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Isozyme studies in *Nicotiana suaveolens, N.glutinosa* and their interspecific hybrid: Genetic control of phosphoglucomutase and glutamic-oxaloacetic-transaminase

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Summary. Two isozyme systems, phosphoglucomutase and glutamic-oxaloacetic-transaminase, have been studied in leaves of *Nicotiana suaveolens, N.glutinosa* and their interspecific hybrid. By analyzing the different isozyme patterns in the hybrid a model for the genetic control of these systems has been proposed. Phosphoglucomutase appears to be controlled by a single locus and glutamic-oxaloacetic-transaminase behaves as a dimeric isozyme system, being controlled by at least two loci.
Key words. Nicotiana suaveolens; N.glutinosa; interspecific hybrid; glutamic-oxaloacetic-transaminase; phosphoglucomutase.

Except in rare cases[2,3] it is very difficult to study the genetic control of certain isozyme systems in species of the genus *Nicotiana* through the methods of formal genetics, which use the crossing among plants of different isozyme patterns, and statistical analysis of the hybrid plant segregations. The major reason for this difficulty is the absence of variability in these species[4]. This problem has been avoided in other species[5] through aneuploid genetics, which analyzes the effects produced by dosage differences in chromosomes or chromosome segments to obtain genetic information[5]. The reasoning followed in our study of the structure and the genetic control of some isozymes in two *Nicotiana* species, was similar to that used by Hart and other authors. In our case, different genomes have been joined in one individual, the interspecific hybrid, which is used to analyze the relative intensities of isozyme patterns and the presence or absence of bands with intermediate migration.

According to the results obtained and having regard to the fact that the hybrid pattern always correspond to the sum of the parental ones, a model of genetic control for these species is proposed.

Material and methods. Two species of *Nicotiana* were used, *N.glutinosa (glu)*, which is a diploid species (2n = 24), and *N.suaveolens (sua)*, which is an allopolyploid species (2n = 32), the parental species of which are unknown. Seeds of *glu* were obtained from: The Indian Agricultural Research Institute, The University of London Botanical Supply Unit, The Botanic Gardens of the University of Birmingham and The Hortus Botanicus Bergianus of Stockholm. In all cases, *glu* plants showed the same isozyme patterns. Seeds of *sua* were also obtained from different sources, but only in one case, namely seeds received from Instituto Tecnológico del Tabaco de Sevilla, did *sua* plants have 32 chromosomes; in the other cases they have 64 chromosomes. These latter ones were not used. The interspecific hybrid *(sua × glu)* was obtained from crossing *sua* from Instituto Tecnológico del Tabaco and *glu* from Hortus Botanicus Bergianus. Isozyme analysis was carried out with a crude extract of leaves. Phosphoglucomutase (PGM) and glutamic-oxaloacetic-transaminase (GOT) isozymes were studied using 0.015 M Tris-citric acid, pH 7.75 as the gel buffer (12% starch) and 0.3 NaOH-boric acid, pH 7.0 as the electrode buffer. All the crude leaf extracts were analyzed simultaneously for all the above mentioned systems, by using 1 cm thick gels, spliced into 2-mm slabs before staining.

The slabs were stained after Bewer and Sing[6] (PGM), Selander et al.[7] and Schwartz et al.[8] (GOT).

Each gel activity zone has been called by the name of the isozyme system studied and numbered in order from the faster to the slower mobility. The implicated loci have been given the same name as the activity zones, and their alleles have sub-indices added to the locus name, the number 1 corresponding to the allele with the faster mobility, 2 to the next one and so on. Each sub-index is followed by the letter s or g to indicate whether the allele corresponds to *sua* or *glu* loci.

Results and discussion. Phosphoglucomutase (PGM): One activity zone has been observed in both species. *Sua* showed two bands numbered 1 and 2 and *glu* only band 1 (fig.). The electrophoretic pattern of the hybrid shows both isozymes. In rye[9] and barley[10] this isozyme system has been described as monomeric with a monogenic control.

It seems likely that the system is controlled by only one locus (PGM) with at least two alleles (PGM_{1s} and PGM_{2s}) for *sua* and one (PGM_{1g}) for *glu*. In the case of *glu*, PGM_{1g} appeared to be fixed, while *sua* had two alleles, being a fixed heterozygote. This last species has been previously described as an amphiploid; therefore it can maintain the heterozygosity in this locus, in spite of its autogamous character; as Adams and Allard[11] demonstrated by electrophoretic techniques, allopolyploids can maintain heterozygosity in homozygous autogamous plants because they can have different alleles fixed in each genome.

Glutamic-oxaloacetic-transaminase (GOT): In both parental species it is possible to distinguish the isozyme pattern into two different activity zones, namely GOT-1 and GOT-2 (fig.).

GOT-1 presented three bands, numbered 1, 2 and 3. *Sua* showed all the three isozymes, band 2 always being more intense than 1

Glutamic-oxaloacetic-transaminase system (GOT)

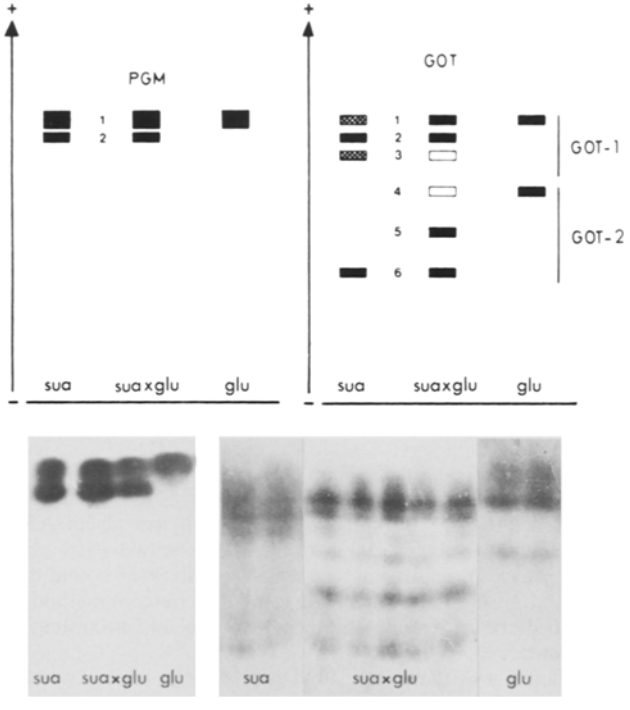
A) GOT genes involved in each genome

Species	*sua*		*sua* × *glu*			*glu*
Genomes	S_1	S_2	S_1	S_2	G	G
GOT-1 genes*	GOT-1_{1s}	GOT-1_{2s}	GOT-1_{1s}	GOT-1_{2s}	GOT-1_{1g}	GOT-1_{1g}
Subunit coded	α	β	α	β	γ	γ
GOT-2 genes*	GOT-2_{2s}	GOT-2_{2s}	GOT-2_{2s}	GOT-2_{2s}	GOT-2_{1g}	GOT-2_{1g}
Subunit coded	ε	ε	ε	ε	μ	μ

B) Theoretical intensities of *sua* × *glu* bands

	Band	Subunits	Theoretical intensities
GOT-1	1	$\alpha\alpha$ $\alpha\gamma$ $\gamma\gamma$	4
GOT-1	2	$\alpha\beta$ $\gamma\beta$	4
GOT-1	3	$\beta\beta$	1
GOT-2	4	$\mu\mu$	1
GOT-2	5	$\mu\varepsilon$	4
GOT-2	6	$\varepsilon\varepsilon$	4

*GOT-1_{1s} and GOT-1_{2s} would be allelic genes. GOT-1_{1g} would be a gene related to GOT-1_{1s} and GOT-1_{2s}, belonging to the same gene family, but no necessary allelic. The same in the case of GOT-2_{2s} and GOT-2_{1g}.



Phosphoglucomutase and glutamic-oxaloacetic-transaminase patterns of *Nicotiana suaveolens (sua), N.glutinosa* (*glu*) and their interspecific hybrid *(sua* × *glu)* leaves.

and 3, and with a intermediate migration. *Glu* showed only isozyme 1. The hybrid also had the three isozymes but with a different staining relationship to *sua*. In all the zymograms, bands 1 and 2 always showed a much higher intensity than band 3 when the *sua* × *glu* pattern was analyzed (fig.). According to these data, the patterns of the parental species were in accordance with the hypothesis of one single locus controlling GOT-1 in each species, the diploid *glu* being homozygous GOT-1_{1g} GOT-1_{1g} and the amphiploid species *sua* being a fixed heterozygote GOT-1_{1s} GOT-1_{1s} GOT-1_{2s} GOT-1_{2s}, where GOT-1_{1g} and GOT-1_{1s} codified for the fastest migrating subunits of a dimeric enzyme, and GOT-1_{2s} for the slowest one (table). According to this hypothesis, and having regard to the amphiploid nature of *sua,* the *sua* × *glu* hybrid has three different genomes, two from *sua* and one from *glu* (table). The two genomes contributed by *sua* have two different alleles, one in each and the genome contributed by *glu* carried the gene GOT-1_{1g}. So, the expected pattern of dimeric association must show the three isozymes with a staining relationship of 4:4:1 (table); as can be seen in the figure, the hybrid pattern agreed with this hypothesis.

GOT-2 (fig.) showed only one band in each species, number 4 in the case of *glu* and number 6 in *sua*. The hybrid *sua* × *glu* presented both bands as well as a hybrid band with intermediate mobility, number 5, that could be interpreted as an heterodimer formed by the monomers of bands 4 and 6. According to the isozyme patterns obtained, GOT-2 seemed to have at least one allele in each species, GOT-2_{1g}, which is fixed in *glu,* and GOT-2_{2s}, which is fixed in *sua* (GOT-2_{1g} and GOT-2_{2s} have not necessary to be allelic between them, see table). Taking into account such hypothesis, as well as the possible dimeric character of GOT-2 indicated by the presence of hybrid isozyme in *sua* × *glu,* and the amphiploid character of *sua*, it is possible to calculate the expected relation of intensities for the three bands shown in the *sua* × *glu* pattern (table). This relation must be 1:4:4 corresponding to the bands 4, 5 and 6 respectively, because the genotype of *sua* × *glu* must be GOT-2_{1g} GOT-2_{2s} GOT-2_{2s}. The analyzed data from the *sua* × *glu* zymograms are in agreement with the postulated hypothesis, showing bands 5 and 6 similarly stained and always with more intensity than band 4 (fig.).

Our results are in agreement with those of other authors. Thus, it has been reported that GOT behaves in other plant species as a functional dimer. That is the case in *Zea mays*[12,13], *Triticum*[14-16], *Hordeum*[10,17] and *Secale*[9,16]. Similarly, different activity zones in GOT have been described[9,13,16].

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Generation of multiple molecular forms of juvenile hormone binding protein from *Galleria mellonella* hemolymph[1]

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Summary. The hemolymph fraction containing juvenile hormone binding protein (JHBP) has been shown to generate multiple molecular forms of JHBP under given experimental conditions. They differ in affinity toward ionic exchanger and in pI values, but exhibit unchanged Stokes radius, sedimentation coefficient and K_d for the hormone. This process is inhibited by phenylmethylsulfonyl fluoride or high ionic strength and presumably reflects an initial step of proteolytic modification of the JHBP.
Key words. Galleria mellonella; juvenile hormone; juvenile hormone binding protein; hemolymph; proteolysis.

It is generally accepted that juvenile hormone (JH) is transported in the hemolymph of insects complexed with binding proteins. At present, two general classes of hemolymph JH binding proteins can be distinguished on the basis of binding affinity: 1) low affinity, low specificity, high capacity, high molecular weight proteins, and 2) high affinity, high specificity binding proteins. Based on molecular weight the latter class can be divided into two groups, those greater than 100,000, and those below 30,000[2]. The only example of such a protein with a mol.wt below 30,000, which has been purified to apparent homogeneity, is JHBP from the hemolymph of *Manduca sexta*[3-5]. Kramer and coworkers[3] obtained two preparations of JHBP with isoelectric points 4.95 and 5.25 from the hemolymph of mid-fifth instar larvae of *Manduca sexta*. It was shown that purified JHBP, CP-α, with pI 4.95 has the same pI as JHBP in the hemolymph subjected to Sephadex G 100 chromatography. The second component, CP-β, of pI 5.25 which can be partially converted to CP-α form, was claimed to be an artifact of unknown origin.

During our attemps to isolate JHBP from the hemolymph of *Galleria mellonella* we observed the generation of new JHBP molecular forms[6]. In this paper the effect of salt concentration and prolonged incubation of the JHBP crude sample on the generation of new molecular forms of the protein is studied. The developmental period of maximal JHBP concentration (the 5th day of the last instar larvae) was used for the collection of the hemolymph[7].

Materials and methods. DEAE Sephacel and Sephadex G 200 were obtained from Pharmacia. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma. JH homologs JH II and JH III were purchased from Sigma and radiolabeled (10-^{3}H) JH II and (10-^{3}H) JH III was purchased from NEN Chemicals. The concentration of JH solution was determined at 220 nm using extinction coefficient of 13,830 M^{-1} cm^{-1} according to Trautmann[8].

The *Galleria mellonella* (Lepidoptera, Pyrylidae) larvae were reared under standard conditions[9]. The hemolymph from last instar larvae (5th day) was collected and stored as described previously[7].

The crude sample of low mol.wt JHBP was isolated using Sephadex G 200 column chromatography[10]. The centrifuged hemolymph (3.6 ml) was applied to a Sephadex G 200 gel column (K 16/100, Pharmacia) equilibrated with 10 mM Tris-HCl buffer containing 100 mM NaCl and 0.25 mM 1-phenyl-2-thiourea, pH 7.3. Proteins were eluted at 5 °C with the same buffer at a flow rate of 12 ml/h; 2-ml fractions were collected. Low mol.wt fractions containing JHBP activity were combined, and then concentrated and equilibrated with 10 mM Tris-HCl, pH 8.5 by ultrafiltration (YM 10 membrane, Amicon).

Chromatofocusing was performed according to Sluterman and Elgersma[11] using the Pharmacia chromatofocusing kit. JH binding activity was measured as described previously[7], with slight modifications. To a glass tube coated with polyethylene glycol, 1 µl of 6.4×10^{-5} M JH III in hexane (18,000 dpm/µl) was added. After solvent evaporation, 200 µl of protein sample was added to each tube and carefully vortexed; after 30 min of incubation at 5 °C, 25 µl of freshly prepared charcoal suspension was added (0.15 g charcoal and 0.08 g dextran T 70 were added to 10 ml of 10 mM Tris-HCl buffer containing 100 mM NaCl, pH 8.5). This solution was incubated for 10 min at 5 °C and then centrifuged at 10,000 × g for 2 min also at 5 °C. The amount of bound hormone was determined by measuring the radioactivity in 150 µl of the supernatant.

Equilibrium constants, K_d, were determined as follows; increasing amounts of JH II ranging from 4.1×10^{-8} M to 1.4×10^{-6} M were added to glass tubes coated with polyethylene glycol. After evaporation of solvent, 200 µl of the JHBP sample was added. Then, after 5 h incubation at 5 °C, a separation of bound and unbound hormone was achieved using the charcoal method as described above. Results obtained were analyzed according to Scatchard[12].

JH esterase activity was measured as described previously[7]. Molecular weight was determined by gel filtration[13] using a Sephadex G 100 column (K16/100, Pharmacia) equilibrated with 10 mM Tris-HCl, 100 mM NaCl, pH 7.3, and using standards of known mol.wts (MS II, Serva).

Sedimentation coefficients were determined using ovalbumin as the external standard. 100 µl of protein sample was layered on top of a linear 5–20% sucrose gradient prepared in 10 mM Tris-HCl, pH 8.5 and centrifuged at 45,000 rpm at 5 °C in a SW 50 rotor for 21 h.

Results and discussion. It has previously been shown that a low mol.wt JHBP fraction can be completely resolved from high mol.wt JH binding activity using Sephadex G 200 chromatography[10]. In this paper, the low molecular weight fraction obtained from Sephadex G 200 column chromatography was used for further experimentation. When this fraction was transferred into 10 mM Tris-HCl, pH 8.5, and immediately applied to a DEAE Sephacel column, a single, slightly asymmetrical, peak of JHBP activity was eluted (fig. A). The prolonged incubation of a crude JHBP sample at 5 °C in the above low ionic strength buffer resulted in formation of new molecular forms of JH binding activity, with decreasing affinity for the DEAE Sephacel column