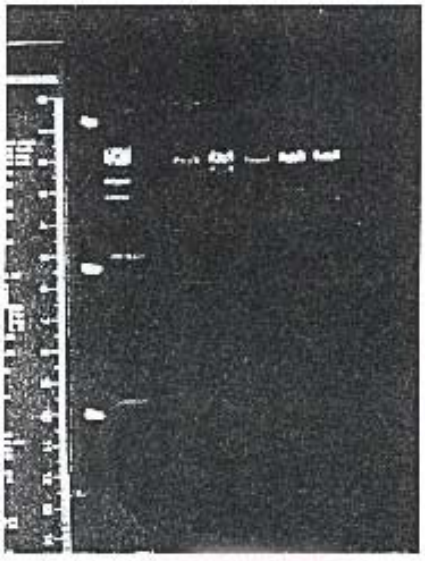

Fig.4 Electrophoresis gel of digested DNA from recombinant clone n0 8_2

Fig.5 Electrophoresis gel of digested DNA from recominant clone no. 9γ

CONCLUSION

1. In AO biosynthesis, the main enzyme responsible for AA biodegradation, there are involved at least four genes (AO_1, AO_2, AO_3 and AO_4).
2. Since genes AO_1, AO_2 and AO_3 had previously been sequenced and mapped, our effort was directed to sequencing and mapping of AO_4 gene. The genomic DNA for AO_4 is bordered at both ends by restriction sites of *Eco RI* and has a length of 2.9 kb.
3. The sequencing of genomic DNA for AO_4 showed a real length of 3072 bp, very close to that measured based on *Eco RI* restriction sites at the both ends of the respective fragment (2.9 kb).
4. Genomic DNA for AO_4 shows a high similarity (94.459%) to cDNA from cucumber. At the 3' end, the melon DNA comprises a sequence of 802 bp nonhomologous to that of cDNA from cucumber. Additionally, the AO_4-DNA does not show the entire length of the similar phragment in cucmber, missing a sequence of 1163 bp.

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[1] University of Iraklion, Greece
[2] mardelean@personal.ro, * to whom correspondence should be addressed
Faculty of Horticulture, Univ.of Agric.Sci., Cluj-Napoca, Romania
Phone: (40).64.196384
Fax: (40).64.193792
[3] Faculty of Agriculture, Univ.of Agric.Sci., Cluj-Napoca, Romania
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