BIOLOGIA PLANTARUM 34 (5-6): 387-394, 1992

Activity and isoenzyme composition of peroxidase and esterase in fertile and male-sterile lines of tomatoes (*Lycopersicon esculentum* Mill.)

M. MARKOVA, M. ANCHEVA and B. ATANASOVA

Institute of Genetics "Academician Doncho Kostov", Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract

A comparative study of the isoenzyme patterns of esterase and peroxidase and overall peroxidase activity in stamens of male-sterile (MS) lines of Pearson ms-35 and P ms-35aa and of the respective male-fertile (MF) tomato plants have been conducted. The study has been made at two stages of stamens development - tetrad and pollen. Higher activities of the esterase isoenzymes in the MF stamens than that of MS in both ontogeny stages have been found. The slow moving esterase isoenzymes both of the MF and the MS stamens are the major isoenzymes in the early stage and are connected with tapetum development while the fast moving esterase isoenzymes are connected with pollen formation in the later ontogeny stage. Overall peroxidase levels in the MS stamens were higher than those of MF. The peroxidase patterns of the MS lines are also characterized by the greater number of isoenzymes and also the presence of specific isoenzymes, the contrast between the MF and the MS stamens being more strongly expressed at the later stage of development. A strong similitude between esterase and peroxidase patterns behaviour in both MS lines has been found.

Introduction

Male sterility (MS) in plants has recently become a problem of great importance to selection programs. It has found wide application in maize, onion *etc.* where cytoplasmic male-sterile (CMS) lines are used. However, of no lesser importance is gene male sterility (GMS) which can be used in such crops as tomatoes where CMS has not been observed. While CMS has been studied in detail in terms of biochemistry and molecular genetics, GMS has been the subject of some biochemical studies (Markova and Daskaloff 1976, Bhadula and Sawhney 1987).

Received 10 October 1990, *accepted* 26 February 1991.

Tomatoes are an interesting subject of biochemical studies because of the availability of all types of GMS: functional pollen, stamenless.
Better studied have been the stamenless mutants (Bhadula and Sawhney 1987). Relatively less attention has been focussed on the activity of genes conditioning pollen sterility which, at least for the time being, finds wider application in hybrid seed production.

According to some researchers, stamens development is closely linked to changes in activity and peroxidase and esterase isoenzymes (Ahokas 1976, Jaiswal and Kumar 1980, Nave and Sawhney 1986, Sawhney and Nave 1986). Considering this, we have sought in our work the connection between esterase and peroxidase isoenzyme composition and overall peroxidase activity on one hand and GMS manifestations in two pollen-sterile lines of tomatoes whose sterility depends on the gene ms-10^{35} on the other.

Material and methods

Stamens of male-sterile (MS) lines of Pearson ms-35 (of the TGC collection) and P ms-35aa (selected at the Institute of Genetics, Sofia) and the respective male-fertile (MF) tomatoes (*Lycopersicon esculentum* Mill.) were studied. The MS lines do not produce pollen. It is well known that the gene ms-35 is allellic with ms-10 (Philouze 1970) and is localized in chromosome II. The study was carried out in two stages of stamens development, tetrad and pollen. Stamens were fixed in alcohol acetic acid (3:1) and their stage was established by squash preparations stained with 4% acetocarmine. Plants were grown in greenhouse. The enzyme extractions were obtained with 0.05 M Tris-HCl buffer (pH 7.2) containing 6 mM ascorbic acid, 6 mM cystein hydrochloride and 0.5 M saccharose. Electrophoretic resolution was done by the method of vertical disc electrophoresis on 7% polyacrylamide gel (pH 8.9) according to Davis (1964). Electrophoresis was carried out at 2 mA per tube for the first 15 min and at 3 mA for an additional 2 h. On each tube were loaded 100 μg protein for the peroxidase isoenzymes and 300 μg for the esterase isoenzymes. Protein content was estimated after Lowry *et al.* (1951). Staining of peroxidase isoenzymes has been done by the procedure described by Ladygina *et al.* (1970) and of esterase isoenzymes as proposed by Macko *et al.* (1967). The isoenzymes were registered at 540 nm on *ERY-65* automatic densitometer *F* (*Zeiss, Jena*). Overall peroxidase activity was determined by the method of Herzog and Fahimi (1973). The method is based on peroxidase oxidation of the diaminobenzidine (DAB) substrate to a red-brown compound. Reaction kinetics has been traced by measuring absorbance increase at 465 nm where absorption maximum of reaction product's oxidized DAB, is located. The enzyme units have been defined as μM (H_2O_2 destructed) min^{-1} mg^{-1} (protein). Overall peroxidase activity determination was repeated four times and isoenzyme analysis - six times. Statistical deviation was calculated according to Plokhinskii (1970).

Results

The densitometer tracings of the esterase isoenzymes of MF and MS stamens at both stages studied are illustrated in Fig. 1.

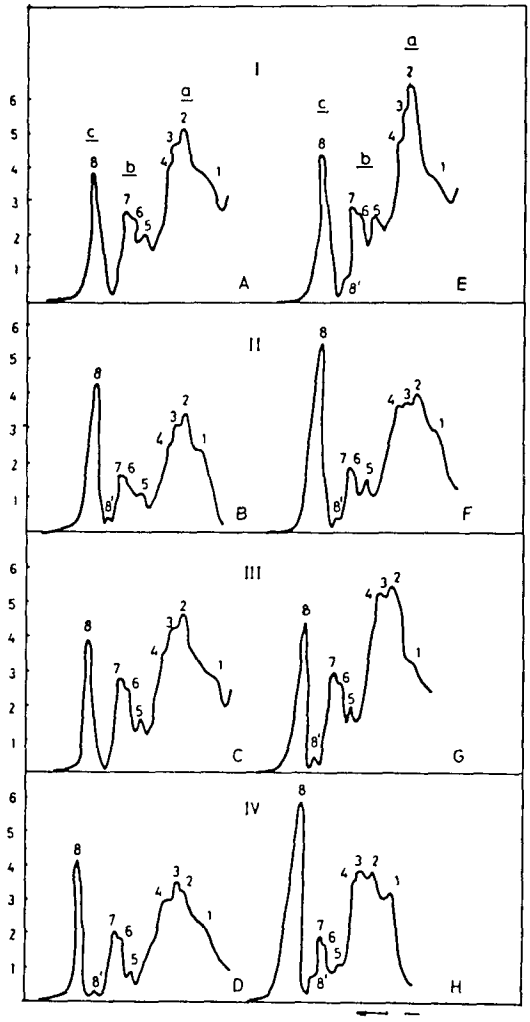

Fig. 1. A-H. Densitometer tracings of esterase isoenzymes in stamens of tomatoes (*L. esculentum* Mill.): (A,B - male-sterile line Pearson ms-35; (C,D) - male-sterile line P ms-35aa; (E,F) - male-fertile plants Pearson ms-35; (G,H) - male-fertile plants P ms-35aa.
I-IV - stamen development stages: (I,III) - tetrad stage; (II,IV) - pollen stage.
a- area of slow moving esterase isoenzymes; *b* - area of moderately moving esterase isoenzymes; *c* - area of fast moving esterase isoenzymes.

As evident, in terms of phenotype form of the densitometric profiles of the individual esterase isoenzymes three areas, based on their electrophoretic mobility, are visible. The area of the slow moving isoenzymes contains four molecular forms, peaks 1 to 4, and is marked as *a*.

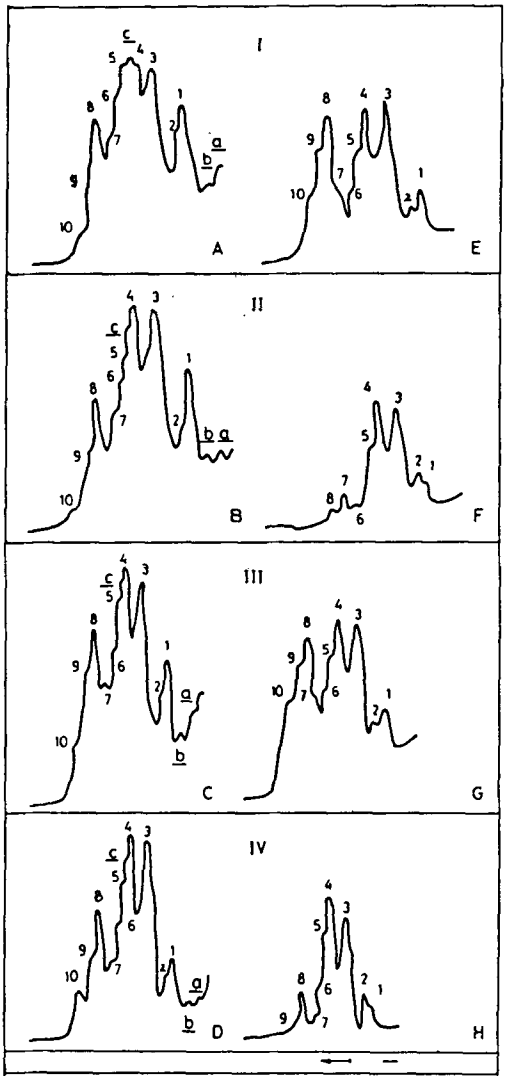

Fig. 2. A-H. Densitometer tracings of peroxidase isoenzymes in stamens of tomatoes (*L. esculentum* Mill.): (A,B) - male-sterile line Pearson ms-35; (C,D) - male-sterile line P ms-35aa; (E.F) - male-fertile plants Pearson ms-35; (G,H) - male fertile plants P ms-35aa.
I-IV - stamen development stages; (I,III) - tetrad stage; (II, IV) - pollen stage.

The area of the moderately moving isoenzymes contains three bands, peaks 5 to 7, and is marked as *b*. The area of the fast moving isoenzymes, consisting of two isoenzymes, peaks 8' and 8 (or 8 only), is marked as *c*. The described arrangement of the individual isoesterases along the gel refers to all genotypes studied in both ontogeny stages. Isoenzyme composition of esterase in MF and MS stamens remains the same with the exception of the sterile plants in the first stage (I-A, III-C) where the least active isoenzyme No 8' is absent in area *c*. In fact, this is the only difference in quality in the patterns between MF and MS stamens. Therefore, peak No 8' is specific for the patterns of the fertile stamens in the tetrad phase. However, differences have been observed in isoesterase activity in the various areas of the ontogeny process. The most characteristic difference is that all MF stamens generally exhibit a higher esterase activity compared to MS as evidenced by the height of their peaks in areas *a* and *c* (although they are the same in area *b*). In the tetrad stage (I, III) the isoenzymes of area *a* show the highest activity both in MF and MS stamens and for that reason area *a* is called major area. In the second stage studied (II, IV) a significant change in activity in areas *a* and *c* occurs. The isoenzymes in area *a* decrease their activities and this is more contrasting in MF stamens. Peak No 8 (area *c*) becomes the major isoenzyme and increases considerably its activity in MF stamens and slightly in MS line Pearson ms-35; however, it remains almost unchanged in the case of the P ms-35aa line. The change in area *b* is not specific - it decreases its activity both in MS and in MF stamens.

Peroxidase activities in MF stamens are lower than those in MS stamens (Table 1). This relationship holds for both ontogeny stages, however, in the pollen stage of the Pearson ms-35 line this decrease is almost 20-fold. During the ontogeny, the peroxidase levels drop both in the MF and the MS stamens.

Table 1. Peroxidase activity [μM H_2O_2 destructed min^{-1} mg^{-1}(protein)] in male-sterile (MS) and male-fertile (MF) stamens of tomatoes in the tetrad and the pollen stages.

Genotype	Tetrad stage		Probability	Pollen stage		Probability
	MS	MF		MS	MF	
Pearson ms-35	5.46	0.82	0.99	4.43	0.23	0.99
P ms-35aa	3.34	1.43	0.95	2.11	0.66	0.99

At the stage of tetrads the MF stamens have 10 isoperoxidases each (Fig. 2. I-E, III-G). In the patterns of the sterile lines (I-A, III-C), apart of these 10 isoperoxidases, there are also 3 isoenzymes which are specific for them only and are marked as *a*, *b* and *c*. Therefore, total number of peroxidase isoenzymes in the sterile lines is 13. Activity of the slow and the moderately moving peroxidase isoenzymes, up to No 8, is higher in the MS stamens. In the pollen stage isoenzymes composition in the MS stamens has been entirely preserved (II-B, IV-D). In the fertile lines it is still more reduced (II-F, IV-H) where the patterns of Pearson ms-35 have two components less and those of P ms-35aa one component less. It is noteworthy that

this isoenzyme is the fastest moving one (No 10) and is common for both sterile lines. All isoperoxidases in the MS stamens are more highly active.

Discussion

The esterases (EC 3.1.1.1) are specifically associated with tapetum development and pollen walls formation (Vithanage and Knox 1976). The higher activities of the isoenzymes (in areas *a* and *c*) found in MF stamens at both ontogeny stages are an indication of their normal development. In the tetrad stage of all genotypes the major area is *a* which can be definitely linked with tapetum development. The fact that the major isoenzyme at the pollen stage is No 8 of area *c* suggests its links with the formation of pollen walls in the fertile stamens where it increases its activity significantly. In MS stamens it almost does not change its intensity and this might be an indication of the absence of pollen grains (Karim *et al.* 1984). Absence of specific esterase isoenzymes, less isoenzymes or lower activity have been also found in CMS lines as compared to their fertile counterparts (Markova and Daskaloff 1976; Van Marrewijk *et al.* 1986). The latter authors are of the opinion that changes in esterase activity and its molecular composition are the result of degeneration processes in tapetum. Our results show certain quantitative and qualitative differences as well as a specific isoenzyme in the MF stamens, namely in the early stage of their development. For that reason we support the opinion of Bhadula and Sawhney (1987) that differences in patterns between MF and MS stamens are not the result of degeneration processes but are due to male sterility itself. Cytological studies of the MS line of tomatoes with gene ms-35 have also indicated that abnormalities in the tapetum cannot be the cause of sterility (Kruleva and Georgiev 1979). A serious argument in support of our contention is the observation that in CMS maize restored to fertility in the presence of the Rf gene the esterase pattern returns to normal (Abbott *et al.* 1984).

The peroxidases (EC 1.11.1.7) in connection with CMS have been studied in great detail with respect to their total activity and their molecular composition but the results are strongly contradictory in character. The higher levels of peroxidase activity found in the early stage are probably connected with the thickening of cell walls of the endothecium (Sawhney and Nave 1986). Differences in overall peroxidase activity and isoenzymes pattern between MS and MF stamens, found by us, are particularly contrasting in the late stage studied. The reason for that is the absence of pollen in the MS stamens. It is believed that plant development processes are controlled by selective gene expression which exerts its effects in part through synthesis and/or activation of enzymes and other proteins. Therefore, it is reasonable to assume that the structural changes observed during ontogeny of plant organs must be preceeded by/or concomitant with changes in the activities of various enzymes (Nave and Sawhney 1986). The greater number of isoperoxidases and the presence of specific peroxidase isoenzymes is considered to correlate with anthers degeneration (Iwasaki 1972). Studies cited above, as well as those in the discussion of esterase patterns, have been carried out entirely with CMS lines. The reason for the analogy with CMS is that GMS has been scarcely studied from biochemical

viewpoint. Besides, there is evidence for a great similitude in the behavior of GMS and CMS lines with respect to the activity of some enzymes (Markova and Daskaloff 1976) as well as with respect to the disturbances of the normal formation of the callosa (Georgieva and Slavova 1985). Based on the study carried out and the results obtained the following conclusions can be made.

Esterase isoenzymes activity is higher in the MF stamens in both stages studied. A differentiated role of the slowly moving and of the fast moving esterase isoenzymes during stamens ontogeny has been established. The esterases can be included in the expression of male sterility in tomatoes but probably as the consequence and not as the cause.

The MS stamens have higher levels of peroxidase activity and a greater number of isoperoxidases. Contrast between MF and MS plants is more strongly expressed in the second case studied when no pollen is formed in the MS stamens. The similarity in behavior of peroxidase patterns in both MS lines is preconditioned by the activity of gene ms-10^{35}, as is in the case of esterase patterns, and depending on the genotype insignificant differences have been also observed.

References

Abbott, A.G., Ainsworth, C.C., Flavell, R.B.: Characterization of anther differentiation in cytoplasmic male sterile maize using a specific isozyme system (esterase). - Theor. appl. Genet. **67**: 469-473, 1984.

Ahokas, H.: Evidence of a pollen esterase capable of hydrolizing sporopollenin. - Experientia **32**: 175-177, 1976.

Bhadula, S.K., Sawhney, V.K.: Esterase activity and isozymes during the ontogeny of stamens of male sterile *Lycopersicon esculentum* Mill., a male sterile stamenless-2 mutant and low temperature-reverted mutant. - Plant Sci. **52**: 187-194, 1987.

Davis, B.J.: Disc electrophoresis. I. Method and application to human serum protein. - Ann. New York Acad. Sci. **121**: 404-427, 1964.

Georgieva, J.D., Slavova, M.: [Cytochemical study of callose during microsporogenesis of *Capsicum annuum* L. with various types of sterility.] - Fiziol. Rast. (Sofia) **7**: 303-306, 1985. [In Bulg.]

Herzog, V., Fahimi, H.D.: A new sensitive colorimetric assay for peroxidase using 3, 3-diaminobenzidine as hydrogen donor. - Anal. Biochem. **55**: 554-562, 1973.

Iwasaki, F.: The characteristics of isozyme in male sterile rape (*Brassica napus* L.). - Jap. J. Breed. **22**: 247-276, 1972.

Jaiswal, V.S., Kumar, A.: Change in peroxidase and its multiple forms in relation to sex differentiation in *Coccinia indica*. - Biochem. Physiol. Pflanz. **175**: 578-581, 1980.

Karim, M.A., Mehta, S.L., Singh, P.M.: Studies on esterase isoenzyme patterts in anthers and seeds of male sterile wheats. - Z. Pflanzenzücht. **93**: 309-319, 1984.

Kruleva, M., Georgiev, H.: [Microsporogenesis and tapetum development of tomato sterile counterparts carriers of the genes ms-32 and ms-35.] - Genet. Selekt. (Sofia) **12**: 129-135, 1979. [In Bulg.]

Ladygina, M.E., Taimla, E.A., Rubin, B.A.: [Isoenzyme composition of peroxidase and polyphenoloxidase during tobacco viral deseases.] - Fiziol. Rast. **17**: 928-936, 1970. [In Russ.]

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randale, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Macko, V., Honald, G.R., Stahmann, R.: Soluble proteins and multiple enzyme forms in early growth of wheat. - Phytochemistry **6**: 465-471, 1967.

Markova, M., Daskaloff, S.: Biochemical investigation of male sterile mutant forms of pepper (*Capsicum annuum* L.). - Z. Pflanzenzücht. **77**: 296-303, 1976.

Nave, E.B., Sawhney, V.K.: Enzymatic changes in post-meotic anther development in *Petunia hybrida*. I. Anther ontogeny and isozyme analysis. - J. Plant Physiol. **125**: 451-465, 1986.

Philouze, J.: Further studies with male-sterile mutants ms-32 and ms-35. - Rep. TGC **20**: 45, 1970.

Plokhinskii, N.A.: [Criteria of probability of differences.] - In: Biometriya. Pp. 33-36. Izd. Moskov. Universiteta, Moskva 1980. [In Russ.]

Sawhney, V.K., Nave, E.B.: Histochemical localization of esterase, peroxidase, malate- and alcohol dehydrogenase. - J. Plant Physiol. **125**: 467-473, 1986.

Van Marrewijk, G.A.M., Bino, R.G., Suurs, L.C.J.M.: Characterization of cytoplasmic male sterility in *Petunia hybrida*. I. Localization, composition and activity of esterase. - Euphytica **35**: 77-88, 1986.

Vithanage, H., Knox, R.B.: Pollen wall proteins: quantitative cytochemistry of the origins of intine and exine systems in *Brassica oleracea*. - J. Cell Sci. **21**: 423-435, 1976.