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Effects on Grain and Malting Quality of Genes Altering Barley Starch Composition

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ABSTRACT

In this study inbred barley lines carrying waxy and/or high amylose genes were obtained from a cross between Waxy Hector and a breeders' line BE285 (high amylose Glacier × Midas) and assessed for malting quality. Inbred lines were assayed and classified as having none, one or both genes. After malting, waxy lines had a slightly lower hot water extract than normal starch lines. Large effects were demonstrated for both grain nitrogen content and hot water extract in high amylose lines and, particularly, in lines with both genes. Endosperm modification during malting was reduced by both starch mutations. Electron microscopy showed that the phenotype with both genes was characterised by a highly compacted endosperm. During malting, this structure was extremely resistant to modification.

Keywords: barley starch, malting quality, waxy gene, high amylose gene.

INTRODUCTION

Starch is the main seed reserve polysaccharide of green plants[1]. Despite the importance of starch in agriculture and human nutrition, little direct effort has been placed on improving starch qualities to meet specific applications[2]. A large number of existing and potential industrial uses exist for starch and its derivatives, including the manufacture and production of paper, chemicals, fuels and plastics[3]. The functional properties of starch depend on its structure and this can be modified chemically, but such procedures may be environmentally hazardous[2]. In addition, modified barley starch may have other applications such as the replacement of high amylose maize starch in special human diets[4].

Genetic engineering of modified starches is currently being attempted in a number of species including wheat, maize and potato, but a major Scandinavian effort is being applied to spring barley[2]. Spring barley is also the major cereal crop in Scotland[5], although wheat predominates in the southern half of the U.K.

In maize, there are a number of separate mutations which increase the amylose content of starch. Many combinations of mutant genes, and their effects on starch composition, have been studied[6]. Only one high amylose gene, however, has been found in barley[7], but lines have been produced in which this gene has been combined with the waxy gene to produce a starch with around 15% amylose[8].

Modification of starch may widen the opportunity to utilise barley for industrial purposes, but the need to satisfy existing outlets will remain. The commercial value of genotypes with modified starch would be greatly increased if they could also be used for malting and brewing. To achieve good malting quality, extensive endosperm breakdown, or modification, must accompany the germination phase of malting, with cell walls and protein being degraded and the starch granules exposed. A hot water extract of the resultant malt should yield a high level of fermentable sugars from readily gelatinised starch granules that are depolymerised by malt amylases. To date, there

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is limited published information on the malting quality of barley starch variants. In this study, inbred lines from a cross between a waxy and a high amylose parent are classified into lines carrying either, both or no starch modifying genes and their malting quality is assessed.

EXPERIMENTAL

Materials

The waxy mutation has been readily induced in a number of North American cultivars[9]. Waxy Hector, which carries this mutation, was crossed with the breeders' line BE285, produced from a cross between high amylose Glacier and Midas at the former Scottish Plant Breeding Station, now part of the Scottish Crop Research Institute (SCRI)[10]. BE285 carries the dwarfing gene termed *GPert*, originally observed in the cultivar Golden Promise[11]. This mutation, characterised by a very erect seedling habit, was also present in Midas. Inbred lines, which segregated into *GPert* or tall (i.e. without the dwarfing gene) types, were produced by single seed descent (SSD)[12]. This enabled rapid production of pure breeding lines, representative of the genetic variation present within the cross, and eliminated problems associated with heterogeneity or differences in genetic background which might otherwise limit comparisons between populations. These lines were grown as paired rows, 2 m long, in 1992 at SCRI, Dundee.

Grain tests and malting

Following harvest, grain samples from Waxy Hector × BE285 were screened over a 2·2 mm sieve and the grain retained was assessed for thousand corn weight (TCW), grain nitrogen (NIT) and grain milling energy (GME)[13]. Milling energy was determined by measuring the velocity of a flywheel at the time the seed was dropped into the mill chamber and twice more at 2 s intervals. Most of the milling occurs between the first and second assessments, the milling energy A (ME: A) period. The difference between the second and third measurements, the ME: B period, does not usually give high values or vary greatly between samples of cultivars[13]. Consequently, milling energy results are usually presented for the A period, but, here, in an experimental situation, results for both ME: A and ME: B are given. Samples were malted and extracted as described by Swanston and Taylor[14]. Water uptake during steeping was calculated by drying and weighing the grain after the steeping phase[14].

Starch classification

Samples were classified into the various starch types using a convenient technique described by Hovenkamp-Hermelink *et al.*[15], with the modification that 100 mg of barley flour was dispersed in distilled water (2 ml), then the starch was dissolved by the addition of 2M sodium hydroxide[16] (2 ml). After 30 min, the solutions were neutralised by the addition of 1M hydrochloric acid (4 ml) and diluted by a factor of 40 with distilled water. An aliquot of each solution was added to an iodine/potassium iodide solution and the absorbance of the starch/iodine complex was read at 2 wavelengths in a spectrophotometer, as described by Schondelmaier *et al.*[8]. These workers showed that lines from a cross between a waxy and a high amylose barley fell into four distinct classes based on the ratio of absorptions. They were also able to verify the presence of mutant genes, by the use of cDNA clones as probes. Consequently, it is possible to deduce the alleles present in inbred lines from the proportion of amylose to amylopectin.

Malt modification tests and microscopy

Malt samples were assessed for milling energy as described above for the grain samples and the percentage cell wall modification was determined by the fluorescence method in Aastrup *et al.*[17]. Malted grains from each starch type were also taken for examination by scanning electron microscopy, following preparation as described by Camm *et al.*[18]. The grains, which had been cut in a longitudinal plane, were examined initially under low power to ensure that a comparable area of endosperm, close to the scutellum, was studied in each sample.

RESULTS

Amylose:amylopectin ratio

Sixty inbred lines from the cross Waxy Hector × BE285 were separated into four groups based on the ratios of the absorptions at the two wavelengths (Table I). Initial results for the proportion of amylose, calculated by the formula used by Hovenkamp-Hermelink *et al.*[15], were, how-

Table I Ratio of amylose to amylopectin in four starch groups from the cross Waxy Hector × BE285

Starch group	Genotype	Ratio of readings (620:535 nm) Mean	s.d.	Amylose (%)
Normal	AMO1 AMO1 Wx Wx	1·06[a]	0·028	21·47
Waxy	AMO1 AMO1 wx wx	0·77[b]	0·027	3·59
High amylose	amo1 amo1 Wx Wx	1·18[c]	0·027	33·01
Waxy + high amylose	amo1 amo1 wx wx	0·88[d]	0·019	9·43
Parents				
Waxy Hector		0·76	0·013	3·13
BE285		1·17	0·010	32·91

Mean values followed by a different letter are significantly different at the 0·1% level.

Table II Ratios of amylose:amylopectin in grain and malt samples of normal and high amylose barley inbred lines from the cross Waxy Hector × BE285, following precipitation of the starch with ethanol. Ratios for malt starch samples without ethanol precipitation are also given

Amylose type	Ratio of readings (620:535 nm) Mean	s.d.	Amylose (%)
Grain samples (ethanol)			
Normal	1·13	0·019	27·77
High	1·26	0·012	43·02
Malt samples (ethanol)			
Normal	1·14	0·027	28·53
High	1·26	0·018	43·02
Malt samples (no ethanol)			
Normal	1·07	0·019	22·31
High	1·17	0·012	32·91

ever, considerably lower than those observed for the four starch groups in barley by Schondelmaier *et al.*[8]. Samples were therefore taken from the normal and high amylose lines, the two groups with the highest amylose contents. They were re-tested, with the starch being precipitated with ethanol[19] and, subsequently, re-dissolved in sodium hydroxide. The test was applied to flour of both grain and malt samples. The results from both were very similar (Table II). In addition, amylose contents of both the normal and high amylose starches were similar to those reported elsewhere[20]. Malt samples, in which the starch was extracted and dissolved without the ethanol precipitation step (Table II), gave apparent amylose contents similar to those of the grain samples (Table I).

Constituents of the starch granule, other than amylose and amylopectin may interfere with iodine tests, and therefore precise measurements of amylose content can only be made following extraction and purification of the starch granules. However, such a process is time-consuming and not readily applicable to the screening of populations of inbred lines. It was therefore considered that the clear differences observed here and by other groups using this technique[8,15] provided sufficiently accurate discrimination between the four starch groups.

Grain quality results

Division of the inbred lines into four classes resulted in an apparent, but not statistically significant, surplus of waxy types, i.e. 21, compared to 11 with normal starch, 11 high amylose and 17 with both genes. The *GPert* locus, however, showed a distribution very close to the expected 1:1 ratio as there were 28 *GPert* and 32 tall types among the inbred lines. This confirmed that the cross population was segregating as expected. The *GPert* gene does, however, exert an effect on thousand corn weight[21] so TCW results were recorded for *GPert* and tall types separately (Table III).

In the absence of the *GPert* gene, both the waxy and high amylose genes gave a reduction in grain size. This is consistent with the reduction in starch content and granule size associated with both mutants[20]. Lines with normal starch carrying the *GPert* gene did not have larger grains than the high amylose lines or waxy lines with the *GPert* gene, suggesting that the reduction in grain size associated with the waxy[22] or high amylose type can be modified by genetic background. Lines

Table III Thousand corn weights of four different starch groups from the cross Waxy Hector × BE285, with and without the *GPert* gene

	Thousand corn weight (g)			
	With *GPert*		Without *GPert*	
Starch group	Mean	S.D.	Mean	S.D.
Normal	46·44[a]	1·102	55·12[c]	0·897
Waxy	46·39[a]	1·041	50·78[e]	0·840
High amylose	47·17[a]	1·361	52·48[d]	0·760
Waxy + high amylose	44·43[b]	0·908	50·49[e]	0·944

Means followed by a different letter are significantly different at the 0·1% level.

Table IV Grain nitrogen and milling energy values for the four starch groups from the cross Waxy Hector × BE285

	Grain nitrogen (%)		Milling energy: A (J)		Milling energy: B (J)	
Starch group	Mean	S.D.	Mean	S.D.	Mean	S.D.
Normal	1·88[a]	0·042	743·3[a]	37·66	82·4[a]	25·06
Waxy	1·95[a]	0·038	809·5[b]	32·83	159·2[b]	40·66
High amylose	2·10[b]	0·044	832·2[c]	26·56	175·1[b]	30·92
Waxy + high amylose	2·13[b]	0·052	924·6[d]	33·87	387·9[c]	35·26

Mean values followed by a different letter are significantly different. All differences are significant at the 0·1% level except for Waxy vs. High amylose for ME: A, significant at the 1% level.

carrying both starch mutant genes did, however, display a much reduced grain size compared with normal starch lines among both tall and *GPert* types. For all starch groups, there was a highly significant difference between tall and *GPert* lines for TCW.

Presence of the high amylose gene produced an increase in grain nitrogen content (Table IV). The lines carrying both genes did not show significantly higher grain nitrogen levels than the high amylose types. In contrast, the milling energy values for both mutants showed a large increase compared with the normal starch lines, and the effect of combining the two genes appeared to be additive. Both starch variants showed a high ME:B in addition to the increased ME: A, suggesting that greater time, as well as mechanical energy, was required to disrupt the endosperm structure. Both starch variants were therefore associated with deleterious grain quality components, and these effects appeared to be increased by combining the two genes.

Malting quality assessments

Water uptake during steeping showed an additive effect of the two starch mutant genes (Table V). The increase in water uptake associated with the waxy gene has been documented previously[22] but, in this study, its association with the high amylose gene was observed to accompany an increased level of grain nitrogen. This conflicts with the view of MacGregor[23] that increased levels of protein, especially in the outer endosperm, would inhibit water uptake.

Since neither cultivars Hector[22] nor Midas[24] offered the genetic background for good malting quality, and both grain nitrogen and milling energy results were high, hot water extracts of the normal starch lines were generally of low to moderate levels (Table V). The waxy gene had a small, but significant, deleterious effect on extract. In particular, there was a very large deleterious effect of the high amylose gene. The lines carrying both mutant genes had exceptionally low levels of hot

Table V Water uptake during steeping and hot water extracts of the four starch groups from the cross Waxy Hector × BE285

Starch group	Water uptake weight after steep (g)		Moisture content (%)	Hot water extract (L°/kg)	
	Mean	s.d.		Mean	s.d.
Normal	19·31[a]	0·071	44·08	287·8[a]	3·63
Waxy	19·75[b]	0·072	45·32	277·0[b]	3·29
High amylose	19·92[b]	0·114	45·78	240·9[c]	6·49
Waxy + high amylose	20·55[c]	0·072	47·45	219·8[d]	4·22

Mean values followed by a different letter are significantly different at the 0·1% level.

Table VI Malt milling energies and cell wall modification in the four starch groups from the cross Waxy Hector × BE285

Starch group	Malt milling energy (J)		Cell wall modification (%)	
	Mean	s.d.	Mean	s.d.
Normal	353·9[a]	15·27	66·6[a]	4·83
Waxy	425·8[b]	12·40	44·1[b]	5·09
High amylose	436·2[b]	23·17	46·0[b]	4·41
Waxy + high amylose	530·6[c]	16·81	33·6[c]	4·86

Mean values followed by a different letter are significantly different at the 0·1% level.

water extract, significantly below those of the high amylose lines. These results, plus those for milling energy (Table IV) suggested that the various starch types might have different endosperm structural features or patterns of modification. Malt milling energy and cell wall modification results (Table VI) showed that the lines carrying both mutant genes modified much less extensively than lines carrying only one of the genes. There was, however, no significant difference between the waxy and high amylose lines for either of these characters.

Scanning electron microscopy

Possible differences in endosperm structure among the four starch types were investigated using scanning electron microscopy of a representative sample of each type. By the end of malting very different patterns and extents of modification were observed. In the normal starch type [Fig. 1(a)], cell walls and protein have been extensively degraded, enabling the starch granules to be clearly observed. However, unlike the pattern observed with a good malting barley[25], large numbers of B-type small starch granules remain and there is no visual evidence of degradation in A-type large granules. By contrast, the waxy type [Fig. (1b)] shows some residual cell wall and protein matrix. However, small starch granules are not observed and there is some evidence of enzymic attack on the surface of the large granules. This shows a similarity, although in not such a pronounced manner, to the pattern of modification observed in malted sorghum[26] where there is extensive pitting of the starch granules while the cell walls remain substantially intact. This is consistent with the findings of Goering *et al.*[27] that waxy starches are more readily solubilised and degraded. Hence, waxy genotypes can give moderate levels of extract despite poor endosperm modification. The survival

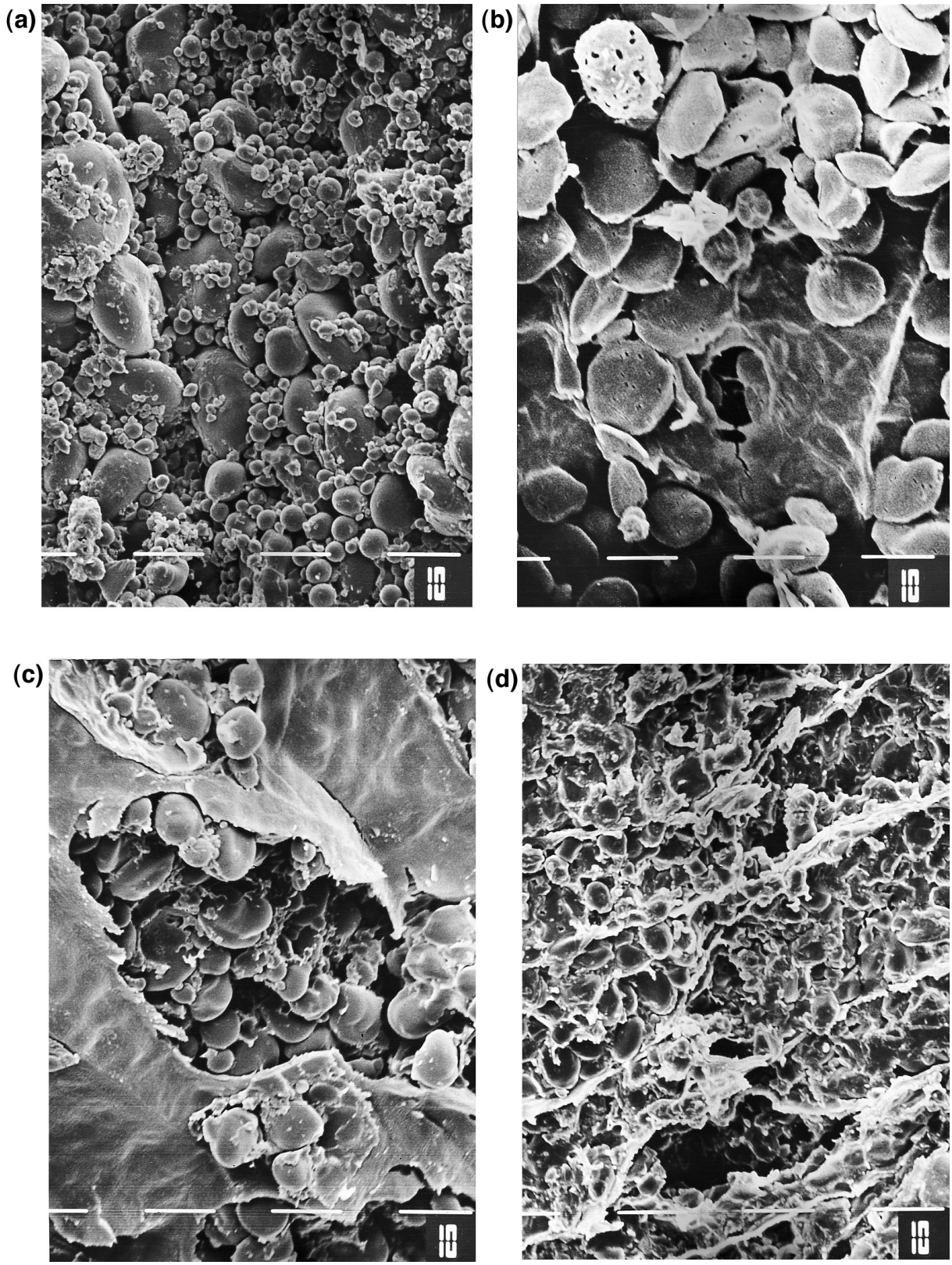

Figure 1 Scanning electron micrographs of malted grains from inbred lines from the cross Waxy Hector × BE285. Bar indicates 10 μm. (a) Normal starch line; (b) waxy starch line; (c) high amylose starch line; (d) combined waxy and high amylose line.

of cell wall material, however, confirming the results of Calcofluor fluorescence (Table VI), may result in filtration problems as the extracted malt will have a higher viscosity. These results suggest that a waxy starch version of a modern malting barley could be of some value but it would have to be selected for high (1→3, 1→4)-β-D-glucanase activity.

The high amylose line [Fig. (1c)] shows very limited modification. The increase in grain nitrogen associated with this gene appears to result in a matrix of storage protein which is not readily degraded. This would also explain why the high amylose types had very high milling energies, although soluble (1→3, 1→4)-β-D-glucan levels did not appear to be particularly high[28]. There is no evidence of pitting of the starch granules. This lack of physical modification is consistent with the earlier finding (Table II) of amylose at similar levels in the starch (with and without ethanol precipitation) of both malted and unmalted high amylose lines. This suggests that the chemical composition of the granules was also preserved throughout malting.

The line combining both mutant genes shows scarcely any modification during the malting process [Fig. (1d)]. In addition, it can be seen that the starch granules are very much smaller than in either of the single gene starch variants. The starch has a very different amylose:amylopectin ratio from the individual mutant or normal starch types and is deposited in very small granules embedded into an exceptionally compacted endosperm. This may lead to problems in extracting the starch and the combined mutant phenotype is likely to be of little use in conventional malting. There may, however, be other uses for this type, e.g. in low alcohol brewing, especially if it could be combined with high protease activity.

DISCUSSION

The effects of genes for high amylose and waxy starches in barley show some similarities to those encountered in maize. The waxy gene, for example, which results in the elimination of type I granule bound starch synthase[29], is assumed to function in a similar manner in both species[30], although amylose content in waxy barley is slightly higher than in waxy maize[31]. Lipid content of the starch granules is also positively correlated with amylose content in both barley and maize[30]. In barley, amylose has been shown to exist in two forms, one of which is complexed with lipid, this complex being insoluble and stable above 90°C[32]. If this complex is not disrupted during the malting process, a proportion of the amylose, which will increase with amylose content, will not be extracted in the first stage of brewing. Results presented here suggest that a proportion of amylose remains insoluble in dilute alkali at the completion of malting, indicating that the complex of amylose and lipid may have remained intact.

Genes that increase the proportion of amylose in maize starch reduce thousand corn weight and total starch content, e.g. the amylose-extender gene, which increases the amylose proportion to 50–60% also reduces starch content by approximately 25%[33]. In barley, the grain size (thousand corn weight) is also reduced by the high amylose gene, but the reduction in starch content is only around 6%[34]. The evidence presented here also suggests that the individual starch variant genes may have less effect on grain size than the dwarfing gene *GPert*, which is present in high yielding, commercial cultivars[11]. Consequently, it should be possible, by selection, to produce high amylose or waxy lines without incurring a loss in yield potential.

The major problems with these genotypes, however, appear to be the associations with deleterious quality factors which are not broken despite recurrent selfing generations during inbred line production. These may result from tight linkage, but it is also possible that a single genetic change may simultaneously influence more than one of the synthetic pathways operating during grain filling. Smith *et al.*[35], for example, suggested that poor malting cultivars may be characterised by a diversion of greater quantities of photosynthate from starch synthesis into soluble (1→3, 1→4)-β-D-glucan. Changes in starch may thus be accompanied by changes in other endosperm components and the manner in which these are assembled into the grain.

Ullrich *et al.*[36] suggested that waxy types had an associated increase in (1→3, 1→4)-β-D-glucan content and the reduced cell wall modification observed here would seem to support that view. Ellis[37] observed a range of deleterious malting factors associated with the high amylose gene and combining the two genes appeared to have an additive effect on the deleterious features of the two single gene starch variants. This results in a phenotype with very small A-type starch granules

tightly embedded into a protein matrix, giving a very highly compacted endosperm structure which is extremely resistant to both mechanical and enzymic disruption.

The mutations of maize and barley, which alter the amylose:amylopectin ratio, are usually associated with reduced levels of particular starch synthetic enzymes[30]. The evidence for maize and, here, for barley suggests that the modification of starch composition, if accompanied by any reduction of total starch synthesis, is likely to produce genotypes with deleterious quality characteristics. Barley mutants with increased levels of starch synthetic enzymes are not available. Therefore, it is not possible, by conventional plant breeding techniques, to modify starch composition in association with increased starch synthesis. Consequently, in future, the process of genetic manipulation[2] may be necessary to develop useful germplasm variations in starch content.

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