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Molecular and whole-plant responses to selection for enzyme activity in alfalfa root nodules: evidence for molecular compensation of aspartate aminotransferase expression *

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Summary. The enzyme aspartate aminotransferase (AAT) plays a key role in the assimilation of fixed-N in alfalfa (*Medicago sativa* L.) root nodules. AAT activity in alfalfa nodules is due to the activity of two dimeric isozymes, AAT-1 and AAT-2, that are products of two distinct genes. Three forms of AAT-2 (AAT-2a, -2b, and -2c) have been identified. It was hypothesized that two alleles occur at the AAT-2 locus, giving rise to the three AAT-2 enzymes. In a prior study bidirectional selection for root nodule AAT and asparagine synthetase (AS) activities on a nodule fresh weight basis in two diverse alfalfa germ plasms resulted in high nodule enzyme activity subpopulations with about 20% more nodule AAT activity than low enzyme activity subpopulations. The objectives of the study presented here were to determine the inheritance of nodule AAT-2 production and to evaluate the effect of bidirectional selection for AAT and AS on AAT-2 allelic frequencies, the relative contributions of AAT-1 and AAT-2 to total nodule activity, nodule enzyme concentration, and correlated traits. Two alleles at the AAT-2 locus were verified by evaluating segregation of isozyme phenotypes among F_1 and S_1 progeny of crosses or selfs. Characterization of subpopulations for responses associated with selection was conducted using immunoprecipitation of in vitro nodule AAT activity, quantification of AAT enzyme protein by ELISA, and AAT activity staining of native isozymes on PAGE. Results indicate that selection for total AAT activity specifically altered the expression of the nodule AAT-2 isozyme. AAT-2 activity was significantly greater in high compared to low activity subpopulations, and high AAT subpopulations from both germ plasms had about 18% more AAT-2 enzyme (on a nodule fresh weight basis). No significant or consistent changes in AAT-2 genotypic frequencies in subpopulations were caused by selection for AAT activity. Since changes in AAT activity were not associated with changes in AAT-2 genotype, selection must have affected a change(s) at another locus (or loci), which indirectly effects the expression of nodule AAT.

Key words: Glutamate oxalate transaminase - Isozymes – Nitrogen fixation – *Medicago sativa* L. – *Rhizobium meliloti*

Introduction

Activities of certain plant enzymes involved in C and N metabolism in root nodules have been shown to be associated with N_2-fixation (Groat and Vance 1984; Jessen et al. 1987). Jessen et al. (1987) observed significant positive correlations between acetylene reduction activity (ARA) and the activities of alfalfa root nodule phosphoenolpyruvate carboxylase (PEPC:EC 4.1.1.31) and glutamate synthase (GOGAT:EC 1.4.1.14). With the goal of enhancing overall N_2-fixation Jessen et al. (1988) conducted bidirectional selection for nodule PEPC and GOGAT activities and developed subpopulations that were significantly different for high and low nodule GOGAT and PEPC activities. Even though selection for nodule enzymes was effective, selection for nodule enzyme activity did not result in a correlated response for the amount of N fixed.

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Recently fixed N in alfalfa root nodules is transported primarily as aspartate (asp) and asparagine (asn) (Maxwell et al. 1984; Ta et al. 1986). Degenhart et al. (1992) hypothesized that the synthesis of asp and asn by aspartate aminotransferase (AAT:EC 2.6.1.1) and asparagine synthetase (AS:EC 6.3.5.4), respectively, might have been limiting in populations developed by Jessen et al. (1988). Using Jessen et al.'s high PEPC/high GOGAT populations as a base, Degenhart et al. (1992) conducted simultaneous bidirectional selection for AAT and AS activities and produced subpopulations that were significantly different for the AAT and AS enzyme activities. However, those subpopulations did not differ for the amount of N fixed by plants. Apart from all other attributes, the subpopulations developed by Degenhart et al. (1992) show significant divergent expression of AAT and are of particular interest to our research group.

Alfalfa AAT activity is due to two distinct dimeric isozymes: AAT-1, which is constitutive in roots, and AAT-2, which is a nodule-enhanced isozyme (Farnham et al. 1990a, 1990b; Griffith and Vance 1989). We produced polyclonal antibodies to both isozymes and showed that antibodies against AAT-1 do not cross-react with AAT-2 and that antibodies against AAT-2 do not cross-react with AAT-1 (Farnham 1990b). Thus, the AAT-1 and AAT-2 isozymes of alfalfa are distinct alfalfa polypeptides and presumably the products of different genetic loci (Farnham et al. 1990b). Three subforms (a, b, and c) of AAT-2 have been identified. We hypothesize that two alleles at the AAT-2 locus produce two different subunit polypeptides resulting in three distinct holoenzymes. In an autotetraploid species like alfalfa, two alleles at the AAT-2 locus would give rise to a total of five genotypes, each of which should produce different ratios of the two subunit polypeptides and hence different phenotypes. The objectives of this study were to determine the inheritance of nodule AAT-2 production and to evaluate the effect of bidirectional selection for AAT and AS activity on AAT allelic frequencies, the relative contributions of AAT-1 and AAT-2 to total nodule activity, nodule enzyme concentration, and correlated traits.

Materials and methods

Inheritance of AAT-2 enzymes

Individual alfalfa clones of known AAT-2 genotype (Fig. 1) were used as parents in a crossing program. Particular crosses were chosen that would confirm whether or not a two-allele model for the AAT-2 locus was correct. The females in all crosses were emasculated using vacuum suction. Foreign pollen was introduced on the stigma immediately following emasculation to produce F_1 seed (Viands et al. 1988). Similar sets of reciprocal crosses were made among parent clones from each of two unrelated germ plasms, Saranac AR and MNPL10 × MNNC7. Approximately ten florets per parental clone were maintained as emasculated, unpollinated controls to determine the effectiveness of emasculation in preventing S_1 progeny. Each parental clone was also self-pollinated by gently rolling racemes between fingers as described by Viands et al. (1988) to produce S_1 seed.

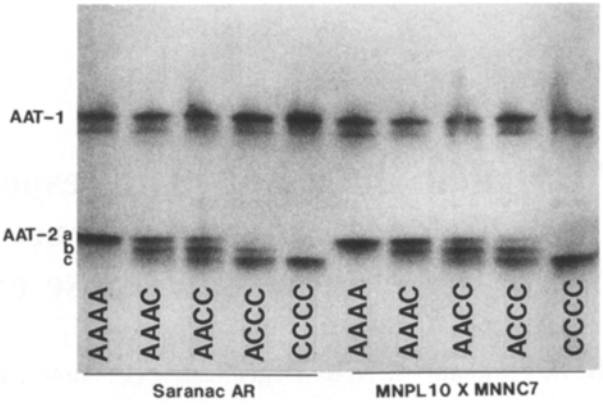

Fig. 1. Zymogram of AAT activity of cell-free nodule extracts of five individual plants with distinct AAT-2 banding pattern phenotypes and hypothesized AAT-2 genotypes from the Saranac AR and MNPL10 × MNNC7 germ plasms. The proposed genotypes represent the possible combinations of two alleles at a single gene locus for an autotetraploid species. Note that no genetic variation for AAT-1 occurs with these individuals. All lanes of the native gel were loaded with 20 nmoles/min AAT activity

In preparation for planting, the F_1 or S_1 seeds were scarified, and then inoculated with mixed strains of *Rhizobium meliloti* acquired from Nitragin Co (Milwaukee, Wis.). All progeny were grown under N_2-dependent conditions in glasshouse sandbenches as previously described (Jessen et al. 1987). Root systems of each plant were kept separate using plastic sleeves pushed into the sand. Plants were cut back to a height of 4 cm at early flower (8–9 weeks after seeding). Approximately 3–4 weeks later, at early flower, plants were removed from the sand benches, roots were rinsed in water, and nodules were removed. Nodules from each plant were placed in an eppendorf tube and stored at −70 °C until the sample was evaluated for AAT-2 phenotype.

The nodule sample from each plant was ground in a glass homogenizer with a ratio of 100 mg nodules per 1.0 ml MES-NaOH buffer (Groat and Vance 1984). Homogenates were centrifuged for 15 min at 10000 *g*, and supernatants were stored on ice in microfuge tubes prior to the AAT enzyme assay and electrophoresis. AAT activity was determined using a NADH-dependent, malate dehydrogenase-linked reaction described by Griffith and Vance (1989).

Nondenaturing-PAGE was performed according to Ornstein (1964). A constant amount of nodule in vitro AAT activity (20 nmoles product per minute) was loaded onto each lane of the nondenaturing gels. AAT activity was identified on gels following the electrophoresis of samples by incubating the gels in 100 m*M* TRIS-HCL (pH 8.0), 40 m*M* aspartate, 5 m*M* a-Ketoglutarate, and fast violet B salt (1 mg/ml buffer) (Shaw and Prasad 1970). The phenotype and hypothetical genotype of each plant was classified as shown in Fig. 1. The segregation ratios of observed F_1 and S_1 progeny in each phenotypic class were compared to the expected segregation ratios by the chi-square analysis to measure goodness of fit. Although the collection of nodules from individual plants makes this evaluation a laborious task, no other plant tissue gives as clear and consistent staining of gels, allowing for the clear typing of individuals for AAT-2 banding pattern.

Evaluation of bidirectional selection for AAT activity

Alfalfa subpopulations with high and low levels of in vitro AAT activity on a nodule fresh weight basis were developed in the Saranac AR and MNPL10 × MNNC7 germ plasms (Degenhart et al. 1992). To synthesize their divergent populations, Degenhart et al. evaluated 300 plants from each of the two germ plasms for AAT and AS activities and conducted one cycle of bidirectional selection for high activities of both enzymes or low activities of both. They used a selection intensity of 12.5% in both directions. Selected plants were intermated to produce Syn1 generation seed. Seed from the unselected base populations and from the high and low AAT activity subpopulations from each germ plasm were inoculated with rhizobia, planted in sand benches, and managed according to procedures described in the inheritance study. The six subpopulations were planted in a randomized complete block design with 25 blocks; a block was made up of a single plant from each subpopulation. Nodules were collected from each plant at approximately 4 weeks of first regrowth. Nodule fresh weight, nodule soluble protein, total nodule AAT activity, AAT-2/AAT-1 activities as proportions of total AAT activity, AAT-2 enzyme protein concentration in nodules, and AAT banding pattern on nondenaturing-PAGE were determined for each plant in the 25 block design. An additional 15 plants from each group were grown using the same conditions described above, and nodules were evaluated for AAT-2 phenotype.

Total nodule AAT activity and AAT-2 phenotype of individual plants were determined as previously described in the inheritance study. Nodule soluble protein content was measured using the method of Lowry et al. (1951). The proportion of total AAT activity that was due to AAT-1 and AAT-2 was determined by immunoprecipitating in vitro nodule AAT activity using AAT-1-specific antibodies. A constant quantity of AAT activity (30 nmoles product per minute) in extracts from nodules was incubated for 1.5 h at 4 °C with AAT-1 antiserum (30 µl). The volume of antiserum needed to immunoprecipitate 30 nmoles product per minute of activity of purified AAT-1 had been determined in previous studies (Farnham et al. 1990b). Immune complexes were precipitated by the addition of 60 µg of goat anti-rabbit IgG, followed by an additional 1.5 h incubation at 4 °C. Remaining AAT activity (which was entirely due to AAT-2) in the supernatant following centrifugation was measured as described before. Preimmune serum assays were run on every sixth sample as controls.

AAT-2 concentration in nodule extracts was measured with an indirect ELISA coupled with AAT-2-specific antibodies. Extracts were diluted 1:200 in MES-NaOH extraction buffer, aliquots were pipetted into wells on Immulon 1 microtiter plates (Dynatech Laboratories, Chantilly, Va.), and plates were incubated 4 h at 37 °C. After nodule protein and antigen were bound to wells, the following steps with washes in between were performed to complete the ELISA:(1) unbound sites on wells were blocked with 0.5% γ-globulin protein and incubated 2 h; (2) polyclonal antibodies specific for AAT-2 (1:2000 dilution) were added to wells and the plates incubated overnight; (3) goat anti-rabbit IgGs conjugated with alkaline phosphatase (1:1000 dilution) were added to wells and the plates incubated 4 h; (4) alkaline phosphate substrate (1.5 mg/ml) was added and the plates incubated 3 h; and (5) absorbance of solution in wells was read at 405 nm using an ELISA plate reader. The AAT-2 concentration of a bulk crude nodule extract was estimated using rocket immunoelectrophoresis as described by Farnham et al. (1990a). That extract was used as a standard on microtiter plates to calculate AAT-2 concentrations for each nodule extract.

Results

Inheritance of AAT-2 enzymes

The AAT banding pattern phenotypes of alfalfa plants from two unrelated germ plasms were evaluated on nondenaturing-PAGE. No variation for the AAT-1 banding (staining) region was observed (Fig. 1). In contrast, the AAT-2 banding region, where three isoforms (a, b, and c; Fig. 1) occur, was variable among individual plants. In general, five phenotypes for the AAT-2 staining region occurred in the two unrelated germ plasms (Fig. 1). The existence of three forms of the dimeric AAT-2 isozyme and five AAT-2 phenotypes are consistent observations if two alleles occur at the AAT-2 locus. Thus, we estab-

Table 1. Segregation of nodule AAT-2 genotypes for S_1 and F_1 families produced from clones of two alfalfa germ plasms

Parental genotypes[a]	Generation	Number of families	Observed offspring genotype segregation					Expected segregation ratio	Goodness of fit	
			AAAA	AAAC	AACC	ACCC	CCCC		χ^2	P
			No. of plants							
AAAA (selfed)	S_1	2	28	0	0	0	0	1:0:0:0:0	–	–
CCCC (selfed)	S_1	2	0	0	0	0	15	0:0:0:0:1	–	–
AACC (selfed)	S_1	2	1	11	26	11	1	1:8:18:8:1	0.360	>0.975
AAAA × CCCC	F_1	2	0	0	30	0	0	0:0:1:0:0	–	–
CCCC × AAAA	F_1	2	0	0	27	0	1[b]	0:0:1:0:0	–	–
AAAC × CCCC	F_1	2	0	0	14	16	0	0:0:1:1:0	0.133	>0.500
AAAA × AACC	F_1	2	6	26	6	0	0	1:4:1:0:0	0.053	>0.950
AACC × AAAA	F_1	2	5	30	4	0	0	1:4:1:0:0	1.92	>0.250
AACC × CCCC	F_1	2	0	1[b]	5	25	9	0:0:1:4:1	1.34	>0.500
CCCC × AACC	F_1	1	0	0	10	7	3	0:0:1:4:1	6.330	>0.025
CCCC × AACC	F_1	1	0	0	6	12	2	0:0:1:4:1	2.800	>0.250

[a] Genotypes and phenotypes were set equal as shown in Fig. 1

[b] These were assumed to occur due to selfing and were not considered in the χ^2 analysis

lished a hypothesis that assigned two alleles (A and C) to the AAT-2 locus. We also proposed a genotype for each of the five AAT-2 phenotypes (Fig. 1). To test our hypothesis, F_1 crosses were made between clones with the distinctive phenotypes, and the parental clones were also selfed to produce S_1 progeny.

Seven individual crosses and three selfs within each of the Saranac AR and MNPL10 × MNNC7 germ plasms were evaluated in the F_1 or S_1 generations for AAT-2 segregations. Results for the two germ plasms were similar for all segregations except for one cross, so the data from both germ plasms were combined for simplicity of presentation (Table 1). The observed segregations of progeny in the S_1 and F_1 generations generally fit the expected segregations. Unexpected progeny resulted in only two crosses (CCCC × AAAA and AACC × CCCC), and in both cases the unexpected individuals could be explained by selfing of the female parent. It was assumed that in all crosses introduced pollen had a competitive advantage and was almost exclusively responsible for fertilization. However, 10 of 100 emasculated control florets set pods, indicating that a few selfs can occur during emasculation.

Only one other cross (CCCC × AACC) failed to fit the expected ratio. It is noteworthy that the lack of fit was not due to the appearance of unexpected classes; the three segregating phenotypes were those that the model predicted. The odds of at least one poor fit occurring was relatively high due to the relatively small numbers of progeny evaluated in each cross.

Table 2. Mean in vitro nodule AAT-1, AAT-2 and total AAT activities on a nodule fresh weight basis for the unselected base populations and subpopulations resulting from bidirectional selection for high and low nodule AAT activity in two alfalfa germ plasms

Germ plasm source	Subpopulation	Nodule AAT activity		
		AAT-1	AAT-2	Total
		nmoles/min per g fw		
Saranac AR	High AAT	2,145	8,343	10,488
	Unselected	2,126	6,564	8,690
	Low AAT	2,213	6,452	8,665
MNPL10 × MNNC7	High AAT	2,095	9,601	11,696
	Unselected	2,100	8,717	10,817
	Low AAT	2,303	7,624	9,927
$LSD_{0.05}$		ns	1,646	1,612

AAT activity of selected subpopulations

Bidirectional selection for nodule AAT and AS activity resulted in high AAT activity subpopulations that expressed about 20% more total AAT activity on a nodule fresh weight basis than low AAT activity subpopulations (Table 2). In both germ plasms under study, high and low activity subpopulation means were significantly different from one another, and the unselected germ plasm mean activities fell between selected subpopulation means.

The individual contribution of AAT-1 and AAT-2 to total AAT activity was measured by selectively immunoprecipitating AAT-1 from crude nodule extracts. The results of the immunoprecipitations show that the differences in total AAT activity among selected subpopulations was almost exclusively due to differences in the amount of AAT-2 enzyme activity in nodules (Table 2). The AAT-1 activities were similar for all subpopulations.

AAT-2 genotypic frequencies in selected subpopulations

Nodule extracts of plants from each of the six subpopulations studied were analyzed by nondenaturing-PAGE to determine their AAT-2 phenotypes and genotypes. Genotype frequencies were different in subpopulations of Saranac AR compared to those of MNPL10 × MNNC7 (Table 3). Generally, AACC and AAAC genotypes were most common in Saranac AR, while AAAA and AAAC were more frequent in MNPL10 × MNNC7. These differences reflect a higher frequency of the A allele (0.72

Table 3. Frequencies of AAT-2 genotypes and alleles in unselected base populations and subpopulations resulting from bidirectional selection for high and low nodule AAT activities in two alfalfa germ plasms

Germ plasm source	Subpopulation	Plants evaluated	AAT-2 genotype frequency					AAT-2A allele	
			AAAA	AAAC	AACC	ACCC	CCCC	Frequency	95% C.I.[a]
Saranac AR	High AAT	38	0.026	0.368	0.421	0.158	0.026	0.55	0.47–0.63
	Unselected	39	0.077	0.333	0.359	0.231	0.000	0.56	0.49–0.64
	Low AAT	39	0.026	0.256	0.538	0.154	0.026	0.52	0.45–0.61
MNPL10 × MNNC7	High AAT	40	0.225	0.425	0.175	0.125	0.050	0.66	0.59–0.74
	Unselected	40	0.375	0.375	0.225	0.025	0.000	0.77	0.71–0.84
	Low AAT	40	0.325	0.375	0.175	0.125	0.000	0.72	0.65–0.80

[a] 95% Confidence Interval (C.I.) assumes a binomial distribution and a sample size of 4n

versus 0.55) in the MNPL10 × MNNC7 germ plasm compared to the Saranac AR germ plasm. Although germ plasms exhibited different genotypic and allelic frequencies, high and low AAT subpopulations within germ plasms did not differ substantially from one another. It is noteworthy that there were more CCCC genotypes and fewer AAAA genotypes in the high subpopulation of MNPL10 × MNNC7 compared to the low subpopulation. However, due to the limited sample size for each subpopulation these were considered to be minor differences.

Nodule AAT-2 enzyme concentrations in selected subpopulations

Bidirectional selection for AAT activity altered the amount of total activity contributed by AAT-2 and similarly altered the concentration of AAT-2 enzyme protein in nodules (Table 4). Averaged across both germ plasms plants from high AAT subpopulations had approximately 19% more AAT-2 protein per fresh weight of nodules than low activity subpopulations. No significant change in the specific activity (on an enzyme protein basis) of the AAT-2 enzyme pool was associated with selection (Table 4). However, the AAT-2 pool in nodules of the MNPL10 × MNNC7 germ plasm had a greater enzyme specific activity than the AAT-2 pool in nodules of Saranac.

Table 4. Mean AAT-2 concentration in nodules (on a fresh weight basis) and nodule AAT-2 specific activity (on an enzyme protein basis) for the unselected base populations resulting from bidirectional selection for high and low nodule AAT activity in two alfalfa germ plasms

Germ plasm source	Subpopulation	AAT-2 concentration µg/g nodule fw	AAT-2 specific activity nmole/min per µg AAT-2
Saranac AR	High AAT	385	24.0
	Unselected	341	20.6
	Low AAT	331	20.7
MNPL10 × MNNC7	High AAT	331	30.9
	Unselected	286	36.1
	Low AAT	273	33.4
$LSD_{0.05}$		49	7.0

Correlated responses due to selection for enzyme activity

Selection for high enzyme activity was associated with a decrease in plant nodule mass, while selection for low activity was associated with an increase in mass (Table 5). No differences in total nodule AAT-2 produced per plant (the product of nodule mass and AAT-2 concentration on nodule fresh weight basis) were observed between high and low activity subpopulations. In general, MNPL10 × MNNC7 subpopulations exhibited greater mean nodule fresh weight and nodule AAT-2 content per plant than did Saranac AR subpopulations.

The average nodule soluble protein concentration was consistently greater in high compared to low activity subpopulations although the differences were not statistically significant (Table 5). When AAT-2 concentration is expressed on a nodule soluble protein basis there are no differences between subpopulations within a germ plasm. The nodule soluble protein content was greater in MNPL10 × MNNC7 subpopulations, while the nodule AAT-2 specific concentration on a soluble protein basis was greater in Saranac AR subpopulations.

Discussion

Selection for nodule AAT activity on a nodule fresh weight basis resulted in high AAT activity subpopulations with about 20% more total activity than low activity subpopulations. Differences in total nodule AAT activity observed in this study were almost exclusively due

Table 5. Means for nodule fresh weight, nodule AAT-2 content of plants, nodule soluble protein, and AAT-2 concentration (on a soluble protein basis) from base populations and subpopulations resulting from bidirectional selection for nodule AAT activity in two alfalfa germ plasms

Germ plasm source	Subpopulation	Nodule fresh weight mg/plant	Nodule AAT-2 content per plant µg/plant	Nodule soluble protein mg/g fw	AAT-2 concentration (protein basis) µg/mg nsp
Saranac AR	High	74.9	29.5	18.7	20.9
	Unselected	100.5	34.4	17.1	20.9
	Low	106.0	35.7	17.4	20.0
MNPL10 × MNNC7	High	134.2	45.3	20.3	17.3
	Unselected	136.7	39.0	18.8	15.2
	Low	159.6	43.2	19.2	15.0
	$LSD_{0.05}$	26.6	10.4	2.3	3.0

to differences in the activity of the AAT-2 isozyme. In addition, the observed alterations in AAT-2 activity were attributable to changes in the amount of AAT-2 enzyme protein in root nodules.

By evaluating segregating F_1 and S_1 populations for AAT-2 phenotypic banding pattern on nondenaturing-PAGE, evidence was obtained to support a hypothesis that there are two alleles at the autotetraploid AAT-2 locus and that five distinct phenotypic banding patterns correspond to five different genotypes. Few other reports (Quiros 1982; Martinez-Zapater and Oliver 1984; Krebs and Hancock 1989) have described the segregation of alleles for isozyme loci in autotetraploid crop species. Martinez-Zapater and Oliver (1984) and Krebs and Hancock (1989) described the inheritance of two alleles at dimeric isozyme loci in the autotetraploid species *Solanum tuberosum* L. and *Vaccinium corybosum* L., respectively. Their results were very similar to those described herein for alfalfa AAT-2.

Since progeny evaluation confirmed that distinct AAT-2 phenotypes represent distinct genotypes, it was possible to determine if selection changed the frequency of AAT-2 genotypes in high versus low activity subpopulations. There was no consistent evidence that increased or decreased enzyme activity of subpopulations was associated with changes in AAT-2 genotypic frequencies.

Selection for AAT activity must have successfully exploited heritable variation in activity due to AAT-2. This is consistent with the results of Griffith and Vance (1989) and Farnham et al. (1990a), who reported that the AAT-2 isozyme is a nodule-enhanced polypeptide that accounts for about 80% of total AAT activity in alfalfa root nodules. It is not possible to determine whether increased AAT-2 protein levels in high versus low AAT subpopulations result from a decreased rate of AAT-2 enzyme breakdown or from increased amounts of AAT-2 mRNA and hence translation of AAT-2. However, Gantt et al. (1992) have shown that as in vitro AAT activity and enzyme protein activity increase during the development of effective alfalfa root nodules, similar increases in AAT-2 mRNA are observed. Additionally, ineffective alfalfa nodules that express lower AAT activity and amounts of AAT-2 polypeptide compared to effective nodules also express decreased amounts of AAT-2 mRNA. Thus, it is likely that higher or lower relative levels of AAT-2 enzyme protein could reflect relative differences in amounts of AAT-2 mRNA.

Since selection for AAT and AS activity altered expression of the AAT-2 enzyme but did not appear to alter frequencies of AAT-2 genotypes in selected subpopulations, the genetic changes that occurred due to selection must have involved one or more loci, other than the AAT-2 locus, that are indirectly associated with the expression of nodule AAT. It is possible that selection had an effect on a gene(s) that exerts a general regulatory effect on nodule enzyme expression. This is supported by the fact that Degenhart et al. (1992) also observed greater GOGAT and PEPC activities in high AAT activity subpopulations compared to low AAT activity subpopulations.

Although selection for high enzyme activity resulted in high activity plants that have relatively more AAT-2 enzyme protein for a given nodule mass, the total nodule AAT-2 polypeptide content per plant was no different in high compared to low activity plants. This is explained by the negative-correlated response for nodule mass that accompanied the alteration of enzyme activity. This observed negative response due to selection exemplifies plant compensation, a phenomenon often encountered by plant breeders. The classic example of compensation is illustrated when selection for a crop yield component (e.g., seed weight) effectively alters that component, but correlated negative changes in another component (e.g., number of seeds per plant), impede any net positive gain in total plant yield due to direct selection. In this study, plant compensation was observed at the molecular level. This compensation for nodule AAT-2 expression by plants, which is associated with the correlated response for nodule mass, is surprising since when Degenhart et al. (1992) selected for enzyme activity, they culled plants from their base populations that had a total nodule mass that was greater or less than one standard deviation from the mean of the population. Stabilizing selection for total nodule mass did not appear to be effective.

Selection for increased nodule enzyme activity on a fresh weight basis resulted in subpopulations of alfalfa plants with less nodule mass but with nodule tissue that appears to be more efficient. The lower nodule mass in high activity plants performs the function of fixing and assimilating N as well as a greater nodule mass in low activity plants (Degenhart et al. 1992). This observation is significant because attempts to enhance N_2-fixation by legumes almost invariably result in plants with an increased capacity to fix N due to overall greater nodule mass (Vance and Heichel 1991). Rarely is the capacity of a given nodule mass to fix N increased. Perhaps the selection for increased enzyme activities in alfalfa root nodules that was evaluated in this study would have had a more positive effect on whole plant N_2-fixation if enzyme activity had been selected simultaneously with nodule mass.

Conclusions

This present study is unique in that the effect of phenotypic selection for a biochemical/physiological whole-plant trait (nodule AAT activity/nodule N-assimilation) was evaluated at the molecular level. We have shown that enzyme activity selection can specifically effect a particu-

lar isozyme; the AAT-2 isozyme was preferentially affected, while the AAT-1 isozyme was not. Additionally, the results indicate clearly that the effect of selection on plant gene expression can be indirect; AAT-2 expression was altered, but allelic expression at the AAT-2 locus was not the target of selection. Lastly, it was shown that plant compensation due to selection can occur at the molecular level; AAT-2 expression on a nodule fresh weight basis was altered, but the total production of nodule AAT-2 polypeptide was unaffected. These observations provide insight into the problems frequently encountered when breeding for improved biochemical/physiological traits in field crops.

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