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NADP$^+$ glyceraldehyde-3-phosphate dehydrogenase in soybeans [*Glycine max* (L.) Merr.]: genetics and developmental expression*

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Summary. Chloroplastic (NADP$^+$) glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.9) catalyzes the second reaction in photosynthesis after the fixation of carbon by RuBisCO. Chloroplast-bound (NADP$^+$) G3PDH was resolved in soybean by starch gel electrophoresis using L-histidine-citrate buffer (pH 5.7). Histochemical staining revealed zymogram patterns indicative of a tetramer. A survey of soybean genotypes revealed differences in zymogram patterns between the principal cytoplasmic sources of the northern and southern US germplasms. In the soybean pedigree, an allelic frequency shift toward a five-banded pattern was observed. G3PDH polymorphism was due to allele associated with gene expression at the slow locus. No linkage was found between the slow locus of (NADP$^+$) G3PDH and *ACO2, ACO3, ACO4, ACP, DIA1, IDH1, IDH2, PGM1,* and *PGM3*. Developmental expression in the above-ground tissues was identical, whereas roots as a rule did not express (NADP$^+$) G3PDH activity. The importance of chloroplast-bound (NADP$^+$) G3PDH in photo-synthesis and its interesting mode of inheritance warrants further exploration of this enzyme in soybean.

Key words: (NADP$^+$) glyceraldehyde-3-phosphate dehydrogenase – Zymogram – Allelic shift – Inheritance – Developmental expression

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) has been biochemically characterized in many species including *Sinus alba*, *Pisum sativum*, and *Phaseolus vulgaris* (Pupillo and Piccari 1975; Ceriff 1978; McGowan and Gibbs 1974; Wara-Aswapati et al. 1980).

There are five forms of G3PDH in plants: (1) three NAD-dependent G3PDH that are involved in respiration; (2) a nuclear-encoded, chloroplast-bound NADP-dependent G3PDH that is part of the Calvin cycle; and (3) a nonreversible, phosphate-inhibited NADP-dependent cystolic enzyme (Kelly and Gibbs 1973). Chloroplast-bound (NADP$^+$)-G3PDH is the predominant form, whereas the phosphate-inhibited enzyme comprises approximately 1% of the total (NADP$^+$) G3PDH in the cell (Kelly and Gibbs 1973).

Chloroplast-bound (NADP$^+$) G3PDH (E.C. 1.2.1.9) is believed to be a tetramer (Pupillo and Piccari 1975; Cerff 1978). Cerff (1979) demonstrated that spinach, pea, and barley have two distinct subunits (A and B), which have an apparent molecular weight of between 36000 and 42000 Da. The subunits were found to be arranged in tetramers composed of A_4 and A_2B_2. Cerff (1982) described the evolutionary relationship of (NADP$^+$) G3PDH, based on the protein mobilities of 13 angiosperm species, concluding that chloroplast-bound (NADP$^+$) G3PDH has undergone rapid evolutionary change towards increased molecular diversity. Therefore, soybean G3PDH might be expected to show some differences from its counterparts characterized in *Pisum sativum* (McGowan and Gibbs 1974) and *Phaseolus vulgaris* (Wara-Aswapati et al. 1980).

Chloroplastic (NADP$^+$) G3PDH is the second enzyme in the photosynthetic pathway after the fixation of carbon by ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO). G3PDH converts 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. Expression of chloroplast-bound (NADP$^+$) G3PDH has been shown to be strongly light dependent in *Sinus alba* (Cerff 1978). Interestingly, Gowri and Campbell (1989) found that nitrate could stimulate enzyme production in the absence of functional chloroplasts.

Although the enzymatic pathway of photosynthesis has been characterized, only two enzymes, RuBisCO and sucrose-

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phosphate synthetase (SPS), have been shown to regulate the net production of photosynthate in a crop species (Murthy and Singh 1978; Losa-Tavera et al. 1987, 1990; Rocher et al. 1989). The importance of the genetic variation found in the other enzymes involved in photosynthesis is unknown. However, given the importance of this pathway and previous work with RuBisCO and SPS, any reduction in overall activity could have an effect on the yield potential of the plant.

In the study reported here we describe the detection of (NADP$^+$) G3PDH resolved by starch gel electrophoresis, determine the genetic diversity of this enzyme in soybean germ plasms, and examine the inheritance and developmental expression of (NADP$^+$) G3PDH in soybean.

Methods

Plant material

Germplasm for this study was obtained from the USDA Soybean Germplasm Collection at Urbana-Champaign, Illinois. Dr. E. Shipe (Clemson University) provided an additional source of the cultivar 'Peking'. The lines PI424020A and PI163453 are *Glycine soja* (L.) Siab. and Zucc.; all other lines used are *Glycine max* (L.) Merr. One hundred lines (five seeds per line) were analyzed for zymogram pattern. Crosses were made at Clemson University using greenhouse-grown plants. F_1 plants were grown at the USDA Winter Nursery, Puerto Rico. For the inheritance and linkage studies, F_1, F_2 and F_3 seed from interspecific ('Essex' × PI424020A and PI88788 × PI424020A) and intraspecific ('Peking' × 'Essex') crosses were germinated on germination paper at 28 °C for 72–96 h to allow primary roots to emerge.

Developmental analyses were conducted on 17 soybean lines. Six types of tissue were collected for study: dry seed, 3-day-old cotyledon, young expanding trifoliolate leaf, fully expanded trifoliolate leaf, and young roots. Cotyledons were germinated, and the seedlings were transplanted to the greenhouse and grown until the first trifoliolate leaf developed.

Sample collection

All samples were homogenized in a sample buffer consisting of 16.7% sucrose and 8.3% ascorbic acid adjusted to pH 7.4 with NaOH. Dry seeds were prepared by allowing crushed seed material to soak in extraction buffer at 4 °C for 1 h prior to homogenization. Fresh samples (cotyledons, leaves, and roots) were frozen at − 70 °C until used.

Electrophoresis

Starch gel electrophoresis was carried out with L-histidine-citrate buffers (pH 5.7 and pH 6.5) and 12.5% starch gels as described by Rennie et al. (1989). (NADP$^+$) (G3PDH) was resolved on L-histidine-citrate (pH 5.7). Aconitase (ACO), acid phosphatase (ACP), diaphorase (DIA), and isocitric dehydrogenase (IDH) were resolved on L-histidine-citrate (pH 6.5), whereas phosphoglucomutse (PGM) was resolved on L-histidine-citrate (pH 5.7) (Rennie et al. 1989).

In order to assay (NADP$^+$) G3PDH in soybeans, we modified the procedure described by Wendel and Weeden (1989). Solutions (I and II) were prepared prior to staining and mixed before pouring over the gel. Solution I consisted of 20 ml of 50 m*M* TRIS-HCl (pH 8.0), 75 mg arsenic acid, 100 mg fructose 1,6-diphosphate (Na salt), 10 mg NADP, 10 mg MTT, 2 mg PMS, and 100 units of aldolase (Sigma A1893). Solution II consisted of 1.5% Bacto agar melted in 50 m*M* TRIS-HCl (pH 8.0).

The assay for (NADP$^+$) G3PDH involves the enzymatic generation of the substrate glyceraldehyde 3-phosphate. To determine if other enzymatic pathways are contributing to the banding pattern observed, four control assays were conducted. In control assay I, TRIS buffer was replaced with 0.2 *M* sodium phosphate buffer (pH 8.0). This assay tests for possible nonreversible (NADP$^+$) G3PDH, which is inhibited by the presence of inorganic phosphate (Kelly and Gibbs 1973). Control assay II, the aldolase (ALD) assay, tested for a possible side-reaction with the substrate fructose 1,6-diphosphate. Control assay III, the triose phosphate isomerase (TPI) assay, tested for a cross-reaction with the generated substrate glyceraldehyde 3-phosphate. The ALD and TPI assays were conducted as described by (Delorme and Skorupska 1992). In control assay IV, purified glyceraldehyde 3-phosphate (Sigma G5251) was substituted for fructose 1,6-diphosphate.

Linkage analysis

Linkage analyses were conducted using the maximum likelihood method. Pair-wise comparisons, recombinant frequencies, and standard error were calculated using the statistical software *Linkage3* (Suiter et al. 1987).

Results and discussion

Detection

Since G3PDH has not previously been reported in soybeans, comparisons of enzymatically generated substrate and purified glyceraldehyde 3-phosphate were conducted. No difference was observed between the enzymatic generated and purified glyceraldehyde 3-phosphate. Furthermore, to insure that the bands observed were due to the action of G3PDH, additional staining for aldolase and triose phosphate isomerase was performed. We found no correlation between G3PDH bands and any bands observed for ALD or TPI. In the experiment conducted to eliminate the possibility of detecting nonreversible NADP-dependent G3PDH, we observed no reduction in enzyme activity upon the addition of inorganic phosphate. The retention of activity in the presence of phosphate indicated that the bands observed were not due to cystolic G3PDH. Since the detected enzyme was present in abundance and retained activity in the presence of inorganic phosphate, we concluded that the bands were due to the nuclear-encoded, chloroplast-bound (NADP$^+$) G3PDH (Fig. 1).

Among the 100 lines of *G. max* screened for expression of G3PDH, zymogram patterns of three and five evenly spaced bands were observed; the two-banded pattern was found only in *G. soja* plants being used for genetic tests (Fig. 2). A zymogram pattern of five evenly spaced bands is expected for a tetramer with two electrophoretically distinct forms.

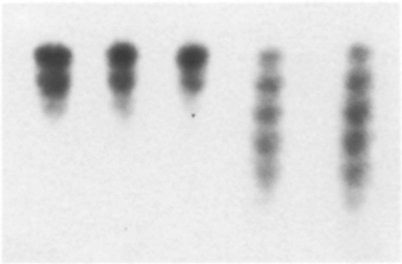

Fig. 1. Detection of the nuclear-encoded, chloroplast-bound (NADP$^+$) glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.9) in soybean

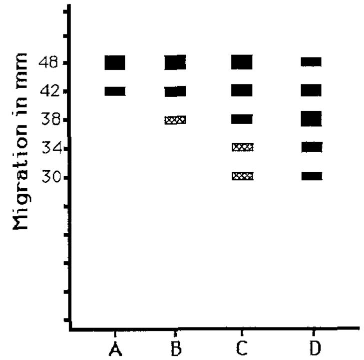

Fig. 2. Zymogram patterns of (NADP$^+$) glyceraldehyde-3-phosphate dehydrogenase in soybean (E.C. 1.2.1.9). *A* two-banded pattern observed in *G. soja*, *B* three-banded pattern observed in *G. max*, *C and D* five-banded weak (5w) pattern and five-banded strong pattern present in *G. max*, respectively

Since soybean is a diploidized tetraploid, the five-banded pattern could result from the interaction of two homologous loci with different electrophoretic forms.

There is a variant of the five-banded pattern that has weaker slow bands. We designated this pattern as 5W, with the 'W' standing for weak slow bands. This pattern can occur in tetramers when one form of the subunit is expressed at a higher concentration than the other form. For example, Scandalios (1974) described the differential expression of maize catalase gene loci that resulted in zymogram patterns of one to five bands. The unequal expression of allelic forms has been observed in many species and may be a common phenomenon in species diploidization (Weeden and Wendel 1989; Vodkin and Scandalios 1981; Goodman et al. 1980). In soybean (NADP$^+$) G3PDH, differences in the banding patterns were probably due to subunit availability, which shifts the equilibrium towards those forms that are comprised of A_4 and A_3B_1, with (A) representing the abundant subunit. The fast locus encodes for subunit A, and its expression was observed in all of the zymograms. In homozygous two- and three-banded soybean germplasms, slow bands were not observed. Therefore, the variation in (NADP$^+$) G3PDH was associated with the expression of the slow locus encoding subunit B (Fig. 2).

Allelic shift in soybean ancestry

The pedigrees of US soybean cultivars have been reported previously (Cheng and Hadley 1986; Allen and Bhardwaj 1987; Bernard et al. 1988). The principal cytoplasmic sources of the northern germplasm, 'Mandarin', 'Manchu' and 'A.K.' have three-banded patterns, whereas 'Mukden' has the five-banded pattern. In contrast, most of the cytoplasmic sources of the southern collection have the five-banded pattern or are heterozygous for G3PDH. The principal cytoplasmic sources in the southern collection which could have contributed alleles to the five-banded pattern are 'S100', and 'Roanoke'; for the three-banded pattern, 'Dunfield'. Cultivar 'Tokyo' could be a source for both patterns. When considering the cytoplasmic sources of the southern collection, we may need to separate 'S100' from 'A.K.'. Although 'S100' was a rogue from 'A.K.', this line shows allelic differences at at least four isoenzyme loci, including (NADP$^+$) G3PDH, and thus could represent an outcross or a misplaced seed.

Allen (1986) described the generation of the North American soybean cultivars based on the number of breeding cycles the cultivars had undergone. Since southern soybean ancestry exhibited more (NADP$^+$) G3PDH diversity, we analyzed southern soybean lines for G3PDH zymogram patterns over breeding cycles. The frequency of the zymograms was determined for each generation of cultivars. The three-banded pattern was present in 31.3% of the ancestral genotypes (Table 1). Interestingly, the percentage of heterogenous lines in a generation ranged from 32% for ancestral introductions to 0% for second- and third-generation

Table 1. Zymogram patterns of (NAPD$^+$) G3PDH in pedigrees of public soybean cultivars in the Southern United States

Zymogram	Percentage of tested lines per generation					
Pattern	Ancestral	First	Second	Third	Fourth	Fifth
3	31.3	14.3	25.0	14.3	0.0	0.0
5W	18.7	28.5	25.0	14.3	41.7	9.5
5	18.7	42.9	50.0	71.4	41.7	76.2
H^a	31.3	14.3	0.0	0.0	16.7	14.3
h^b	0.3385	0.3193	0.2599	0.1439	0.2571	0.1772

[a] Includes heterogenous lines for: 3 and 5; 3 and 5W; 5W and 5 banding patterns

[b] Diversity index calculated as $\frac{-\sum f_i l_n f_i}{n}$ where f_i = frequency of genotype and n = divergence of zymogram patterns

cultivars. In over 50 years of soybean breeding, there was a shift in frequencies with every generation away from the three-banded pattern, and simultaneously there was a corresponding increase in the frequency of the five-banded pattern. In fourth-generation cultivars, there was an increase in heterogeneity and in occurrence of 'five weak' zymograms. This situation could result from the increased number of backcrosses to ancestral and first-generation lines for the introgression of resistance genes from primitive germplasms.

The five-banded pattern can be traced to four cultivars: 'Hill' (most likely from 'Haberlandt'), 'Volstate' (through heterozygous 'Tokyo'), 'S100', and 'Roanoke'. The selection pressures which have resulted in the accumulation of five-banded G3PDH alleles from these ancestors could be due to the presence of tightly linked agronomically favorable genes, or an intrinsic agronomic disadvantage associated with the three-banded pattern, or both.

Since (NADP$^+$) G3PDH is part of a five enzyme complex located in the chloroplast and comprised of RuBisCO, phosphoribulokinase (PRK), ribose-phosphate isomerase (RPI), phosphoglycerate kinase (PGK), and G3PDH (Gontero et al. 1988), the interactions of G3PDH with another complex protein during photosynthesis may influence allelic selection in soybean. In the green alga, *Scenedesmus obliquus*, PRK and (NADP$^+$) G3PDH were found to form a multimeric protein complex (Nicholson et al. 1987). Light depolymerizes this PRK-G3PDH complex, which in turn activates the enzymes. In sugar beets the maximum activation states of PRK and (NADP$^+$) G3PDH occurs early in the light cycle, whereas there is a lag time for RuBisCO (Servaites et al. 1991). PGK has been reported to be highly active regardless of the light conditions (Heber et al. 1982). The light activation of PRK and G3PDH affects the substrate-to-product equilibrium of the carbon fixation reaction. The substrate concentration (ribulose 1,5-bisphosphate) increases through the action of PRK, while simultaneously the concentration of product (3-phosphoglyceric acid) decreases through the action of PGK and G3PDH. The 'improved' substrate-to-product ratio produces a highly favorable condition for the fixation of carbon by RuBisCO. Specific G3PDH alleles might interact differentially with other enzymes to create this favorable condition, which could be of selection value in plant breeding.

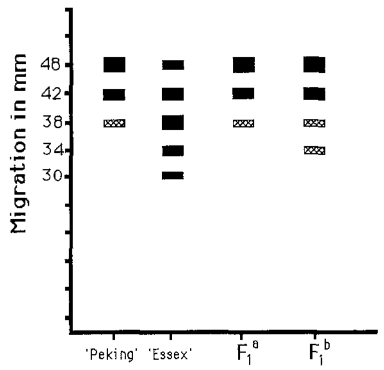

Fig. 3. Zymogram patterns of (NADP$^+$) glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.9) of 'Peking' × 'Essex' and their hybrids. F_1^a represents zymogram from reciprocal crosses, F_1^b appearance of the fourth band due to developmental expression in the hybrid plants

Inheritance and gene linkages

Intra- and interspecific crosses revealed that the two- and three-banded patterns were codominant with the five-banded pattern. Expression of G3PDH was found to be the same in ten F_1 plants from reciprocal crosses of cvs 'Peking' (three banded) and 'Essex' (five banded) (Fig. 3). This provides additional evidence for the nuclear origin of (NADP$^+$) G3PDH. In all crosses, segregation of $F_{2:3}$ progenies gave a good fit to a 1:2:1 ratio, indicating a single locus (Mahalingam et al., 1993). We did not examine the relationships between two- and three-banded patterns.

Glycine max and *Glycine soja* have 20 pairs of chromosomes ($2n = 4x = 40$). Palmer and Kiang (1990) published a morphological linkage map for soybeans that lists eighteen linkage groups (LG). Fifteen isoenzyme loci have been assigned to nine linkage groups 1, 4, 5(16), 8, 9, 11, 15, 17, and 18 (Palmer and Kiang 1990; Kiang 1990). Our analysis of recombination values did not reveal linkages for *ACO2*, *ACO3 ACO4*, *ACP*, *DIA1*, *IDH1*, *IDH2*, *PGM1*, and *PGM3*. As well, our study eliminates four (1, 9, 11, 17) of the nine linkage groups with mapped isoenzyme loci and possible linkages to five unassigned isoenzyme loci. Since over half of the linkages established on the conventional soybean map have a recombination value of 20 or more centiMorgans (cM), and eight linkage groups are comprised of only two loci, there is a possibility that the slow locus of (NADP$^+$) G3PDH may be located on the same chromosome as one of the eliminated linkage groups. This study points out the need for a high density molecular map to establish the genetic relationships of chromosomes in soybeans.

Developmental expression

Tissue from various plant organs and developmental stages were examined to determine the expression of (NADP$^+$) G3PDH during development. The genotypes tested represented all zymogram variations of G3PDH determined for cultivated soybeans and F_1 heterozygous plants. Dry seeds, 3-day-old cotyledons, and young and mature leaves had identical zymogrms in the genotypes tested. In contrast, roots as a rule did not express G3PDH. A low expression of G3PDH was detected in only 1 cultivar; the roots of this line had high numbers of nodules, whereas other lines had not developed extensive nodules before sample collection. In maize leaves, $(NADP^+)$ G3PDH mRNA levels increase proportionally to nitrate reductase mRNA levels (Gowri and Campbell 1989). Sequence analysis of cDNA clones in maize showed nitrate reductase and $(NADP^+)$ G3PDH to be closely related (Gowri and Campbell 1989). Thus, the expression of $(NADP^+)$ G3PDH in roots may be due to an evolutionary relationship between these enzymes that has left some residual linkage in gene expression. The relationship between nitrate reductase and G3PDH in plant developments needs to be examined.

We observed that in cotyledons which were collected during the early stages of greening additional weak bands appeared in F_1 heterozygous plants (Fig. 3). These additional bands were not present in green cotyledons or in the leaves. Since the present of transition to the photosynthetic state involves the active synthesis and assembly of the photosynthetic apparatus, it may be assumed that G3PDH synthesis is elevated. The G3PDH protein is part of a five-enzyme complex that includes RuBisCO and other dark reaction enzymes (Gontero et al. 1988). The appearance of additional bands during a period in which G3PDH is in high demand suggests incomplete dominance during periods of active G3PDH gene transcription.

Conclusion

Based on the genetic analysis and developmental expression, we suggest that the observed polymorphism of $(NADP^+)$ G3PDH is due to the reduced expression at the slow locus. Given the importance of $(NADP^+)$ G3PDH in photosynthesis it would be worthwhile to further explore the physiological importance of preferential allelic selection in soybean.

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