

Tetrazolium Staining, Mitochondria, and Barley Quality

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ABSTRACT

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Triphenyl tetrazolium chloride (TTC) staining of individual isolated barley aleurone layers was carried out under aseptic conditions and the amount of formazan generated under a standardized incubation period was quantified. The nature of the tests minimized or excluded the influences of grain damage, ageing or contaminating microorganisms. Embryos from selected Triumph barley grains, having intensely or poorly staining aleurone layers, were grown on to maturity. Collected grain was analysed and embryos from the highest-staining progeny from the high-staining aleurone line and the lowest-staining progeny of the low-staining aleurone line were grown on to yield grain of the second selected generations. Aleurone layers of the high-staining line continued to stain intensely, respired strongly, and generated large amounts of α -amylase in response to gibberellic acid and embryos from these grains respired and grew vigorously, as did whole grains. In contrast, the aleurone layers of grains from the low-staining line continued to stain poorly and respired weakly, generated little α -amylase in response to gibberellic acid, the embryos respired slowly and lacked vigour and, like the whole grains, grew poorly. It is proposed that these results are the consequence of the selection procedure separating lines with superior and inferior mitochondria. If this apparently novel approach using TTC staining is confirmed as being valid, it opens a new way for plant breeders to select for more vigorous cereal lines.

Key words: Barley, mitochondria, novel plant-breeding possibilities, tetrazolium chloride (TTC) staining, uniformity, vigour.

INTRODUCTION

2,3,5-Triphenyl tetrazolium chloride (TTC) staining has been used for many years as an indirect indicator of the viability of the embryos of cereal grains^{4,13,17,19} and more recently in a test for pregerminated grain¹¹. Uneven modification in malting some samples of Galant, a mutant of Triumph barley, was associated with the failure of aleurone tissue to stain uniformly and strongly, indicating that patches of the tissue were defective or dead²³. Indications

that aleurone layers of different barley cultivars stained to varying extents led to this project, which had the primary objective of discovering whether the intensity of tetrazolium staining of aleurone layers could be used to guide the breeding of barleys with superior malting qualities. It was already clear that the failure to stain or irregular partial staining indicated that the barley would not modify well and was probably unfit for malting. In the course of this work observations were made that are potentially of great importance for providing means for improving the quality of barleys and other cereals.

In the test, the colourless soluble triphenyl-tetrazolium salt is reduced to an insoluble scarlet formazan. In plants, the reducing hydrogen/electrons originate mainly from the electron transport systems of the mitochondria^{14,28}. Plant mitochondria have more complex electron transport systems than those of animal mitochondria, and include a pathway from cytoplasmic NADH, and a salicyl hydroxamic acid (SHAM)-sensitive, cyanide-resistant oxidation pathway which is an alternative to the cyanide-sensitive cytochrome oxidase¹⁸. Sites of tetrazolium reduction in various plant mitochondria include the cytochrome oxidase system, the cyanide and azide resistant SHAM-sensitive oxidase and the succinate oxidation system^{14,28}. We have confirmed (data not presented) that, as expected, SHAM, potassium cyanide, sodium azide, sodium malonate (a competitive inhibitor of succinate dehydrogenase), and 2,4-dinitrophenol (an uncoupling agent) all inhibit TTC reduction and the respiration of aleurone layers to greater or lesser extents, but in roughly the same proportions. The results confirm the expected involvement of the mitochondrial respiratory pathways in TTC reduction in barley aleurone layers. The roles of mitochondria in cells include electron transport, which indirectly drives oxidative phosphorylation and generates most of the ATP in aerobic cells, the metabolic inter-conversion of carbon skeletons particularly in the tricarboxylic acid cycle, a section of the photorespiration pathway, the synthesis of some amino acids and vitamins and the synthesis of some proteins using a novel system coded by mitochondrial DNA (mtDNA). However, many proteins located in mitochondria are coded for in the nuclear genome, are synthesized in the cytoplasm and are transmitted into the mitochondria. The remainder are coded for by the mitochondrial DNA and are synthesized in the mitochondria.

The correct functioning of mitochondria is essential to the survival of aerobic cells; faults in mitochondria give rise to “petite” mutants in yeast² and to a number of diseases in man^{25,26}. The genetics of plant mitochondria are complex^{18,27} (See discussion).

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MATERIALS AND METHODS

Chemicals

All chemicals used were of high grade and were supplied by the Sigma Chemical Co., Fisons Chemical Co., Aldrich Chemical Co, or by BDH Ltd.

Barleys

Barley samples (including spring and winter forms, proanthocyanidin-free barleys and high and low NIAB malting grades) were from Crisp Malting Ltd., Great Ryburgh or from New Farm Crops, Market Stainton. On arrival most of the samples were dried for three days in a rapid airflow at 43°C to a moisture content of about 8%, to improve their stability during subsequent storage. Storage was in sealed containers, often initially for periods of 4 to 12 weeks at 40°C to minimise dormancy, then at room temperature. Usually samples were screened and grains of between 2.78 and 2.18 mm were used. Exceptions are noted in the text.

Preparation of grains and aleurone layers (final methods)

For preparing aleurone layers, barley was decorticated by immersion in sulphuric acid (50%, v/v) at 25°C^{20,21}. As soon as a sample was ready (usually after 60–90 min) the grain was washed in running tap water and gently rubbed to remove lodicules and adhering fragments of husk and pericarp. Final neutralization was by immersion and frequent mixing for 30 min in ice-cold water containing suspended ice and calcium carbonate (*ca.* 0.1%). Residual calcium carbonate was rinsed away. For many purposes subsequent sterilization was essential. Acid treatment alone was inadequate⁵, as it does not kill microbial spores. Subsequent operations were carried out under aseptic conditions in a sterile chamber. A suspension of calcium hypochlorite (2%), cooled to 5°C, was filtered through Whatman No. 1 paper. After a 30 min immersion the decorticated grain was rinsed with hydrochloric acid (1 mM; to destroy residual hypochlorite), washed with sterile distilled water, drained on absorbent paper and used.

Aleurone layers, with testae still attached, were prepared from decorticated grains, which were usually obtained from grains 2.18 to 2.78 mm wide⁷. Damaged or blemished grains were discarded. Intact grains were transversely cut into two immediately behind the embryo. The embryo end might be discarded or saved at 4°C for up to 3 days, and subsequently “grown on” to obtain a mature plant and the grain it produced. The de-germed grain was cut longitudinally along the rounded, dorsal side to expose the starchy endosperm and facilitate the activity of the enzyme in the next, starchy-endosperm removal stage. The samples were treated individually, in plastic microwell plates. When required the separated embryos were also held in microwell plates, so that those that had been attached to “interesting” aleurone layers could be recovered and germinated. For the clean removal of starchy endosperm it was necessary to use a limited exposure to a purified enzyme preparation to partially degrade the sub aleurone cell walls⁷. The purified enzyme (Biofase, 50 µL) was added to each well, the plates were closed and incubated for at least 20 h at 18°C. Softened starchy endo-

sperm was removed by squeezing with sterile forceps. The isolated aleurone preparations were rinsed with sterile distilled water and were used immediately. The integrity of the aleurone cell walls of some preparations was checked using both light and scanning electron microscopy, to ensure that the exposure to the enzyme had not been excessive and physically damaging⁷. Biofase (donated by Biocon (U.K.) Ltd) contains a mixture of enzymes from *Aspergillus niger*.

Unwanted potentially damaging low-molecular-weight components were removed as follows. Bovine serum albumen (0.5 g to add bulk) was added to 10 mL Biofase. Solid ammonium sulphate was added to a final concentration of 95% saturation and after an overnight stand at 4°C the precipitated material was collected by centrifugation. The pellet was rinsed twice with saturated ammonium sulphate solution, then dissolved in the minimum volume of bicine buffer (approx. 50 mL, 1 mM, pH 8.5, 4°C) and dialysed against more buffer overnight, at 4°C. The buffer was replaced with distilled water containing some toluene (an antimicrobial agent), and a trace of ammonium carbonate to increase the pH above 8. Dialysis was continued overnight. Alkaline pH was used to check the enzymic degradation of the dialysis tubing. The preparation was freeze-dried, then suspended in MES buffer (1 mM, 30 mL, pH 5.5) containing sodium succinate (10 mM), calcium chloride (1 mM) and a mixture of antibiotics intended to combat all likely contaminating microbes (benzyl penicillin, 800 mg/L; nystatin, 400 mg/L; amphotericin B, 50 mg/L; streptomycin sulphate, 800 mg/L)^{10,21}. Aliquots were stored frozen until required.

Whole, husked grains were sterilised by immersion in ice-cold, filtered calcium hypochlorite solution (usually 2%/1 h). The rinsed and drained grains were then steeped in a solution of a mixture of antibiotics (as above) for 15 h at 15°C and used after draining.

EMBRYO CULTURE

Embryos for heritability studies, cut from grains selected with reference to the staining characteristics of their associated aleurone layers, were grown to maturity. Initially the embryos from decorticated grains, with a small amount of attached aleurone and starchy endosperm tissue, were grown on agar (10 mL, 2%) in Petri dishes. The agar was prepared in White's medium (Flow Laboratories, Woodcock Hill) with added sucrose (1%) and potassium nitrate (6 mM). Dishes were illuminated with artificial light for 3–4 days at 18°C until roots and green shoots had formed. The seedlings were transferred to vermiculite, watered with Vitafeeds fertiliser (Vitax Ltd., Lancs.). After 2–3 weeks the plants were transferred to sterile compost in peat pots and grown in a greenhouse with 18 h/day illumination. When established, grown plants were placed in 6 inch pots, with sterile John Innes compost no. 1, and grown to maturity in a protective plastic tunnel, surrounded by guard rows of barley plants growing in pots.

Harvesting grain

Ripe ears were collected by hand and dried in a rapid airflow (43°C/3 days, to a moisture content of about 8%).

To avoid the harmful damage caused by mechanical threshing, the grain was recovered by gently disrupting the ears by rubbing them between two rubber mats, and by grading the grains and fragments of the ears on slotted sieves. The grain was usually stored initially at 40°C for at least 4 weeks to reduce any residual dormancy and then at room temperature⁴.

Germinative capacity was determined by the hydrogen peroxide method of the Institute of Brewing and Distilling, using two samples of 200 grains¹³.

Germinative energy tests were carried out on agar by the 1 mL method⁸, using three 100 grain samples from each lot.

Tetrazolium (TTC) staining of aleurone layers

To obtain reliable numerical results it was necessary to stain individual, isolated aleurone layers (not groups) and not aleurone layers with starchy endosperm still attached. The "standardised" method finally adopted was chosen as being convenient and avoiding problems caused by contaminating micro-organisms. Aleurone layers were individually held in microfuge tubes (1.5 mL capacity). TTC reagent (1 mL) was added and the tubes were incubated in a waterbath for 4 h, in the dark at 35°C. The reagent consisted of triphenyl tetrazolium chloride (0.2%) dissolved in buffer, adjusted to pH 5.5, containing phosphate (10 mM), sodium succinate (10 mM), calcium chloride (1 mM) and glucose (2 mM). Evidence was subsequently obtained that the nutrients succinate and glucose were not essential, but their use was retained for consistency.

If the solutions used for decorticating the grains were degassed before use, the TTC values of the aleurone layers prepared from them were, on average, slightly higher than those prepared using oxygenated solutions, suggesting that oxygen competed with the TTC for the reducing power generated within the aleurone tissue. However, the differences were relatively small and not statistically significant.

At the end of the incubation period aleurone layers were washed with distilled water, blotted and added to dimethyl formamide (1 mL) in microfuge tubes. Formazan extraction, with agitation, was complete in about 2 h. The extracted aleurone layers were collected, dried and weighed to within 0.1 mg. The absorbance of the formazan solution was measured at 485 nm using a 1 cm light path and a small-volume, flow-through cell. Aleurone staining intensity is expressed as absorbance/mg dried aleurone. Results are given as the arithmetic mean, standard deviation ($n-1$), and n (the number of observations).

Measuring the respiration of aleurone layers

Respiration was determined by following the decline in oxygen concentration in buffer (2 mL, as used for TTC staining but without the TTC) in which aleurone layers were suspended at 25°C. Measurements were made in Hansatech type D.W.1 cells, which contained Clark-type oxygen electrodes (Hansatechnology, Norfolk)²⁴. Each cell was routinely cleaned and sterilised before use. At 25°C, 2 mL of water in equilibrium with a standard atmosphere contains 0.506 μ mole of oxygen; the value used was routinely corrected for differences in the atmospheric pressure. Results are mostly expressed as μ moles

O₂/h/mg dry aleurone layer. In some cases the tissue was stained with TTC immediately after removal from the respirometer cell.

Gibberellic acid and α -amylase in degermed grains

Gibberellic acid was applied to degermed grains using our standard techniques^{12,16}. Mature, husked grains were steeped in filtered calcium hypochlorite solution (5%, 1 h at 15°C), washed twice in distilled water and finally steeped in a solution of the mixture of antibiotics (see above) for 47 h. The steeping liquor was replaced after 23 h. Grains were drained and transferred to damp paper in a sterile cabinet. For each grain, the lemma covering the embryo was peeled back and the embryo removed using a sharpened, sterile stainless-steel needle. The peeled and de-germed grains were placed in sterile PTFE blocks so that the recesses revealed by the removal of the embryos' scutella faced upwards. GA₃ was applied in 1 μ L drops of warmed, low melting point agarose. Dosed degermed grains were incubated in a humid atmosphere at 15°C for a chosen time. They were then frozen and freeze-dried.

The estimation of α -amylase

The enzyme was assayed with commercial Phadebas amylase test tablets (Pharmacia Diagnostics, Uppsala), using a modified version of the Barnes and Blakeney method¹. The original, commercial method was designed to assay the human enzyme by measuring the release of soluble coloured degradation products from the synthetic, cross-linked and covalently coloured starch substrate. Complications arise because the barley enzyme has appreciably different properties from those of the human, mammalian enzyme. Barley extracts contain several other starch-degrading enzymes and the reaction progress curves and the relationships between enzyme concentration and colour release are not linear. The method finally adopted was as follows, giving results in arbitrary units. Ground barley (0.8 g) was added to extraction buffer (10 mL) in a boiling tube. The extraction buffer contained calcium acetate (50 mM) and maleic acid adjusted to pH 6.8. The mixture was finely ground with a Polytron homogeniser, which was rinsed with more buffer (10 mL). The mixture (20 mL) was agitated for 1 h at room temperature. Tubes were then warmed in a water-bath and held at 70°C for 10 min to complete enzyme extraction, to kill microbes and to inactivate other starch degrading enzymes³. With the Phadebas substrate, the inactivation of the other starch-degrading enzymes was probably not essential for the specific assay of α -amylase. Each tube was cooled to 5°C and the contents were centrifuged. Enzyme activity was determined in the supernatant, diluted with Phadebas buffer as needed. Diluted extract (2 mL) was warmed to 40°C in a boiling tube in a shaking water bath. At zero time pre-warmed, well-mixed Phadebas reagent (4 mL) was added and mixed in, and the tubes were shaken continuously. Phadebas reagent contained 4 Maker's tablets/10 mL buffer (calcium acetate, 10 mM and maleic acid, 10 mM, pH 6.2). Samples (1 mL) were taken at 5 min intervals up to 25 min and were added to stopping reagent (5 mL) immediately. The stopping reagent was

sodium hydroxide (0.5 M) containing disodium ethylene diamine tetra-acetic acid (Na₂ EDTA; 50 mM). Samples were centrifuged and the absorbances of the clear supernatants determined at 620 nm, using a 1 cm light path, measured against an enzyme-free blank. A standard calibration curve was prepared using commercial malt. Enzyme extract was diluted with Phadebas buffer and enzyme activity then determined. A calibration graph was constructed using the varying incubation times and the weight of extracted malt in the diluted solution. The calculated “reduced Phadebas arbitrary units” (RPU) were plotted against absorbance at 620 nm, giving an upward curve. Enzyme activities were calculated by converting the absorbances to RPUs using the calibration graph. The α -amylase activity was given as RPUs/incubation time (min).

RESULTS

The results of TTC staining tests

Following the development of the TTC aleurone staining method various tests were made. Triumph barley (TN 1.71%) was graded into width classes by sieving. The dry weights (mg) of the aleurone layers recovered from the different fractions declined with decreasing grain width, but the TTC values for these fractions (TTC reducing power/mg tissue) did not differ significantly (Table I). This result was confirmed using Maris Otter barley (data not shown).

TTC reducing power of aleurone layers from samples of Triumph barley (n = 40), which differed in their bulk nitrogen (TN) content, was determined (Table II). All samples were 99% or 100% viable. The mean values considered alone suggest a trend, but it is clear that the considerable overlapping standard deviations of the results make any conclusions unsafe. It was desirable to analyse the nitrogen content of the barley grains individually and non-destructively by NIR before the aleurone layers were prepared and analysed. Collaboration with a commercial company showed that the use of NIR in this way was feasible, but the high cost of the equipment and the unwillingness of the commercial organisation to proceed

Table I. Grain width and aleurone layer staining. The results are for groups of 20 aleurone layers prepared from Triumph barley.

Grain width (mm)	Aleurone wt (mg)	TTC staining (abs/mg)
>2.74	2.24±0.36	0.074±0.032
2.74–2.51	1.90±0.44	0.072±0.028
2.51–2.18	1.34±0.26	0.084±0.040
<2.18	1.25±0.40	0.063±0.022

Table II. The TTC aleurone staining characteristics of samples of Triumph barley (n = 40) differing in their bulk nitrogen contents.

Sample TN%	TTC values (abs/mg)
1.47	0.064±0.024
1.49	0.080±0.035
1.51	0.082±0.029
1.71	0.093±0.024
1.83	0.110±0.036
1.86	0.084±0.032
1.89	0.058±0.016

with a limited collaborative trial compelled us to abandon this approach.

Respiration rates of aleurone layers from Triumph barley were determined and immediately afterwards they were stained using the TTC method. There was a reasonable, but not perfect, proportionality between the two measurements. Other results indicated that the two measurements were not exactly equivalent (see below). It is significant that the wide scatter of results obtained using the TTC method with individual aleurone layers from one variety of grain was mirrored by the wide scatter of the respiration rate measurements.

Aleurone layers were prepared from decorticated Triumph barley, using selected mealy and steely grains. TTC values were mealy, 0.065±0.024 and steely, 0.052±0.020, values that are not significantly different.

Samples of Blenheim, Triumph, Camargue and Prisma barleys were received fresh. Half of each sample was stored in the un-dried state in sealed jars at room temperature for 5 weeks. The other half-samples were dried in a rapid airflow for 3 days at 43°C, then stored in sealed jars for 4 weeks at 40°C, then at room temperature (a treatment intended to minimise dormancy). All samples had germinative capacities of 98–99%. The drying and warm storage treatments reduced dormancy and improved the germination rates and final 3-day germination percentages, but had no significant influence on the TTC values (Table III).

In a separate trial, part of a sample of dormant Doublet was dried and stored at 40°C for 12 weeks. The G. E. (%) of the dried, cool-stored and heat-treated samples were 18% and 85% respectively and the TTC values were 0.042±0.013 and 0.058±0.019 respectively. In a similar trial with a sample of Triumph, the G. E. values were 32% and 99% and the TTC values were 0.129±0.034 and 0.104±0.023. These results indicate that warm, dormancy-reducing treatments have no significant influence on the tetrazolium reducing capabilities of aleurone layers.

A sample of Triumph (ca. 2000 grains) was surface-sterilised with calcium hypochlorite for 1 h then steeped in an antibiotic solution for 16 h. The grains were drained and put to germinate on agar in approx. 25 cm square plastic “bioassay” Petri dishes. The first 1% to germinate was collected and the last 1% to germinate was collected.

Table III. The effects of drying and warm storage on the germination (3 × 100 corns; 1 mL agar test) of four barleys and TTC staining of aleurone layers (n = 40).

	Germination (%)			TTC values (abs/mg)
	Day 1	Day 2	Day 3	
Camargue				
Undried	24	66	77	0.091±0.018
Dried	40	91	96	0.091±0.012
Prisma				
Undried	26	82	90	0.096±0.013
Dried	50	97	98	0.104±0.014
Blenheim				
Undried	27	64	79	0.078±0.018
Dried	36	94	98	0.089±0.014
Triumph				
Undried	22	64	75	0.109±0.019
Dried	36	91	93	0.106±0.022

The grains (20) were manually dehusked by peeling, and aleurone layers, contaminated with residual husk and pericarp, were prepared and stained with TTC. The absorbance values/20 aleurone layers were 0.487 ± 0.051 for those from the rapidly germinating grain and 0.499 ± 0.119 for the slow germinating grain. Evidently the rate of chitting was not simply related to the intensity of TTC staining. The scatter of the results of the slow germinating grains was very much greater than that of the fast germinating grains.

Grains were taken from the tops, the middles and the bases (nearest the stems) of ears of three different barley varieties. Aleurone layers from the grains in each group were investigated by the TTC test (Table IV). Despite the apparent trend with the results of Sarah, there were no regular, significant differences between the TTC values of the aleurone layers from grains from different parts of the ears.

A collection of barleys was obtained that included winter (W) and spring (S) forms having a mixture of poor (NIAB grade 1) and good (NIAB grade 9) malting qualities. Aleurone layers were prepared and 40–45 were individually assayed by the TTC method. The results are in Table V. The highest result was given by Gerbel, a variety with a poor malting grade. There is no simple relationship between TTC values and malting quality, even though very low staining in Galant barley correlates with poor malting performance²³. There is no relationship between TTC staining and varieties being winter or spring types.

Table IV. TTC values of aleurone layers prepared from grains taken from different places on the ears of three barley varieties.

Variety	Position on ear	TTC values (abs/mg)	No. grains (n)
Posaune	Top	0.049±0.014	11
Posaune	Middle	0.049±0.007	11
Posaune	Bottom	0.043±0.013	9
Sarah	Top	0.088±0.022	7
Sarah	Middle	0.106±0.020	8
Sarah	Bottom	0.129±0.034	10
Maya	Top	0.129±0.033	9
Maya	Middle	0.127±0.022	11
Maya	Bottom	0.115±0.026	11

Table V. TTC values of aleurone layers from a range of barley varieties (n = 40–45).

Variety	Winter or spring	NIAB grade	TTC values (abs/mg)
Vixen	S	1	0.097±0.027
Panda	W	1	0.111±0.029
Target	W	1	0.110±0.035
Gerbel	W	1	0.142±0.035
Igri	W	1	0.087±0.018
Mimosa	S	1	0.093±0.030
Pirate	S	1	0.112±0.034
Puffin	W	9	0.101±0.027
Finesse	W	9	0.104±0.027
Halcyon	W	9	0.097±0.028
Pipkin	W	9	0.112±0.056
Natasha	S	9	0.120±0.034
Blenheim	S	9	0.115±0.028
Corniche	S	9	0.119±0.025
Doublet	S	9	0.129±0.035
Triumph	S	9	0.137±0.032

Different samples of the variety Galant have sometimes failed to malt well, but not always. This variety lacks anthocyanin pigments and proanthocyanidins²³. Three small samples of Galant were obtained. Sample A had been grown in the UK from seed that had been grown from seed produced in the UK. Sample B had been grown in Spain from seed that had been produced in Spain, while sample C was grown in the UK from seed that had been grown in Spain. The TTC values of the different samples were as follows: sample A, 0.029 ± 0.011 (n = 14); sample B, 0.056 ± 0.016 (n = 29) and sample C, 0.020 ± 0.009 (n = 10). Before the formazan was extracted from the aleurone layers, these were inspected. Sample B stained uniformly while A and C had patches of unstained tissue. Despite the small numbers of grains available, it is clear that Galant grown in the UK had a significantly lower TTC staining-capacity than that grown in Spain, indicating that the quality of the aleurone layer in this variety was strongly influenced by the growing conditions encountered by the mother plants. Although the aleurone samples of B stained uniformly, the colour yield was still lower than that of many of the other cultivars investigated.

Most of the grains used in these trials were of high quality. However, an old sample of Doublet of uncertain history and provenance yielded a TTC value about 50% less than those usually attained with this cultivar, so deterioration with ageing possibly inhibited mitochondrial functions.

Samples of four varieties were investigated. Blenheim contains both anthocyanin pigments and proanthocyanidins. Eminant and Dramant, like Galant, lack both of these families of phenolic substances while 88088-1 is proanthocyanidin-free but contains anthocyanin pigments. Aleurone layers were prepared and tested for their TTC values and respiration rates. The results are in Table VI. Eminant and Dramant, like Galant, stained relatively poorly in comparison with the other samples. However, when the respiration rates of aleurone layers were determined the differences were not significant (Table VI). Thus, perhaps surprisingly, but as noted above, TTC staining and respiration measurements do not reflect exactly the same characteristics of the mitochondria and the aleurone layers.

The heritability of TTC staining

The spreads of the TTC staining results were wider than were easily explained by experimental error, and numerous factors that might have influenced the results (dormancy, position in the ear, nitrogen content, physical grain damage, micro-organisms, drying damage, grain size) were excluded or shown not to be significant. In addition high-quality grain samples were used, reducing the chances that grain deterioration had influenced the

Table VI. TTC values (n = 50) and respiration rates (n = 20) of barley aleurone layers.

Variety	Antho-cyanins	Proantho-cyanidins	TTC values (abs/mg)	Respiration (µmole O ₂ /h/mg)
Blenheim	+	+	0.117±0.023	0.114±0.015
Eminant	-	-	0.078±0.018	0.108±0.020
Dramant	-	-	0.066±0.017	0.106±0.017
88088-1	+	-	0.100±0.021	0.101±0.030

results. Other explanations were looked for and it was decided to test if the observed variability had a genetic component, even though the grain samples were of "pure varieties." It has long been apparent that "pure varieties" are not wholly genetically homogeneous^{4, 22} and it seems virtually certain that typically plant mitochondria are genetically inhomogeneous^{18, 27}.

Samples of aleurone layers ($n = 200$) from Triumph barley (the "parental stock") were analysed. The TTC values were 0.072 ± 0.028 . Embryos from grains having "high-staining" aleurone layers (>0.105) and "low-staining" aleurone layers (<0.041), i.e., those above and below the standard deviation range of the results, were saved and eight of each were grown on to maturity. Selection was in February-March. The grains from these plants were harvested, dried and stored. They were called the 1st generation, high-stain and 1st generation low-stain progeny. Aleurone layers ($n = 100$) were prepared from these samples and were TTC stained. The aleurones from the high-stain progeny analysed as 0.086 ± 0.034 , while those from the low-stain progeny gave the results 0.066 ± 0.022 . Although the standard deviations of these results overlapped and they were not significantly different, the upward range of the high-staining sample extended to substantially higher values (0.120 and above) than the downward extent of the low-staining progeny (0.044 and below).

A second round of selection was carried out the next March on the 1st generation progeny. Embryos from the high-staining line grains that had TTC values above the standard deviation above the mean (i.e., >0.120) and from the low-staining line below the standard deviation below the mean (i.e., <0.044) were selected and grown on to maturity. Aleurone layers ($n = 20$) were prepared from 1st generation high- and low-staining progenies and the respiration rates and TTC values were determined. The spreads of the results were similar, but on average the high-staining progeny respired and stained more strongly than the low-staining material. Once again it was noted that the TTC and respiration rate values were not exactly in proportion and so, for example, aleurone layers with almost the same respiration rates had different TTC values.

The selection process was repeated in March-April, of the next year. Aleurone layers ($n = 40$) were prepared from the 2nd generation grains. The aleurones from the high-staining line gave TTC values of 0.118 ± 0.023 (uncorrected for dry weight), while those of the low-staining line gave 0.046 ± 0.022 (uncorrected for dry weight). The respiration rates of the aleurone layers from the high- and low-staining lines were 0.12 ± 0.02 and 0.03 ± 0.01 $\mu\text{mole O}_2/\text{h/mg}$ respectively. The respiration rates of 10 groups of 3 isolated embryos from these grains were 0.068 ± 0.009 and 0.028 ± 0.011 respectively, while the value for the parental (unselected) grain was 0.059 ± 0.006 $\mu\text{mole O}_2/\text{h/mg}$. Thus the difference between the two selected lines was now very striking. Embryos from the high-staining line with aleurone TTC values >0.230 (uncorrected for dry wt.) grew vigorously, and 20 out of 20 embryos grown on survived initial cultivation on vermiculite and reached a height of 4–5 cm after 2 weeks. However embryos from low-staining grains with aleurone TTC values <0.018 (uncorrected) grew badly. The vigour of the low-staining

Table VII. The α -amylase (arbitrary units; mean \pm s.d., $n = 5$) produced by peeled and degermed grains of barley, dosed or not dosed, with gibberellic acid (GA_3 , 5 ng/grain) and incubated at 15°C for the times shown^a.

	Incubation time (h)		
	48	72	96
Control			
GA ₃ -ve	0.8 \pm 0.4	2.2 \pm 0.5	3.6 \pm 0.1
GA ₃ +ve	3.2 \pm 0.3	11.7 \pm 2.7	35.9 \pm 2.3
High-stain			
GA ₃ -ve	2.3 \pm 0.8	14.6 \pm 4.1	21.9 \pm 1.1
GA ₃ +ve	4.9 \pm 2.0	20.2 \pm 3.5	43.1 \pm 3.1
Low-stain			
GA ₃ -ve	0.4 \pm 0.4	0.4 \pm 0.3	1.1 \pm 0.7
GA ₃ +ve	1.2 \pm 0.7	1.0 \pm 0.6	1.4 \pm 0.8

^aThe grain samples were unselected, parental Triumph barley (control) and samples of the second selected generation with high-staining and low-staining aleurone layers. Enzyme was absent from all 24h samples.

grains was very low indeed; only 3 out of 96 embryos survived and after 2 weeks these were 1 cm in average height. When ten grains from each sample were germinated on damp paper all grains from the high-staining line germinated and grew strongly. Of the grain from the low-staining line, nine did not germinate at all and one grain produced roots but no shoot. The wide standard deviation of the high-staining, second generation grain indicated that more selection would be advantageous, but circumstances did not allow us to continue this work. In field plots, seed from the second-generation, low-staining progeny died out. High-staining progeny grew well, and were judged to be superior to the unselected controls.

Samples of grains of the 2nd generation, high- and low-staining progenies were surface-sterilised (using hypochlorite and antibiotics) then degermed. Half of each batch was dosed with gibberellic acid (0.5 ng/grain). The samples were incubated at 15°C and at intervals sub-samples were frozen and freeze-dried. The α -amylase was extracted from the dried samples and assayed (Table VII). The amount of enzyme produced by the low-staining samples was negligible. In contrast to most of our previous studies with Proctor barley, the aleurone layers of both the high-staining and control grains generated some α -amylase in the absence of added gibberellic acid. This was much more striking with the high-staining sample. This result may have been due to gibberellin release from the embryos during the 48 h steep which preceded embryo removal, and which was not used in our earlier experiments. With added GA_3 , both control and high-staining aleurone layers produced enhanced levels of enzyme, the high-staining samples making the most (Table VII), although the % increase in enzyme yield in response to added GA_3 was greater in the control grains.

DISCUSSION AND CONCLUSIONS

Using a standardised method for staining isolated individual aleurone layers with triphenyl tetrazolium chloride (TTC), it was shown that while poor staining occurred in low quality samples of Galant barley (but less so in better grown samples) in a range of surface-sterilised barleys, staining capacity was not simply related to total nitrogen content, grain size, cultivar, malting quality, spring- or

winter-variety, mealiness or steeliness, grain being freshly harvested or dried and warm stored, fast or slow germination or position of grains in the ear. The nature of the trials excluded the effects of other factors that influence grain heterogeneity, including mixture of varieties, contamination with microbes, physical damage and dormancy. There was a partial and approximate (but imperfect) correlation between TTC staining and the respiration rates of aleurone layers, both of which depend on functioning mitochondria. The authors regard TTC staining as an indirect, partial measure of the functionality of mitochondrial electron transport chains. In all cases it was apparent that the scatter of the TTC staining values for aleurone layers prepared from grains of any variety of barley (as measured by the standard deviation of the results) was surprisingly large.

By staining aleurone layers with TTC and growing on the embryos of high-staining and low-staining grains, it has been shown that the variability of the results has a significant genetic component. This genetic variability is probably located in the mitochondrial DNA, and may now, apparently for the first time, be exploited by plant breeders. Following two selection cycles, and comparing the results with unselected, control samples of grain, it was clear that low-staining grains lack vigour in the field and germinate poorly and when degermed produced little or no α -amylase in response to a dose of gibberellic acid. Their aleurone layers respired slowly. Embryos from the low-staining grains themselves stained weakly and respired relatively weakly. In contrast, the progeny of high-staining grains germinated well and grew strongly in the field, while their aleurone layers and embryos stained strongly and respired vigorously. The degermed, high-staining grains produced large amounts of α -amylase in response to a dose of gibberellic acid. It is clear that the selection processes produced weak or strong lines of the one variety of barley, with weak or superior malting quality and vigour characteristics. It seemed likely, from the large standard deviations of the results, that further selection stages to the high-staining line would have improved the line further, while attempts at further selection for low-staining would result in a wholly non-viable line. It is believed that TTC staining in some way assesses the overall performance of mitochondrial electron transport chains which, among several functions, drive oxidative phosphorylation and ATP production and which may differ between different grains of any cultivar. The selection for high-staining aleurone layers, and so indirectly for higher staining embryos, seems to achieve the selection of "superior" mitochondria, and perhaps more "superior" mitochondria per cell.

The possible genetic basis for the results of TTC staining selection is complex and certainly not fully understood. With grains from a single cultivar, variations in the nuclear genome are expected to be small but not necessarily negligible^{4, 22}. Both nuclear and mitochondrial DNA (mtDNA) are involved in coding for mitochondrial proteins. The modern view of the behaviour of plant mitochondria and mtDNA is surprising and complex and our information is inadequate¹⁷. Mitochondria move around within hydrated, living cells, changing their shapes and sizes, sometimes fusing together and at other times divid-

ing and probably exchanging mtDNA. Plant mtDNA, in contrast to mammalian mtDNA, is said not to be subject to a high mutation frequency¹⁸. On the other hand, plant mtDNA is subject to frequent rearrangements; it migrates between mitochondria; in one plant and apparently in one cell at various times it physically occurs in large and small rings and in different sized straight chains and possibly branched chains. Thus mitochondria within one plant species are expected to contain multiple copies of genetic material yet differ in their complements of mtDNA and it is thought that some of the mitochondria in a cell may only have incomplete copies of the mitochondrial genome. To compensate for this it has been proposed that the mitochondrial genome should be considered to be multiple copies of one unit, divided between all the mitochondria of a cell. If this picture is correct then it follows that mitochondria will probably differ in the efficiency of their genetically regulated electron transport chains and the distribution of these mitochondria between cells, following cell-division, will probably be more or less random. This variability between cells may well have provided the variability on which our selection process is based. It is well known that mitochondrial variability between people is a basis for a number of metabolic faults and weaknesses in humankind^{25,26} and faults in the mitochondria of yeasts give rise to the "petite" mutant forms². There are some further complications to be considered in the case of barley, or other cereal grains. These follow from the fact that mitochondria (and plastids) are chiefly or entirely transmitted maternally via the egg cell and not the pollen. Furthermore the grain is the product of a double fertilization⁴. The diploid embryo, from which the next generation of plants will grow, is the product of the fusion of two haploid nuclei, one the egg, the other from the pollen, while the aleurone layer and the starchy endosperm are the triploid products of the fusion of three different haploid nuclei, viz two maternal polar nuclei and one pollen nucleus. So the diploid embryo from which the next generation plants will be derived, considering both the nuclear and mitochondrial DNAs, is genetically distinct from the triploid aleurone layer, which is destructively analysed as the basis of our selection system. Fortunately the predominant self-fertilisation in barley ensures that the genetics of the mitochondria are likely to be similar, but probably not identical, in the two tissues.

Plant breeders are aware of cytoplasmic inheritance, which is based on the maternal inheritance of the DNA of the mitochondria and the DNA of the plastids. However, we know of no indication that any breeder has succeeded in selecting for "superior" mitochondria. The application of our TTC selection system opens possibilities that appear to be completely novel and even astounding. All the barleys we investigated have very variable aleurone TTC staining ranges. This seems to indicate that **all** barley varieties are genetically heterogeneous and could be improved by repeated, TTC-based selection for high-staining mitochondria, and the elimination of lines containing "weak" organelles, probably applied by selection in three or more successive generations. The result would be the appearance of more vigorous, better malting quality sub-lines of established cultivars, perhaps with more agronomic vigour and of greater uniformity. Clearly the appli-

cation of the TTC selection method to Galant, Eminant, Dramant and to any malting quality barley is likely to be of value. The greater uniformity would be of value to breeders, experimentalists and maltsters and the superior characteristics would be preferred by maltsters and by breeders in choosing parents when making crosses to obtain new varieties. The possibilities do not end with barley. In principle the TTC-aleurone staining approach described could be applicable to all cereals. Furthermore, if trials show that TTC staining is applied to samples of the cotyledons of dicotyledonous plants and embryos from seeds that had high-staining cotyledon tissue are propagated, it seems probable that "high-vigour" strains of dicotyledonous plants could also be selected.

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