Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5360–5364, September 1980
Genetics

Genetic regulation of the catalase developmental program in maize scutellum: Identification of a temporal regulatory gene

(gene expression/enzyme turnover/additive *trans*-acting gene)

J. G. SCANDALIOS, D.-Y. CHANG, D. E. MCMILLIN, A. TSAFTARIS, AND R. H. MOLL

Department of Genetics, North Carolina State University, Raleigh, North Carolina 27650

Communicated by Frank H. Ruddle, May 23, 1980

ABSTRACT **Genetic and biochemical analyses suggest that the developmental program of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) activity in maize scutella is controlled by a temporal regulatory gene (*Car1*) that is distinct from the structural genes thus far identified. Recombination data show that *Car1* is located about 37 map units from the *Cat2* structural gene on the chromosome 1S. Turnover studies indicate that *Car1* may act by regulating the rate of catalase synthesis.**

The enzymes in specific tissues of an organism usually express a particular developmental pattern (1). Developmental mutants altering the timing for the expression of a given enzyme encoded by a specific gene can be used to study the genetic control of gene expression in eukaryote development. Such mutants have been identified in mice and other organisms (2–6).

In maize, catalase (H_2O_2:H_2O_2 oxidoreductase, E.C. 1.11.1.6) is an enzyme for which the genetic background and developmental program have been thoroughly characterized (7–9). Catalase in maize scutella is coded by two loci, *Cat1* (expressed during kernel maturation) and *Cat2* (expressed in the scutellum during germination) (9, 10). The expression of these two structural genes is regulated by several factors (10, 11); among these are different synthesis and degradation rates for the catalase gene products (CAT-1 and CAT-2) of *Cat1* and *Cat2* (10). One inbred line of maize, R6-67, has been found to express an altered developmental program for catalase in the scutellum as compared with the standard profile exemplified by the inbred strain W64A. Herein, we report on the genetic analysis and regulation of the catalase developmental program in maize scutellum.

MATERIALS AND METHODS

The highly inbred maize strains W64A, R6-67, and R6-43 were used in these experiments; these lines are maintained by our laboratory. Seeds were surface-sterilized by soaking in 1% sodium hypochlorite solution for 10 min and were germinated on moist germination paper at a constant temperature of 25°C.

Isolated scutella were ground with a mortar and pestle in 25mM glycylglycine buffer (pH 7.4). The homogenate was centrifuged at 17,000 × *g* for 30 min.

Enzyme Assays. The supernatant fraction was used to assay for catalase (12), malate synthetase (13), isocitrate lyase (13), aminopeptidase (14), endopeptidase (15), alcohol dehydrogenase (16), malate dehydrogenase (17), glutamic oxalacetic transaminase (18), and superoxide dismutase (19) activity. Protein concentration was determined by the method of Lowry (20). Starch-gel electrophoresis and staining procedures were performed as described (8).

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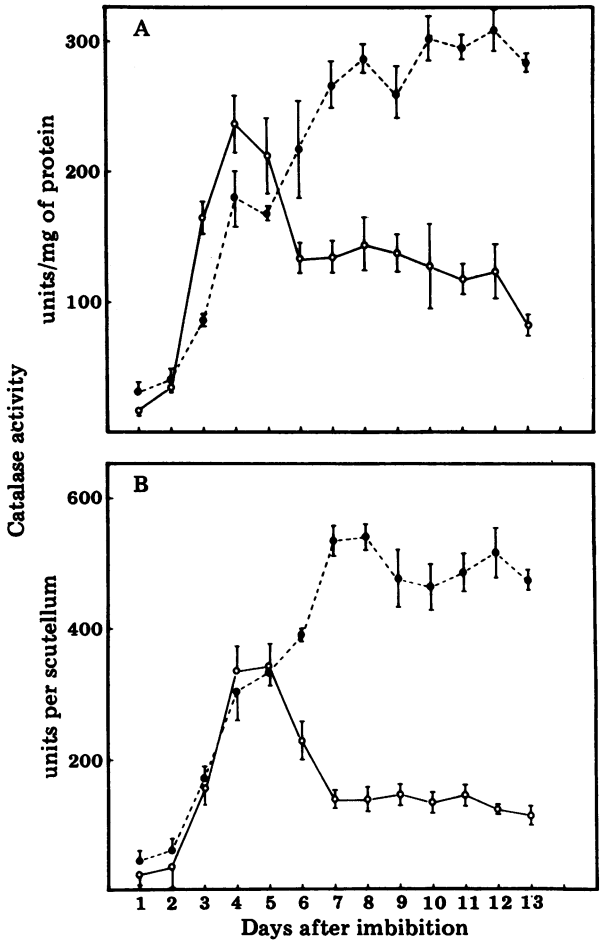

FIG. 1. The time course of catalase activity in W64A (○) and R6-67 (●) scutella during germination. Scutella isolated from seedlings were ground with 25 mM glycylglycine buffer (pH 7.4). The homogenate was centrifuged at 17,000 × *g* for 30 min. The supernatant fraction was used for the catalase activity assay. (*A*) Catalase activity per mg of protein. (*B*) Catalase activity per scutellum.

Determination of Catalase Synthesis and Degradation Rates. Five scutella were incubated in 5 ml of 0.08 M 2-allyl-2-isopropylacetamide/0.2 M sodium acetate, pH 5.6, for 24 hr. As a control, five scutella were incubated in the same buffer without the 2-allyl-2-isopropylacetamide. Both incubation mixtures were supplemented with 100 units of penicillin and 20 mg of mycostatin per ml to prevent microbial contamination and were shaken at room temperature.

2-Allyl-2-isopropylacetamide is an inhibitor of catalase synthesis (21). The degradation rate of catalase can be calculated by measuring the decrease in catalase activity during the period that the scutella were incubated in the 2-allyl-2-isopropylacetamide solution. The amount of catalase synthesis can

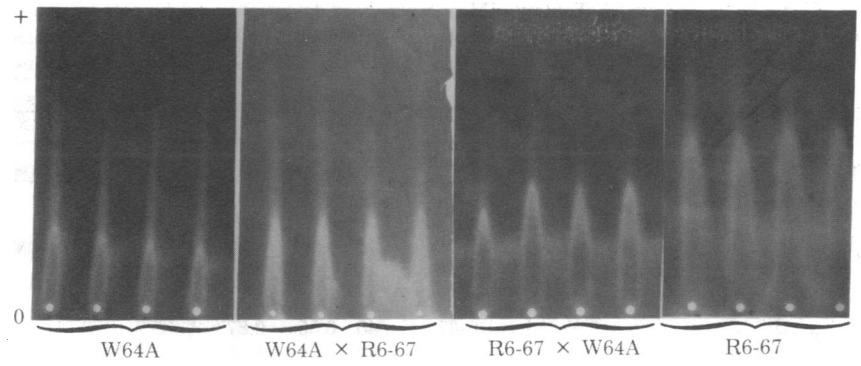

FIG. 2. Rocket immunoelectrophoresis with CAT-2 antiserum. The gel was stained for catalase. The height of each rocket illustrates the amount of CAT-2 protein present in each lane (one scutellum per lane). Migration is anodal. 0, point of sample insertion.

be obtained from the sum of net synthesis and degradation during the incubation period. The rate constants of synthesis (k_s) and degradation (k_d) were determined as described (22).

Rocket Immunoelectrophoresis. The technique of rocket immunoelectrophoresis (23) was modified as follows: Fifty milliliters of 1% Agarose (SeaKem, Rockland, ME)/0.05 M Tris·HCl, pH 8.6, was boiled and then cooled to 57°C, and 25 μl of CAT-2 antiserum was added. The gel was poured onto a 23 × 13 cm glass plate. Samples (5 μl) were added to 1-mm-diameter wells. Electrophoresis was conducted at 4°C with 60 V for 24 hr. At the end of the run, the gel was pressed and washed (but not dried) as described (23), except that the electrophoresis buffer was used for the washes and the whole pressing/washing procedure was conducted at 4°C to avoid enzyme inactivation. The gel was stained for catalase (8) by taking advantage of the fact that immunoprecipitated catalase is still active in decomposing H_2O_2.

Statistical Procedure. Frequency distributions expected for the contrasting homozygous genotypes and their heterozygotes were computed for normal distribution from the parental lines and their F_1 progeny means and standard deviations. Using these calculated values, points of minimum overlap among the distributions representing these three genotypes were identified as boundaries for classification (24). From these expected frequencies based on the normal distributions of the parental lines and the F_1 crosses, expected frequencies were computed for the F_2 and backcross generations. The F_2 and backcross data were compared with the expected frequencies by goodness-of-fit χ^2 tests.

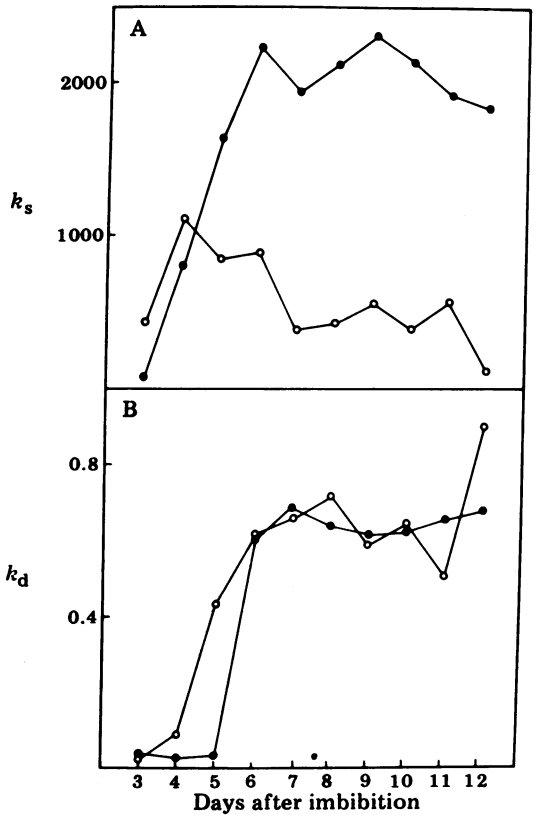

FIG. 3. The rate of catalase synthesis (*A*) and degradation (*B*) in W64A (○) and R6-67 (●) scutella during the period of germination examined. Five scutella were incubated in 5 ml of 0.08 M 2-allyl-2-isopropylacetamide/0.2 M sodium acetate, pH 5.6, for 24 hr. The rate of catalase degradation was calculated by measuring the decrease of catalase activity per day per g of scutellum (fresh weight) during the period of incubation. The amount of catalase synthesis was obtained from the sum of net synthesis and degradation during the incubation period. K_d, fraction of catalase units degraded per day; K_s, catalase units synthesized per scutellum per day.

RESULTS

Catalase Time Course in Inbred Lines R6-67 and W64A. The catalase activity in scutella of W64A and R6-67 differed significantly. Unlike its activity in W64A, which peaked at days 4–5, catalase activity in R6-67 scutella continued to increase beyond day 5 after germination and maintained an even higher level at later days (Fig. 1 *A* and *B*). When the catalase activity was expressed as units per scutellum, the activity of catalase in R6-67 after 7 days was about 3-fold that in W64A (Fig. 1*A*). Because the protein content in R6-67 scutella was higher than in W64A, the specific activity of catalase in R6-67 after 7 days

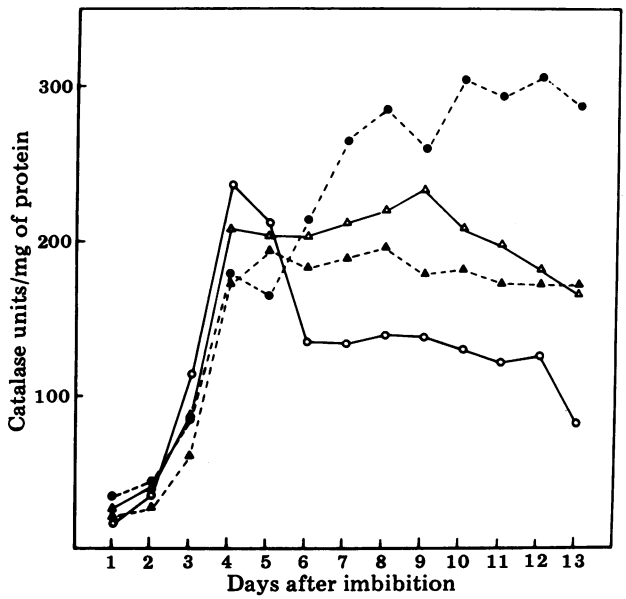

FIG. 4. The developmental profile of catalase activity (catalase units/mg protein) in scutella of R6-67 (●), W64A (○), W64A × R6-67 (△), and R6-67 × W64A (▲) seedlings during the first 13 days of germination.

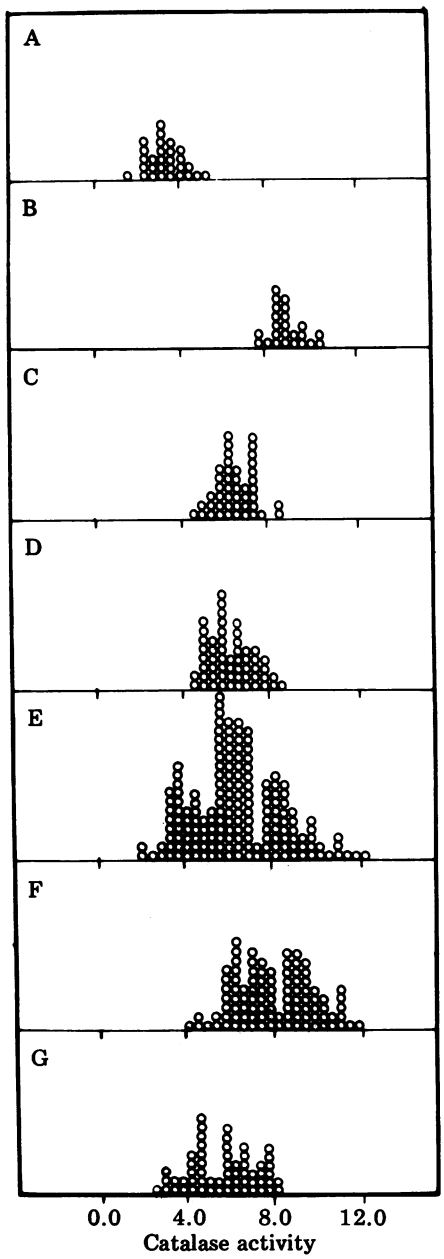

FIG. 5. The segregation of catalase activity (ΔA min/mg protein) in scutella from backcrosses and the F_2. The scutella isolated from 10-day-old seedlings of W64A (*A*); R6-67 (*B*); F_1 progeny W64A × R6-67 (*C*) and R6-67 × W64A (*D*); F_2 generation [W64A × R6-67]⊗ (*E*); and backcross generations [R6-67 × W64A] × R6-67 (*F*) and [W64A × R6-67] × W64A (*G*) were used to measure catalase activity. A circle was used to represent each scutellum and the position of each circle on the horizontal axis represents the catalase activity of each scutellum.

was about twice that in W64A (Fig. 1*B*). Electrophoretic, immunoprecipitative, and genetic analyses indicated that R6-67 and W64A have the same alleles at both structural loci—namely, *Cat1V* and *Cat2Z* (data not shown).

The isozyme pattern shift from CAT-1 to CAT-2 was identical in R6-67 and W64A, because they both possessed the same allele for *Cat1* and *Cat2* (data not shown). As has been reported (8–10), this shift is due to differences in the turnover rates between the two gene products, leading to the elimination of CAT-1 and the predominant expression of CAT-2. Furthermore, data from rocket immunoelectrophoresis with CAT-2 antibody showed that the increase in CAT-2 activity was due to an increase in CAT-2 protein rather than to mere activation of the CAT-2 enzyme (Fig. 2), which suggests that line R6-67

Table 1. Means, standard deviations, and expected frequency distributions of catalase activity in 10-day scutella in samples of parental inbreds, F_1 crosses, F_2 and backcross generations

Line	Catalase activity N	Mean	SD	Activity class <4.6	4.6–7.8	>7.8
				Frequency expected*		
Parents						
W64A	29	3.50	0.77	0.9150	0.0846	0
R6-67	24	9.06	0.77	0	0.0520	0.9480
R6-43	23	3.20	0.66	0.9773	0.0227	0
F_1 Crosses						
W64A × R6-67	45	6.55	0.87	0.0227	0.9174	0.0520
R6-67 × W64A	56	6.26	1.00	0.0227	0.9174	0.0520
R6-43 × R6-67	48	5.80	0.62	0.0227	0.9769	0.0004
				Segregation frequency expected†		
F_2 Generations						
(W64A × R6-67) ⊗	168	6.51	2.04	0.2402	0.4928	0.2670
(R6-43 × R6-67) ⊗	182	6.20	2.27	0.2557	0.5071	0.2372
Backcrosses						
(R6-67 × W64A) × R6-67	98	8.04	1.81	0	0.4954	0.5046
(W64A × R6-67) × W64A	59	5.60	1.52	0.4698	0.5302	0
(R6-43 × R6-67) × R6-67	122	7.97	1.80	0	0.5247	0.4742
R6-67 × (R6-43 × R6-67)	95	8.08	1.53	0	0.5247	0.4742

* Frequencies computed for normal distributions based on observed estimates of means and variances.

† Frequencies computed for single locus segregation from the expected distributions for parental lines and their F_1.

was producing more CAT-2 protein than W64A was during the period examined. Immunoelectrophoresis with CAT-1 antibodies showed very low and declining CAT-1 protein (data not shown).

Constants of Synthesis and Degradation of Catalase. Six days after imbibition, the degradation rate constants of catalase in the scutella of W64A and R6-67 were similar, but the synthesis rate constants in these two lines showed very significant differences (Fig. 3). These results suggest that the higher level of catalase activity in R6-67 scutella after 6 days was due to a higher K_s value than that found with W64A and not due to a smaller K_d value than that found with W64A. In other words, the catalase molecules in R6-67 scutella were synthesized faster than in W64A scutella, but their degradation rate was similar to that in W64A scutella.

Expression of Other Enzymes in W64A and R6-67 Scutella. The developmental patterns of eight other enzymes were examined in W64A and R6-67 scutella. These were the glyoxysomal enzymes isocitrate lyase and malate synthetase (11); superoxide dismutase; alcohol dehydrogenase; malate dehydrogenase; glutamate oxalacetic transaminase; aminopeptidase; and endopeptidase. The developmental profiles for all these

Table 2. χ^2 tests of the hypothesis that the temporal expression of *Cat2* is controlled by a single locus with additive alleles

Segregating generation	Activity class <4.6	4.6–7.8	>7.8	χ^2	P
(W64A × R6-67) ⊗	38	79	51	1.152	0.56
(R6-43 × R6-67) ⊗	48	87	47	0.689	0.71
(R6-67 × W64A) × R6-67	0	45	53	0.514	0.47
(W64A × R6-67) × W64A	22	37	0	2.225	0.14
(R6-43 × R6-67) × R6-67	0	58	64	1.252	0.26
R6-67 × (R6-43 × R6-67)	0	41	54	3.389	0.07

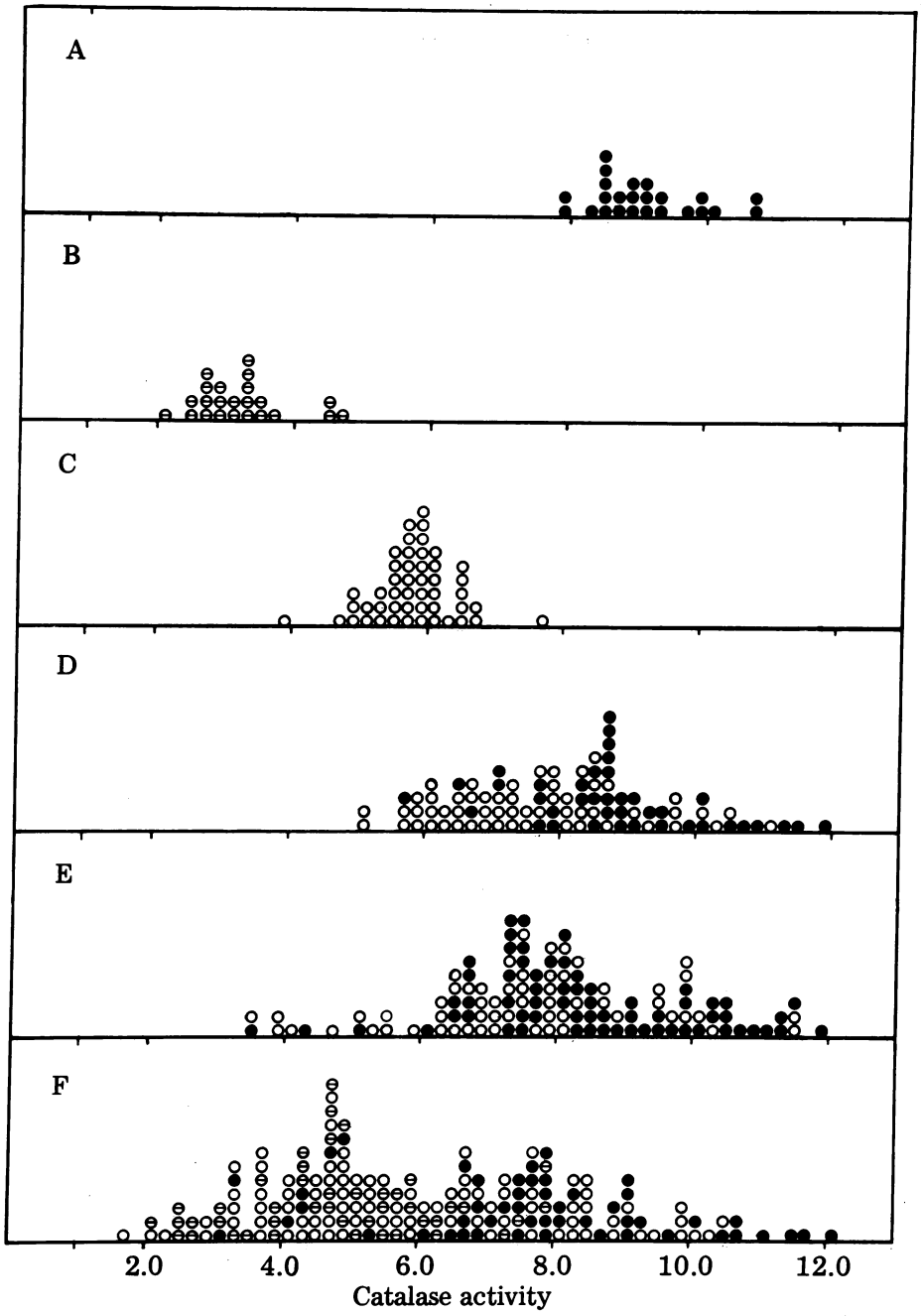

FIG. 6. Genetic analysis of the map distance between *Car1* and *Cat2*. Individual scutella from 10-day-old seedlings were ground in 1 ml glycylglycine buffer (pH 7.4) and centrifuged at 17,000 × *g* for 30 min. The supernatant fraction was used to determine catalase activity. In addition, a sample of the supernatant was used for electrophoresis to determine the CAT-2 genotype. Catalase activity (ΔA per min/mg of protein) in R6-67 (*A*); R6-43 (*B*); F_1 progeny R6-43 × R6-67 (*C*); backcross generations R6-67 × (R6-43 × R6-67) (*D*) and (R6-43 × R6-67) × R6-67 (*E*); and F_2 generation (R6-43 × R6-67)⊗ is represented by the position of each circle on the horizontal axis. The respective *Cat2* genotypes are: ● = *Z/Z*; ⊖ = *P/P*; and ○ = *Z/P*.

enzymes (except alcohol dehydrogenase) were identical between the two lines (data not shown). These results also eliminate the possibility that R6-67 possesses a general protease variant.

Genetic Studies. The time course of catalase activity in progeny from reciprocal F_1 crosses between W64A and R6-67 were intermediate to those of the two parental levels (Fig. 4). Since the difference in catalase activity between W64A and R6-67 at 10 days was significant, the level of catalase 10 days after germination was used as a marker for genetic studies. Scutella isolated from 10-day-old seedlings of W64A, R6-67, R6-43, R6-67 × W64A, W64A × R6-67, R6-43 × R6-67, F_2, and backcrosses were used to measure catalase activity (Table 1). R6-43 was chosen for linkage studies because it possesses the *Cat2P* allele but has the catalase developmental program of W64A.

The means of catalase activity in W64A, R6-67, R6-43, R6-67 × W64A, W64A × R6-67, and R6-43 × R6-67 illustrate that the pattern of scutellar catalase activity is inherited additively. The F_1 scutella tested showed activity levels between those of parental types. Furthermore, the rocket immunoelectrophoresis data with CAT-2 antibody clearly showed that the F_1 was intermediate to the parental lines (Fig. 2), which supports the hypothesis that the alleles are additive. By using the statistical approach described, approximate points of minimal overlap were determined for the crosses involving R6-67 and W64A or R6-67 and R6-43 (Figs. 5 and 6). The expected distributions generated to test the hypothesis involved assumptions that the alleles are additive and that errors of measurement of catalase activity are randomly distributed variables (Table 1). The χ^2-analysis (Table 2) supports the hypothesis that a major part of the developmental activity program differences between W64A and R6-67, and between R6-43 and R6-67, is due to allelic differences at a single locus.

Inbred line R6-43 has the P allele (electrophoretically distinct from the Z allele) at the *Cat2* locus and exhibits the same level

Table 3. Map distance of *Car1* from *Cat2*

Cross	Geno-type	Catalase activity: Low	Intermediate	High	% recombination
R6-43 ⊗	*PP*	23			
R6-67 ⊗	*ZZ*			24	
R6-43 × R6-67	*ZP*		48		
(R6-43 × R6-67) ⊗	*PP*	18	24	1	
	ZP	22	46	21	
	ZZ	6	19	25	Avg. 38.3
R6-67 × (R6-43 × R6-67)	*ZP*		33	23	
	ZZ		8	31	Avg. 32.6
(R6-43 × R6-67) × R6-67	*ZP*		33	25	
	ZZ		25	39	Avg. 41.0

of catalase activity as the standard line W64A (Table 1). The F_1 hybrid, and the segregating generations (F_2 and backcrosses) were analyzed for zymogram phenotypes and for catalase activity (Fig. 6). The zymogram analysis suggested that the P and Z Cat-2 forms were affected similarly. Distributions of data clearly showed a preponderance of parental types when the *Cat2* genotype and catalase activity were considered jointly. Goodness of fit χ^2 tests indicated that independent assortment between these loci is unlikely. Analysis of the data showed that the two genes (*Car1* and *Cat2*) may be loosely linked with a recombination value of approximately 37% (Table 3). This was also supported by data based upon units of catalase activity per mg (fresh weight) of scutellum (data not shown).

DISCUSSION

The developmental profile of catalase activity in the scutellum of W64A and R6-67 differ significantly (Fig. 1). This is due to a higher activity, as a result of increased synthesis, of the CAT-2 isozyme in R6-67 (Figs. 2 and 3).

Examination of the time courses of other enzymes indicated that only catalase, and to a lesser degree alcohol dehydrogenase, express altered developmental activity profiles in the scutellum of R6-67. In addition, the possibility that R6-67 is a mutant expressing a low protease activity was eliminated. We also have recently demonstrated that the increased alcohol dehydrogenase activity in R6-67 is due to a regulatory gene (*Adr1*) that acts independently and is unlinked to *Car1* (25). The identification of *Car1* and *Adr1* in R6-67 was merely fortuitous, because we have identified two other inbred strains (59 and D10) with high catalase activity, like R6-67, but with normal (low) alcohol dehydrogenase activity levels, similar to W64A. The finding that isocitrate lyase and malate synthetase do not vary between the two lines suggests that the higher catalase activity in R6-67 is not due to the promotion of glyoxysome development in the scutellum.

Genetic studies showed that the inheritance of the factor controlling catalase activity was additive. The time course of catalase activity in the F_1 crosses W64A × R6-67 and R6-67 × W64A is intermediate to the two parental levels after the 6th day of germination (Fig. 4). In addition, the means of the F_1 progeny of the crosses R6-67 × W64A, W64A × R6-67, and R6-43 × R6-67 are intermediate to the parental means (Table 1). This is illustrated further by the distribution of F_1 progeny in Figs. 5 and 6. In addition, rocket immunoelectrophoresis with CAT-2 antibody confirmed that the F_1 CAT-2 protein level was between the parental protein levels (Fig. 2).

The crosses were analyzed statistically as described. The values obtained support the hypothesis that the developmental activity program differences between W64A and R6-67 and between R6-43 and R6-67 are due largely to a single gene (Table 2; Figs. 5 and 6). Linkage analysis showed that *Car1* is approximately 37 map units from the *Cat2* structural gene (Table 3).

The fact that *Car1* and *Cat2* appear to be linked further indicates that there is only one gene coding for the higher catalase activity in the day 10 scutellum of R6-67. We have located the *Cat2* gene on the distal-half of chromosome 1S (unpublished data). *Car1* is not on chromosome 1L, since *Car1* is not linked to *Amp1* (data not shown), which is located approximately 27 map units from the centromere on chromosome 1L (26).

Thus, our results show that *Car1* is linked with *Cat2* and is located on chromosome 1S. Since the regulatory gene is not located adjacent to the structural gene, its action on *Cat2* gene expression may be mediated by diffusible substances (trans-acting) which are produced by the regulatory gene.

This work was supported by Grant GM22733 from the National Institutes of Health to J.G.S. This is paper no. 6276 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC.

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