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Research Article

Structural and regulatory differences in amylase isoenzymes in germinating Brazilian barley cultivars

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Abstract

The amylase electrophoretic patterns of 10 Brazilian brewing-barley varieties with different malting grades and diastatic power were analyzed during the 7-day germination which occurs during the malting process. Intra and inter-variety genetic variability was observed at both the structural and regulatory level. In the first few days after germination all varieties showed a few active loci, all of them with low activity. In subsequent days, new loci became active and those already detected since early germination showed increased activity. All varieties showed a continuous increase in amylase synthesis until the 3rd and/or 4th day after germination. Some varieties maintained high amylase activity until the last day of germination, while others showed a decrease in activity on the 5th or 6th day. No specific band increased or decreased its intensity independently of the others. A total of 14 loci were detected, out of which only one locus was polymorphic, indicating very low structural genetic variability, with only 2.8% polymorphic loci, an average of 1.04 alleles per loci, and an average expected heterozygosity of only 0.7%. The mean intra-variety Jaccard similarity coefficient complement ($1 - S_J$) was 0.009. The mean intra-variety difference based on regulatory differences was higher ($1 - S_J = 0.17$) than that obtained based on structural differences, suggesting differential gene activation. Inter-variety differentiation also showed low structural variability, with $1 - S_J = 0.026$ and a Nei genetic distance (D) value of 0.0076, and a remarkable increase in divergence caused by differential gene activation ($1 - S_J = 0.34$). These results indicate that regulatory polymorphism is the principal agent responsible for amylase variability in the barley varieties analyzed.

Key words: barley, amylases, isoenzymes, structural and regulatory genetic differences.

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Introduction

The central problem in plant developmental biology is to understand the role structural and regulatory genes play in differentiation. Structural genes codify RNA molecules, including mRNA, which is responsible for the synthesis of the structural proteins and enzymes necessary for cell structure and metabolic activities. Regulatory genes control structural gene expression at the temporal, spatial, and quantitative level. The effect of gene expression is illustrated by the fact that although all the cells of multicellular individuals are derived from a one-celled zygote by equal mitotic divisions, an extraordinary degree of cell and tissue heterogeneity occurs in the differentiated organism, this heterogeneity being the result of the activation and repression of specific genes in different cells. During plant development a well-defined temporal and spatial program for gene expression demands interaction between regulatory and structural genes.

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Developmental studies with isoenzymes have provided considerable insight into the role of structural genes and into the mechanisms that control enzyme synthesis during cell differentiation, consistently supporting the concept of differential gene action during this process.

Starch-degrading enzymes, present in cereal grains, are important in the development of plant embryos. In the case of barley, such enzymes are critical to the malting and brewing industries because they allow good quality malting to be obtained. Among the amylolytic enzymes, α-amylase (1,4-α-D-glucanohydrolase, EC 3.2.1.1) is thought to be the only enzyme which can initiate the cleavage of native starch granules by hydrolyzing α-1,4-linked glucose polymers. The debranching and degradation of the resultant maltodextrins and soluble polymers are completed by β-amylase, limit dextrinase, β-glucosidase, and α-amylase (Dunn, 1974), of which β-amylase (1,4-D-glucan malto-hydrolase, EC 3.2.1.2) is considered the principal enzyme responsible for starch hydrolysis, which it achieves by catalyzing the reaction that releases β-maltose from the non-reducing chain end of starch and starch-related compounds (Harris, 1962).

This paper describes the intra and inter-variety structural and regulatory genetic variability of the amylases in 10 Brazilian barley varieties.

Material and Methods

Plant material and germination

Table 1 indicates the 10 Brazilian barley varieties (8 cultivars and 2 lines) and their genealogical data, and Table 2 their malting quality and diastatic power. Both measures were determined and supplied by Ambeve Brewery, Navegantes Malting Subsidiary, Porto Alegre, RS, Brazil. Diastatic power (a measure of starch-degrading activity) was measured in malt kilned and ground, according to EBC-Analytic 4.12 (EBC Analytica, 1997). Malting quality scores of each variety were determinated on the basis of 16 malt characteristics (fine milling, extract, diastatic power, total proteins, β-glucan concentration, friability, and viscosity being the most important ones) and 10 plant characteristics (germinative power, total proteins in dry material, kernel size, to mention but a few).

Grain samples from each variety (grain size 2.2-2.8 mm) were germinated at 16 °C in a micro-malting unit at the AMBEVE Brewery - Navegantes Malting Subsidiary, after 13 h of stepping at 20 °C. Samples were removed on day 1, 2, 3, 4, 5, 6 and 7 after germination and frozen until the electrophoretic analysis.

Table 1 - Genealogy of the ten Brazilian barley varieties.

Variety[1]	Genealogy
Cultivar MN-682	MN-610 X MN-599
Cultivar MN-698	MN-599 X MN-635
Cultivar MN-656	[(SG4279 X FM-404) X (4b Bacco X Union)] X FM-434
Cultivar MN-684	Ant-5 X MN-577
Cultivar MN-668	[(SG4279 X FM-404) X (4b Bacco X Union) X Delisa
Cultivar BR-2	NORBERT X FM-424
Cultivar MN-694	MN-610 X MN-578
Cultivar MN-691	(Bonita X MN-377) X MN-599
Line MN-705	MN-599 X BR-2
Line MN-706	MN-607 X MN-640
	MN-610: FM-434 X Duvehot
	MN-599: (Ariana X Volla) X FM-462
	FM-462: {[(Alpha X Pirolina) X Dunajaski] X Mansholtz}
	FM-404: seleção WISA-WB
	FM-434: [(Quinn X M.Heda) X WISA-WB]
	MN-635: [(SG4279 X FM-404) X 4b Bacco X Union)] X FM-434
	FM-424: [(Quinn X M.Heda) X WS-5746)
	MN-577: Jlyb.Jlor X FM-404
	MN-578: (CEB.CAPA X Volla) X (Bolivia X FM-404)
	MN-607: FM-434 X FM-462
	MN-640: [(SG4279 X FM-404) X (4b Bacco X Union)] X FM-404

[1]All varieties (except for BR-2) were origined and provided by AMBEVE Brewery, Navegantes Malting Subsidiary, Porto Alegre-RS, Brazil. Cultivar BR-2 was origined and provided by the Centro Nacional de Pesquisa de Trigo, EMBRAPA, Passo Fundo-RS, Brazil.

Table 2 - Malting quality and diastatic power from the Brazilian barley varieties.

Variety[1]	Malting quality	Diastatic power (WK)[1]
Cultivar MN-682	91.2 High	237 High
Cultivar MN-698	94.4 High	181 Low
Cultivar MN-656	85.6 Medium	220 High
Cultivar MN-684	82.4 Medium	269 High
Cultivar MN-668	80.0 Medium	272 High
Cultivar BR-2	76.8 Medium	260 High
Cultivar MN-694	76.8 Medium	209 High
Cultivar MN-691	71.2 Medium	277 High
Line MN-705	74.4 Medium	193 Low
Line MN-706	80.0 Medium	173 Low

[1]Windish Kolbach units.

Electrophoretic analyses

For each day after germination, 11 endosperms from each variety were dissected out and each half endosperm homogenized in 0.05 M Tris-HCl buffer (pH 6.7) supplemented with 1 mM $CaCl_2$. Each sample was analyzed using horizontal and vertical electrophoresis. Horizontal electrophoresis was carried out in 6% acrylamide gel with discontinuous buffer (Scandalios, 1969) in a constant voltage gradient of 10 V/cm until the front line reached 9 cm beyond the origin. Vertical electrophoresis was carried out in 3.1% acrylamide compaction gel and 7.18% acrylamide separation gel with discontinuous buffer (Davis, 1964) at 10 mA until the tracking dye reached the separation gel (about 1 h) and subsequently at 20 mA for 3 h. After electrophoresis, the gels were incubated to develop the amylase isoenzymes (Chao and Scandalios, 1972; Maris, 1992).

Data analysis

The genetic control of isoenzymes was determined by analyzing the patterns of different endosperms from the 10 varieties, considering that barley is an autogamous species. The intra-variety genetic variability was measured based on allele frequencies, assessing the mean number of alleles per loci (A), proportion of polymorphic loci (P) and expected heterozygosity (H). The inter-variety divergence (D) was calculated according to Nei (1972). Intra-variety and inter-variety differences were also measured at the phenotypic (banding-pattern) level using the Jaccard similarity coefficient complement ($1 - S_J$), where $S_J = a/(a+u)$ in which a is the number of characters shared by the two entities and u the number of characters present in one endosperm but absent in the other (Jaccard, 1908).

Results

Characterization of amylase isoenzymes in barley endosperms

Electrophoretic analyses of barley endosperm at different stages after germination allowed the detection of 16 principal amylase bands plus one secondary band. These

bands were considered to be the result of 14 loci, numbered in ascending order, according to the position of the band in relation to the electrophoretic anode (*Amy-1* to *Amy-14*). Only locus *Amy-9* was polymorphic, with two alleles (*Amy-9*^{1} and *Amy-9*^{2}). Locus *Amy-1* presented a band typical of secondary isoenzyme, with constant electrophoretic distance and intensity proportional to the principal. Figure 1 shows amylase banding-patterns and indicates the genetic control of some isoenzymes.

Ontogenetic regulation of genic expression

The general pattern of the temporal distribution of endosperm amylase isoenzymes is shown in Figure 2. All varieties exhibited only a few active amylase loci in the first days after germination, but there was a general increase in activity during the following days, with new loci becoming active and those already detected since early germination showing increased activity. All varieties showed increasing amylase activity up to the 3rd or 4th day after germination. Some varieties (MN-656 and MN-682) maintained this high activity until the last day (7th day) analyzed while others showed a decrease in amylase activity on the 5th or 6th day. No band increased or decreased in intensity independently of the other bands.

Intra-variety variability

The 10 varieties showed two kinds of genetic variability, *i.e.* structural polymorphism due to the occurrence of different alleles for each locus and regulatory polymorphism due to the differential activity of each locus at each developmental stage in the endosperms of the different varieties.

Intra-variety polymorphism was very low when only structural differences were considered because, as has already been stated, only locus *Amy-9* was polymorphic (Table 3). Genetic variability measurements based on allele

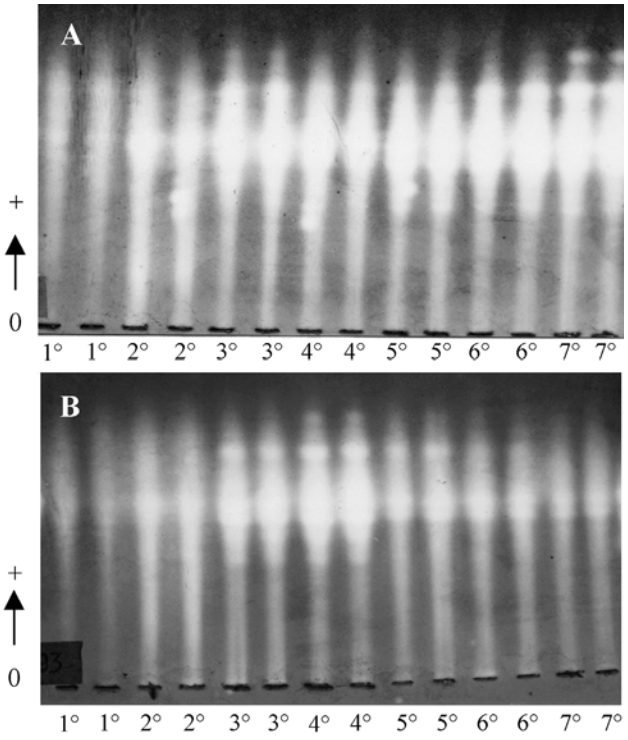

Figure 2 - Ontogenetic changes in banding-patterns of amylases extracted from barley seed endosperms during seven days after germination (vertical electrophoresis). A) Variety MN-656 shows a continuous increase in the intensity of all amylase bands. B) Variety MN-684 shows a higher number of more intense amylase bands on the 3rd and 4th day after germination.

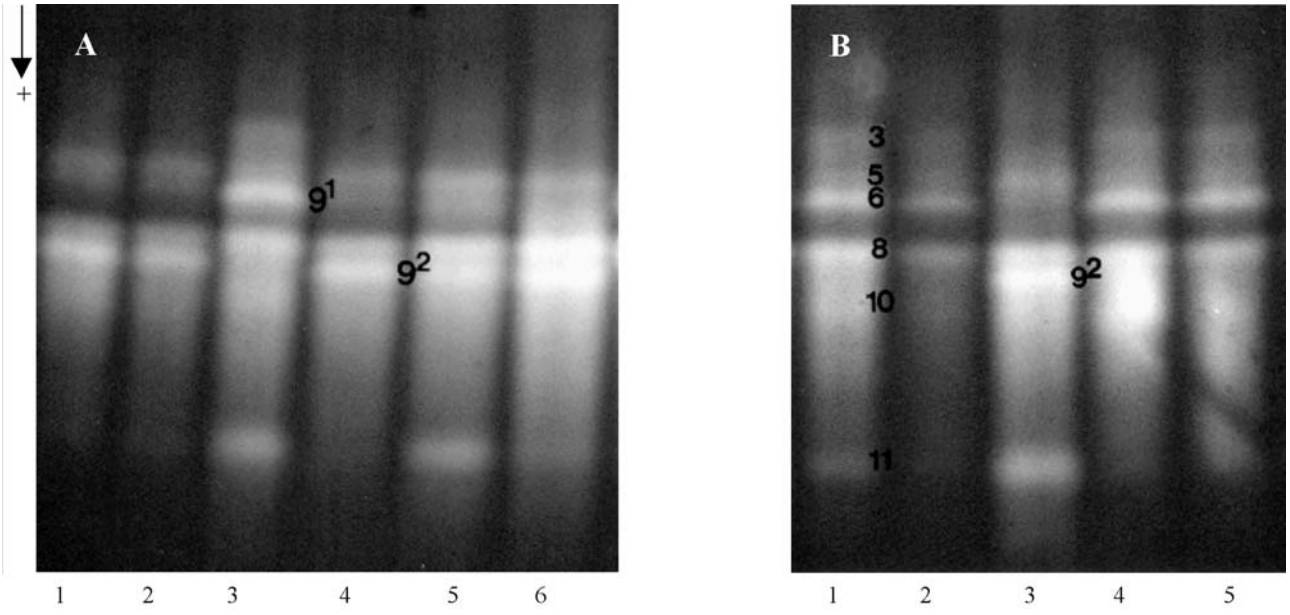

Figure 1 - Vertical electrophoresis banding-patterns of amylases extracted from barley seed endosperms on the 4th day after germination. A) Six varieties: MN-698, MN-656, MN-684, MN-668, BR-2, MN-694. B) Variety MN-684. Some loci and the alleles of loci *Amy-9* are shown.

frequency for the 14 loci are shown in Table 4. Considering all varieties assayed, only 2.8% of loci were polymorphic, with an average number of 1.04 alleles per loci, and an average expected heterozygosity of only 0.7%. Structural variability was also evaluated using the Jaccard similarity coefficient complement ($1 - S_J$). In this case, the occurrence of one or other band produced by the alleles of locus *Amy-9* was considered as the only difference between varieties. For the other loci, only one allele (regardless of whether or not the locus was active) was used to calculate structural variability. The mean intra-variety difference was $1 - S_J = 0.009$ (Table 5), a result which confirms the low intra-variety polymorphism determined by structural genes.

Although low structural polymorphism was observed, higher intra-variety variability was detected between different endosperms due to differential gene activation, *i.e.* the same locus was active or inactive in different endosperms from each variety at the same developmental stage. The intra-variety differences generated by gene activation only were measured using the Jaccard similarity coefficient complement, calculated using the number of active loci shared by each endosperm pair divided by the total number of active loci present in the pair but without considering structural (allele) differences (Table 5). The mean intra-variety difference based on regulatory differences was higher ($1 - S_J = 0.17$) than that obtained based on structural differences, indicating an increase in variability generated by differential gene activation.

The Jaccard similarity coefficient complement was also calculated considering both structural and regulatory differences (Table 5), producing a mean intra-variety difference of $1 - S_J = 0.16$, which is similar to the figure found for regulatory differences. These results suggest that regulatory polymorphism is the principal agent of amylase variability in the barley varieties analyzed.

Inter-variety differentiation

Different varieties presented few structural differences in relation to locus *Amy-9* (Table 3), with most variet-

Table 3 - Frequency of the *Amy-9*^{2} allele.

Varieties	*Amy-9*^{2} frequency*
MN-682	1.00
MN-698	0.96
MN-656	1.00
MN-684	0.30
MN-668	0.99
BR-2	1.00
MN-694	0.97
MN-691	0.75
MN-705	1.00
MN-706	1.00

* *Amy-9*^{1} frequency is complementary.

Table 4 - Intra-variety genetic variability calculated using 673 barley endosperms assessed for 14 amylase loci.

	Geneticvariability		
Variety	A	P	H
MN-682	1.00	0.00	0.000
MN-698	1.07	0.07	0.006
MN-656	1.00	0.00	0.000
MN-684	1.07	0.07	0.030
MN-668	1.07	0.07	0.001
BR-2	1.00	0.00	0.000
MN-694	1.07	0.07	0.004
MN-691	1.07	0.07	0.027
MN-705	1.00	0.00	0.000
MN-706	1.00	0.00	0.000
Mean	1.04	0.028	0.007

A = mean number of alleles per loci.
P = proportion of polymorphic loci.
H = expected heterozygosity.

Table 5 - Intra-variety differentiation over seven days after germination, calculated using the Jaccard similarity coefficient complement ($1 - S_J$).

	Differentiation					
	Structural		Regulatory		Structural & regulatory	
Variety	Mean	Range	Mean	Range	Mean	Range
MN-682	0.000	0.00-0.00	0.27	0.00-0.89	0.22	0.00-1.00
MN-698	0.004	0.00-0.07	0.20	0.00-0.50	0.14	0.00-0.75
MN-656	0.000	0.00-0.00	0.19	0.00-0.60	0.14	0.00-0.60
MN-684	0.035	0.00-0.07	0.17	0.00-0.62	0.23	0.00-0.80
MN-668	0.009	0.00-0.07	0.11	0.00-0.50	0.11	0.00-0.78
BR-2	0.000	0.00-0.00	0.19	0.00-0.60	0.18	0.00-0.60
MN-694	0.009	0.00-0.07	0.14	0.00-0.60	0.15	0.00-0.62
MN-691	0.025	0.00-0.07	0.24	0.00-0.87	0.28	0.00-1.00
MN-705	0.000	0.00-0.00	0.11	0.00-0.56	0.08	0.00-0.56
MN-706	0.000	0.00-0.00	0.10	0.00-1.00	0.09	0.00-0.43
Overall mean	0.009	-	0.17	-	0.16	-

ies having the *Amy-9*^{2} allele fixed or at high frequency, the exception being variety MN-684 in which the *Amy-9*^{1} allele was most common. This low structural gene divergence between varieties is shown in Figure 1A. The inter-variety differences (Table 6) were measured using the Jaccard similarity coefficient complement at the same three levels (structural polymorphism, regulatory polymorphism, and both simultaneously) used to evaluate intra-variety variability. Nei distance (1972), based on allele frequencies, was also calculated to assess structural gene differences (Table 7). Inter-variety divergence was very low for structural genes, with $1 - S_J = 0.026$ and $D = 0.0076$. Analysis of inter-variety variability due exclusively to regulatory differences showed a remarkable increase in inter-variety divergence caused by differential gene activation ($1 - S_J = 0.34$) as compared to the divergence obtained for

Table 6 - Inter-variety differentiation over seven days after germination, calculated using the Jaccard similarity coefficient complement ($1 - S_J$).

	MN-682	MN-698	MN-656	MN-684	MN-668	BR-2	MN-694	MN-691	MN-705	MN-706	Mean	Overall mean
MN-682	-	0.27[1]	0.29	0.48	0.31	0.35	0.36	0.36	0.30	0.34	0.34	
MN-698	**0.002**[2] 0.29[3]	-	0.21	0.42	0.31	0.38	0.27	0.32	0.24	0.38	0.31	
MN-656	**0.000** 0.33	**0.003** 0.25	-	0.43	0.35	0.30	0.27	0.30	0.21	0.40	0.31	
MN-684	**0.048** 0.38	**0.047** 0.27	**0.046** 0.30	-	0.42	0.50	0.45	0.38	0.45	0.48	0.45	
MN-668	**0.007** 0.35	**0.003** 0.38	**0.007** 0.36	**0.045** 0.37	-	0.38	0.34	0.42	0.33	0.15	0.33	0.35
BR-2	**0.000** 0.38	**0.002** 0.32	**0.000** 0.30	**0.053** 0.39	**0.001** 0.44	-	0.26	0.38	0.26	0.39	0.36	
MN-694	**0.003** 0.39	**0.003** 0.27	**0.004** 0.30	**0.039** 0.33	**0.004** 0.40	**0.004** 0.25	-	0.38	0.27	0.37	0.33	
MN-691	**0.016** 0.38	**0.019** 0.30	**0.019** 0.34	**0.041** 0.32	**0.016** 0.46	**0.019** 0.40	**0.020** 0.35	-	0.36	0.47	0.37	
MN-705	**0.000** 0.33	**0.004** 0.23	**0.000** 0.24	**0.044** 0.29	**0.001** 0.40	**0.000** 0.26	**0.004** 0.27	**0.014** 0.33	-	0.33	0.31	
MN-706	**0.000** 0.38	**0.002** 0.45	**0.000** 0.44	**0.044** 0.41	**0.001** 0.17	**0.000** 0.46	**0.004** 0.47	**0.014** 0.52	**0.000** 0.42	-	-	
Mean	**0.009** 0.36	**0.009** 0.31	**0.009** 0.32	**0.045** 0.34	**0.009** 0.37	**0.009** 0.36	**0.009** 0.34	**0.020** 0.38	**0.119** 0.37			
					Overall mean =	**0.026** 0.34						

[1]Values for structural and regulatory polymorphism together are shown above the diagonal line which cuts the table.
[2]Values for structural polymorphism are shown in bold in the first line of each cultivar below the diagonal line which cuts the table.
[3]Values for regulatory polymorphism are shown in normal type below the values for structural polymorphism.

Table 7 - Nei distance between Brazilian barley varieties based on structural differences found for the amylase loci over 7 days after germination.

	MN-682	MN-698	MN-656	MN-684	MN-668	BR-2	MN-694	MN-691	MN-705
MN-698	0.000110								
MN-656	0.000000	0.000110							
MN-684	0.036063	0.032111	0.036063						
MN-668	0.000006	0.000062	0.000006	0.035055					
BR-2	0.000000	0.000110	0.000000	0.036063	0.000006				
MN-694	0.000062	0.000007	0.000062	0.033078	0.000027	0.000062			
MN-691	0.004443	0.003148	0.004443	0.014997	0.004099	0.004443	0.003451		
MN-705	0.000000	0.000110	0.000000	0.036063	0.000006	0.000000	0.000062	0.004443	
MN-706	0.000000	0.000110	0.000000	0.036063	0.000006	0.000000	0.000062	0.004443	0.000000
Mean	0.004520	0.003986	0.004520	0.032850	0.004364	0.004520	0.004097	0.005323	0.004520
				Overall mean = 0.0076					

structural differences alone. The total difference, including structural and regulatory polymorphism, was $1 - S_J = 0.35$, again similar to the figure obtained for regulatory differences.

Discussion

Only one polymorphic locus was detected in the 10 Brazilian barley varieties analyzed in this study. In a previous investigation, Maris (1992) assessed other Brazilian barley varieties for amylases and also detected low variability for the amylase system, finding that out of the four loci analyzed only one was polymorphic with two different alleles. Although both studies have detected relatively high intra-variety structural homogeneity for amylase genes, five varieties analyzed in the present study showed some degree of intra-variety polymorphism, while those varieties analyzed by Maris (1992) did not.

The inter-variety structural divergence detected in the present study (D = 0.0076) was lower than that found by Maris (1992) for amylases (D = 0.096). In the varieties studied by Maris, one variety presented a fixed allele different from that found in the other varieties, while many of the varieties analyzed in the present research were polymorphic for the same alleles.

In the study developed by Maris (1992), amylases showed less polymorphism than other enzymes such as esterases, acid phosphatases, malate dehydrogenases, leucine aminopeptidases, and glutamate oxaloacetate transaminases. Amylases are hydrolases (IUB, 1979) involved in peripheral metabolism, and for this reason are considered potentially polymorphic enzymes (Gillespie and Langley, 1974). The low structural genetic variability found in Brazilian barley variety amylases compared to other enzymes also involved in peripheral metabolism, such as esterases and acid phospatases, must be related to the selection for malting characteristics that breeders impose upon these barley varieties. Such selection restricts the structural variability of amylase genes by selecting only certain allelic forms.

The major differences in amylase patterns found when barley varieties were analyzed at the same developmental stage were at the regulatory level. These differences are probably due to differential gene activation in the different genotypes. It is known that regulatory differences are of greater adaptive and evolutionary significance than structural gene changes (Wilson, 1976), and Soulé (1973) emphasized that the process involved in species differentiation requires changes mainly at the gene regulatory level. Differently, at the intraspecific level, gene regulation follows a standard development program with precisely coordinated sequences, although some regulatory mutants have been detected in different species (Holmes *et al*., 1983; Doane *et al*., 1983; Scandalios, 1983). In self-pollinated plants intraspecific regulatory differences seem to be more common (Motta, 1981; Cavalli-Molina, 1984; Schiengold, 1985; Georg, 1996) because, as pointed out by Cavalli-Molina (1984), self-pollination restricts gene flow, assuring that each line (an individual and its progeny) follows an independent evolutionary path. Conversely, in outbreeding species, individual differences in regulatory genes do not seem to be so frequent because gene flow, heterozygosity and recombination of regulatory genes may lead to sterile and unviable genotypes. The present study confirms the importance of regulatory mechanisms for genetic differentiation at least in self-pollinated species.

The developmental study showed that, at least for the varieties analyzed, there is a high general amylase activity around the fourth day after germination. This finding is very important for malting proposes because it would allow reduction in expenses and the malting of a larger amount of grain in less time. This conclusion was fully examined in Georg-Kraemer et al. (2001).

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