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A somaclonal variant of wheat with additional β-amylase isozymes

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Summary. The progeny of 149 plants regenerated from tissue culture of immature wheat (*Triticum aestivum*) embryos were screened for variation in their grain β-amylase isozyme pattern. One regenerant was found which was heterozygous for a variant pattern characterized by the presence of at least five new isozyme bands, as well as an increased intensity in existing bands in two more positions. The F_2 of a homozygous variant crossed back to the parent segregated in an approximate 3 : 1 ratio but resolution of the gels was not sufficient to distinguish whether this represents a dominant or co-dominant single mutant gene. No chromosome abnormalities were evident in mitosis or meiosis of the homozygous variant or in the F_1 of the variant crossed back to the parent. No recombination has been seen between the variant bands and production of multiple bands from a single locus is consistent with the nature of the known β-amylase loci. However, the variant bands were not evident in a survey of 111 diverse genotypes, nor were they present in developing grain of the parent cultivar. Therefore, this variant could represent a rare mutation leading to expression of a currently unexpressed locus.

Key words: β-amylase – Isozymes – *Triticum aestivum* – Somaclonal variation

Introduction

Somaclonal variation is genetic variation induced during the culture of plant cells (Larkin and Scowcroft 1981). The mechanism(s) causing the variation are not understood but a variety of genetic effects have been reported. These include changes in ploidy level, aneuploidy, chromosome breakage and rearrangement, gene amplification and deamplification, mobilisation of transposable elements, and single gene mutations. Recent reviews illustrating the scope of somaclonal variation include those of King (1984), Maliga (1984) and Larkin et al. (1985).

The potential for somaclonal variation to act as a useful source of genetic variation for plant breeding has been widely flaunted. However, sound judgements on the potential use of somaclonal variation and its efficient exploitation will depend on having specific knowledge about the genetic basis of the variation. The somaclonal variant described here was isolated in an experiment designed to use the β-amylase isozymes of wheat to screen for variants which could be easily analyzed genetically.

Compared to other plant characters, isozymes are quite rapidly screened, are relatively free of environmental variation, and are simply inherited. When combined with the availability of good cytogenetic stocks, such as in wheat, they can become powerful tools in the analysis of genetic variation. The alcohol dehydrogenase isozymes of wheat were thus used in a study of somaclonal variation by Davies et al. (1986), enabling a detailed description of several translocations to be made. Brettell et al. (1986) also used alcohol dehydrogenase isozymes to isolate somaclonal variants of maize. One variant with altered isozyme mobility was analyzed at the molecular level and found to differ from the parent by a single nucleotide substitution.

The β-amylase isozymes were selected for this study because they represent the products of at least four loci on four separate chromosomes (Ainsworth et al. 1983), and because they resolve into many bands when separated by isoelectric focusing. These properties were expected to increase the chances of isolating somaclonal mutants. The reason for the separation of wheat β-amylases into complex patterns is not yet understood. Each locus resolves into a number of isozyme bands but the bands have not been able to be separated by recombination. They thus appear to represent products of a single locus, termed 'complex' by Ainsworth et al. (1983).

Materials and methods

Culture

Plants were regenerated from tissue cultures of wheat (*Triticum aestivum* L.) cv. 'Sunstar' according to Larkin et al. (1984). All cultures were initiated from immature embryos and maintained on SD1 medium for about six weeks. Some cultures were then divided in two. One half was advanced to MS9 regeneration medium and the other half was kept on SD1 for a further six weeks before regeneration. All parent plants, embryos, the cultures and subcultures deriving from them and the regenerated plants were individually and uniquely identified throughout.

Plants and seeds

Plants donating embryos for culture were grown in the greenhouse. All these 'parent' plants and the regenerants (SC_1 generation) were bagged before anthesis to prevent cross-pollination. Spikes from regenerated plants were harvested and threshed individually and these seed, the SC_2 generation, were screened for β-amylase isozyme variation.

β-amylase analysis

Preparation of samples and isoelectric focusing of β-amylase were based on procedures described by Ainsworth et al. (1983). Individual seeds were divided into an embryo half, for storage at 4 °C, and an endosperm half for β-amylase analysis. The endosperm half was extracted overnight at 22 °C with 100 µl of solution containing 10 mM $CaCl_2$, 0.05 M 2-mercaptoethanol and 15 mM sodium azide. The endosperm piece was then removed and the extracts centrifuged in Eppendorf tubes at 4 °C and 10,000 × g for 5 min. The supernatant was dialyzed against distilled water for 30 min then 30 µl of sample was applied to two stacked paper wicks (Whatman 3MM) each 5 mm × 10 mm. The wicks were placed at the cathode end of a prefocused polyacrylamide gel 0.4 mm thick containing equal quantities of pH 4.5–5.4 and pH 5–6 ampholines (Pharmacia). This mixture resulted in a non-linear pH gradient but gave good separation of the many bands with isoelectric points around pH 5.0. An LKB Ultrophor Unit cooled to 4 °C and LKB 2197 constant power supply were used for the isoelectric focusing. Gels were prefocused for 30 min with 5 W applied to the gel. After applying the samples, power was increased to 10 W at a maximum voltage of 2,500 V. The wicks were removed after 30 min and the gel run for a further 2 h.

Greater separation of isozyme bands was obtained by running the gel in the lengthwise direction so that the electrode wicks were 22 cm apart compared to 10 cm in the crosswise direction. A 1 : 1 mixture of pH 5–6 and pH 4–6.5 ampholines was used to ensure development of the bands with higher pI. The extracts from several seeds were combined and 100 µl of this was applied to the gel. Voltage and current settings were as before, and the power was set to 1 W for the prefocus and 2 W for the run. The gel was run for 6.5 h then the pH was measured at 1 cm intervals along the gel with a surface electrode.

The β-amylase bands were visualized by overlaying the polyacrylamide gel onto a starch gel (2% hydrolyzed starch, 2% Sigma agarose) for 30 min, then staining the starch gel with a solution of 1.5×10^{-3} M iodine, 3.5×10^{-3} M potassium iodide and 2% acetic acid.

Since α-amylase isozymes in germinating (mature) grain may differ from those in developing grain (Gale and Ainsworth 1984), the β-amylase isozymes of the variant and the parent were examined in developing grain. Six grains of a homozygous variant line and six grains of the parent were taken at 21, 28 and 35 days after pollination and their individual β-amylase isozyme patterns compared to these in mature grain using the same procedure as described for mature grain.

Results

Amongst the progeny of 149 regenerated plants, one family was found which segregated for a β-amylase isozyme pattern characterized by the presence of additional bands relative to the parent (Fig. 1). Another 22 families segregated for variant patterns displaying loss of isozyme bands. These latter patterns were characteristic of monosomy for chromosomes 4A or 4D and after confirmation of aneuploidy by examining mitotic cells in several cases, these families were not explored further. The remaining families showed no variation from the parental pattern.

The variant pattern which displayed additional bands relative to the parent was a complex one but within the limits of the resolution of the system the following characteristics could be determined (Fig. 2).

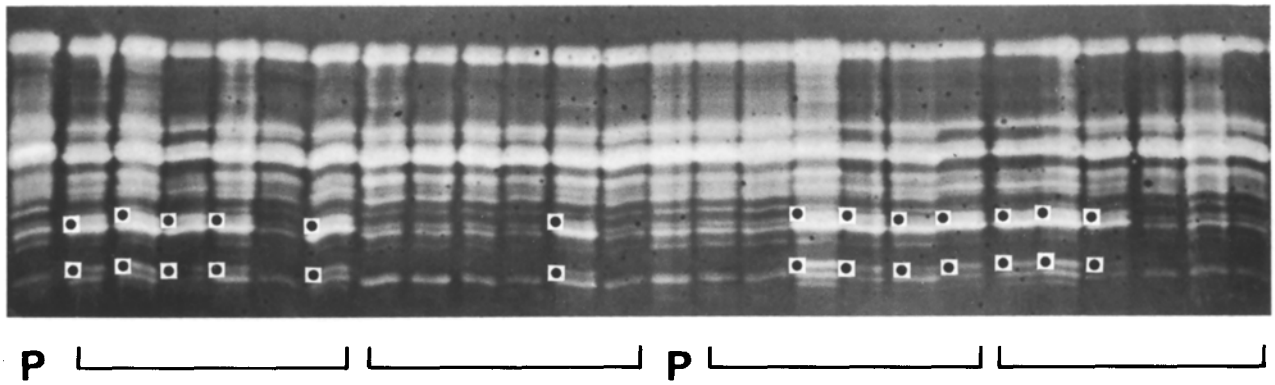

Fig. 1. Segregation for variant bands in four individual spikes of one regenerated plant. P represents the 'Sunstar' parent and the brackets denote SC_2 seed from individual spikes. The major variant bands are marked

The variant displayed all the bands present in the parent except for one band at pI 5.58. In addition the variant displayed new bands in five positions (pI 5.12, 5.30, 5.35, 5.45, 5.60) and increases in intensities of bands at two positions (pI 5.20, 5.50). The parental band at pI 5.50 is very faint and does not reproduce well. Bands at a pI greater than 5.40 did not always develop when the gels were run in the crosswise direction. Therefore, in segregating populations, the variant status of an individual was determined only on the bands at pI's below 5.40. In all segregating populations, and in all homozygous variant lines, the variant bands were either all present together or all absent together. Variants shown to be either homozygous or heterozygous by progeny analysis could not be reliably distinguished from each other on the gels. In some cases heterozygotes appeared to have reduced intensities of the variant bands but resolution of the gels was not sufficient to clearly establish whether the variant gene as acting in a dominant or co-dominant manner.

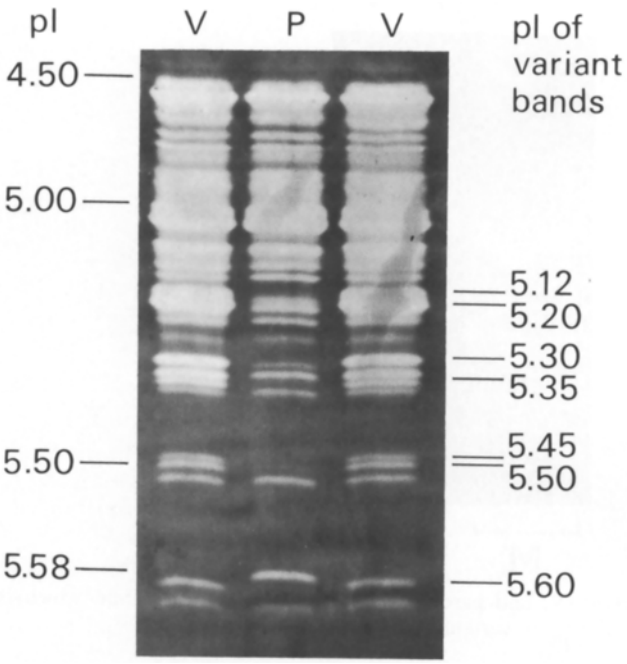

Fig. 2. β-amylase isozyme pattern of the homozygous variant (V) and its parent (P). Non-linear expansion of the isoelectric points between pH 5 and pH 6 was accomplished by using a 1 : 1 mixture of pH 4–6.5 and pH 5–6 ampholines

All eight spikes of the regenerated plant giving this variant segregated for the variant pattern (see Fig. 1 for patterns in four of these spikes). The progeny of seven other regenerants originating from the same embryo were also examined. None of these, including four which had had two culture periods on SD1, displayed the variant pattern. The regenerant with the variant pattern came from a culture which had had a single period of culture on SD1 medium. Progeny of an additional 60 regenerants derived from 19 embryos from the same donor plant were also screened and none showed this variant pattern. A total of 220 single seed of the parent cultivar were also examined and none of these displayed the variant pattern. These included eight seeds from the actual plant contributing the embryo giving rise to the variant.

Embryos from eight individual seeds in the SC_2 family which displayed both the variant pattern and the parental pattern were germinated and their selfed progeny tested. Six variant individuals all gave SC_3 progeny which were segregating, while the two parental individuals gave parental patterns in the SC_3. Five variant individuals were then advanced to the SC_4 generation, and two homozygous variant lines were recovered. One of these homozygous variant lines has been named SV2. Reciprocal crosses were then made between one plant of this line and one plant of the parent cv. 'Sunstar', and the F_2 analyzed for presence of the variant bands. Segregation ratios in each SC generation and in the F_2 are shown in Table 1. In the SC_2 and some of the SC_3 individuals, segregations fitted a 2 : 1 ratio better than a 3 : 1 ratio. However, in one of the SC_3 individuals and in the F_2 of the reciprocal cross with the parent, segregation best fitted a 3 : 1 ratio. The F_2 data clearly suggest that the variant β-amylase bands are under control of a single dominant or co-dominant gene. The aberrant ratios could have

Table 1. Segregation ratios and χ^2 of the variant and parental isozyme patterns

Generation	Isozyme pattern		H_0 2 : 1		H_0 3 : 1	
	Variant	Parental	χ^2	P	χ^2	P
SC_2	43	24	0.19	0.50–0.75	4.18	0.025–0.050
SC_3 # 61	26	15	0.19	0.50–0.75	2.93	0.050–0.100
# 102	12	6	0	>0.99	0.67	0.25 –0.50
# 109	27	9	1.12	0.25–0.50	0	>0.99
F_2 – 'Sunstar' × SV2	23	8	0.79	0.25–0.50	0.01	>0.90
– SV_2 × 'Sunstar'	21	9	0.15	0.50–0.75	0.40	0.50 –0.75

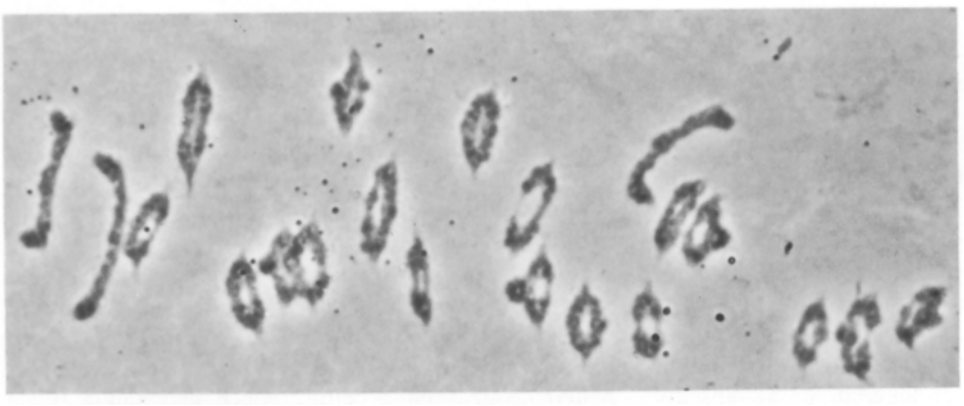

Fig. 3. Metaphase I chromosomes in a pollen mother cell of the F_1 of the cross SV2×'Sunstar'. The selfed progeny of this plant segregated for the parental and variant isozyme patterns

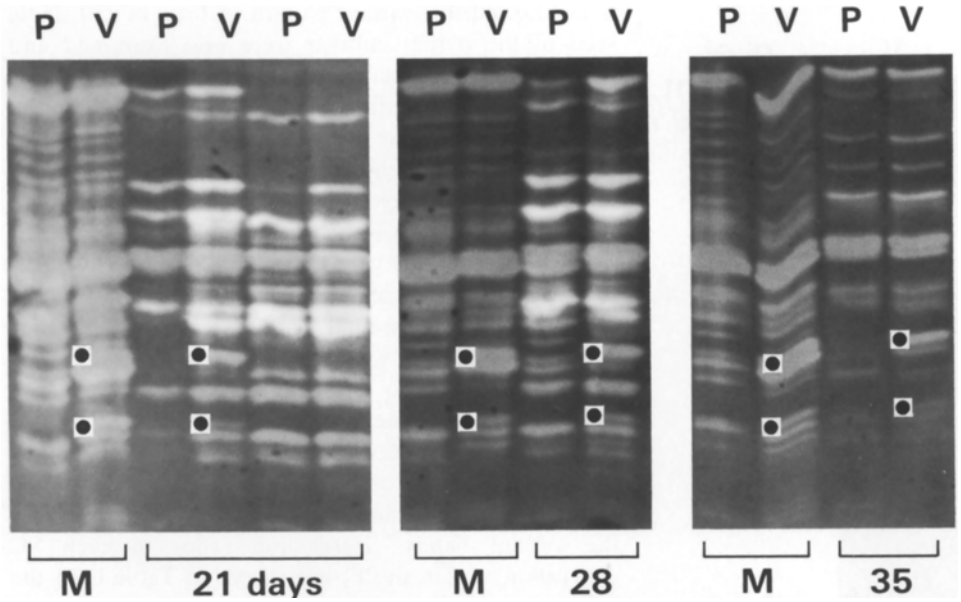

Fig. 4. β-amylase isozyme patterns of maturing grain in the variant (V) and parent (P). M represents mature grain which is compared to grain harvested at 21, 28 and 35 days after pollination. The major variant bands are marked

resulted from linkage of the β-amylase locus with a variant gametophyte or lethal factor in the regenerant. Recombination may then have separated the variant isozyme allele and the gametophyte or lethal allele, leading to the regular segregation observed in some cases. No detailed observations of pollen or ovule fertility were made, but no obvious reduction in seed set was ever observed.

The absence of karyotypic abnormalities was also consistent with the variant being inherited as a single-factor character. Mitotic chromosome spreads of the root tips of seven variant individuals demonstrated a normal $2n=42$ count. Meiotic chromosome examination of the homozygous variant and of the F_1 of the cross between the variant and the parent cultivar also showed a normal count with no abnormal chromosome arrangements (Fig. 3). In the latter case, a total of 117 cells from six individual F_1 plants were examined.

The patterns of β-amylase isozymes in developing grain of the variant and the parent are shown in Fig. 4. The patterns in developing grain did differ from those in mature grain, but at no time did the parent display bands in the positions of the variant bands. In the homozygous variant, the variant bands had appeared in three of the six individuals by 21 days, and were present in all individuals at all subsequent dates. The appearance of the new bands in the variant does not therefore appear to be a consequence of altered developmental expression of parental bands.

Since considerable allelic variation exists for β-amylases (Ainsworth et al. 1983), a set of diverse wheat genotypes was searched for the variant pattern. A total

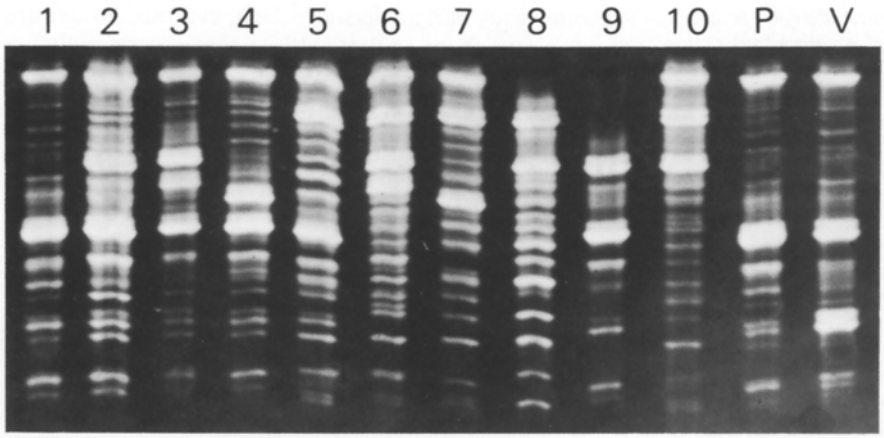

Fig. 5. Allelic variation in β-amylase. Groups marked 1 to 10 represent the groups named in Table 2. Cultivars displayed are: 1 = 'Chinese Spring'; 2 = 'Millewa'; 3 = 'Triple Dirk'; 4 = 'Klein Colon'; 5 = 'Q33'; 6 = 'Bindawarra'; 7 = 'Red Egyptian'; 8 = 'Iassul'; 9 = 'Kewell'; 10 = 'Ciano 67'. P represents the 'Sunstar' parent and V the variant

of 111 genotypes was examined and examples of the allelic variation seen are shown in Fig. 5. These could be arranged into ten groups (Table 2). Although all of group 7 carried a band around pI 5.12, none carried the whole set of bands seen in the variant.

Discussion

The β-amylase variant described here was obtained from tissue culture with strict controls applied to handling all plants and cultures. Outcrossing of parent plants, regenerants, and plants in subsequent generations was prevented by enclosing heads in paper bags before anthesis. Since the SV2 variant was heterozygous in the regenerant, seed contamination is an unlikely source of this variant, particularly as the variant pattern has never been seen in any cultivar ever grown in the same greenhouse, and is apparently very rare in the population of hexaploid wheats. Strictly pedigreed cultures enabled us to examine progeny of seven 'sister' regenerants from the same embryo as the variant. That none of these carried the variant adds support for the claim that this was a culture induced variant.

All the variant bands in SV2 appear to be under the control of a single gene. Segregation patterns in the F_2 of the variant crossed back to the parent were consistent with a single dominant or codominant gene determining the variant pattern and no recombination between the variant bands was observed. The meiotic data show no karyotypic abnormalities, and the variant is otherwise phenotypically indistinguishable from the parent, suggesting that the genetic effect producing this variant was confined to a small length of chromatin. However, the possibility that more than the β-amylase locus was affected by the one mutational event must not be discarded since some aberrant 2 : 1 ratios were obtained which may have indicated the presence of a linked variant gametophyte or lethal gene. The linkage was not strong, however, since recombinants were relatively easily found.

Table 2. Cultivars examined for allelic variation in β-amylase isozyme pattern arranged in phenotypic groups. Representative patterns are displayed in Fig. 5

Group	Cultivar
1.	'Banks', 'Bodallin', 'Canna', 'Chinese Spring', 'Condor', 'Cook', 'D6899', 'Egret', 'Gatcher', 'Genaro 81', 'Highbury', 'Hilgendorf', 'India 56', 'Nacozari', 'Nainari 60', 'Nipigon S'', 'Oxley', 'PI 78012', 'Pato Argentino', 'Sunstar', 'Veery 2', 'WW15'
2.	'Angus', 'Arz', 'Avocet', 'Bandeirantes', 'Bluejay S'', 'Bobwhite S'', 'Carazinho', 'Cargill Trigal 800', 'Centurk', 'Cleopatra', 'DeKalb Tala', 'Dundee', 'Extra Early Blackhull', 'Federation', 'Gabo', 'Gutha', 'Halberd', 'Hartog', 'Hyden', 'IA 78112', 'IWP 19', 'Imuris', 'Jacup', 'Kalkee', 'Kenya K16', 'Kenya W1483', 'Khapstein', 'Kite', 'Mendos', 'Mengavi', 'Mianzi 52', 'Miling', 'Millewa', 'Mivhor 1177', 'ND2', 'Oligo 90', 'Olympic', 'Pavon', 'Penjamo', 'Q89', 'QT 7903', 'Ramona', 'Rescue', 'Selkirk', 'Songlen', 'Teal', 'Timstein', 'Tobari 66', 'Wialki'
3.	'Aroona', 'Bordan', 'Chino 466', 'Idaho', 'India 66', 'Lance', 'NP 799', 'Rescue', 'Selkirk', 'Thatcher', 'Triple Dirk', 'Winglen'
4.	'Froid', 'Klein Colon', 'Klein Progneso', 'Mentana', 'Tex Red', 'VIC 002', 'Xiaoyan # 4'
5.	'Buck Cimarron', 'Harrier', 'Kharchia', 'PI 177971', 'Q33'
6.	'Bindawarra', 'DeKalb Lopacho', 'Eradu', 'IA7873', 'Leisbeck', 'Nap Hal', 'Pitic', 'Yecora'
7.	'Kenya Poa', 'Red Egyptian', 'Xiaoyan # 6'
8.	'Iassul'
9.	'Kewell', 'Leaks Rust Proof', 'Tungurahua'
10.	'Ciano 67'

Although larger scale aberrations seem to be the more common consequence of tissue culture (e.g. Ogihara 1981; Karp and Maddock 1984), single gene mutations have been documented (e.g. Chaleff and Ray 1984), and one has been confirmed as a single point mutation (Brettell et al. 1986). In the latter case, where two loci were screened, one single gene somaclonal variant was found in 645 regenerants. This is comparable to the one single gene variant found here, where 149 regenerants and four loci were screened. However, no single gene somaclonal variants were found in 551 wheat regenerants

screened for variation at the three alcohol dehydrogenase-1 loci (Davies et al. 1986). On the basis of seedling and endosperm mutants, Edallo et al. (1981) calculated that each regenerated maize plant carried an average of one single gene mutation, but in this case the number of loci cannot be defined. These few data are not sufficient to provide a good estimate of the true frequencies of single gene somaclonal mutations. Nevertheless, the rate does appear to be higher than that encountered with spontaneous mutation.

The appearance of a number of isozyme bands coded by a single locus had already been demonstrated for β-amylases of wheat (Ainsworth et al. 1983). These authors were also unable to achieve recombination between different parental patterns coded on the same chromosome, and concluded that β-amylases in wheat are determined by single 'complex' loci, possibly arising from tandem gene duplication followed by mutation and differentiation. However the possibility of post-translational modification as a cause of the complex isozyme pattern was also raised. Whatever the explanation, the isozyme patterns are heritable, and the properties of the SV2 variant bands are consistent with the known β-amylase loci.

The origin of the variant locus in SV2 is not obvious. It does not appear to have derived from an existing expressed locus as was demonstrated for the somaclonal variant of maize alcohol dehydrogenase (Brettell et al. 1986). All the parental bands, with the possible exception of one at pI 5.58, are present in SV2, and the variant bands apparently represent expression of an additional locus. De novo creation of a β-amylase locus, through a mutation conferring β-amylase activity to another existing enzyme, is possible but perhaps not very likely. Alternatively, the variant locus could represent an existing silent locus whose expression is allowed in the mutant. The mutation allowing this expression would not, however, be common. The additional locus was not observed in a diverse set of wheat cultivars, despite the presence of considerable allelic variation at other loci. Nor does the additional locus represent recovery of expression of a null allele at three of the four known loci in the parent, since the parent has the same allelic constitution as 'Chinese Spring' (see Fig. 5), which does not carrry null alleles at the 4A, 4D and 5A loci (Ainsworth et al. 1983). 'Chinese Spring' does, however, carry a null allele at a locus on chromosome 5B. This locus has only been recorded in one synthetic wheat amphiploid (Ainsworth et al. 1983) and possibly represents an ancestral locus not expressed in any modern wheats. It is a possible location for the newly expressed variant locus. Monosomic analysis is in progress to determine the chromosome location of the variant locus. It is also possible that the variant locus completes the homoeologous series on the group 4 or group 5 chromosomes, as is known for alcohol dehydrogenase on group 4, shikimate dehydrogenase on group 5 and other isozymes on other groups (McIntosh 1983).

Possible explanations of activity from an unexpressed gene include a mutation in a repressor gene or movement of a transposable element out of a structural gene. Recent evidence of past activity of transposable elements in wheat has been deduced from the presence of inverted repeats in the sequences of an alcohol dehydrogenase gene (L. Mitchell personal communication) and an α-amylase gene (D. Baulcombe, personal communication). Transposable elements have not otherwise been found in wheat. However, activation of previously silent transposable elements as a result of tissue culture has been implicated in alfalfa (Bingham and McCoy 1985) and maize (Benzion et al. 1985). The homozygous variant has also been returned to culture and progeny of the resulting regenerants will be screened for revertants. Whether mutational events induced by tissue culture are specifically required to produce this type of mutant cannot yet be judged. However, any mechanism that allows increased access to pre-existing genetic variation is potentially useful.

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References

Ainsworth CC, Gale MD, Baird S (1983) The genetics of β-amylase isozymes in wheat. 1. Allelic variation among hexaploid varieties and intrachromosomal locations. Theor Appl Genet 66:39–49

Benzion G, Phillips RL, Rines HW (1986) Case histories of genetic variability in vitro: oats and maize. In: Vasil IK (ed) Plant regeneration and genetic variability. Academic Press, New York (in press)

Bingham ET, McCoy TJ (1985) Somaclonal variation in alfalfa. In: Plant breeding reviews. AVI Publishing (in press)

Brettell RIS, Dennis ES, Scowcroft WR, Peacock WJ (1986) Molecular analysis of a somaclonal mutant of maize alcohol dehydrogenase. Mol Gen Genet 202:235–239

Chaleff RS, Ray TB (1984) Herbicide-resistant mutants from tobacco cell cultures. Science 223:1184–1151

Davies PA, Pallotta MA, Ryan SA, Scowcroft WR, Larkin PJ (1986) Somaclonal variation in wheat: genetic and cytogenetic characterisation of alcohol dehydrogenase 1 mutants. Theor Appl Genet 72:644–653

Edallo S, Zucchinali C, Perenzin M, Salamini F (1981) Chromosomal variation and frequency of spontaneous mutation associated with in vitro culture and plant regeneration in maize. Maydica 26:39–56

Gale MD, Ainsworth CC (1984) The relationship between α-amylase species found in developing and germinating wheat grain. Biochem Genet 22:1031–1036

Karp A, Maddock SE (1984) Chromosome variation in wheat plants regenerated from cultured immature embryos. Theor Appl Genet 67:249–255

King PJ (1984) From single cells to mutant plants. Oxford Surveys of Plant Mol Cell Biol 1:7–32

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor Appl Genet 60:197–214

Larkin PJ, Ryan SA, Brettell RIS, Scowcroft WR (1984) Heritable somaclonal variation in wheat. Theor Appl Genet 67:443–455

Larkin PJ, Brettell RIS, Ryan SA, Davies PA, Pallotta MA, Scowcroft WR (1985) Somaclonal variation: impact on plant biology and breeding strategies. In: Day P, Zaitlin M, Hollaender A (eds) Biotechnology in plant science. Academic Press, New York, pp 83–100

Maliga P (1984) Isolation and characterization of mutants in plant cell culture. Annu Rev Plant Physiol 35:519–542

McIntosh RA (1983) A catalogue of gene symbols for wheat. In: Sakamoto S (ed) Proc 6th Int. Wheat Genet Symp. Kyoto, Japan, pp 1197–1254

Ogihara Y (1981) Tissue culture in *Haworthia*. 4. Genetic characterization of plants regenerated from callus. Theor Appl Genet 60:353–363