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(54) Titre : ORGE POSSEDANT UNE ACTIVITE ENZYMATIQUE SSII LIMITEE ET AMIDON ET PRODUITS CONTENANT DE L'AMIDON A TENEUR LIMITEE EN AMYLOPECTINE

(54) Title: BARLEY WITH REDUCED SSII ACTIVITY AND STARCH AND STARCH CONTAINING PRODUCTS WITH A REDUCED AMYLOPECTIN CONTENT

(57) Abrégé/Abstract:
Barley with reduced SSII activity has a starch structure with reduced amylopectin content and a consequent high relative amylose content. Additionally the grain has can have a relatively high β glucan content. The structure of the starch may also be altered in a number of ways which can be characterised by having a low gelatinsation temperature but with reduced swelling. The viscosity of gelatinised starch of the starch is also reduced. There is a chain length distribution of the amylopectin content and a low crystallinity of the starch. The starch is also characterised by having high levels of lipid associated starch exhibiting very high levels of V form starch crystallinity. The dietary fibre content of the starch is high. This has desirable dietary and food processing characteristics.

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(54) **Title:** BARLEY WITH REDUCED SSII ACTIVITY AND STARCH CONTAINING PRODUCTS WITH A REDUCED AMYLOPECTIN CONTENT

(57) **Abstract:** Barley with reduced SSII activity has a starch structure with reduced amylopectin content and a consequent high relative amylose content. Additionally the grain has can have a relatively high β glucan content. The structure of the starch may also be altered in a number of ways which can be characterised by having a low gelatinsation temperature but with reduced swelling. The viscosity of gelatinised starch of the starch is also reduced. There is a chain length distribution of the amylopectin content and a low crystallinity of the starch. The starch is also characterised by having high levels of lipid associated starch exhibiting very high levels of V form starch crystallinity. The dietary fibre content of the starch is high. This has desirable dietary and food processing characteristics.

BARLEY WITH REDUCED SSII ACTIVITY AND STARCH AND STARCH CONTAINING PRODUCTS WITH A REDUCED AMYLOPECTIN CONTENT.

This invention relates to a barley plant with a reduced SSII enzyme activity leading to a starch having reduced amylopectin content. The invention also relates to starch and grain and food products obtained therefrom.

BACKGROUND OF THE INVENTION

One finding in nutritional science is that resistant starch has important implications for bowel health, in particular health of the large bowel. The beneficial effects of resistant starch result from the provision of a nutrient to the large bowel wherein the intestinal microflora are given an energy source which is fermented to form *inter alia* short chain fatty acids. These short chain fatty acids provide nutrients for the colonocytes, enhance the uptake of certain nutrients across the large bowel and promote physiological activity of the colon. Generally if resistant starches or other dietary fibre is not provided the colon is metabolically relatively inactive.

There has in recent years been a direction to look at providing for resistant starches from various sources to address bowel health. Accordingly high amylose starches have been developed in certain grains such as maize for use in foods as a means of promoting bowel health.

The physical structure of starch can have an important impact on the nutritional and handling properties of starch for food products. Certain characteristics can be taken as an indication of starch structure including the distribution of amylopectin chain length, the degree of crystallinity and the presence of forms of crystallinity such as the V-complex form of starch crystallinity. Forms of these characteristics can also be taken as indicator of nutritional or handling properties of foods containing these starches. Thus short amylopectin chain length may be an indicator of low crystallinity and low gelatinisation and is also thought to have a correlation with reduced retrogradation of amylopectin. Additionally shorter amylopectin chain length distribution is thought to reflect organoleptic properties of food in which the starch is included in significant amounts. Reduced crystallinity of a starch may also be indicative of a reduced gelatinisation temperature of starch and additionally it is thought to be associated with enhanced organoleptic properties. The presence of V-complex crystallinity or other starch associated lipid will enhance the level of resistant starch and thus dietary fibre.

Lines of barley having high amylose starch contents have been identified in the past. These have only resulted in relatively modest increases in amylose content to a maximum of about 45% of total starch such as in the barley variety known as High Amylose Glacier (AC38). Whilst elevated amylose starches of that type are useful a starch with a higher amylose content still is preferred, and certain other species of grain are bred to have higher amylose content starches with levels in the 90 percentile range. These are very resistant to digestion and bring a greater health benefit.

There is a problem with providing the high amylose starches because known high amylose starches also have a high gelatinisation temperature. Gelatinisation temperature is reflective of the comminution energy required to process such foods. Thus higher temperatures are normally required to process grain or flour to manufacture foods from such grains or starches. Thus generally products having high amylose starches are more expensive. Similarly from the point of view of the consumer longer times and higher temperatures may be required to prepare the manufactured foods, or to make foods from flour having high amylose starches. Thus there is a significant disadvantage in the provision of high amylose starches in foods.

Another nutritional component of the grains and in particular of barley as β-glucans. β-glucans consist of glucose units bonded by β (1-4) and/or β (1-3) glycosidic linkages and are also not degraded by human digestive enzymes which makes them suitable as a source of dietary fibre. β-glucans can be partially digested by endogenous colonic bacteria which fermentation process gives rise to short chain fatty acids (predominantly acetate, propionate and butyrate) which are beneficial to mucosal cells lining the intestine and colon (Sakata and Engelhard Comp. Biochem Physiol. 74a:459-462 (1983))

Ingestion of β-glucan also has the effect of increasing bile acid excretion leading to a reduction in total serum cholesterol and low density lipoproteins (LDL) with a lowering of the risk of coronary disease. Similarly β-glucans act by attenuating excursions in postprandial blood glucose concentration. It is thought that both of these effects are based on the increase of viscosity in the contents of the stomach and intestines.

The composition of foods containing starches and the intimate relationship of those starches with other nutritional or other components can have a significant impact on the nutritional value of those foods or on the functional characteristics of those components in the preparation or structure of the foods.

Whilst modified starches or β glucans, for example, can be utilised in foods that provide functionality not normally afforded by unmodified sources, such processing has a tendency to either alter other components of value or carry the perception of being undesirable due to processes involved in modification. Therefore it is preferable to provide sources of constituents that can be used in unmodified form in foods.

The barley variety MK6827 is available from the Barley Germplasma Collection (USDA-ARS National Small Grain Germplasma Research Facility Aberdeen, Idaho 831290 USA). The grain of MK6827 is shrunken and has a highly coloured husk and an elongate shape and, in the hands of the inventors, this grain is very difficult to process including being very resistant to milling. The properties of MK6827 grain had not been characterised before, nor had the nature of the mutation been ascertained nor is it considered suitable for producing food.

SUMMARY OF THE INVENTION

This invention arises from the isolation and characterisation of SSII mutant of barley plants the grain of which is found to contain starch that has reduced amylopectin content and therefore high relative levels of amylose and therefore has elevated levels of dietary fibre.

The grain of the mutant and grain from crosses into certain genetic backgrounds additionally has an elevated level of β glucan. The combination of elevated β glucan level and resistant starch contributing to high dietary fibre is thought by the inventors to be unique to the present invention.

Additionally, at least in some genetic backgrounds, it is found that grain from such mutants contain starch that have high relative levels of amylose, and also have low gelatinisation temperatures. The low swelling charactistics of such starch during and following gelatinisation also has advantages in certain dietary and food processing applications.

Furthermore, grain from such mutants are found to contain starch that have high relative levels of amylose, the amylose levels found are higher than 50% of the starch content which is a level never before found in unmodified starch derived from barley.

The starch of the mutants and backcrossed lines derived from the mutants (to the extent that the backcrosses have been tested) exhibit a resistant starch, with an altered structure indicated by specific physical characteristics including one or more of the group comprising the presence of a high relative amylose content, physical inaccessibility by reason of having a high β-glucan content, altered granule morphology, and the presence of starch associated lipid, and the altered structure being indicated by a characteristic selected from one or more of the group comprising low crystallinity, reduced amylopectin chain length distribution and presence of appreciable starch associated lipid.

Additionally thus far the grain derived from the mutant barley plants can readily be used in food processing procedures.

This invention in one aspect might be said to reside in starch obtained from the of grain of a barley plant the barley plant having a reduced level of SSII activity, said starch granules having a high amylose content by reason of a reduced amylopectin content.

The invention might in another aspect of broadly be said to reside a grain useful for food production obtained from a barley plant the barley plant having a reduced level of SSII activity, starch of said grain having a high amylose content by reason of a reduced amylopectin content.

In a yet further aspect the invention might broadly said to reside in a barley plant with a reduced level of SSII activity, said barley plant capable of bearing grain, starch of said grain having a high amylose content by reason of a reduced amylopectin content, said grain suitable for food production.

Alternatively the invention could be said to reside in an isolated nucleic acid molecule encoding a barley SSII protein said nucleic acid capable of hybridising under stringent conditions with SEQ ID NO 1. or a cell carrying a replicable recombinant vector carrying said nucleic acid molecule. In a yet further form the invention might be isolated nucleic acid molecule capable of hybridising specifically to SEQ ID NO 1.

BRIEF DESCRIPTION OF THE DRAWINGS.

For a better understanding, the invention will now be described with reference to a number of examples.

Figure 1 Analysis of the starch molecular size distribution as determined by HPLC separation of starch in 90% DMSO. (a) Himalaya (b) AC38 (c) 342 (d) 292

Figure 2 Photographs showing the grain morphology of mutant and parental lines. (a) Himalaya (b) AC38 (c) 292 (d) Waxiro (e) 342 (f) Tantangara (g) MK6827 (h) Sloop. The length (L), width (W) and thickness (T) dimensions of the grain are illustrated in panel (a).

Figure 3 Analysis of the chain length distribution of various mutant and wild type starches using FACE. (a) normalised chain length distribution (b) comparison of chain length distributions by difference plot. Samples were 342 (■), 292 (●), Tantangara (s), AC38 (⊕), MK6827 (◆) and Himalaya (+).

Figure 4 RVA analysis of barley starch samples. Samples were Himalaya (⊕), Namoi (Δ), AC38 (O), 342 (∇), 292 (▲) and MK6827 (■). The temperature profile used during the profile is indicated by the unbroken line.

Figure 5 X-ray diffraction data for mutant and wild type lines.

Figure 6 Scanning electron micrographs of isolated barley starches. (a) Himalaya (b) Waxiro (c) AC38 (d) 292 (e) 342 (f) MK6827

Figure 7. Loci on barley chromosome 7H showing the proximity of the *nud*1 and sex6 loci. Diagram after GrainGenes (http://wheat.pw.usda.gov/) Barley morphological genes, 7H map, author; Franckowiak JD.

Figure 8. Relationships between seed dimensions and starch chain length distribution for 292 x Tantangara doubled haploid lines. Lines denoted

by (+) yielded the Himalaya PCR pattern and lines denoted by (O) gave the 292 PCR result. Panel (A), the seed length to thickness ratio plotted against the percentage of starch chains with DP between 6 and 11; Panel (B) seed weight plotted against the percentage of starch chains with DP between 6 and 11

Figure 9 Sequence of a barley SSII cDNA (SEQ ID NO 1) from the cultivar Himalaya

Figure 10 The structure of the SSII genes from (1) *T. tauschii* (diploid wheat), (2) barley cultivar Morex. The thick lines represent exons and the thin lines introns. The straight line underneath each example indicates the region of the gene sequences. The dotted line represents a region of the barley SSII gene, from intron 7, that has not been sequenced but has been determined by PCR analysis to be approximately 3 kb in length.

Figure 11 Comparisons of the predicted SSII cDNAs from MK6827 (SEQ ID NO 2), Morex (SEQ ID NO 3) and 292 (SEQ ID NO 4), and a cDNA sequence of Himalaya (SEQ ID NO 1). Predicted sequences were generated by identifying regions of the genomic sequences present in the Himalaya SSII cDNA. The ATG start codon and wild type stop codon are indicated, as are additional stop codons present in MK6827 (#) and 292 (&) respectively.

Figure 12 Comparison of amino acid sequences deduced from the genes encoding SSII from barley lines 292 (SEQ ID NO 7 and SEQ ID NO 8), Morex (SEQ ID NO 5), MK 6827 (SEQ ID NO 9 and SEQ ID NO 10), Hamalaya (SEQ ID NO 9 and SEQ ID NO 10). Additional stop codons In 292 and MK 6827 are indicated by the symbols (&) and (#) respectively.

Figure 13. Position of the mutations in MK6827 (SEQ ID NO 2) and 292 (SEQ ID NO 4) in the barley SSII gene.

Figure 14. Development and use of a PCR assay for the 292 mutation. (a) schematic representation of an SSII region from Himalaya amplified by the primers ZLSS2P4 and ZLBSSIIP5 (b) representation of the region amplified from the SSII gene from 292 using ZLSS2P4 and ZLBSSIIP5, showing the absence of one NlaIV site (c) agarose gel electrophoresis of NlaIV digested products from barley; Lane M; DNA marker ladder, lane 1: MK6827, lane 2; Himalaya; lane 3, Tantangara; lane 4, 292; lane 5, 342.

Figure 15. SDS-PAGE electrophoresis of starch granule proteins. Panel (A) 8% Acrylamide (37.5:1 Acryl/Bis) SDS-PAGE gel, electroblotted and probed with a SSII antibody produced against purified granule-bound SSII protein from Wheat. (B) 12.5% acrylamide (30:0.135 Acryl/Bis), silver stained. The migration of molecular weight standards of defined mass (units are kd) are indicated on each side of the figure.

Figure 16. A schematic representation of DNA constructs designed to down regulate SSII expression following stable transformation of barley (1) The SSII gene from nucleotides 1 to 2972 (see Figure 9 for sequence) is inserted between the promoter and terminator in the sense orientation. (2) The SSII gene is inserted between the promoter and terminator in the anti-sense orientation from nucleotides 2972 to 1 (see Figure 9 for sequence). (3) Duplex construct in which intron 3 of the barley SSII gene (between nucleotides 1559 and 2851) of the Morex SSII genomic sequence is inserted between exons 2 and 3 from the barley SSII cDNA from Himalaya (nucleotides 363 to 1157 from Figure 9).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Glycaemic Index. Is a comparison of the effect of a test food such as white bread or glucose on excursions in blood glucose concentration. The Glycaemic Index is a measure of the likely effect of the food concerned on post prandial serum glucose concentration and demand for insulin for blood glucose homeostasis.

Resistant Starch. The sum of starch and products of starch digestion not absorbed in the small intestine of healthy humans but entering into the large bowel. Thus resistant starch excludes products digested and absorbed in the small intestine.

Resistant starches can be classified in four groups.

RS1 physically inaccessible starch. Examples of this form of starch arise where the starch is entrapped within a protein or similar matrix or within plant cell wall, or might arise because of the partial milling of grain or in legumes after cooling.

RS2 Resistant granules. These are generally raw starches such as those that arise from raw potato or green banana, some legumes and high amylose starches.

RS3 Retrograded starches. These arise by heat/moisture treatment of starch or starch foods such as occurs in cooked and cooled potato, bread and cornflakes.

RS4 Chemically modified. These arise by reason of chemical modifications such as substitution or cross linking. This form of starch is often used in processed foods.

Dietary fibre. In this specification is the sum of carbohydrates or carbohydrate digestion products that is not absorbed in the small intestine of healthy humans but enters the large bowel. This includes resistant starch, β-glucan and other soluble and insoluble carbohydrate polymers. It is intended to comprise that portion of carbohydrates that are fermentable, at least partially, in the large bowel by the resident microflora.

Gelatinsation is the collapse (disruption) of molecular order within the starch granule with concomitant and irreversible changes in properties such as granular swelling, crystallite melting, loss of birefringence, viscosity development and starch solubilisation.

This invention arises from the isolation and characterisation of SSII mutant barley plants. the grain of which is found to contain starch that has reduced amylopectin content and therefore high relative levels of amylose and therefore has elevated levels of dietary fibre.

Such mutants are found to have a number of quite desirable characteristics, and it has been shown that crosses into various other genetic backgrounds maintains at least some of those characteristics.

The grain of the mutant and grain from crosses into certain genetic backgrounds additionally has an elevated level of β glucan. The combination of elevated β glucan level and high dietary fibre is thought by the inventors to be unique to the present invention.

Additionally at least in some genetic backgrounds it is found that grain from such mutants are found to contain starch that have high relative levels of amylose, and also have low gelatinisation temperatures. The swelling charactistics of the gelatinisation of such starch also has the benefit of being low swelling which has advantages in certain dietary and food processing applications.

Furthermore grain from such mutants are found to contain starch that have high relative levels of amylose, the amylose levels found are higher than 50% of the starch content which is a level never before found in unmodified starch derived from barley.

The starch of the mutants and to the extent that the backcrosses have been tested exhibit a resistant starch, with an altered structure indicated by specific physical characteristics including one or more of the group comprising the presence of a high relative amylose content, physical inaccessibility by reason of having a high β-glucan content, altered granule morphology, and the presence of starch associated lipid, and the altered structure being indicated by a characteristic selected from one or more of the group comprising low crystallinity, reduced amylopectin chain length distribution and presence of appreciable starch associated lipid.

Additionally thus far the grain derived from the mutant barley plants can readily be used in food processing procedures.

Grain from such mutants in one form preferably contain starch that have high relative levels of dietary fibre, more particularly amylose as well as an elevated level of β glucan. The combination of elevated β glucan level and high amylose level is thought by the inventors to be unique to the present invention, and provide for a unique source of a combination of β-glucan and resistant starch that does not, at least in broader forms of the invention require mixing of β glucan and soluble dietary fibre together or modification of the component parts.

To the best of the knowledge of the inventors the barley plant of the present invention is the first time that there has been a barley grain having elevated relative dietary fibre levels in the form of resistant starch having an elevated amylose level, that also has elevated levels of β glucan that are at the higher end of the typical levels of β glucan or that go beyond that level. Grains that have β glucan content that are still higher are of the waxy phenotype and therefore have low levels of amylose.

It is known that there is a wide variation in β glucan levels in barley in the range of about 4% to about 18% by weight of the barley, but more typically from 4% to about 8% (Izydorcyk *et al.*, (2000) *Journal of Agricultural and Food Chemistry* **48**, 982-989; Zheng *et al.*, (2000) *Cereal Chemistry* **77**, 140-144; Elfverson *et al.*, (1999) *Cereal Chemistry* **76**, 434-438; Andersson *et al.*, (1999) *Journal of the Science of Foods and Agriculture* **79**, 979-986; Oscarsson *et al.*, (1996) *J Cereal Science* **24**, 161-170; Fastnaught *et al.*, (1996) *Crop Science* **36**, 941-946). Enhanced barley strains have been developed, Prowashonupana for example, which have between about 15% and about 18% by weight β-glucan but has a waxy phenotype. This is sold commercially under the name Sustagrain™, (ConAgra™ Specially Grain Products Company, Omaha, Neb. USA).

The levels of β glucan contemplated by this invention may depend on the genetic background in which the amylopectin synthesis enzyme activity is reduced. However it is proposed that the reduction of the amylopectin synthesis activity will have the effect of elevating the relative level of dietary fibre which, in part, takes the form of amylose, and at the same time elevating the level of β glucan. One explanation for the concomitant elevation of β glucan with elevated relative amylose levels is that such elevation might be the result of a concentration effect of having reduced endosperm and may be further increased through the diversion of carbon from starch synthesis to β glucan synthesis.

Thus the grain of the barley plant preferably has a β glucan content that is greater than 6% of total non-hulled grain weight or more preferably greater than 7% and most preferably greater than 8%, however levels of β glucan in a waxy mutant has been measured as being as high as 15 to 18% and the present invention may contemplate levels as high, or higher, than that.

In a second preferable form the grain of the barley plant has a reduced gelatinsation temperature (as measured by differential scanning calorimetry)in addition to the relatively high amylose content. On the data shown for the exemplified barley this reduced gelatinisation temperature is not just reduced when compared to starch produced by barley with somewhat elevated amylose content but also when compared with starch produced from barley with starch having normal levels of amylose. Thus whilst the invention contemplates reduced gelatinisation temperatures relative to a corresponding high amylose starch, it may also contemplate a gelatinisation temperature reduced relative to that of starch with normal amylose levels.

Additionally in the genetic backgrounds thus far checked the starch is also characterised by a swelling in heated excess water that is lower than swelling of other starches tested.

In a third preferable form the starch has amylose levels of higher than 50% of the starch content which is a level never before found in unmodified starch derived from barley.

The starch of the present barley plant has a high relative amylose content and much higher than might be anticipated for a mutation in the SSII gene or other starch synthase gene. Thus in wheat mutants in SSII result in relative amylose levels of about 35% of starch. The amylose content of starch might be considered to be elevated when the content is significantly greater than the 25% or so that is present in normal barley grain and thus might be greater than about 30% w/w of total starch. Known barley plants considered to be high amylose have a content of 35-45%. The present invention however provides for barley with an amylose content that is greater than 50%, with is a level never before found in unmodified starch derived from barley.

The relative amylose content might be greater than 60% and more preferably, still greater than 70%. It may be desired to have even higher levels and thus it has been possible to achieve even higher levels in other plants by breeding with single mutations, such levels approach 90%. Thus the invention might encompass amylose levels of greater than 80% or greater than 90%.

In a fourth preferable form the starch also has an altered structure which gives rise to the resistant starch. This might arise from a high amylose content. Resistant starch might also arise because β-glucan is present at elevated levels and is likely to exert protective effects by reason of the association of the β glucan with the starch granule, the intimacy of association potentially provides a protective effect to the starch to thereby provide for a resistance that might be characterised as an RS1 form, being somewhat inaccessible to digestion. Similarly the presence of starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant starch. In this case the resistance is likely to arise because of the physically inaccessible of the starch by virtue of the presence of the lipid and accordingly this might be regarded as an RS1 starch. It is known that retrograded starch that takes up the V-complex configuration is highly resistant to digestion and accordingly it is anticipated that amylopectin that forms part of the V-complex crystalline structure will also be resistant to digestion. The starch of the exemplified barley plant may be resistant to digestion by reason of the structure of the starch granule and accordingly may have RS2 starch. Each of these characteristics might be present separately or as two or more of these characteristics in combination.

The elevated dietary fibre may at least in part take the form of resistant starch which may be characterised by a high amylose content of the starch granules as referred to above.

The relative amylose content might be greater than 60% and more preferably greater than 70%. It may be desired to have even higher levels and thus it has been possible to achieve even higher levels in other plants by breeding with single mutations, such levels approach 90%. Thus the invention might encompass amylose levels of greater than 80% or greater than 90%.

It might be desired that the barley plant additionally expresses an altered level of activity of one or more amylose synthesis enzymes or other enzymes to further enhance the relative level of amylose. Thus the barley plant may carry another mutation that further decreases or alters amylopectin biosynthesis, or a mutation or genetic background that increases amylose biosynthesis. For example the barley plant may exhibit an amylose extender genotype, such as a barley plant carrying the amo1 mutation. An example of such a plant is the variety known as AC38 (also known as High Amylose Glacier).

It will be understood that the relative level of amylose referred to is in relation to total starch content, and thus the remainder of the starch might be predominantly of an intermediate type of starch or it might be predominantly amylopectin or a mixture of both. In the barley analysed the elevated level of amylose results from decreased amylopectin levels, and accordingly the relative level of amylose does not result from an increased synthesis of amylose.

It is known that β glucan has the effect of slowing digestion in the small intestine simply by its presence when together with another food component. Similarly it is known that resistant molecules that have close juxtaposition with starch granules help to mask the starch and contribute to its resistance by making it physically inaccessible. Elevated levels of amylose and other forms of starch as may arise from association with lipid will be further enhanced therefore by the presence and physical juxtaposition to the starch granules. Thus there is provided a significant enhancement of the effects of the resistant starch, as well as a provision of other beneficial effects arising from high β glucan levels.

Additionally it is known that there is a dose response in terms of the beneficial effects of resistant starch and β glucan. It is proposed therefore that the increased level of β glucan together with the increased levels of resistant starch will provide enhanced health benefits.

The combination of the levels of β glucan and resistant starch of at least preferred forms of this invention have not been found before and certainly not from one source without a degree of modification or purification and thus forms of the present invention provide for a single practical source of these benefits.

Another preferred aspect of the starch is that despite the high relative amylose content it also has a low gelatinisation temperature as measured by differential scanning calorimetry. This is in contrast with the general finding that high amylose starches tend to have a raised gelatinisation temperature which introduces restrictions on the manner in which high amylose starches can be utilised. On the data shown for the exemplified barley this reduced gelatinisation temperature is not just reduced when compared to starch produced by lines with somewhat elevated amylose content but also when compared with starch produced from barley with starch having normal levels of amylose. Thus whilst a preferred aspect of the invention contemplates reduced gelatinisation temperatures relative to corresponding high amylose starch it may also contemplate a gelatinisation temperature reduced relative to that of starch with normal amylose levels. For high amylose starches aspects of processing requiring higher temperatures and therefore inherently require a higher energy input which is expensive and can destroy the functionality of other food components. Similarly from the point of view of the ultimate consumer, high amylose starch foods may be less convenient because of a higher temperature or longer time required for preparation. Thus, for example, in this preferred form of the invention it is now possible to provide for a product such as a noodle product requiring the addition of boiling or heated water to a vessel such as a cup and not requiring heating for an extended period of time and at the same time providing for delivery of resistant starches and other constituents of nutritional value to the large bowel.

A major effect of the low gelatinisation temperatures of these starches is the lower temperature requirements and hence comminution energy requirement of the food. A corollary is also that where, as typically might be the case in certain food processing, mixing occurs at room temperature and then the mixture is heated, the lower gelatinisation temperature also reduces the time required to achieve gelatinisation. Additionally at a range of temperatures below the temperature for full gelatinisation of normal starch, there will be more complete gelatinisation of the starch of the present invention than normal starch.

One measure of the gelatinisation capacity is reflected in the thermal properties as measured by DSC (differential scanning calorimetry). The onset of the first peak (gelatinisation peak) of DSC may be at less than 53°C, more preferably at less than 50°C and most preferably at less than about 47°C. The onset of the first peak may be regarded as the onset of gelatinisation. The starch produced from the barley grain may have a first peak at less than about 60°C, more preferably at less than 55°C and most preferably at less than 52°C. The ΔH (enthalpy) of the first peak may be less than about 3.5, more preferably less than about 1.0 and most preferably less than about 0.5.

Another finding of the gelatinsation of flours containing the starches of this invention is that they exhibit a reduced swelling. Swelling volume is typically measured by mixing either a starch or flour with excess water and heating to elevated temperatures, typically greater than 90°C. The sample is then collected by centrifugation and the swelling volume is expressed as the mass of the sedimented material divided by the dry weight of the sample. The swelling volumes of flour from starches of waxy and normal barleys are found to be greater than about 5.5. The swelling volumes of flour made from the grain that is a high amylose grain, (AC38) is about 3.75. Whereas the grains of the mutants and crosses examined are less than 3.2, preferably less than 3.0, but generally higher than about 2.

This low swelling gelatinisation characteristic is particularly useful where it is desired to increase the starch content of a food preparation, in particular a hydrated food preparation. In the present instance it might be desired to increase the dietary fibre content of a sol or other liquid preparation where there would otherwise be a restriction on delivery of the food preparation.

This characteristic in combination with the reduced gelatinisation temperature exhibited by the present starch provides a prospect of significantly enhancing the nutritional benefits of foods where there is a requirement of rapid preparation, such as instant soups and instant noodles.

It is postulated gelatinisation temperature effects are the result of an altered amylopectin structure in the endosperm of its grain, and one measurement of this structure is the distribution of chain lengths (degrees of polymerisation) of the starch molecules following debranching by isoamylase. An analysis of the chain length of the amylopectin content of the starch of the exemplified SSII mutants showed that when debranched they have a distribution of chain length in the range from 5 to 60 that is shorter than the distribution of starch yielded by non-mutant lines upon debranching. Starch with shorter chain lengths will also have a commensurate increase in frequency of branching. Thus the starch may also have a distribution of shorter amylopectin chain lengths. The proportion of starch chains that have a degree of polymerisation that falls in the range of 6 to 11 residues may be greater than 25%, more preferably greater than 30% and most preferably greater than 35%. The proportion of starch chains that have a degree of polymerisation that falls in the range of 12-30 residues may be less than 65%, more preferably less than 60% and most preferably less than about 55 %. The proportion of starch chains that have a degree of polymerisation that falls in the range of 31-60 residues may be less than about 10%, more preferably less than about 8% but also preferably greater than about 5% and more preferably greater than about 6%. Rather than taken individually combination of proportions of the three chain length ranges might be taken as an indicator that a starch is of a type that accords with the present invention.

The reduction in chain length distribution is likely to contribute to lower gelatinsation temperatures. Reduced chain length is also thought to enhance the organoleptic properties of the starch, in particular mouthfeel, thus perhaps contributing to a smooth product. Additionally it has been postulated that reduced amylopectin chain length might decrease the extent of amylopectin degradation, which has an impact on food quality, for example it is thought to be important in bread staling.

The starch structure in the exemplified starch is additionally shown to differ in that the degree of crystallinity is reduced compared to normal starch isolated from barley. When combined with a reduced amylopectin chain length distribution, reduced granular crystallinity may indicate that gelatinsation temperature will be lower. The reduced crystallinity of a starch is also thought to be associated with enhance organoleptic properties and as with shorter amylopectin chain length contributes to a smoother mouth feel. Thus the starch may additionally exhibit reduced crystallinity resulting from reduced levels of activity of one or more amylopectin synthesis enzymes. The proportion of starch exhibiting crystallinity may be less than about 20% and preferably less than about 15%.

A further measure of the properties of the present starch is by measuring viscosity. It is found using a Rapid Visco Analyser that the peak viscosity of the starch of this invention is significantly different to that of normal and waxy starches and high amylose starches obtained from barley. These measurements were made on wholemeal however the properties of the starch will predominate in these measurements. The normal and waxy starches have a peak viscosity of between about 900 and about 500 RVA units, known high amylose starch has a peak viscosity of greater than 200, whereas barley plants according to the present invention have a peak viscosity of less than 100 with a majority being less than about 50 in some plants as low as about 10 RVA units. It will be understood by a person skilled in the art that the parameters cited empirical units and the results cited are intended to indicate the relative performance of these starches in RVA instruments or similar instruments such as the amylograph.

In addition to reduced crystallinity referred to above the present starch may be characterised by the presence of the V-complex form of starch. It is thought by the inventors that this is the first time that this form of starch has been exhibited in appreciable amounts in starch granules of a grain. This form of starch is usually associated with retrograded starch, in particular where there has been contact with lipids. In the case of the present invention it is postulated that the structure of the starch permits the formation of an intimate relationship between plant lipids and starch which results in the V-complex structure. It is thought that this form of starch may have health benefits because it has reduced digestibility and therefore may contribute to resistant starch.

Other forms of structure can also result from lipid-starch interaction and include non crystalline lipid-starch complexes. Thus the invention might also be said to reside in a barley plant exhibiting appreciable amounts of starch-lipid complexes in the starch content of the endosperm of its grain resulting from reduced levels of activity of one or more amylopectin synthesis enzymes. Starches that contain starch lipid complexes, including those that exhibit V-complex structure, are also usually resistant to digestion and thus contribute to the dietary fibre levels. Preferably the proportion of crystalline starch exhibiting a form of crystallinity characteristic of a starch-lipid complex is greater than about 50% and more preferably greater than about 80%.

The starch additional to the presence of the V-complex form of starch may also exhibit no appreciable amounts of A complex forms of starch. Absence of A-complex might be taken as indicator of the presence of a starch of this invention.

It is also found that the pasting temperature of strchs and product made from the grain of thisinvention are considerably elevated. The pasting temperatures in known starches is less than 70°C, and this is for both normal and high amylose starches. The starches of the present invention however preferably exhibit pasting temperatures of higher than about 75°C or more preferably higher than about 80°C. It will be noted that these are empirical measures and might be taken as relative to those measurement of the other starches.

The starch of the exemplified barley plant is found to have significant amounts of dietary fibre and resistant starch, presumably this increase is at least in part as a result of the high relative level of amylose, however there may also be a contribution of dietary fibre by reason of starch/lipid complexes, including V-complex, or because of the intimate associate of amylose or amylopectin with β glucan. Similarly simply the elevated level of β glucan may also make a significant contribution to the elevation of dietary fibre.

The elevated relative amylose levels in the endosperm of the exemplified barley plant in all likelihood results from altered amylopectin production as a result of a reduction in the level of activity of the SSII enzyme.

Mutations in the gene encoding this enzyme might be expected to exhibit increased amylose content and/or a decrease in the level of amylopectin. Where amylopectin synthesis alone is decreased, starch exhibits an increased relative level of amylose.

Reduced activity of the amylopectin synthesis enzyme may be achieved by the appropriate mutations within a respective gene or regulatory sequences of the gene. The extent to which the gene is inhibited will to some degree determine the characteristics of the starch made. The exemplified mutations of this invention being SSII mutations in barley are truncation mutants and these are known to have a significant impact on the nature of the starch, however an altered amylopectin structure will also result from a leaky mutant that sufficiently reduces amylopectin synthesis enzyme activity to provide the characteristic of interest in the starch or grain of barley. Other chromosomal rearrangements may also be effective and these might include deletions, inversions, duplication or point mutations.

Such mutations can be introduced into desirable genetic backgrounds by either mutagenizing the varieties of interest, but more reliably by crossing the mutant with a plant of the desired genetic background and performing a suitable number of backcrosses to cross out the originally undesired parent background. Isolation of mutations might be achieved by screening mutagenised plants.

A molecular biological approach might be taken as an alternative to conventional methods. The SSII sequence is presented in this specification. Vectors carrying the desired mutations and a selectable marker may be introduced into tissue cultured plants, or suitable plant systems such as protoplasts. Plants where the mutation has been integrated into a chromosome to replace an existing wild type allele can be screened by, for example, using a suitable nucleic acid probe specific for the mutation and phenotypic observation. Methods for transformation of monocotyledonous plants such as barley and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, Canadian Patent Application 2092588 by Nehra, Australian Patent Application No 61781/94 by National Research Council of Canada, Australian Patent No 667939 by Japan Tobacco Inc., International Patent Application PCT/US97/10621 by Monsanto Company, US Patent 5589617, and other methods are set out in Patent specification WO99/14314.

Other known approaches to altering the activity of the amylopectin synthesis enzyme, other than the use of mutations may also be adopted. Thus, for example, this could be by expression of suitable antisense molecules that interfere with the transcription or processing of the gene or genes encoding the amylopectin synthesis enzyme. These might be based on the DNA sequence elucidated herein for the barley SSII gene. These antisense sequences can be for the structural genes or for sequences that effect control over the gene expression or splicing event. These sequences have been referred to above. Methods of devising antisense sequences are well known in the art and examples of these are can be found in, for example, United States Patent 5190131, European patent specification 0467349 AI, European patent specification 0223399 AI and European patent specification 0240208.

Methods of introducing and maintaining such sequences in plants are also published and known.

A variation of the antisense technique is to utilise ribozymes. Ribozymes are RNA molecules with enzymic function that can cleave other RNA molecules at specific sites defined by an antisense sequence. The cleavage of the RNA block the expression of the target gene. Reference is made to European patent specification 0321201 and specification WO 97/45545.

Another molecular biological approach that might also be used is that of co-suppression. The mechanism of co-suppression is not well understood, but it involves putting an extra copy of a gene into a plant in the normal orientation. In some instances the additional copy of the gene interferes with the expression of the target plant gene. Reference is made to Patent specification WO 97/20936 and European patent specification 0465572 for methods of implementing co-suppression approaches.

A further method that might be employed using the DNA sequences is duplex or double stranded RNA mediated gene suppression. In this method a DNA is used that directs the synthesis of a double stranded RNA product. The presence of the double stranded molecule triggers a response from the plant defence system that destroys both the double stranded RNA and also the RNA coming from the target plant gene, efficiently reducing or eliminating the activity of the target gene. Reference is made to Australian Patent specification 99/292514-A and Patent specification WO 99/53050 for methods of implementing this technique.

It will be understood that the invention may arise as a result of reducing the levels of activity of two or more of the above genes using a molecular biological approach.

One important product that might be envisaged in particular as a result of the high amylose and high β glucan content is a low calorific product with a reduced glycaemic index. A low calorific product might be based on inclusion of flour produced from milled grain. It might be desired, however, to first pearl the grain removing perhaps 10% or 20% by weight of the grain, thereby removing the aleurone layer and at the greater reduction removing also the germ. The effect of the pearling step is to reduce the lipid content and therefore reducing the calorific value of the food. Such foods will have the effect of being filling, enhancing bowel health, reducing the post prandial serum glucose and lipid concentration as well as providing for a low calorific food product. Use of the pearled product would result in a reduction in nutritional benefits provided by the aleurone layer and the germ. The flour produced from the pearled product is likely to have an enhanced appearance because a product made in that way tends to be whiter.

Aspects of this invention also arise from the combination of aleurone layer and germ in combination with high levels of dietary fibre. Specifically this arises from the somewhat higher relative levels of aleurone or germ present in the exemplified grain. Firstly, barley has a significantly higher aleurone layer than other commercial grains, being a result of having a three cell aleurone layer. Secondly, the exemplified barley grain is also shrunken which means that the endosperm is present in reduced amounts, a corollary of which is that the aleurone layer and the germ are present in elevated relative amounts. Thus the barley has a relatively high level of certain beneficial elements or vitamins in combination in a resistant starch delivery system, such elements include divalent cations such as bioavailable Ca^{++} and vitamins such as folate or antioxidants such as tocopherols and tocotrienols. Thus calcium is established in the provision of material for growth and deposition of bone and other calcified tissue and in lowering the risk of osteoporosis later in life. Folic acid is found to be protective against neural tube defects when consumed periconceptually and decreases the risk of cardiovascular disease thereby enhancing the effects of the combination of resistant starch and β-glucan. Folic acid also is thought to have an effect of lowering the risk of certain cancers. Tocopherol and tocotrienols carry the benefits of antioxidants and are believed to lower the risk of cancer and heart disease, and also have the effect of reducing the undesirable effects of oxidation of components of a food such as fatty acids which can result in rancidity. When these components of this preferred form of barley grain or products made therefrom constitute a convenient packaging with the one grain. One specific form of milled product might be one where the aleurone layer is included in the milled product. Particular milling process might be undertaken to enhance the amount of aleurone layer in the milled product. Such a method is referred to in Fenech *et al.,* ((1999) *J Nutr* **129**:1114-1119). Thus any product derived from grain milled or otherwise processed to include aleurone layer and germ will have the additional nutritional benefits, without the requirement of adding these elements from separate sources.

It will be understood that the barley plant of the present invention is preferably one having grain that is useful for food production and in particular for commercial food production. Such a production might include making of flour or other product that might be an ingredient in commercial food production. A lower level of usefulness might be a starch content greater than about 12% or perhaps greater than about 15%. Or similarly this might include the capacity to mill the grain; thus whilst pearled barley may be produced from most forms of grain certain configurations of grain are particularly resistant to milling. Another characteristic that might have an impact on a variety producing a commercially useable grain is discolouration of the product produced. Thus where the husk or other portion of the grain exhibits significant colouration, for example purple, this will come through with the product and limits its commercial applications to niche applications such as being a component of a bread containing coloured whole or kibbled grains. It is generally also more convenient that the barley plants are naked, because the presence of husks on barley grains introduces greater difficulty in processing the grain. Another aspect that might make a barley plant of higher value is on the basis of starch extraction from the grain, the higher extraction rates being more useful. Grain shape is also another feature the can impact on the commercial usefulness of a plant, thus grain shape can have an impact on the ease or otherwise with which the grain can be milled, thus for example the barley grain of MK6827 plant has an unusually very elongated grain morphology which makes it difficult to mill and process. A convenient measure of this elongate shape and useability is the ratio of two morphological characteristics length of the grain to the thickness of the grain (L/T ratio). This ratio is often dictated by the nature of the starch. It has been found by the inventors that MK6827 has a L/T ratio of greater than 6. Barley plants thus screened carrying the mutant SSII gene have an L/T ratio ranging from about 4 to about 5, although it is anticipated that this might extend over an even greater range and still be useful, perhaps being less than about 5.8 or at least 5.5.

The desired genetic background will include considerations of commercial yield and other characteristics. Such characteristics might include whether it is desired to have a winter or spring type of barley, agronomic performance, disease resistance and abiotic stress resistance. In Australia one might want to cross into barley cultivars such as Sloop, Schooner, Chebec, Franklin, Arapiles, Tantangara, Galleon, Gairdner or Picola. The examples provided are specific for an Australian production region, and other varieties will be suited for other growing regions.

A fuller grain may be desirable in terms of achieving greater yields and certain benefits of the invention might be achieved, such as the production of starch with high levels of amylose, or in the alternative starch with altered chain length distributions. Other aspects of the invention may, however, be better achieved by a grain that is less filled. Thus the proportion of aleurone layer or germ to starch may be higher in less filled grain, thereby providing for a barley flour or other product that is higher in the beneficial constituents of the aleurone layer. The high aleurone layer product might thus be higher in certain vitamins such as folate, or it might be higher in certain minerals such as calcium, and that combined with higher resistant starch levels and/or higher β glucan levels might provide synergistic effects such as providing for enhance uptake of minerals in the large bowel.

In order to maximise the amount of amylose it may be desirable for the barley plant to also have other phenotypic characteristics in addition to a reduced activity of one or more amylopectin synthesising enzymes. The genetic background might therefore include additionally an high amylose phenotype for example the amo1 mutation in AC38 (causal gene unknown) and the waxy mutation (found for example in the Waxiro variety). Additionally it might be desired to make double mutations in other barley mutants available with shrunken endosperms where the causal gene is not known.

In a further aspect the invention could be said to reside in the grain produced from a barley plant as referred to in this specification.

It will also be understood that the invention encompasses a processed grain including a milled, ground, kibbled, pearled or rolled grain or product obtained from the processed or whole grain of the barley plant referred to above, including flour. These products may be then used in various food products, for example farinaceous product such as breads, cakes biscuits and the like, or food additives, such as thickeners or to make malted or other barley drinks, noodles and quick soups.

Alternatively the invention encompasses starch isolated from the grain of the barley plant referred to above. Starch might be isolated by known techniques.

It will be understood that one benefit of the present invention is that it provides for one or more products that are of particular nutritional benefit, and moreover it does so without the need to modify the starch or other constituents of the barley grain.

However it may be desired to make modifications to the starch, β glucan or other constituent of the grain, and the invention encompasses such a modified constituent.

The method of modification are those known, and include the extraction of the starch or β glucan or other constituent by conventional methods and modification of the starches to for the desired resistant form.

Thus the starch or β glucan may be modified either singly of multiply though the use of a treatment selected from group including but not limited to, heat and/or moisture, physically (for example ball milling), enzymatically (using for example α or β amylase, pullalanase or the like), chemical hydrolysis (wet or dry using liquid or gaseous reagents), oxidation, cross bonding with difunctional reagents (for example sodium trimetaphosphate, phosphorous oxychloride), or carboxymethylation.

The dietary fibre content of the exemplified barley grain does not result solely from the increased relative endospermal amylose content. One primary reason is that β-glucan is present at elevated levels and contributes significantly to the dietary fibre level. There are also likely to be protective effects by reason of the association of the β glucan with the starch granule, the intimacy of association potentially provides a protective effect to the starch to thereby provide for a resistance that might be characterised as an RS1 form, being somewhat inaccessible to digestion. Similarly the presence of starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant carbohydrate. In this case the resistance is likely to arise by reason of physical inaccessibility by reason of the presence of the lipid and accordingly this might be regarded as an RS1 starch. Thus it is known that retrograded starch that takes up the V-complex configuration is highly resistant to digestion and accordingly it is anticipated that amylopectin that forms part of the starch granule having the V-complex crystalline structure will have enhanced resistance to digestion. Thirdly the starch of the exemplified barley plant may be resistant to digestion by reason of the structure of the starch granule and accordingly may have RS2 starch.

It will be understood that whilst various indications have been given as to aspects of the present invention, the invention may reside in combinations of two or more aspects of the present invention.

EXAMPLE 1

Background

The synthesis of starch in the endosperm of higher plants is carried out by a suite of enzymes that catalyse four key steps. Firstly, ADPglucose pyrophosphorylase activates the monomer precursor of starch through the synthesis of ADPglucose from G-1-P and ATP. Secondly, the activated glucosyl donor, ADPglucose, is transferred to the non-reducing end of a pre-existing α1-4 linkage by starch synthases. Thirdly, starch branching enzymes introduce branch points through the cleavage of a region of α1,4 linked glucan followed by transfer of the cleaved chain to an acceptor chain, forming a new α1,6 linkage. Finally, genetic studies demonstrate that starch debranching enzymes are essential for the synthesis of normal quantities of starch in higher plants, however, the mechanism through which debranching enzymes act is unresolved (Myers *et al.*, 2000).

While it is clear that at least these four activities are required for normal starch granule synthesis in higher plants, multiple isoforms of each of the four activities are found in the endosperm of higher plants and specific roles have been proposed for individual isoforms on the basis of mutational analysis (Wang *et al,* 1998, Buleon *et al.*, 1998) or through the modification of gene expression levels using transgenic approaches (Abel *et al.*, 1996, Jobling *et al.*, 1999, Scwall *et al.*, 2000). However, the precise contributions of each isoform of each activity to starch biosynthesis are still not known, and it is not known whether these contributions differ markedly between species. In the cereal endosperm, two isoforms of ADPglucose pyrophosphorylase are present, one form within the amyloplast, and one form in the cytoplasm (Denyer *et al.*, 1996, Thorbjornsen *et al.*, 1996). Each form is composed of two subunit types. The shrunken (*sh2*) and brittle (*bt2*) mutants in maize represent lesions in large and small subunits respectively (Girouz and Hannah, 1994). Four classes of starch synthase are found in the cereal endosperm, an isoform exclusively localised within the starch granule, granule-bound starch synthase (GBSS), two forms that are partitioned between the granule and the soluble fraction (SSI, Li *et al.*, 1999a, SSII, Li *et al.*, 1999b) and a fourth form that is entirely located in the soluble fraction, SSIII (Cao *et al*, 2000, Li *et al.*, 1999b, Li *et al,* 2000). GBSS has been shown to be essential for amylose synthesis (Shure *et al.*, 1983), and mutations in SSII and SSIII have been shown to alter amylopectin structure (Gao *et al*, 1998, Craig *et al.*, 1998). No mutations defining a role for SSI activity have been described.

Three forms of branching enzyme are expressed in the cereal endosperm, branching enzyme I (BEI), branching enzyme IIa (BEIIa) and branching enzyme IIb (BEIIb) (Hedman and Boyer, 1982, Boyer and Preiss, 1978, Mizuno *et al.*, 1992, Sun *et al.*, 1997). In maize and rice, high amylose phenotypes have been shown to result from lesions in the BEIIb gene (Boyer and Preiss, 1981, Mizuno *et al.*, 1993). In these mutants, amylose content is significantly elevated, and the branch frequency of the residual amylopectin is reduced. In addition, there is a significant pool of material that is defined as "intermediate" between amylose and amylopectin (Boyer *et al.*, 1980, Takeda, *et al.*, 1993). Mutations defining the roles of BEIIa and BEI have yet to be described, although in potato down regulation of BEI alone causes minimal affects on starch structure (Filpse *et al.*, 1996). However, in potato the combination of down regulation of BEII and BEI provides a much higher amylose content than the down-regulation of BEII alone (Schwall *et al.*, 2000). Two types of debranching enzymes are present in higher plants and are defined on the basis of their substrate specificities, isoamylase type debranching enzymes, and pullulanase type debranching enzymes (Myers *et al.*, 2000). Sugary-1 mutations in maize and rice are associated with deficiency of both debranching enzymes (James *et al.*, 1995, Kubo *et al.*, 1999) however the causal mutation maps to the same location as the isoamylase-type debranching enzyme gene. In the *Chlamydomonas* sta-7 mutant (Mouille *et al.*, 1996), the analog of the maize *sugary-1* mutation, isoamylase activity alone is down regulated.

Known variation in barley starch structure is limited relative to the variation available in maize. The most highly characterised mutations are waxy and a high amylose mutation identified as AC38. Double mutants have also been constructed and analysed (Schondelmaier *et al.*, 1992, Fujita *et al*, 1999). A broad range of characteristics of the variation in starch structure and properties (Czuchajowska *et al.*, 1992; Schondelmaier *et al.*, 1992; Vasanthan and Bhatty, 1995; Morrison *et al.*, 1984; Gerring and DeHaas, 1974; Bankes *et al.*, 1971; Persson and Christerson, 1997;. Vasanthan and Bhatty, 1998; Czuchajowska *et al.*, 1998; Song and Jane, 2000; Andreev *et al.*, 1999; Yoshimoto *et al.*, 2000), and grain properties (Swantson 1992, Ahokas 1979;, Oscarsson *et al*, 1997; Oscarsson *et al.*, 1998; Andersson *et al.*, 1999; Elfverson *et al.*, 1999; Bhatty 1999; Zheng *et al.*, 2000; Izydorczyk *et al.*, 2000; Andersson *et al.*, 2000), have been reported and the utility of the mutants in animal feeding trials (Xue *et al.*, 1996; Newman *et al.*, 1978; Calvert *et al.*, 1976; Wilson *et al.*, 1975; Sundberg *et al.*, 1998; Bergh *et al.*, 1999), human foods (Swanston *et al.*, 1995; Fastnaught *et al.*, 1996; Persson *et al.*, 1996; Pomeranz *et al.*, 1972) and human nutrition investigated (Pomeranz 1992; Granfeldt *et al.*, 1994; Oscarsson *et al.*, 1996; Akerberg *et al.*, 1998.)

In the present example, we have isolated a novel class of high amylose mutant from barley. The mutant lines contain amylose contents (65-70%) above those known from the well characterised High Amylose Glacier (AC38) mutant (45-48%)(Walker *et al.*, 1968), and have starch with an amylopectin structure that has an increase in starch branch frequency, this is in contrast to the reduced branch frequency associated with the amylose extender mutant in maize (Takeda, *et al.*, 1993).

The grain and starch characteristics of the present mutant have been investigated in detail and the causal mutation mapped. The mutations isolated are allelic to the previous known shrunken mutant in barley, sex6, and the causal mutation has been shown to be located within the starch synthase II gene. The effects of this mutation shed new light on the process of starch biosynthesis and illustrate how mutations in specific genes can have differing impacts on starch structure from one species to another.

Materials and Methods

Mutagenesis and Screening

The hull-less barley variety "Himalaya" was mutagenised using sodium azide according to Zwar and Chandler (1995). Selection of variants with altered grain morphology was carried out according to Green *et al.*, (1997). A total of 75 lines with shrunken endosperm phenotypes were identified and maintained according to Green *et al.*, (1997).

Starch Isolation

Starch was isolated from barley grain using the method of Schulman *et al.* (1991).

Methods for Amylose Determination

Determinations of the amylose/amylopectin ratio by an HPLC method for separating debranched starches, and an iodine binding method, were carried out as described by Batey and Curtin, (1996). Analysis of the amylose/amylopectin ratio by the analysis on non-debranched starches was carried out according to Case *et al.*, (1998).

Starch Content Measurement

Starch was determined using the total starch analysis kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland).

Protein Content

Nitrogen was determined by the Kjeldahl method, and protein contents were calculated using a factor of 5.7.

β-Glucan Levels

β-Glucan was determined using the kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland).

Starch Chain Length Distribution

Starches were debranched and chain length distributions analysed using flurophore assisted carbohydrate electrophoresis (FACE) using a capillary electrophoresis according to Morell *et al* (1998).

DSC

Gelatinisation was measured in a Pyris 1 differential scanning calorimeter (Perkin Elmer, Norwalk CT, USA). Starch was mixed with water in the ratio of 2 parts water: 1 part starch and this mixture (40-50mg, accurately weighed) was placed in a stainless steel pan and sealed. The sample was scanned at 10°C per minute from 20°C to 140°C with an empty stainless steel pan as a reference. Gelatinisation temperatures and enthalpy were determined using the Pyris software.

RVA Analysis

Viscosity was measured on a Rapid-Visco-Analyser (RVA, Newport Scientific Pty Ltd, Warriewood, Sydney) using conditions as a reported by Batey et al., 1997 for wholemeal flours. In order to inhibit α-amylases, silver nitrate was included in all assays at a concentration of 12 mM. The parameters measured were peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature. In addition, breakdown (peak viscosity minus holding strength) and setback (final viscosity minus holding strength) were calculated.

Flour Swelling

Flour swelling volume was determined according to the method of Konik-Rose *et al* (2001).

X-ray Data

X-ray diffraction data was collected using standard techniques (Buleon *et al.*, 1998).

Scanning Electron Microscopy

Scanning electron microscopy was carried out on a Joel JSM 35C instrument. Purified starches were sputter coated with gold and scanned at 15 kV at room temperature.

Doubled haploid production

Doubled haploids were produced from F1 plants derived from crosses between 292 and *Hordeum vulgare* cv Tantangara, and between 342 and *H. vulgare* cv Tantangara by Dr P. Davies, Waite Institute, Adelaide, Australia.

Linkage Analysis

Genetic linkage data was calculated using MapManager.

Construction of barley cDNA library

Five mgs of polyA+ mRNA from 10, 12 and 15 days post-anthesis of barley endosperm tissues was used for cDNA synthesis according to the protocols (Life Technology). The *Not*I-(dT)18 primer (Pharmacia Biotech) was used for the first stand of cDNA synthesis. The double strand cDNAs were ligated with a *Sal*I-*Xho*I adapter (Stratagene) and cloned to the *Sal*I-*Not*I arms of ZipLox (Life Technology) after digestion of cDNAs with *Not*I followed by size fractionation (SizeSep 400 spun Column of Pharmacia Biotech). The ligated cDNAs were packaged with Gigapack III Gold packaging extract (Stratagene). Titre of the library was $2x10^6$ pfu tested with Y1090(ZL) strain of *E.coli.*

Cloning of specific cDNA regions of barley starch synthase II using PCR

The cDNA clone, wSSIIp1, was used for the screening of a cDNA library of barley. The cDNA clone, wSSIIp1 was generated by PCR using the primers ssIIa (TGTTGAGGTTCC ATGGCACGTTC SEQIDNO 11) and ssIIb (AGTCGTTCTGCCGTATGATGTCG SEQ. ID NO 12), amplifying the region between nucleotide positions 1,435 and 1,835 of wSSIIA (GenBank accession no: AF155217).

The amplification was performed using a FTS-1 thermal sequencer (Corbett, Australia) for 1 cycle of 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 1 minutes, 72°C for 2 minutes and 1 cycle of 25°C for 1 minute. The fragment wSSIIp1 was cloned into a pGEM-T vector (Promega)

Screening of barley cDNA library

A cDNA library, constructed from RNA from the endosperm of barley cv Himalaya, was screened with a 347-bp cDNA fragment, wSSIIp1 at the hybridisation conditions as previously described (Rahman *et al.*, 1998). Hybridisation was carried out in 50% formamide, 6 x SSPE, 0.5% SDS, 5 x Denhardt's and 1.7 μg/mL salmon sperm DNA at 42°C for 16 h, then washed 3 x with 2 x SSC containing 0.1% SDS at 65°C for 1 h per wash.

Screening of a barley genomic library.

A barley (barley cv Morex) genomic library was constructed and screened essentially as described in Gubler et al (2000) using the barley SSII cDNA as a probe.

Sequencing of genomic clones

The Morex SSII gene was subcloned into plasmid vectors and sequenced. The292 and MK6827 genes were sequenced by PCR amplification of overlapping regions of the gene using primers designed on the basis of the Morex sequence. PCR fragments were either sequenced directly or subcloned and sequenced from plasmids

Identification of Expressed Regions

Regions of the 292 and MK6827 genomic sequences predicted to be present in cDNAs were defined by reference to the Himalaya cDNA sequence and Morex genomic sequence.

PCR analysis of the G to A mutation in the SSII gene

PCR primers were designed that amplify the region containing the G to A transition mutation identified in 292. The primer sequences are: ZLSS2P4 (CCTGGAACACTTCAGACTGTACG SEQ. ID NO 13) and ZLBSSII5 (CTTCAGGGAGAAGTTGGTGTAGC SEQ ID NO 14). The amplification was performed using a FTS-1 thermal sequencer (Corbett, Australia) for 1 cycle of 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 1 minutes, 72°C for 2 minutes and 1 cycle of 25°C for 1 minute.

SDS-PAGE Analysis of barley endosperm proteins

Starch was prepared from the developing and mature endosperm of barley and wheat and the surface proteins were removed by proteinase K as described (Rahman et al,1995). Starch granule proteins were extarcted from 20mg of starch dry wt., using 0.5ml of an extraction buffer containing 50mM Tris pH 6.8, 10%SDS and 10% 2-mercaptoethanol. After gelatinization by boiling for 10 min, and collection of the starch by centrifugation, 15 microliters of the supernatant was loaded on each lane.

Doubled haploid production

Doubled haploids were produced from F1 plants derived from crosses between 292 and *Hordeum vulgare* cv Tantangara, and between 342 and *H. vulgare* cv Tantangara by Dr P. Davies, Waite Institute, Adelaide, Australia.

Backcrossing Strategy

Crosses were made between 292 and *Hordeum vulgare* cv Sloop to generate F1 seed. Plants derived from the F1 seed were selfed to generate a population of F2 seed. The plants growing from these F2 seed were tested using a PCR assay and plants homozygous for the 292 mutation were backcrossed to Sloop (BC1). The F1 plants resulting from BC1 were again tested by PCR and plants heterozygous for the 292 mutation selected, and crossed back to Sloop (BC2). The F1 plants derived from BC2 were again analysed by PCR and plants heterozygous for the 292 mutation selected. These plants were either selfed to generate a BC2F2 population, or crossed again to Sloop (BC3). The F1 plants derived from BC3 were again analysed by PCR and plants heterozygous for the 292 mutation selected. These plants were selfed to generate a BC3F2 population. Plants derived from these seed were tested by PCR and plants homozygous for the 292 mutation selected for single seed descent and seed increase.

Results

Selection of Mutants

The identification of a range of mutants in the hull-less or naked barley variety "Himalaya" induced by a sodium azide treatment has been previously reported by Zwar and Chandler (1995). A group of 75 shrunken grain mutants were identified by the inventors and the amylose content of the starch from the shrunken seed was determined by HPLC (Figure 1). Two lines, 292 and 342, were found to have amylose contents of 71 and 62.5% respectively (Table 1). The amylose contents of 292 and 342 were substantially higher than the previously well characterised AC38 line (47% amylose, see Table 1). This study defines the genetic basis of the novel high amylose phenotype displayed by 292 and 342, and describes effects of the causal mutation on grain and starch structure and functionality.

<u>Grain Characteristics</u>

Grain size and morphology:

The effects of the mutation on grain weight and morphology are marked (Table 2). The grain weight is reduced from 51 mg for the parent line Himalaya, to 32 mg for 292 and 35 mg for

342. The mutants retain the length and width of the wild type, but in comparison are flattened (from 2.82 mm average thickness in Himalaya to 1.58 and 1.75 mm in 292 and 342 respectively) and have an essentially unfilled central region. Figure 2 shows photographs of the mutant and wild-type grain. The dimensions of the grain were routinely measured, the length of the grain (L), the width of the grain at the widest point (W), and the thickness (T) as indicated in Figure 2. The ratio of length (L) to thickness (T) of the grain is a useful diagnostic for the mutation, with values of >3.5 typically found for seed carrying the 292 or 342 mutations, and values <3.5 for non-mutant barleys.

Grain composition:

The starch content of the mutant lines is reduced from 49.0% for Himalaya to 17.7 and 21.9% for 292 and 342 respectively (see Table 1). Subtraction of the starch weight from total grain weight to give a total non-starch content of the grain, showed that the loss of starch content accounted for the loss of grain weight, with non-starch weights of 26.0, 26.3 and 27.3 mg for Himalaya, 292 and 342 respectively.

The protein content of 292 and 342 is increased relative to the parent line, Himalaya (Table 1) however, this effect is due to the loss of starch from the grain and is not due to any increase in protein synthesis per caryopsis.

The β-glucan levels of the 292 and 342 mutants are also increased, and are higher than would be expected from the effect of the reduction of starch content (Table 1). In both cases, β-glucan content is increased about 20% per caryopsis, possibly representing diversion of a small proportion of incoming carbon from starch synthesis to β-glucan synthesis.

<u>Starch Composition and Functionality</u>

Amylose and amylopectin content

Amylose content was determined using two techniques, firstly, size exclusion HPLC in 90% (v/v) DMSO, and secondly, iodine blue value. The amylose contents determined by each method were similar and the HPLC data are given in Table 1.

From grain weight and amylose content data for mutant and wild type lines, calculations of the amount of amylose deposited per grain can be made. This analysis shows that there is a decrease in amylose amount per grain from 6.2 mg/caryopsis in Himalaya, to 4.0

mg/caryopsis in 292 and 4.8 mg/caryopsis in 342. In contrast, there is a dramatic reduction in amylopectin synthesis per caryopsis, from 18.7 mg in Himalaya, to 1.6 mg in 292 and 2.9 mg in 342.

Chain Length Distribution

The chain length distribution of the starch following isoamylase debranching was carried out using fluorophore-assisted carbohydrate electrophoresis (FACE). The chain length distribution of the 292 and 342 mutants, and Himalaya, are shown in Figure 3a. Figure 3b shows a difference plot in which the normalised chain length distributions for the 292 and 342 mutants are subtracted from the normalised distribution of Himalaya. The percentages of chain lengths from DP 6-11, DP 12-30 and DP 31-65 have been calculated and are presented in Table 3. There is a marked shift in the 292 and 342 mutants in chain length distribution such that there is a higher percentage of chains in the region from DP6-11 compared to DP12-30.

Differential Scanning Calorimetry

The gelatinisation temperature of the mutants was investigated using differential scanning calorimetry, and the data is shown in Table 4. Both 292 and 342 yield starches that have markedly lower gelatinisation temperatures than the Himalaya starches, with respect to onset, peak and final temperatures for the gelatinisation peak. The enthalpy for the gelatinisation peak for the 292 and 342 mutants is also dramatically reduced in comparison to the wild type. The amylose/lipid peak onset temperature is also reduced for the 292 and 342 mutants, however, the enthalpy is increased, consistent with the increased amylose content of the mutants.

Starch Viscosity by RVA

RVA analysis of barley wholemeal samples was conducted in order to examine their pasting viscosity. Previous studies have shown that analysis of wholemeal samples is strongly correlated with the analysis of isolated starches (Batey *et al.,* 1997). The analysis showed that there are major differences between the barley genotypes studied (see Table 5 and Figure 4). Two barley varieties containing wild type starch, Himalaya and Namoi, showed typical RVA profiles in which there was a prominent peak viscosity, followed by a decline in viscosity to a holding strength, followed by an increase in viscosity as the temperature is reduced to a final viscosity. As is generally observed for barley starches, the final viscosities for the wild type starches were equivalent to, or less than, the peak viscosities (Table 5). In AC38, a prominent peak viscosity was obtained, however, because of the elevated amylose content of this line, the final viscosity obtained was higher than the peak viscosity. However, in 292, 342 and MK6827, a very different profile was obtained. No marked initial increase in viscosity corresponding to the peak viscosity in other barley starches was obtained, and therefore no value for breakdown could be calculated. The values for peak viscosity given in Table 5 for 292, 342 and MK6927 were the viscosities registered at the time of peak viscosity for Himalaya. In 292, 342 and MK6827, viscosity increased throughout the analysis to reach a final viscosity comparable to the other wholemeal samples. When normalized on the basis of starch content, the 292 and 342 starches had very high final viscosities (see Table 5).

Swelling volume is a method of measuring the properties of flour and starch that probes the behaviour of the material on exposure to heat and excess water. Increased uptake of water is measured by weighing the sample prior to and after mixing the sample in water at defined temperatures and following collection of the gelatinized material. The analysis showed that the control samples, Himalaya and Tantangara, swell to 6 to 8 times their dry weight, in contrast, 292 and 342 swell to just 2-3 times their dry weigh (Table 9).

Crystallinity

The structure of the starches was further investigated by X-ray crystallography (see Table 6 and Figure 5). Himalaya shows the expected pattern for a cereal starch, having predominantly "A" type crystallinity, and both AC38 and Waxiro showed very similar X-ray diffraction patterns, although the levels of crystallinity were lower for AC38 and higher for Waxiro. For the 292 and 342 mutants, the X-ray diffraction pattern shifted to a mixture of V and B pattern. In addition to the shift in diffraction pattern, the amount of crystallinity was sharply reduced in the 292 and 342 mutants, to 9 and 12% respectively. This result is consistent with the low amylopectin content of the 292 and 342 starches.

Granule Morphology

Starch granule morphology was investigated using scanning electron microscopy (Figure 6). The size and shape for granules from Himalaya (Figure 6, panel A), waxy barley (Waxiro, Figure 6 panel b), and AC38 (Figure 6, panel c) were consistent with previously published observations of starch granules in normal barley lines. The morphology of "A" type starch granules in the mutant lines 292 (Figure 6, panel d), 342 (Figure 6, panel e), and MK6827 (Figure 6, panel f), is clearly altered with the granules having a convoluted surface in comparison to the smooth lenticular shape of the normal barleys.

Dietary Fibre

Dietary fibre analysis was conducted according to the AOAC procedure and showed that there was an increase in dietary fibre in 292 and 342, and that this increase in dietary fibre was due to an increase in insoluble dietary fibre rather than soluble dietary fibre (Table 1), consistent with components of the dietary fibre being resistant starch and β-glucan. It is to be noted that this measure of dietary fibre is a chemically determined one which is quite distinct form the physiological measure relevant from a nutritional point of view.

Genetic Basis of the Mutation

Segregation ratio

Crossing of the mutation to barley varieties not displaying the shrunken endosperm phenotype of 292 or 342 demonstrated that the mutation is a straightforward recessive mutation, displaying a 3 normal : 1 shrunken ratio in the F2 seed of outcrossed populations, and 1 normal : 1 shrunken ratio in the seed of a doubled haploid population developed following a single outcross (see Table 6). Normal is defined as seed with an L/T ratio of <3.5, shrunken seed as seed with an L/T ratio of >3.5.

Allelic nature of mutants

The 292 and 342 mutations were shown to be allelic through the analysis of progeny from crosses of 292 and 342. All F1 seed derived from reciprocal crosses showed grain weight and grain morphology phenotypes within the range of sizes and shapes observed for the parental 292 and 342 lines, and outside of the range of seed size and shape found for the parental Himalaya line. Furthermore, all F2 seed derived from 292 x 342 F1 plants showed the typical shrunken seed phenotype of the 292 and 342 mutants.

Analysis of the grain morphology and starch characteristics of a series of shrunken grain mutants available from the Barley Germplasm Collection (USDA-ARS, National Small Grains Germplasm Research Facility, Aberdeen, Idaho 83210, USA) suggested that the line MK6827 (BGS31, also referred to as GSHO 2476), carrying the *sex6* mutation showed a highly similar set of starch and grain characteristics to the 292 and 342 mutations. Crosses were established between 292 and MK6827 and all F1 grain showed the typical 292 phenotype with respect to grain weight and shrunken seed phenotype. F2 seed derived from the 292 x MK6827 F1 plants all showed shrunken endosperm phenotype with L/T ratios of >4. In contrast, F2 seed from a cross between 292 and the commercial barley cultivar Sloop yielded a bimodal distribution showing a 3:1 segregation ratio between shrunken and filled seed (Table 6). F1 seed generated from crosses of 292 and 5 other lines with shrunken endosperm phenotypes (BGS 380, shrunken endosperm 4, 7HL (Jarvi *et al.*, 1975); BGS 381, shrunken endosperm 5, 7HS (Jarvi *et al.*, 1975); BGS 382, sex1, 6HL (Eslick and Ries 1976); BGS 396, Shrunken endosperm 6, 3HL (Ramage and Eslick 1975); BGS 397, Shrunken endosperm 7, not mapped, (Ramage and Eslick 1975) all yielded grain with a filled seed morphology. On this basis, the 292, 342 and MK6827 mutations are considered to be allelic, and on the basis of previously published map locations for the sex6 locus, the 292 and 342 mutations would be predicted to map to the short arm of chromosome 7H in barley, about 4 cM from the centromere (Netsvetaev, 1990, Netsvetaev and Krestinkov, 1993, Biyashev *et al.*, 1986, Netsvetaev, 1992).

Linkage Analysis

A doubled haploid population was generated from a cross between 292 and the commercial malting barley variety, Tantangara, which contained 90 progeny lines (Table 8).

The lines were scored for seed morphology (filled versus shrunken seed), chain length distribution by FACE (percentage of chains with DP 6 to 11), seed covering (naked or husked), and for a PCR marker (see below). This data is given in Table 8. The shrunken seed character and 292 FACE distribution co-segregated precisely in this population, as would be expected if the altered grain size and shape were a consequence of altered starch deposition. The co-segregation of characters is illustrated in Figure 8. Panel A shows the relationship between starch chain length (illustrated by the percentage of chains between DP 6 to 11) and the length to thickness ratio. The open circles indicate lines that have the PCR marker for the 292 mutation, the crosses indicate lines that carry the wild type PCR marker. There is a clear definition between the two groups of lines. Figure 8 Panel B shows the relationship between starch chain length and seed weight, and shows that seed weight is less diagnostic for the mutation that the length to thickness ratio.

In barley the presence or absence of the husk is controlled by the *nud* locus located on chromosome 7H, and as Tantangara is a husked barley and 292 is a naked type, this character could be scored in the doubled haploid progeny. Analysis of the linkage between the naked/husked character and FACE data showed that in this cross the 292 mutation was mapped within 16.3 cM of the *nud* locus. This location is consistent with previous mapping data for the allelic *sex*6 mutation (Netsvetaev, 1990, Netsvetaev and Krestinkov, 1993, Biyashev *et al.*, 1986, Netsvetaev, 1992).

Identification of the causal gene

The *nud* gene has been demonstrated to be located on barley chromosome 7H (Figure 8, Fedak *et al.*, 1972). In wheat, three starch synthases (GBSS, SSI and SSII), and an isoamylase-type debranching enzyme (S. Rahman, personal communication) are located on the short arm of chromosome 7, the homologous chromosome (Yamamori and Endo, 1996, Li *et al.*, 1999a, Li *et al.*, 1999b, Li *et al.*, 2000). The close linkage to the *nud* locus suggested that the most probable candidate gene was the SSII gene. The wheat SSII gene has been cloned at the cDNA level (Li *et al*, 1999b; Genbank Accession No. AF155217) and at the genomic level (Li *et al.*, personal communication), and a barley cDNA has been isolated and cloned (Figure 9). The sequencing of barley and wheat SSII genomic sequences shows that the genes have very similar exon/intron structures, however, the lengths of the intron regions differ between sequences (Figure 10). Comparison of the Morex genomic sequence and the sequence of a cDNA from Himalaya (Figure 9) lead to the identification of deduced cDNA sequences from Morex, 292 and MK2827.

A G to A transition mutant was found in the SSII gene from 292 at a position that corresponds to 1829 of the alignment shown in Figure 11. This mutation introduces a stop codon into the 292 SSII open reading frame (Figure 12). Sequence analysis of Tantangara and Himalaya showed that both wild type genes were identical in this region and both 292 and 342 contained the same G to A transition mutation. The introduced stop codon would truncate the gene product such that the entire C-terminal catalytic domain of the starch synthase II gene would not be translated , and it is therefore highly likely that all SSII activity is abolished by this mutation.

A G to A transition was also present in MK6827, at position 242 of the alignment shown in Figure 11 and the Himalaya cDNA sequence in Figure 9. This mutation also introduces a stop codon into the 292 SSII open reading frame (Figure 12) and would prevent translation of over 90% of the SSII gene, abolishing SSII activity encoded by this gene.

The G to A transition mutation in 292 disrupted a restriction site (NlaIV) in the barley SSII gene. The location of the diagnostic NlaIV site is shown in Figure 14, panels (a) and (b). Figure 14c shows agarose gel electrophoresis of NlaIV digested products from barley showing that the diagnostic pattern for the 292 mutation is in 292 and 342 but not MK6827, Himalaya or Tantangara.

The PCR marker for the G to A transition was scored in the 90 lines of the 292 x Tantanagara doubled haploid population and found to co-segregate precisely with the shrunken seed and FACE chain length distribution phenotypes, indicating that the 292 mutation is perfectly linked to this starch phenotype and that it is highly probable that this mutation is the causal mutation underlying the 292 phenotype. Figure 14d shows the analysis of lines from the 292 x Tantangara doubled haploid population.

Biochemical Evidence for loss of SSII activity

The composition of starch biosynthetic enzymes in the mutant and normal barley lines was investigated using a range of gel electrophoresis techniques. Analysis of the soluble fraction of the developing endosperm demonstrated that all lines contained BEI, BEIIa, BEIIb, SSI and SSIII and that the content of these isoforms of BE and SS respectively were essentially unaltered . However, analysis of the starch granule indicated that several bands were missing. Firstly, SDS-PAGE analysis (Figure 16, panel B) showed that a band of 90 kD was not present in 292, 342 or MK6827 that was present in Himalaya, Tantangara and AC38. This band was shown by immunoblotting to contain SSII (Figure 16, panel B) and BEIIa and BEIIb. The finding that BEIIa and BEIIb are present in the soluble fraction but not the granule indicates that there has been an alteration in the distribution of these enzymes in the 292, 342 and MK6827 mutants, rather than a mutation abolishing expression. In contrast, no evidence was found for SSII expression in either the soluble or the granule fraction (Figure 16, panels A and B), consistent with the genetic evidence directly linking the SSII mutation to the observed phenotypes in 292, 342 and MK6827.

Breeding of lines carrying the 292 mutation

Two strategies have been used to transfer the 292 mutation into alternative barley genotypes.

In the first example, doubled haploid lines were generated from a cross between 292 and Tantangara. Data for seed covering, seed weight, L:T ratio, chain length distribution and SSII

DNA marker status is given in Table 8. More comprehensive analysis of the composition of these lines is given in Table 9, including RVA analysis, β-glucan content and flour swelling volume. The data shows that the lines carrying the 292 mutation have significantly different RVA parameters (as exemplified by the Peak/Final Viscosity ratio), higher β-glucan content, and altered flour swelling volumes.

In the second example, the mutation was transferred by performing two backcrosses from 292 to a cultivar with normal starch properties (cv Sloop). The F2 seed from three backcross 2 F1 plants was collected for analysis. The F2 seed were categorized into seed with an L:T ratio of >3.5 and an L:T ratio of <3.5. The distribution of seeds between these classes was consistent with expectations for a single recessive gene. Flour swelling volume data for the categories of seeds derived from each plant are shown in Figure 10 and shows that the starch swelling trait was clearly transferred through the breeding process into lines with an average of 75% Sloop background.

Discussion

We describe the isolation of novel mutants, 292 and 342, in barley that have a shrunken endosperm phenotype. Analysis of grain composition demonstrates that the shrunken phenotype is due to a significant decrease in starch content, and analysis of starch composition shows that this decrease is manifested as a high amylose phenotype that arises because of a decrease in amylopectin synthesis.

The 292 and 342 mutants possess a unique combination of grain and starch properties, in containing both increased β-glucan levels and resistant starch. The β-glucan levels of the lines are increased approximately 15% above that expected by the effect of reduced starch content, suggesting that carbon unable to be converted to starch is diverted to β-glucan synthesis. Determinations of dietary fibre levels demonstrate that the grain from the mutants have increased levels of dietary fibre, and that this increase is due to an increase in insoluble dietary fibre.

This combination of properties indicates that these mutants may have very interesting potential as components of the human diet. First, the elevated β-glucan levels suggests that the lines may be useful in lowering cholesterol through the well established action of β-glucan in reducing cholesterol levels. Secondly, the presence of resistant starch indicates that the lines may be beneficial from a bowel health perspective through the well established ability of resistant starches to promote colonic fermentation (Topping *et al.*, 1997, Topping 1999). Thirdly, the grain composition indicates that the lines will have low energy density and that they may be slowly digested, indicating that they may contribute to the formulation of foods with a reduced glycaemic index.

The starch properties of the exemplified lines are also unique in that they combine a high amylose starch that also has a low gelatinisation temperature. This contrasts with high amylose mutations resulting from mutations in branching enzyme IIb in which gelatinisation temperature typically increases, such as the *amylose extender* mutation in maize (Ng *et al.*, 1997, Katz *et al.*, 1993, Krueger *et al.*, 1987, Fuwa *et al.*, 1999). While the amylose content of 292 is comparable to *amylose extender* lines, the structure of the amylopectin component of the starches differs dramatically (Wang *et al.*, 1993). In 292 and 342, the chain length distribution of amylopectin shifts towards lower degree of polymerisation, whereas in *amylose* extender, the chain length distribution shifts towards increased degree of polymerisation. This suggests that amylopectin, rather than amylose content, is the primary determinant of gelatinisation temperature and that this effect is mediated through the strength of the interaction between the external chains of the amylopectin molecule. Similar effects were noted for a range of starches by Jane *et al.*, 1999.

The viscosity data from the RVA analysis indicate that the starch from the SSII mutant lines is marked different from normal barleys and AC38. The SSII mutant barleys have essentially no peak of viscosity typically seen as the temperature is ramped up to 95°C at the beginning of an RVA temperature profile. Instead, in these mutants, the viscosity increases steadily until a final viscosity if present. These data are consistent with the low amylopectin content of the granules, the low level of amylopectin crystallinity in the granule, and the low gelatinisation temperature and enthalpy observed in the diffential scanning calorimeter. A high final viscosity is reached once the amylose has been released from the granule through heating in excess water, and stirring. These RVA characteristics are unique for a cereal starch and provide a novel source of starch for food and industrial applications where low pasting viscosity yet high final viscosity is required.

The observations made on gelatinisation temperature in the DSC are reflected in results from X-ray diffraction studies. The granules of 292 and 342 have reduced levels of crystallinity and the crystal form shifts from the A type typical of cereal starches to a mixture of V and B types. The V type is typical of amylose and reflects the amylose component of the starch complexed with fatty acids, while the B form is derived from amylopectin and presumably reflects the residual amylopectin content of the starch (Buleon *et al*, 1998).

Analysis of the genetic basis of the 292 and 342 mutations demonstrates that the mutations are simple recessive mutations that give typical Mendelian ratios in outcrossing experiments. Crossing studies demonstrated that 292 and 342 are allelic. Further analysis of the interaction between 292 and other shrunken endosperm mutations in crossing experiments demonstrated that the 292/342 mutations were also allelic with the Sex6 mutation in the line MK6827. This mutation has previously been mapped and shown to be located within 3 cM of the centromere on the short arm of chromosome 7H (Netsvetaev, 1990, Netsvetaev and Krestinkov, 1993, Biyashev *et al.*, 1986, Netsvetaev, 1992).

A doubled haploid population between the husked barley Tantangara and the naked 292 mutant was established and the shrunken endosperm mutation mapped to the short arm of chromosome 7HS, to within 16 cM of the *nud* gene, a location consistent with the map location of the Sex6 mutation.

The localisation of the gene to the region adjacent to the centromere on the short arm of chromosome 7HS demonstrates that the causal mutation (sex6) is in a different gene to the mutation that causes the high amylose phenotype in AC38 (*amo1*) which has been mapped to chromosome 1H (Schondelmeier *et al* 1992). The map location suggested that one candidate for the gene disrupted in the sex6/292 mutation was starch synthase II, known in wheat to be localised in the same region of the chromosome (Yamamori and Endo 1996, Li *et al*, 1999b). Sequence analysis of the 292 and 342 mutants showed that there was a G to A transition mutation in the gene which would cause truncation of the gene such that the C-terminal region containing the active site of the enzyme would not be translated, presumably leading to the synthesis of a completely inactive protein. Furthermore, the sequencing of the SSII gene from MK6827 showed a G to A transition mutation at position 242 which would also cause truncation of the gene. This result confirms the allelic nature of the 292 and MK6827 mutations.

The identification of mutations in the SSII gene lead to the development of a PCR marker diagnostic for the mutation in 292. This PCR marker was scored across the 91 progeny of the 292 x Tantangara population and shown to 100% co-segregate with the shrunken endosperm phenotype and the reduced chain length distribution phenotype of starch. The discovery of allelic mutations in the SSII genes from barleys of diverse backgrounds (292 and MK6827) which give rise to similar phenotypes, and the perfect linkage of the mutation to the shrunken grain phenotype provides a high degree of certainty that the mutations present in the SSII genes of 292, 342 and MK6827 are the causal mutations leading to the shrunken endosperm character.

The phenotype observed here for the SSII mutation in barley is similar to the phenotypes of SSII mutations in other plants in some respects, however, SSII mutations do not give rise to amylose contents as high as those found in 292/342. SSII mutants are known in pea (*rug*5, Craig *et al.*, 1998) and Chlamydomonas (Fontaine *et al.*, 1993) and give rise to amylopectins with reduced chain length distributions, as observed here. There is also evidence to suggest that the Shrunken-2 mutation in maize arises through mutation of the SSII gene although this has yet to be conclusively demonstrated (Harn *et al.*, 1998, Knight *et al.*, 1998). In maize, the Shrunken-2 mutation gives rise to starches with reduced gelatinisation temperatures (Campbell *et al.*, 1994). In wheat, Yamamori has developed a triple null line that lacks the Sgp-1 protein (Yamamori 1998) that has been shown by Li *et al* (Li *et al*, 1999b) to be the product of the SSII gene. In wheat, amylose content is increased to about 35% and abnormal starch granules, altered crystallinity and altered gelatinisation temperature are observed (Yamamori 1998). The differences in properties between the barley SSII mutants and SSII mutants from other species are quite unexpected.

The SSII mutation has been shown to be able to be transferred by breeding from one genetic background to another and yield diagnostic grain morphology and composition typical of the original 292, 342 and MK6827. In table (9) data from 292 x Tantangara doubled haploid lines for the L/T ratio, β-glucan content, chain length distribution, RVA and flour swelling volume parameters demonstrate that lines carrying the 292 mutation show phenotypes typical of the 292 parent. In a further demonstration, the segregation of seed from the selfed progeny of a second backcross of 292 to Sloop showed a segregation ratio consistent with 3:1 segregation for the normal (74 seeds with L/T ratio <3.5) and shrunken phenotypes (21 seeds L/T ratio >3.5).

The availability of the sequence of the SSII gene and barley transformation systems provides the tools required to knock out the SSII gene using gene suppression technologies, in order to produce a comparable phenotype to that found with the SSII mutations. A recently developed highly effective strategy is to produce a hairpin construct designed to produce a double stranded RNA which would suppress the endogenous SSII activity. While complete knock out mutants analogous to the mutations described here would be of interest, the use of DNA constructs with differing promoters, and the recovery of transgenes with differing levels of hairpin construct expression, would allow the impact of titrating the expression of the gene from normal levels to complete knockdown levels to be assessed.

The mutations were shown to be able to be transferred from 292 into alternative barely genetic backgrounds, while retaining essential features of the original 292 mutation. In Tables 9 and 10, phenotypic data for 292 x Tantangara doubled haploid progeny, and the seed from a second backcross to Sloop, are shown, and indicate that the phenotypes are transferred through the breeding process.

Table 1
Barley Grain Composition

	Starch Content (%)[a]	Amylose Content By HPLC (%)[b]	Amylose Content by iodine binding (%)	Protein Content (%)[a]	β-glucan (%)[a]	Total Dietary Fibre[a] (%)	Insoluble Dietary Fibre[a] (%)	Soluble Dietary Fibre[a] (%)
Glacier	n.d.	31.0	n.d.	11.5	4.3	21.6	16.6	5
AC38	47	47.4	60.6	10.4	5.8	24.9	28.8	6.1
Himalaya	49	25	25.4	10.0	4.8	27.1	18.1	9
292	17.7	71	68.9	15.0	9.5	30.3	21.4	8.9
342	21.9	62.5	71.7	15.7	8.3	28.3	19.4	8.9
MK6827	10.2	n.d.	44.4	21.3	n.d.	n.d.	n.d.	n.d.
Waxiro	42.8	n.d.	5.0	14.6	n.d.	19.8	12.7	7.1.
Tantangara	51.6	n.d.	29.5	14.6	n.d.	17.2	12.7	4.5.

[a] % grain weight, 14% moisture
[b] % of total starch content
n.d. not determined

Table 2
Grain Dimensions

	Grain weight (mg)	Grain Length (mm)	Grain Width (mm)	Grain Thickness (mm)	L/T Ratio
Himalaya	51.01 ± 6.63[a]	7.01 ± 0.51	3.58 ± 0.34	2.82 ± 0.36	2.48
Tantangara	50.40 ± 6.51[a]	7.22 ± 0.98	3.60 ± 0.25	2.73 ± 0.21	2.64
Waxiro	45.71 ± 5.21	7.54 ± 0.47	3.40 ± 0.20	2.67 ± 0.19	2.82
AC38	50.79 ± 8.22	7.62 ± 0.65	3.35 ± 0.27	2.64 ± 0.25	2.89
292	32.13 ± 4.67[a]	7.05 ± 0.49	3.63 ± 0.55	1.58 ± 0.20	4.46
342	35.45 ± 6.01	7.28 ± 0.55	3.76 ± 0.38	1.75 ± 0.18	4.16
MK6827	44.89 ± 3.78	11.20 ±0.58	3.63 ± 0.27	1.77 ± 0.33	6.33

N=50, except where indicated by [a], n=200

Table 3
Chain Length Distribution of Isoamylase Debranched Starches

Dp[a]	Himalaya %[b]	Tantangara %[b]	AC38 %[b]	342 %[b]	292 %[b]	MK6827 %[b]
DP 6-11	24.15	22.40	26.33	38.18	38.96	37.98
DP 12-30	69.12	67.59	67.62	54.14	53.42	55.60
DP 31-60	6.73	10.01	6.05	7.68	7.62	6.42

[a] degree of polymerisation

[b] percentage of distribution of oligosaccharides expressed on a molar basis

Table 4
Barley Starch Thermal Properties Measured by DSC

		Peak 1				Peak 2		
	Onset	**Peak**	**End**	**ΔH**	**Onset**	**Peak**	**End**	**ΔH**
Glacier	55.4	59.3	65.3	4.2	93.9	101.4	107.7	0.87
AC38	55.0	62.2	68.2	3.9	89.3	100.1	106.9	1.195
Himalaya	56.8	60.9	68.0	4.5	93.1	101.8	108.3	0.78
292	46.0	51.2	58.1	0.29	88.7	97.7	104.9	1.34
342	45.2	50.4	56.8	0.47	86.5	97.0	105.0	1.59

Table 5
RVA Parameters for Barley Starches

	Peak Viscosity	Breakdow n	Holding Strength	Setback	Final Viscosity	Normalised Final Viscosity*	Pasting Temp (C)
Himalaya	871.5	653.1	218.4	235.8	454.2	926	64.9
Namoi	621.7	367.5	254.2	375.3	629.5	1284	65.9
AC38	226.7	87.3	139.4	188.4	327.8	697	68.9
292	92.1**	***	133.9	230	363.9	2055	89.5
342	110.9**	***	144.9	264.5	409.4	1869	87.9
MK6827	18.2**	***	25.7	43.3	69	676	n.d.

* Final viscosity divided by starch content of wholemeal
** Value registered at time of peak viscosity for Himalaya
*** Value was less than zero
n.d. not determined

Table 6
Starch Crystallinity Data

Sample	% H2O (W.B)	Crystallinity %*	A %*	B %*	V %*
292	29.6	9	-	13	87
342	35.8	12	-	18	81
AC38	26.1	19	93	7	(traces)
Himalaya	27.7	27	93	7	(traces)
Waxiro	29.7	41	94	6	-

(* _ ± 5%)

Table 7
Progeny Analysis

Cross	Shrunken	Full	Calculated χ^2 value[c]
292 x Sloop[a]	45	155	χ^2 (3:1) = 1.0
292 x Tantangara[b]	45	46	χ^2 (1:1) = 0.01

[a] progeny from standard cross
[b] doubled haploid progeny
[c] in each case, χ^2 (0.05), df=1 = 3.84, hence the 292 x Sloop population fits a 3:1 segregation and 292 x Tantangara doubled haploid population fits a 1:1 segregation

Table 8
Scoring of 292 x Tantangara Doubled Haploid Lines

Line Number[a]	Husk[b]	Seed Weight (mg)	L/T Ratio[c]	DP6-11 (%)[d]	Amylose Content[e]	PCR[f]
1	N	26	3.8	35.87	50.2	292
2	N	24	4.21	36.87	56.2	292
3	H	43	3.32	25.45	18.3	Wt
5	N	40	4.58	39.47	55.5	292
7	N	34	4.28	19.63	43.0	292
8	H	48	3.02	21.6	46.7	Wt
9	N	31	2.76	22.89	25.9	Wt
10	N	26	3.02	27.56	21.1	Wt
11	N	34	3.55	37.90	44.7	292
12	H	50	2.94	26.37	32.8	Wt
13	N	27	4.29	38.68	48.4	292
14	H	56	3.07	22.98	20.8	Wt
15	H	46	2.74	24.88	22.9	Wt
16	H	43	2.78	25.40	18.3	Wt
17	N	31	3.8	37.37	54.2	292
18	N	31	4.51	37.46	57.5	292
19	H	26	3.1	29.57	22.7	Wt
20	H	53	3.04	25.42	23.8	Wt
21	N	31	4.5	38.51	59.1	292
22	N	27	4.63	37.25	27.2	292
23	H	47	2.73	24.11	21.2	Wt
24	N	27	4.58	36.89	42.0	292
26	H	35	3.57	19.50	15.1	Wt
27	H	22	4.3	36.81	48.6	292
28	N	31	4.34	38.88	37.0	292
30	N	30	4.04	38.05	48.4	292
31	N	23	4.25	37.07	51.7	292
32	H	48	2.62	20.67	13.0	Wt
33	N	25	4.92	35.68	33.3	292
34	N	31	4.01	38.34	46.1	292
35	H	43	3.16	20.07	23.6	Wt
36	N	26	4.33	36.93	29.7	292
38	H	38	3.01	21.11	9.1	Wt
39	H	33	2.92	20.49	23.5	Wt
40	H	36	2.99	19.57	2.2	Wt
41	N	30	4.05	37.82	40.9	292
42	H	47	2.95	20.80	11.9	Wt
43	N	40	3.24	21.97	18.1	Wt
45	H	52	2.78	19.97	14.5	Wt
46	N	29	4.44	35.87	32.1	292
47	N	35	3.69	36.34	92.9	292
48	H	31	2.54	20.27	13.4	Wt
49	H	54	2.94	22.29	19.3	Wt
50	H	50	2.94	21.92	20.6	Wt

51	H	43	3.73	20.59	18.1	Wt
53	N	31	4.12	36.52	55.3	292
54	N	34	4.02	35.17	57.1	292
55	H	32	4.19	41.35	60.4	292
56	N	29	3.17	21.48	18.1	Wt
57	H	30	4.85	36.66	46.3	292
58	N	32	2.97	23.83	13.8	Wt
59	N	46	2.91	24.15	9.2	Wt
60	H	44	2.74	22.39	13.5	Wt
61	N	31	4.47	35.67	61.3	292
63	N	32	4.3	36.94	39.4	292
64	H	39	2.93	21.95	20.5	Wt
65	N	26	3.87	37.51	20.7	292
66	N	30	4.03	36.89	48.7	292
67	H	36	3.17	20.24	14.4	Wt
68	N	43	2.65	22.53	8.4	Wt
69	N	32	3.93	36.34	54.7	292
70	H	43	2.77	22.28	17.6	Wt
71	N	29	3.73	38.73	31.5	292
72	H	47	2.65	22.00	20.8	Wt
73	N	36	4.09	39.58	49.0	292
74	N	24	4.18	36.15	47.8	292
75	H	34	2.99	24.42	14.2	Wt
76	N	31	4.35	35.95	49.9	292
77	H	49	3.19	21.22	17.0	Wt
78	H	33	2.78	21.27	15.6	Wt
79	H	31	2.85	23.04	21.2	Wt
80	H	38	3.18	19.88	18.9	Wt
81	H	37	2.84	24.22	16.2	Wt
82	H	33	4.64	39.99	45.3	292
84	N	28	3.62	36.98	28.9	292
85	N	26	6.44	44.43	41.3	292
86	H	32	2.87	30.73	16.1	Wt
88	N	26	4.62	46.12	39.3	292
89	H	38	2.88	31.25	16.3	Wt
90	H	32	3.19	31.11	13.8	Wt
91	N	31	4.17	42.86	37.3	292
92	N	27	3.99	45.30	44.6	292
93	H	37	2.99	30.77	12.5	Wt
94	H	43	3.67	29.46	21.9	Wt
96	N	33	5.69	47.34	52.2	292
97	N	23	3.41	31.36	17.1	Wt
98	N	32	5.95	45.27	52.4	292
99	N	19	3.68	38.36	1.7	292
100	H	36	3.1	31.92	15.4	Wt
101	N	58	3.29	24.71	2.9	Wt

[a] 292 x Tantangara doubled haploid line

[b] Husk phenotype. N, naked; H, husked

[c] L/T ratio: length to thickness ratio

[d] Percentage of chains in debranched starch with DP6 to DP11, calculated on a molar basis as a percentage of chains eluting between DP6 and DP65

[e]Amylose content determined by iodine blue value

[f] PCR score. 292, PCR reaction yields band which yields 169 bp band plus 103 bp on NlaIV digestion;Wt, PCR reaction yields band which yields 111 bp, 103 bp and 57 bp band on NlaIV digestion

Table 9
Detailed Analysis of Doubled Haploid Lines

Line	L/T Ratio	FACE	RVA Peak Viscosity (RVA Units)	RVA Final Viscosity (RVA Units)	Ratio Peak/Final Viscosities	β-glucan Content (%)	Flour Swelling Volume
Control							
Sloop	2.78	23.5	535.8	483.5	1.11	2.3	7.54
Tantangara	2.64	22.4	507	395.1	1.28	5.16	5.97
Himalaya	2.48	24.2	873.9	449.3	1.94	8.53	8.18
AC38	2.89	26.33	226.7	327.8	0.69	5.8	3.75
292	4.46	38.9	92.1	363.9	0.25	13.09	2.00
MK6827	6.33	37.98	18.2	69	0.26	n.d.	2.11
Doubled Haploid Line							
Wild Type							
8	3.02	21.6	527.9	431.3	1.22	8.9	6.47
43	3.24	25.4	566.6	527.4	1.07	7.77	6.04
56	3.17	24.9	703.1	523.5	1.34	7.81	6.95
58	2.97	27.9	726.8	588.8	1.23	9.65	6.23
59	2.91	27.0	655	435.8	1.50	7.16	7.21
68	2.65	22.5	876.3	465.5	1.88	8.87	8.63
101	3.29	34.71	471.3	410.3	1.15	6.54	6.26
Mutant SSII							
5	4.58	39.5	68.7	316.6	0.217	9.87	2.55
11	3.55	48.2	51.5	240.8	0.21	8.36	2.58
13	4.29	38.7	43.7	265.5	0.16	11.13	2.92
27	4.30	36.8	20.3	96.6	0.21	13.11	2.71
30	4.04	38.05	57.3	251.1	0.23	10.56	2.27
31	4.25	37.1	17.6	124.5	0.14	11.35	2.48
33	4.92	35.7	11.7	83.5	0.14	7.22	2.13
36	4.33	36.9	14.5	93.6	0.15	7.20	2.20
46	4.44	35.9	31.3	175.8	0.18	10.02	2.32
91	4.17	42.9	35.8	189.5	0.19	11.3	2.43

n.d. not determined

Table 10
Flour Swelling Data for BC2F2 seed

Line	Swelling Volume
C5/1 Plant 1 L:T>3.5	2.118
C5/1 Plant 1 L:T<3.5	6.913
65/2 Plant 1 L:T>3.5	2.382
65/2 Plant 1 L:T<3.5	7.565
65/2 Plant 2 L:T>3.5	2.409
65/2 Plant 2 L:T<3.5	6.707

EXAMPLE 2.

Design and Construction of Vectors

Regions of the barley SSII gene (as defined in Figure 15) were cloned into vectors for transformation. Three constructs were prepared for each gene target, addressing the gene suppression strategies, (1) sense cosuppression, (2) antisense and (3) duplex-mediated suppression.

Figure 16 illustrates the configuration of sequences in DNA constructs designed to suppress the expression of the endogenous target gene. The promoter may be selected from either endosperm-specific (such as High Molecular Weight Glutenin promoter, the wheat SSI promoter, wheat BEII promoter) or promoters not specific for the endosperm (such as ubiquitin or 35S). The construct may also contain other elements that enhance transcription such as the nos 3 element of OCS. The regions of DNA illustrated will be incorporated into vectors containing suitable selectable marker gene sequences and other elements, or into vectors that are co-transformed with vectors containing these sequences.

Cereal Transformation

Methods for the transformation of barley (Tingay *et al.*, 1997; Wan *et al,* 1994) oats (Somers *et al.*, 1992, 1994; Gless *et al.*, 1998; Zhang *et al.*, 1999, Cho *et al.*, 1999) and rye (Castillo *et al.*, 1994; Pena *et al.*, 1984) have been described and can be used to transfer DNA constructs generating transgenic plants.

Analysis of transgenics

Identification of transgenic plants is carried out through identification of the DNA of the DNA construct through PCR or through Southern hybridisation. The levels of the expression of the individual barley starch biosynthetic genes is measured at both the mRNA and protein levels through standard techniques such as Northern hybridisation and Western blotting respectively. The starch and grain content and composition is measured using standard techniques such as those exemplified in Example 1.

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Claims

1. Processed grain from a barley plant, said grain comprising a loss of starch synthase II (SSII) as a result of a truncation mutation or a deletion mutation in the SSII gene, and a relative amylose content of at least 50% (w/w) of the total starch content of the grain, wherein the processed grain is milled, ground, rolled, kibbled or cracked grain, wherein the grain before processing has a length to thickness ratio of less than 5.8.

2. Grain according to claim 1, wherein said mutated SSII gene comprises a truncation mutation such that the C-terminal catalytic domain of the mutated SSII gene is not translated.

3. Grain according to claim 1, wherein the cDNA sequence of said mutated SSII gene is that set forth in SEQ ID NO: 4.

4. Grain according to claim 1, 2 or 3, wherein

   a) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 6 to 11 residues is at least 30%, and/or
   b) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 12-30 residues is less than 60%.

5. Processed grain from a barley plant, said grain having a loss of starch synthase II (SSII) as a result of a truncation mutation or a deletion mutation in the SSII gene, and a relative amylose content of at least 50% (w/w) of the total starch content of the grain, wherein

   a) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 6 to 11 residues is at least 30%, and/or
   b) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 12-30 residues is less than 60%,

   and wherein the processed grain is milled, ground, rolled, kibbled or cracked grain, and wherein the grain before processing has a length to thickness ratio of less than 5.8.

6. Grain according to claim 4 or 5, wherein

   a) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 6 to 11 residues is at least 35%, and/or
   b) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 12-30 residues is less than 65%, and/or
   c) the proportion of amylopectin chains contained in the starch of said grain which have a length in the range of 31-60 residues is less than 8%.

7. Processed grain according to any one of claims 1 to 6 wherein flour or wholemeal made from the grain has a swelling volume of less than 3.2.

8. Processed grain according to any one of claims 1 to 7 wherein flour or wholemeal made from the grain has a swelling volume of at least 2.

9. Processed grain according to any one of claims 1 to 8 wherein the starch of said grain is characterized by at least one of the following:

   a) the onset of the first gelatinization peak detected by differential scanning calorimetry is lower than 53°C,
   b) the apex of the first gelatinization peak detected by differential scanning calorimetry is lower than 60°C, and
   c) the enthalpy (ΔH) of the first gelatinization peak detected by differential scanning calorimetry is lower than 3.5.

10. Grain according to any one of claims 1 to 9, wherein the starch of said grain has a pasting temperature higher than 75°C.

11. Grain according to any one of claims 1 to 10, wherein the proportion of the starch of said grain exhibiting crystallinity is less than 20%.

12. Grain according to any one of claims 1 to 11, wherein the proportion of the crystalline starch contained in the starch of said grain exhibiting a form of crystallinity characteristic of a starch-lipid complex is greater than 50%.

13. Grain according to any one of claims 1 to 12, wherein the total starch content of said grain comprises at least 60% (w/w) amylose.

14. Grain according to any one of claims 1 to 13, wherein the proportion of amylopectin chains contained in the starch of said grain that have a length in the range of 31-60 residues is greater than 5%.

15. Starch comprising starch associated lipid, wherein the starch has a relative amylose content of at least 50% (w/w) and wherein the starch with associated lipid exhibits a V-complex crystalline form wherein the V-complex crystalline form represents at least 10% of the crystalline starch.

16. Starch of claim 15, wherein the V-complex crystalline form represents at least 50% of the crystalline starch.

17. Starch of claim 15 or 16, wherein

   a) the proportion of amylopectin chains contained in the starch which have a length in the range of 6 to 11 residues is at least 35%, and/or
   b) the proportion of amylopectin chains contained in the starch which have a length in the range of 12-30 residues is less than 65%, and/or
   c) the proportion of amylopectin chains contained in the starch which have a length in the range of 31-60 residues is less than 8%.

18. Barley flour comprising a loss of starch synthase II (SSII) as a result of a truncation mutation or a deletion mutation in the SSII gene, wherein the total starch content of the flour has a relative amylose content of at least 50% (w/w).

19. A food product or food additive comprising the starch of any one of claims 15 to 17 or the flour of claim 18.

20. A food product or food additive according to claim 19 wherein said food product or additive is selected from the group consisting of breads, cakes, biscuits, thickeners, malted drinks, barley drinks, noodles, instant noodles and quick soups.

21. Use of the starch according to any one of claims 15 to 17, the barley flour according to claim 18, or a processed grain according to any one of claims 1 to 14 for the preparation of a food product.

22. Use of an SSII mutant of a barley plant having a loss of SSII to produce a barley grain comprising a loss of SSII, as a result of a truncation mutation or a deletion mutation in the SSII gene, the total starch content of said grain having a relative amylose content of at least 50% (w/w), and wherein the grain before processing has a length to thickness ratio of less than 5.8.

23. Use of a DNA construct to down regulate the expression of a SSII gene in a barley plant, such that grain of said plant has a loss of SSII and a relative amylose content of at least 50% (w/w) in the total starch content of said grain, the grain before processing has a length to thickness ratio of less than 5.8 and wherein said DNA construct comprises a sequence which encodes

    a) an antisense RNA molecule capable of interfering with the transcription or processing of RNA encoding SSII,
    b) a double-stranded RNA molecule, or
    c) a hairpin construct designed to produce a double-stranded RNA molecule capable of suppressing the endogenous SSII activity.

24. Use of a nucleic acid molecule capable of hybridizing in vivo specifically to SEQ ID NO: 1 to inhibit expression of SSII in a barley plant, such that grain obtained from said plant has a loss of SSII, the grain before processing has a length to thickness ratio of less than 5.8, and the total starch of said grain has a relative amylose content of at least 50% (w/w), wherein said nucleic acid molecule is

    a) an antisense RNA molecule capable of interfering with the transcription or processing of RNA encoding SSII,
    b) a double-stranded RNA molecule, or
    c) a hairpin construct designed to produce a double-stranded RNA molecule capable of suppressing the endogenous SSII activity.

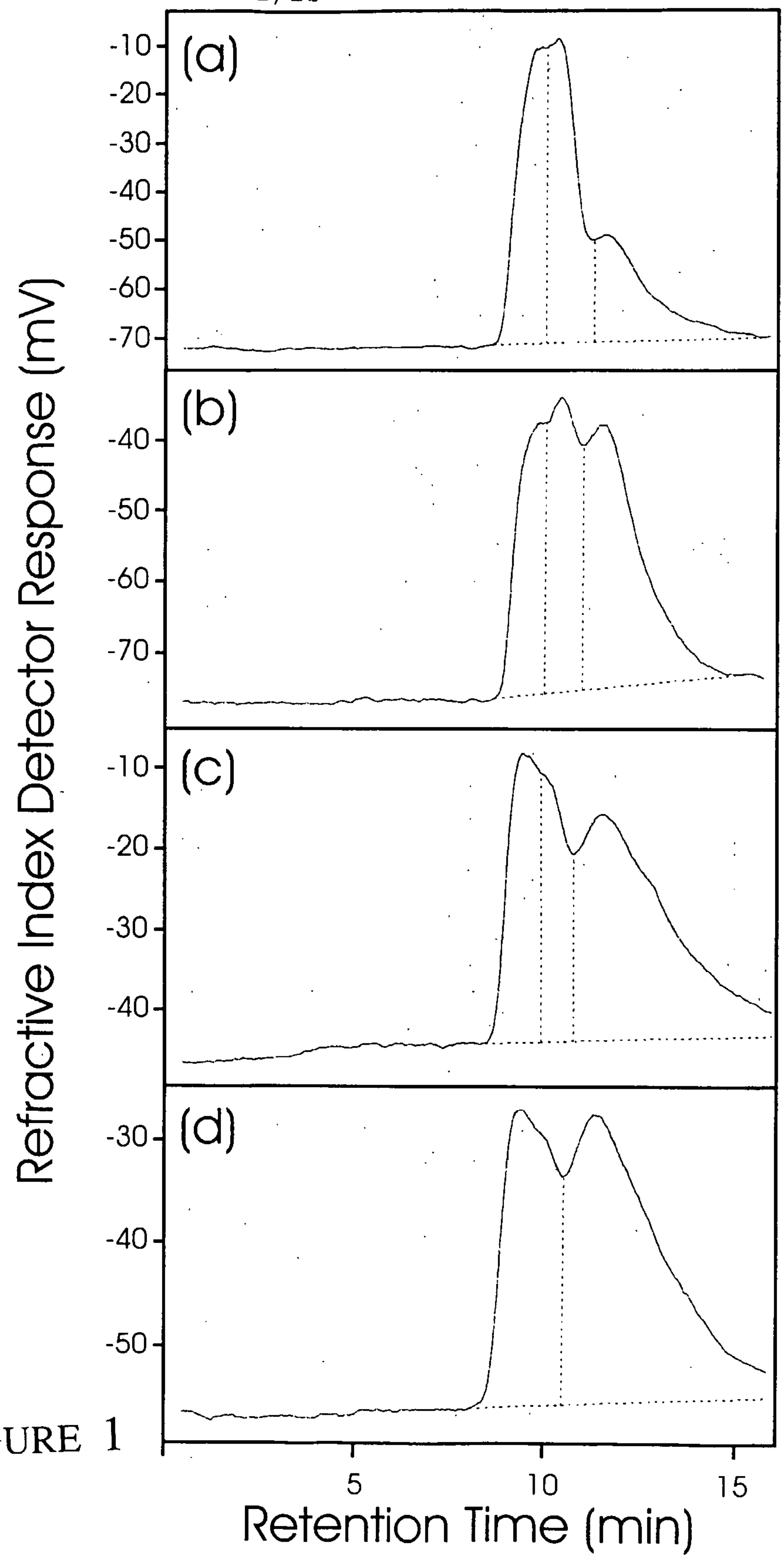
(a)
(b)
(c)
(d)
-10
-20
-30
-40
-50
-60
-70
Refractive Index Detector Response (mV)
5
10
15
Retention Time (min)

FIGURE 1

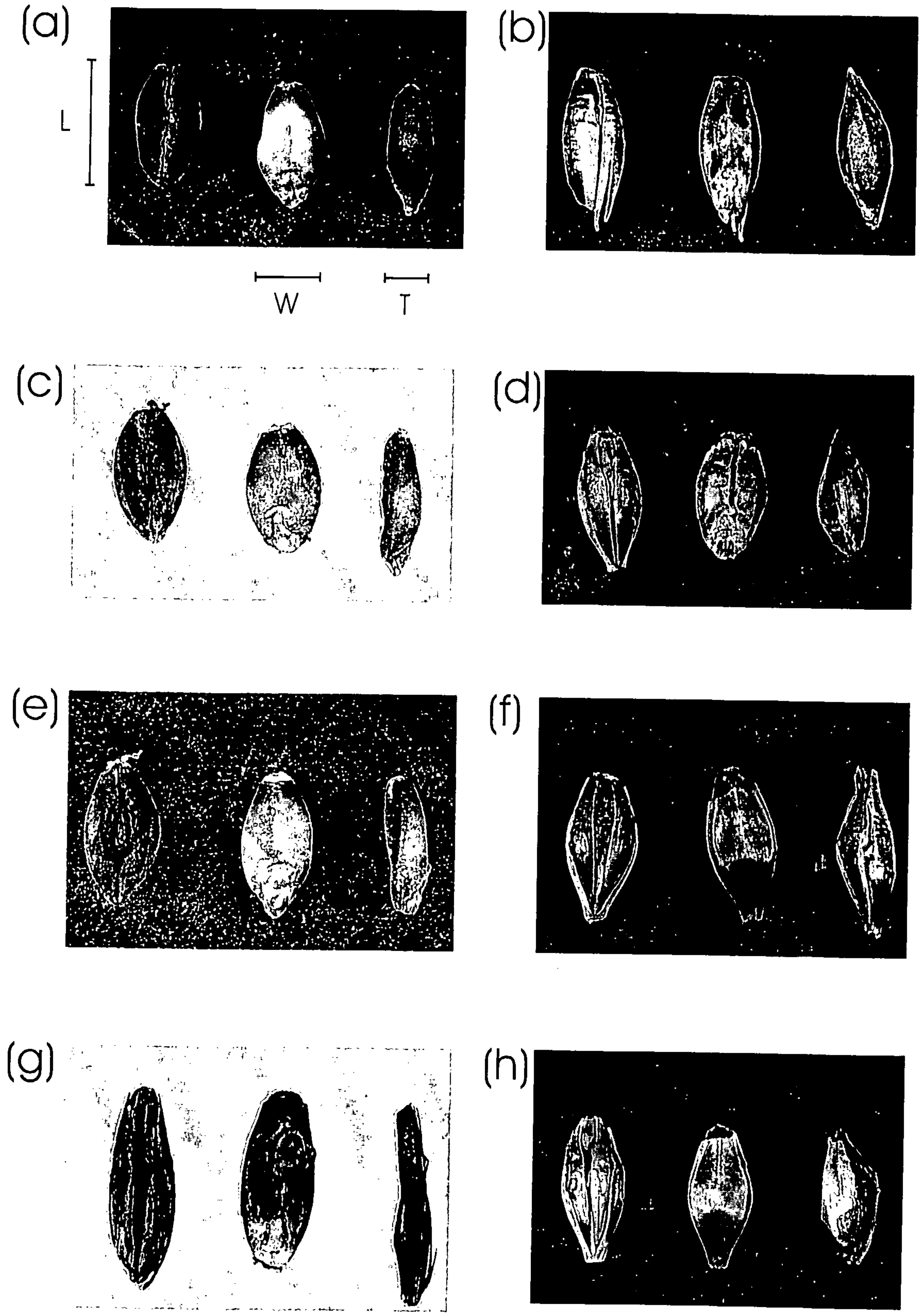

FIGURE 2

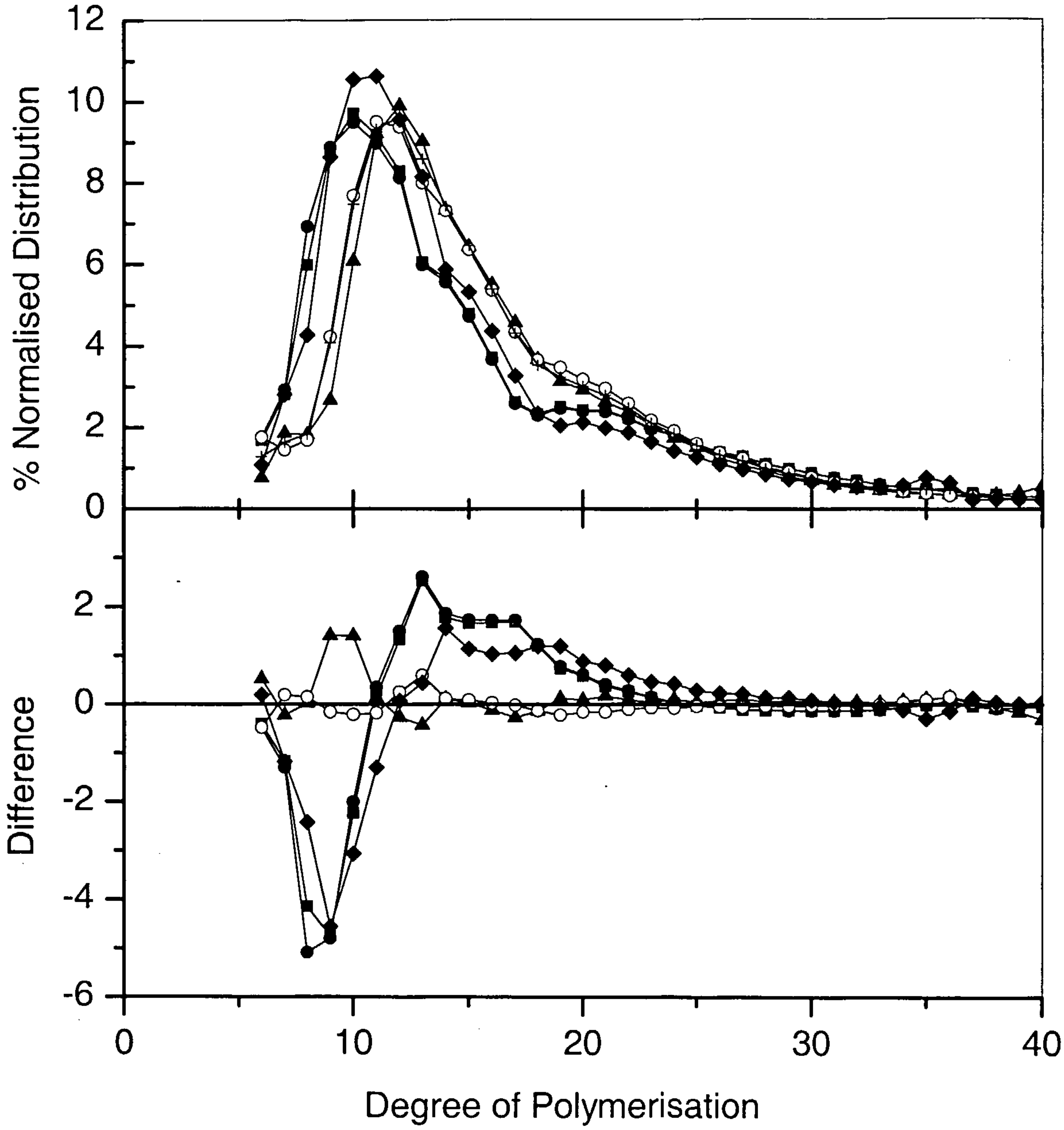
% Normalised Distribution
12
10
8
6
4
2
0
Difference
2
0
-2
-4
-6
0
10
20
30
40
Degree of Polymerisation

FIGURE 3

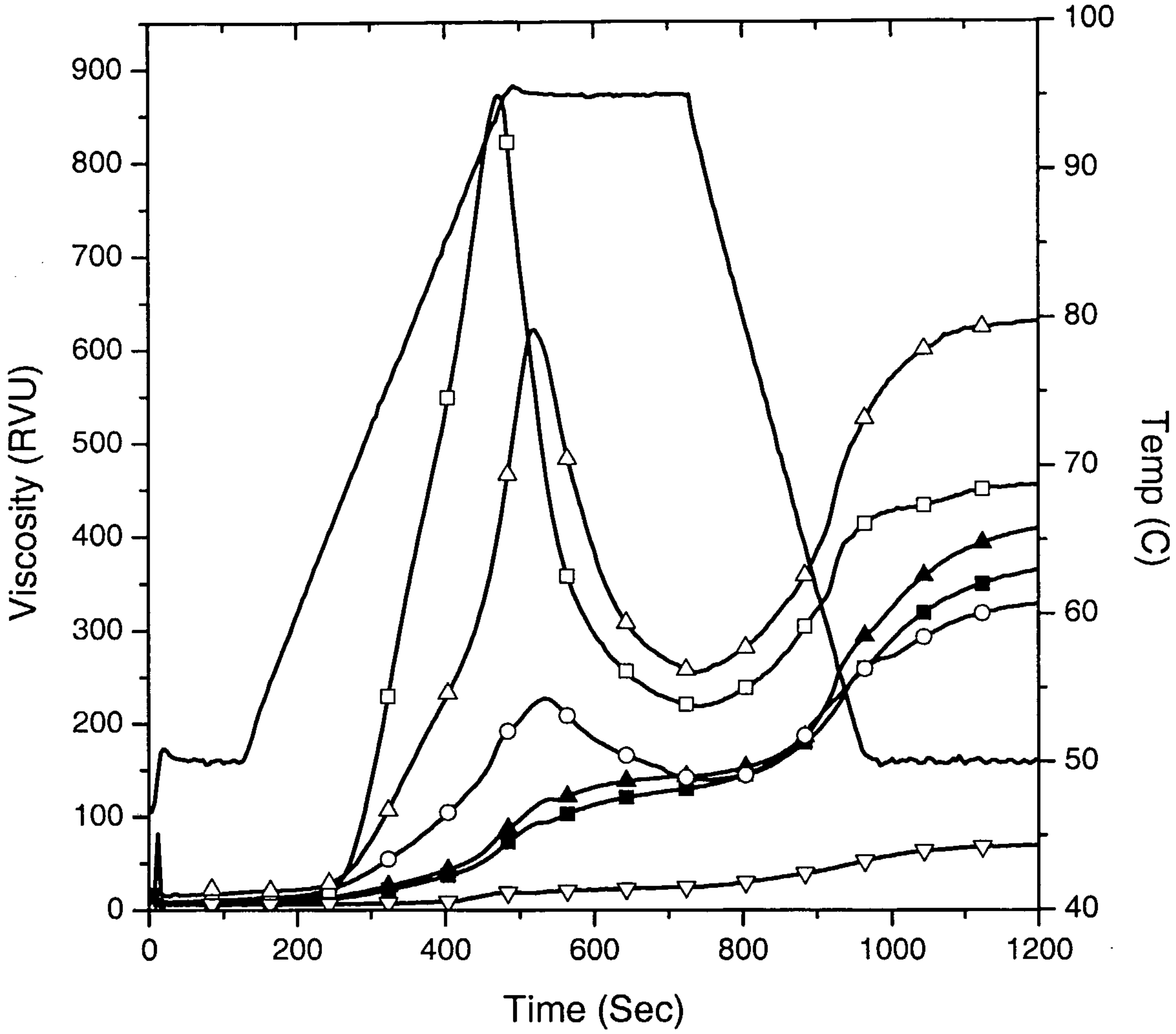
Viscosity (RVU)
Temp (C)
Time (Sec)
0
100
200
300
400
500
600
700
800
900
40
50
60
70
80
90
100
0
200
400
600
800
1000
1200

FIGURE 4

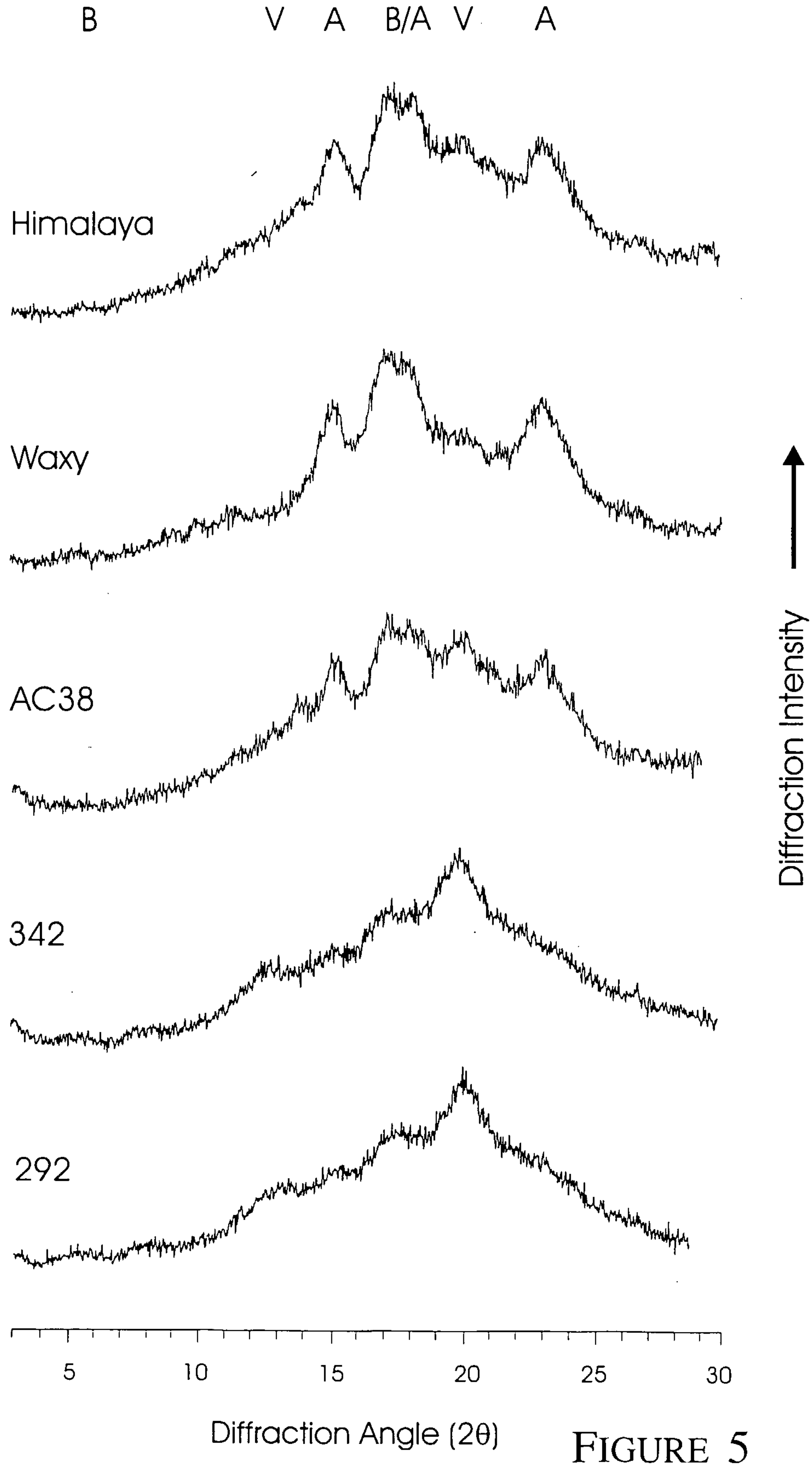
B
V
A
B/A
V
A
Himalaya
Waxy
AC38
342
292
Diffraction Intensity
5
10
15
20
25
30
Diffraction Angle (2θ)

FIGURE 5

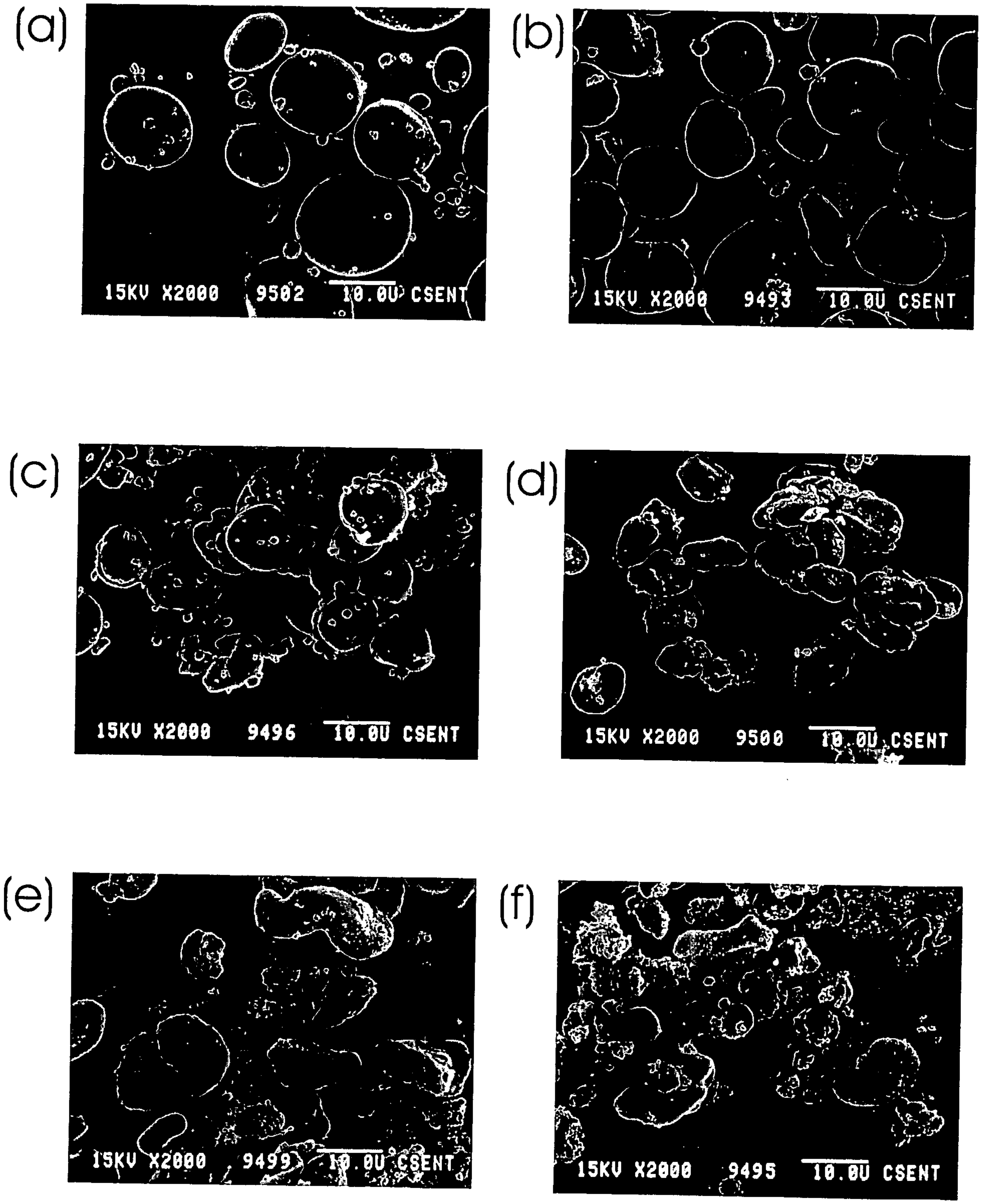
(a)
15KV X2000 9502 10.0U CSENT
(b)
15KV X2000 9493 10.0U CSENT
(c)
15KV X2000 9496 10.0U CSENT
(d)
15KV X2000 9500 10.0U CSENT
(e)
15KV X2000 9499 10.0U CSENT
(f)
15KV X2000 9495 10.0U CSENT

FIGURE 6

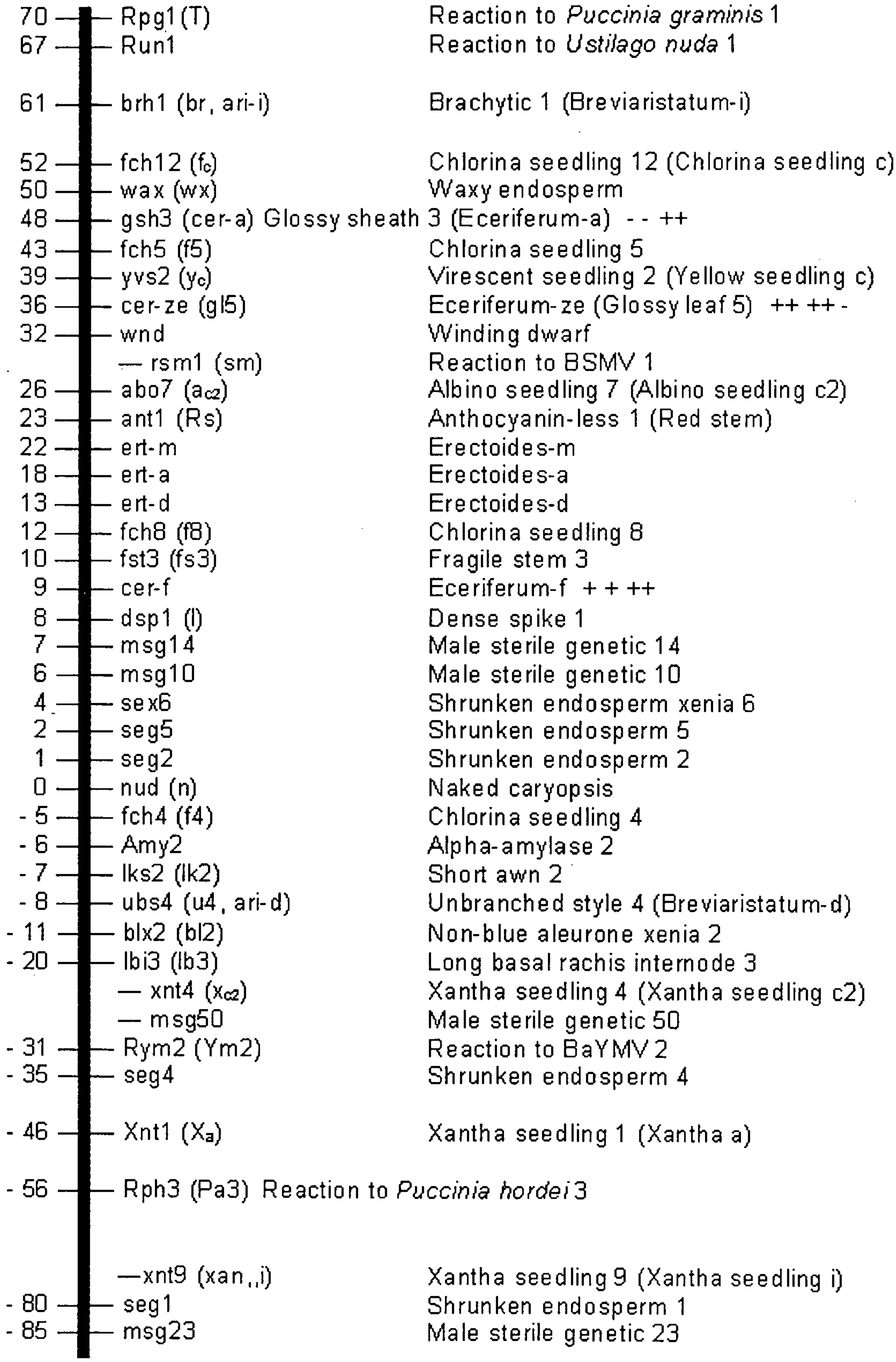
70 — Rpg1 (T) Reaction to *Puccinia graminis* 1
67 — Run1 Reaction to *Ustilago nuda* 1
61 — brh1 (br, ari-i) Brachytic 1 (Breviaristatum-i)
52 — fch12 (f_c) Chlorina seedling 12 (Chlorina seedling c)
50 — wax (wx) Waxy endosperm
48 — gsh3 (cer-a) Glossy sheath 3 (Eceriferum-a) - - ++
43 — fch5 (f5) Chlorina seedling 5
39 — yvs2 (y_c) Virescent seedling 2 (Yellow seedling c)
36 — cer-ze (gl5) Eceriferum-ze (Glossy leaf 5) ++ ++ -
32 — wnd Winding dwarf
— rsm1 (sm) Reaction to BSMV 1
26 — abo7 (a_{c2}) Albino seedling 7 (Albino seedling c2)
23 — ant1 (Rs) Anthocyanin-less 1 (Red stem)
22 — ert-m Erectoides-m
18 — ert-a Erectoides-a
13 — ert-d Erectoides-d
12 — fch8 (f8) Chlorina seedling 8
10 — fst3 (fs3) Fragile stem 3
9 — cer-f Eceriferum-f + + ++
8 — dsp1 (l) Dense spike 1
7 — msg14 Male sterile genetic 14
6 — msg10 Male sterile genetic 10
4 — sex6 Shrunken endosperm xenia 6
2 — seg5 Shrunken endosperm 5
1 — seg2 Shrunken endosperm 2
0 — nud (n) Naked caryopsis
- 5 — fch4 (f4) Chlorina seedling 4
- 6 — Amy2 Alpha-amylase 2
- 7 — lks2 (lk2) Short awn 2
- 8 — ubs4 (u4, ari-d) Unbranched style 4 (Breviaristatum-d)
- 11 — blx2 (bl2) Non-blue aleurone xenia 2
- 20 — lbi3 (lb3) Long basal rachis internode 3
— xnt4 (x_{c2}) Xantha seedling 4 (Xantha seedling c2)
— msg50 Male sterile genetic 50
- 31 — Rym2 (Ym2) Reaction to BaYMV 2
- 35 — seg4 Shrunken endosperm 4
- 46 — Xnt1 (X_a) Xantha seedling 1 (Xantha a)
- 56 — Rph3 (Pa3) Reaction to *Puccinia hordei* 3
—xnt9 (xan,,i) Xantha seedling 9 (Xantha seedling i)
- 80 — seg1 Shrunken endosperm 1
- 85 — msg23 Male sterile genetic 23

FIGURE 7

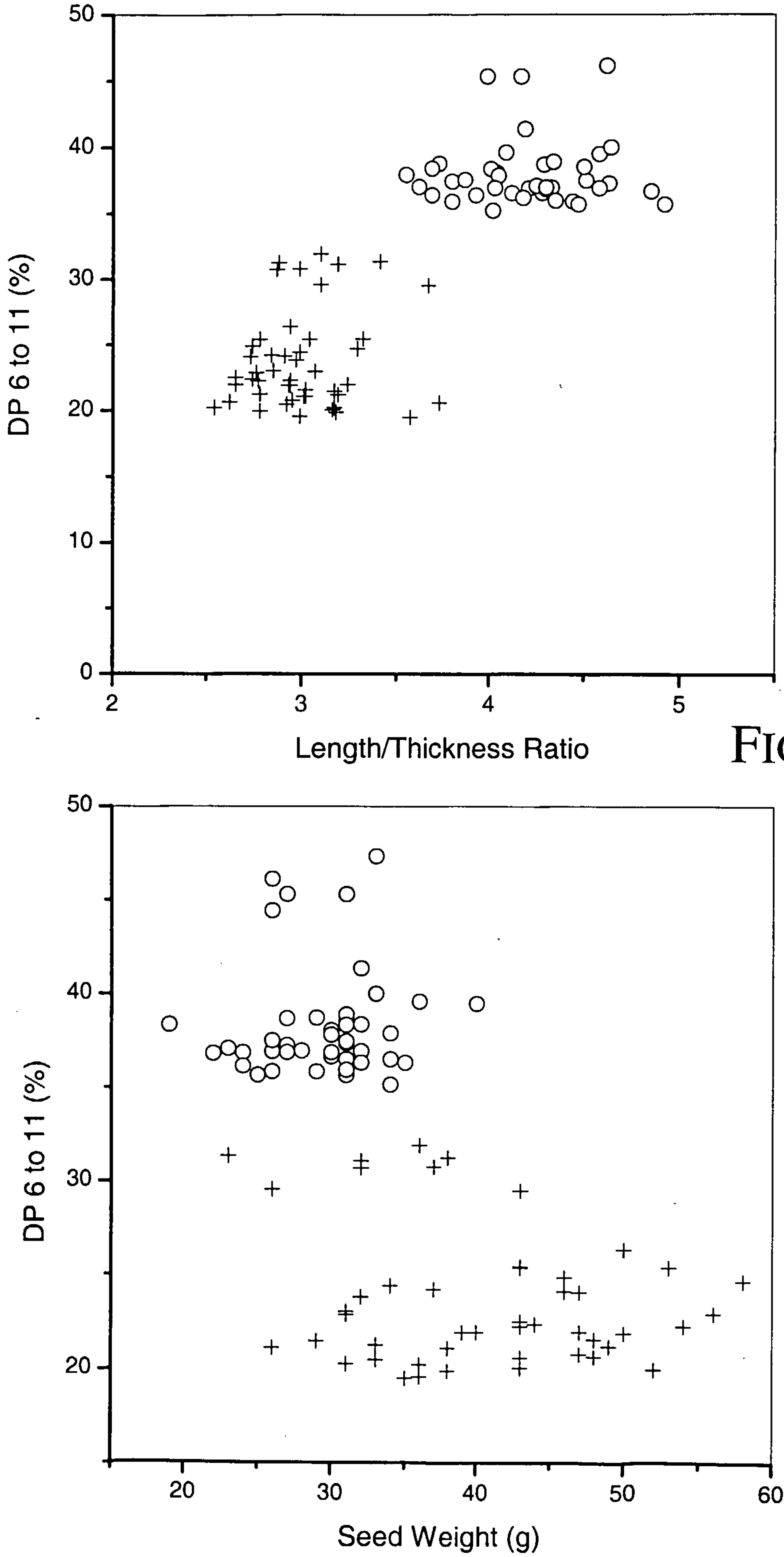
DP 6 to 11 (%)
50
40
30
20
10
0
2
3
4
5
Length/Thickness Ratio
DP 6 to 11 (%)
50
40
30
20
20
30
40
50
60
Seed Weight (g)

FIGURE 8

Barley SSII cDNA Sequence FIGURE 9

```
   1  CCTCGAGGTG CGTTTACCCC ACACAGAGTA CACTCCAACT CCAGTCCAAT
  51  CCAGCCCACT GCCGCTTCTG CCCGCCCATC GTACCGTCGC CCGCCCCGAT
 101  CCCGGCCGCC GCCATGTCGT CGGCGGTCGC GTCCCCCGCG TCCTTCCTCG
 151  CGCTCGCGTC CGCCTCGCCC GGGAGATCAT CACGGAGGAG GGCGAGGGTG
 201  GGCGCGTCGC CAACCCGCGC TGGGGCCGGC AGGCTGCAAT GGCGGCCGTC
 251  GCCGCTGCAG CGCACGGCTC GCGACGGAGC GGTGGCCGCG CGCGCCGCCG
 301  GGATCGACGA CGCCGCGCCC GGTAGGCAGC CCCGCGCTCG CCGCTATGGC
 351  GCCGCCACCA AGGTCGCGGA TCCCGTCAAG ACGCTCGATC GCGACGCCGC
 401  GGAAGGTGGT GGGCCGTCCC CGCCGGCACC GAGGCAGGAC GCCGCCCGTC
 451  TGCCGAGTAA GAACGGCACG CTGATCAACG GTGAGAACAA ACCTACCGGC
 501  GGCGGTGGCG CGACTAAAGA CAGCGGGCTT GCCACACCCG CACGCGCGCC
 551  CCATCTGTCA ATCCAAAACA GAGTACCGGT GAACGGTGAA AACAAACATA
 601  AGGTCGCCTC GCCGCCGACC AGCATAGTGG ATGTCGCGTC TCCGGGTTCC
 651  GCAGCTAACA TTTCCATCAG TAACAAGGTG CCGCCGTCCG TTGTCCCAGC
 701  CAAGAAGACG CCGCCGTCGT CCGTTTTCCC GGCCAAGAAG ACGCTGCCGT
 751  CGTCCGGCTC AAATTTTGTG TCCTCGGCCT CTGCTCCCAG GCTGGACACT
 801  GTCAGCGATG TGGAACTTGC ACAGAAGAAG GATGCGCTGA TTGTCAAAGA
 851  AGCTCCAAAA CCAAAGGCTC TTTCGGCCCC TGCAGCCCCC GCTGTACAAG
 901  AAGACCTTTG GGATTTCAAG AAATACATTG GTTTCGAGGA GCCCGTGGAG
 951  GCCAAGGATG ATGGCTCGGC TGTTGCAGAT GATGCGGGTT CCTTTGAACA
1001  TCACCAGAAT CATGATTCCG GACCTTTGGC AGGGGAGAAC GTCATGAACG
1051  TGGTCGTCGT TGCTGCTGAA TGTTCTCCCT GGTGCAAAAC AGGTGGTCTT
1101  GGAGATGTTG CGGGTGCTTT GCCCAAGGCT TTGGCTAAGA GAGGACATCG
1151  TGTTATGGTT GTGGTACCAA GGTATGGGGA CTATGAGGAA GCCTACGATG
1201  TCGGAGTCCG AAAATACTAC AAGGCTGCTG GACAGGATAT GGAAGTGAAT
1251  TATTTCCATG CTTATATCGA TGGAGTGGAT TTTGTGTTCA TTGACGCTCC
1301  TCTCTTCCGA CACCGTCAGC AAGACATTTA TGGGGGCAGC AGACAGGAAA
1351  TTATGAAGCG CATGATTTTG TTCTGCAAGG CCGCTGTCGA GGTTCCTTGG
1401  CACGTTCCAT GCGGCGGTGT CCCTTACGGG GATGGAAATC TGGTCTTCAT
1451  TGCAAATGAT TGGCACACGG CACTCCTGCC TGTCTATCTG AAAGCATATT
1501  ACAGGGACCA TGGTTTGATG CAATACAGTC GCTCCGTTAT GGTGATACAT
1551  AACATCGCTC ACCAGGGCCG TGGCCCTGTA GATGAATTCC CGTTCACCGA
1601  GTTGCCTGAG CACTACCTGG AACACTTCAG ACTGTACGAC CCCGTCGGCG
1651  GTGAGCACGC CAACTACTTC GCCGCCGGCC TGAAGATGGC GGACCAGGTT
1701  GTCGTCGTGA GCCCCGGGTA CCTGTGGGAG CTGAAGACGG TGGAGGGCGG
1751  CTGGGGGCTT CACGACATCA TACGGCAGAA CGACTGGAAG ACCCGCGGCA
1801  TCGTGAACGG CATCGACAAC ATGGAGTGGA ACCCTGAGGT GGACGTCCAC
1851  CTGAAGTCGG ACGGCTACAC CAACTTCTCC CTGAAGACGC TGGACTCCGG
1901  CAAGCGGCAG TGCAAGGAGG CCCTGCAGCG CGAGCTGGGG CTGCAGGTCC
1951  GCGGCGACGT GCCGCTGCTC GGGTTCATCG GGCGGCTGGA CGGGCAGAAG
2001  GGCGTGGAGA TCATCGCGGA CGCGATGCCC TGGATCGTGA GCCAGGACGT
2051  GCAGCTGGTG ATGCTGGGCA CGGGGCGCCA CGACCTGGAG AGCATGCTGC
2101  AGCACTTCGA GCGGGAGCAC CACGACAAGG TGCGCGGGTG GGTGGGGTTC
2151  TCCGTGCGCC TGGCGCACCG GATCACGGCG GGCGCCGACG CGCTCCTCAT
2201  GCCCTCCCGG TTCGAGCCGT GCGGGCTGAA CCAGCTCTAC GCGATGGCCT
2251  ACGGCACCGT CCCCGTCGTG CACGCCGTCG GCGGCTTGAG GGATACCGTG
2301  CCGCCGTTCG ACCCCTTCAA CCACTCCGGG CTCGGGTGGA CGTTCGACCG
2351  CGCCGAGGCG CACAAGCTGA TCGAGGCGCT CGGGCACTGC CTCCGCACCT
2401  ACCGGGACCA CAAGGAGAGC TGGAGGGGCC TCCAGGAGCG CGGCATGTCG
2451  CAGGACTTCA GCTGGGAACA TGCCGCCAAG CTCTACGAGG ACGTCCTCGT
2501  CCAGGCCAAG TACCAGTGGT GAACGCTGCT ACCCGGTCCA GCCCCGCATG
2551  CGTGCATGAG AGGATGGAAA TGCGCATTGC GCACTTGCAG ATTTGGCGCA
2601  CGCAGGAACG TGCCGTCCTT CTTGATGAGA ACGCCGGCAT CCGCGAGGTT
2651  GAGACGCTGA TTCCGATCTG GTCCGTCGCA GAGTAGAGTG AAACGCTCCT
2701  TGTTGCAGGT ATATGGGAAT GTTTTTTTTC CTTTTTTTTT GCGAGGGAGG
2751  TATATGGGAA TGTTAACTTG GTATTGTAAT GTGGTATGCT GTGTGCATTA
2801  TTACATCGGT TGTTGTTGCT TATTCTTGCT AGCTAAGTCG GAGGCCAAGA
```

2851 GCGAAAGCTA GCTCACATGT CTGATGTATG CAAGTGACAT GGTTGGTTTG
2901 AAAAAAAAAA AAAAAAAAAA

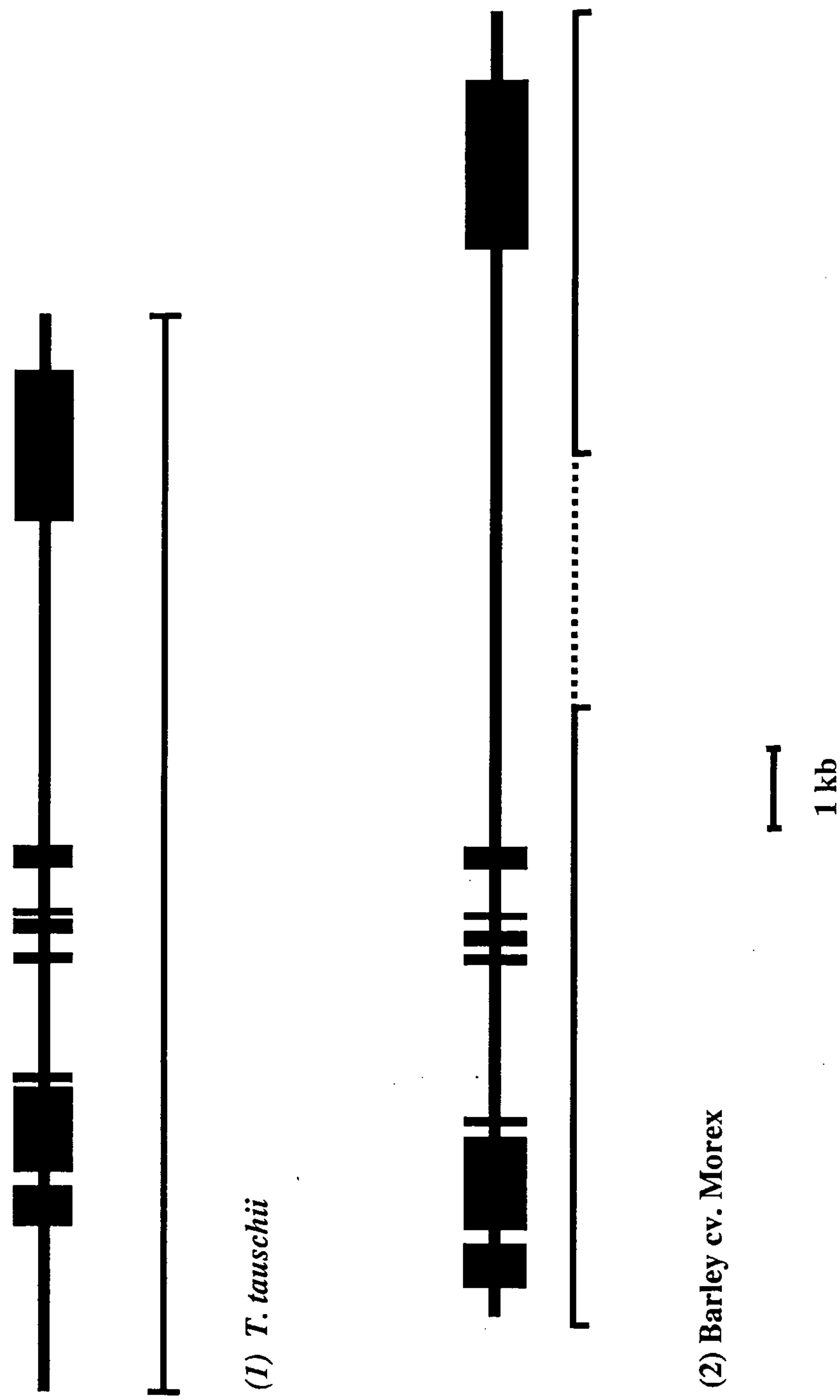

FIGURE 10

Comparison of cDNA Sequences

	1				50
MK6827	-------GTG	CGTTTACCCC	ACACAGAGTA	CACTCCAACT	CCAGTCCAGT
MOREX	-------GTG	CGTTTACCCC	ACACAGAGTA	CACTCCAACT	CCAGTCCAGT
292	-------GTG	CGTTTACCCC	ACACAGAGTA	CACTCCAACT	CCAGTCCAGT
HIMALAYA	CCTCGAGGTG	CGTTTACCCC	ACACAGAGTA	CACTCCAACT	CCAGTCCAAT

	51				100
MK6827	CCAGCCCACT	GCCGCTTCTG	CCCGCCCATC	GTACCGTCGC	CCGCCCCGAT
MOREX	CCAGCCCACT	GCCGCTTCTG	CCCGCCCATC	GTACCGTCGC	CCGCCCCGAT
292	CCAGCCCACT	GCCGCTTCTG	CCCGCCCATC	GTACCGTCGC	CCGCCCCGAT
HIMALAYA	CCAGCCCACT	GCCGCTTCTG	CCCGCCCATC	GTACCGTCGC	CCGCCCCGAT

	101	*** start codon			150
MK6827	CCCGGCCGCC	GCC**ATG**TCGT	CGGCGGTCGC	GTCCCCCGCG	TCCTTCCTCG
MOREX	CCCGGCCGCC	GCC**ATG**TCGT	CGGCGGTCGC	GTCCCCCGCG	TCCTTCCTCG
292	CCCGGCCGCC	GCC**ATG**TCGT	CGGCGGTCGC	GTCCCCCGCG	TCCTTCCTCG
HIMALAYA	CCCGGCCGCC	GCC**ATG**TCGT	CGGCGGTCGC	GTCCCCCGCG	TCCTTCCTCG

	151				200
MK6827	CGCTCGCGTC	CGCCTCGCCC	GGGAGATCAT	CACGGAGGAG	GGCGAGGGTG
MOREX	CGCTCGCGTC	CGCCTCGCCC	GGGAGATCAT	CACGGAGGAG	GGCGAGGGTG
292	CGCTCGCGTC	CGCCTCGCCC	GGGAGATCAT	CACGGAGGAG	GGCGAGGGTG
HIMALAYA	CGCTCGCGTC	CGCCTCGCCC	GGGAGATCAT	CACGGAGGAG	GGCGAGGGTG

	201				#
MK6827	GGCGCGTCGC	CAACCCGCGC	TGGGGCCGGC	AGGCTGCAAT	G**A**CGGCCGTC
MOREX	GGCGCGTCGC	CAACCCGCGC	TGGGGCCGGC	AGGCTGCAAT	GGCGGCCGTC
292	GGCGCGTCGC	CAACCCGCGC	TGGGGCCGGC	AGGCTGCAAT	GGCGGCCGTC
HIMALAYA	GGCGCGTCGC	CAACCCGCGC	TGGGGCCGGC	AGGCTGCAAT	GGCGGCCGTC

	251				300
MK6827	GCCGCTGCAG	CGCACGGCTC	GCGACGGAGC	GGTGGCCGCG	CGCGCCGCCG
MOREX	GCCGCTGCAG	CGCACGGCTC	GCGACGGAGC	GGTGGCCGCG	CGCGCCGCCG
292	GCCGCTGCAG	CGCACGGCTC	GCGACGGAGC	GGTGGCCGCG	CGCGCCGCCG
HIMALAYA	GCCGCTGCAG	CGCACGGCTC	GCGACGGAGC	GGTGGCCGCG	CGCGCCGCCG

	301				350
MK6827	GGATCGACGA	CGCCGCGCCC	GGTAGGCAGC	CCCGCGCTCG	CCGCTATGGC
MOREX	GGATCGACGA	CGCCGCGCCC	GGTAGGCAGC	CCCGCGCTCG	CCGCTATGGC
292	GGATCGACGA	CGCCGCGCCC	GGTAGGCAGC	CCCGCGCTCG	CCGCTATGGC
HIMALAYA	GGATCGACGA	CGCCGCGCCC	GGTAGGCAGC	CCCGCGCTCG	CCGCTATGGC

	351				400
MK6827	GCCGCCACCA	AGGTCGCGGA	TCCCGTCAAG	ACGCTCGATC	GCGACGCCGC
MOREX	GCCGCCACCA	AGGTCGCGGA	TCCCGTCAAG	ACGCTCGATC	GCGACGCCGC
292	GCCGCCACCA	AGGTCGCGGA	TCCCGTCAAG	ACGCTCGATC	GCGACGCCGC
HIMALAYA	GCCGCCACCA	AGGTCGCGGA	TCCCGTCAAG	ACGCTCGATC	GCGACGCCGC

	401				450
MK6827	GGAAGGTGGT	GGGCCGTCCC	CGCCGGCACC	GAGGCAGGAC	GCCGCCCGTC
MOREX	GGAAGGTGGT	GGGCCGTCCC	CGCCGGCACC	GAGGCAGGAC	GCCGCCCGTC
292	GGAAGGTGGT	GGGCCGTCCC	CGCCGGCACC	GAGGCAGGAC	GCCGCCCGTC
HIMALAYA	GGAAGGTGGT	GGGCCGTCCC	CGCCGGCACC	GAGGCAGGAC	GCCGCCCGTC

	451				500
MK6827	TGCCGAGTAA	GAACGGCACG	CTGATCAACG	GTGAGAACAA	ACCTACCGGC
MOREX	TGCCGAGTAA	GAACGGCACG	CTGATCAACG	GTGAGAACAA	ACCTACCGGC
292	TGCCGAGTAA	GAACGGCACG	CTGATCAACG	GTGAGAACAA	ACCTACCGGC
HIMALAYA	TGCCGAGTAA	GAACGGCACG	CTGATCAACG	GTGAGAACAA	ACCTACCGGC

FIGURE 11

```
            501                                                   550
MK6827      GGCGGTGGCG CGACTAAAGA CAGCGGGCTG CCCACACCCG CACGCGCGCC
MOREX       GGCGGTGGCG CGACTAAAGA CAGCGGGCTG CCCACACCCG CACGCGCGCC
292         GGCGGTGGCG CGACTAAAGA CAGCGGGCTG CCCACACCCG CACGCGCGCC
HIMALAYA    GGCGGTGGCG CGACTAAAGA CAGCGGGCTT GCCACACCCG CACGCGCGCC

            551                                                   600
MK6827      CCATCTGTCA ATCCAGAACA GAGTACCGGT GAACGGTGAA AACAAACATA
MOREX       CCATCTGTCA ATCCAGAACA GAGTACCGGT GAACGGTGAA AACAAACATA
292         CCATCTGTCA ATCCAGAACA GAGTACCGGT GAACGGTGAA AACAAACATA
HIMALAYA    CCATCTGTCA ATCCAAAACA GAGTACCGGT GAACGGTGAA AACAAACATA

            601                                                   650
MK6827      AGGTCGCCTC GCCGCCGACC AGCATAGTGG ATGTCGCGTC TCCGGGTTCC
MOREX       AGGTCGCCTC GCCGCCGACC AGCATAGTGG ATGTCGCGTC TCCGGGTTCC
292         AGGTCGCCTC GCCGCCGACC AGCATAGTGG ATGTCGCGTC TCCGGGTTCC
HIMALAYA    AGGTCGCCTC GCCGCCGACC AGCATAGTGG ATGTCGCGTC TCCGGGTTCC

            651                                                   700
MK6827      GCAGCTAACA TTTCCATCAG TAACAAGGTG CCGCCGTCCG TTGTCCCAGC
MOREX       GCAGCTAACA TTTCCATCAG TAACAAGGTG CCGCCGTCCG TTGTCCCAGC
292         GCAGCCAACA TTTCCATCAG TAACAAGGTG CCGCCGTCCG TTGTCCCAGC
HIMALAYA    GCAGCTAACA TTTCCATCAG TAACAAGGTG CCGCCGTCCG TTGTCCCAGC

            701                                                   750
MK6827      CAAGAAGACG CCGCCGTCGT CCGTTTTCCC GGCCAAGAAG GCGCCGCCGT
MOREX       CAAGAAGACG CCGCCGTCGT CCGTTTTCCC GGCCAAGAAG GCGCCGCCGT
292         CAAGAAGACG CCGCCGTCGT CCGTTTTCCC GGCCAAGAAG GCGCCGCCGT
HIMALAYA    CAAGAAGACG CCGCCGTCGT CCGTTTTCCC GGCCAAGAAG .......... ..........

            751                                                   800
MK6827      CGTCCGTTGT CCCGGCCAAG AAGACGCTGC CGTCGTCCGG CTCAAATTTT
MOREX       CGTCCGTTGT CCCGGCCAAG AAGACGCTGC CGTCGTCCGG CTCAAATTTT
292         CGTCCGTTGT CCCGGCCAAG AAGACGCTGC CGTCGTCCGG CTCAAATTTT
HIMALAYA    .......... .......... ...ACGCTGC CGTCGTCCGG CTCAAATTTT

            801                                                   850
MK6827      GTGTCCTCGG CCTCTGCTCC CAGGCTGGAC ACTGTCAGCG ATGTGGAACT
MOREX       GTGTCCTCGG CCTCTGCTCC CAGGCTGGAC ACTGTCAGCG ATGTGGAACT
292         GTGTCCTCGG CCTCTGCTCC CAGGCTGGAC ACTGTCAGCG ATGTGGAACT
HIMALAYA    GTGTCCTCGG CCTCTGCTCC CAGGCTGGAC ACTGTCAGCG ATGTGGAACT

            851                                                   900
MK6827      TGCACAGAAG AAGGATGCGC TGATTGTCAA AGAAGCTCCA AAACCAAAGG
MOREX       TGCACAGAAG AAGGATGCGC TGATTGTCAA AGAAGCTCCA AAACCAAAGG
292         TGCACAGAAG AAGGATGCGC TGATTGTCAA AGAAGCTCCA AAACCAAAGG
HIMALAYA    TGCACAGAAG AAGGATGCGC TGATTGTCAA AGAAGCTCCA AAACCAAAGG

            901                                                   950
MK6827      CTCTTTCGGC CCCTGCAGCC CCCGCTGTAC AAGAAGACCT TTGGGATTTC
MOREX       CTCTTTCGGC CCCTGCAGCC CCCGCTGTAC AAGAAGACCT TTGGGATTTC
292         CTCTTTCGGC CCCTGCAGCC CCCGCTGTAC AAGAAGACCT TTGGGATTTC
HIMALAYA    CTCTTTCGGC CCCTGCAGCC CCCGCTGTAC AAGAAGACCT TTGGGATTTC

            951                                                  1000
MK6827      AAGAAATACA TTGGTTTCGA GGAGCCCGTG GAGGCCAAGG ATGATGGCTC
MOREX       AAGAAATACA TTGGTTTCGA GGAGCCCGTG GAGGCCAAGG ATGATGGCTC
292         AAGAAATACA TTGGTTTCGA GGAGCCCGTG GAGGCCAAGG ATGATGGCTC
HIMALAYA    AAGAAATACA TTGGTTTCGA GGAGCCCGTG GAGGCCAAGG ATGATGGCTC
```

	1001				1050
MK6827	GGCTGTTGCA	GATGATGCGG	GTTCCTTTGA	ACATCACCAG	AATCATGATT
MOREX	GGCTGTTGCA	GATGATGCGG	GTTCCTTTGA	ACATCACCAG	AATCATGATT
292	GGCTGTTGCA	GATGATGCGG	GTTCCTTTGA	ACATCACCAG	AATCATGATT
HIMALAYA	GGCTGTTGCA	GATGATGCGG	GTTCCTTTGA	ACATCACCAG	AATCATGATT
	1051				1100
MK6827	CCGGACCTTT	GGCAGGGGAG	AACGTCATGA	ACGTGGTCGT	CGTTGCTGCT
MOREX	CCGGACCTTT	GGCAGGGGAG	AACGTCATGA	ACGTGGTCGT	CGTTGCTGCT
292	CCGGACCTTT	GGCAGGGGAG	AACGTCATGA	ACGTGGTCGT	CGTTGCTGCT
HIMALAYA	CCGGACCTTT	GGCAGGGGAG	AACGTCATGA	ACGTGGTCGT	CGTTGCTGCT
	1101				1150
MK6827	GAATGTTCTC	CCTGGTGCAA	AACAGGTGGT	CTTGGAGATA	TTGCGGGTGC
MOREX	GAATGTTCTC	CCTGGTGCAA	AACAGGTGGT	CTTGGAGATG	TTGCGGGTGC
292	GAATGTTCTC	CCTGGTGCAA	AACAGGTGGT	CTTGGAGATG	TTGCGGGTGC
HIMALAYA	GAATGTTCTC	CCTGGTGCAA	AACAGGTGGT	CTTGGAGATG	TTGCGGGTGC
	1151				1200
MK6827	TTTGCCCAAG	GCTTTGGCTA	AGAGAGGACA	TCGTGTTATG	GTTGTGGTAC
MOREX	TTTGCCCAAG	GCTTTGGCTA	AGAGAGGACA	TCGTGTTATG	GTTGTGGTAC
292	TTTGCCCAAG	GCTTTGGCTA	AGAGAGGACA	TCGTGTTATG	GTTGTGGTAC
HIMALAYA	TTTGCCCAAG	GCTTTGGCTA	AGAGAGGACA	TCGTGTTATG	GTTGTGGTAC
	1201				1250
MK6827	CAAGGTATGG	GGACTATGAG	GAAGCCTACG	ATGTCGGAGT	CCGAAAATAC
MOREX	CAAGGTATGG	GGACTATGAG	GAAGCCTACG	ATGTCGGAGT	CCGAAAATAC
292	CAAGGTATGG	GGACTATGAG	GAAGCCTACG	ATGTCGGAGT	CCGAAAATAC
HIMALAYA	CAAGGTATGG	GGACTATGAG	GAAGCCTACG	ATGTCGGAGT	CCGAAAATAC
	1251				1300
MK6827	TACAAGGCTG	CTGGACAGGA	TATGGAAGTG	AATTATTTCC	ATGCTTATAT
MOREX	TACAAGGCTG	CTGGACAGGA	TATGGAAGTG	AATTATTTCC	ATGCTTATAT
292	TACAAGGCTG	CTGGACAGGA	TATGGAAGTG	AATTATTTCC	ATGCTTATAT
HIMALAYA	TACAAGGCTG	CTGGACAGGA	TATGGAAGTG	AATTATTTCC	ATGCTTATAT
	1301				1350
MK6827	CGATGGAGTG	GATTTTGTGT	TCATTGACGC	TCCTCTCTTC	CGACACCGTC
MOREX	CGATGGAGTG	GATTTTGTGT	TCATTGACGC	TCCTCTCTTC	CGACACCGTC
292	CGATGGAGTG	GATTTTGTGT	TCATTGACGC	TCCTCTCTTC	CGACACCGTC
HIMALAYA	CGATGGAGTG	GATTTTGTGT	TCATTGACGC	TCCTCTCTTC	CGACACCGTC
	1351				1400
MK6827	AGCAAGACAT	TTATGGGGGC	AGCAGACAGG	AAATTATGAA	GCGCATGATT
MOREX	AGCAAGACAT	TTATGGGGGC	AGCAGACAGG	AAATTATGAA	GCGCATGATT
292	AGCAAGACAT	TTATGGGGGC	AGCAGACAGG	AAATTATGAA	GCGCATGATT
HIMALAYA	AGCAAGACAT	TTATGGGGGC	AGCAGACAGG	AAATTATGAA	GCGCATGATT
	1401				1450
MK6827	TTGTTCTGCA	AGGCCGCTGT	CGAGGTTCCT	TGGCACGTTC	CATGCGGCGG
MOREX	TTGTTCTGCA	AGGCCGCTGT	CGAGGTTCCT	TGGCACGTTC	CATGCGGCGG
292	TTGTTCTGCA	AGGCCGCTGT	CGAGGTTCCT	TGGCACGTTC	CATGCGGCGG
HIMALAYA	TTGTTCTGCA	AGGCCGCTGT	CGAGGTTCCT	TGGCACGTTC	CATGCGGCGG
	1451				1500
MK6827	TGTCCCTTAC	GGGGATGGAA	ATCTGGTCTT	CATTGCAAAT	GATTGGCACA
MOREX	TGTCCCTTAC	GGGGATGGAA	ATCTGGTCTT	CATTGCAAAT	GATTGGCACA
292	TGTCCCTTAC	GGGGATGGAA	ATCTGGTCTT	CATTGCAAAT	GATTGGCACA
HIMALAYA	TGTCCCTTAC	GGGGATGGAA	ATCTGGTCTT	CATTGCAAAT	GATTGGCACA

```
          1501                                                 1550
MK6827    CGGCACTCCT GCCTGTCTAT CTGAAAGCAT ATTACAGGGA CCATGGTTTG
MOREX     CGGCACTCCT GCCTGTCTAT CTGAAAGCAT ATTACAGGGA CCATGGTTTG
292       CGGCACTCCT GCCTGTCTAT CTGAAAGCAT ATTACAGGGA CCATGGTTTG
HIMALAYA  CGGCACTCCT GCCTGTCTAT CTGAAAGCAT ATTACAGGGA CCATGGTTTG

          1551                                                 1600
MK6827    ATGCAATACA GTCGCTCCGT TATGGTGATA CATAACATCG CTCACCAGGG
MOREX     ATGCAATACA GTCGCTCCGT TATGGTGATA CATAACATCG CTCACCAGGG
292       ATGCAATACA GTCGCTCCGT TATGGTGATA CATAACATCG CTCACCAGGG
HIMALAYA  ATGCAATACA GTCGCTCCGT TATGGTGATA CATAACATCG CTCACCAGGG

          1601                                                 1650
MK6827    CCGTGGCCCT GTAGATGAAT TCCCGTTCAC CGAGTTGCCT GAGCACTACC
MOREX     CCGTGGCCCT GTAGATGAAT TCCCGTTCAC CGAGTTGCCT GAGCACTACC
292       CCGTGGCCCT GTAGATGAAT TCCCGTTCAC CGAGTTGCCT GAGCACTACC
HIMALAYA  CCGTGGCCCT GTAGATGAAT TCCCGTTCAC CGAGTTGCCT GAGCACTACC

          1651                                                 1700
MK6827    TGGAACACTT CAGACTGTAC GACCCCGTCG GCGGTGAGCA CGCCAACTAC
MOREX     TGGAACACTT CAGACTGTAC GACCCCGTCG GCGGTGAGCA CGCCAACTAC
292       TGGAACACTT CAGACTGTAC GACCCCGTCG GCGGTGAGCA CGCCAACTAC
HIMALAYA  TGGAACACTT CAGACTGTAC GACCCCGTCG GCGGTGAGCA CGCCAACTAC

          1701                                                 1750
MK6827    TTCGCCGCCG GCCTGAAGAT GGCGGACCAG GTTGTCGTCG TGAGCCCCGG
MOREX     TTCGCCGCCG GCCTGAAGAT GGCGGACCAG GTTGTCGTCG TGAGCCCCGG
292       TTCGCCGCCG GCCTGAAGAT GGCGGACCAG GTTGTCGTCG TGAGCCCCGG
HIMALAYA  TTCGCCGCCG GCCTGAAGAT GGCGGACCAG GTTGTCGTCG TGAGCCCCGG

          1751                                                 1800
MK6827    GTACCTGTGG GAGCTGAAGA CGGTGGAGGG CGGCTGGGGG CTTCACGACA
MOREX     GTACCTGTGG GAGCTGAAGA CGGTGGAGGG CGGCTGGGGG CTTCACGACA
292       GTACCTGTGG GAGCTGAAGA CGGTGGAGGG CGGCTGGGGG CTTCACGACA
HIMALAYA  GTACCTGTGG GAGCTGAAGA CGGTGGAGGG CGGCTGGGGG CTTCACGACA

          1801                                                 1850
MK6827    TCATACGGCA GAACGACTGG AAGACCCGCG GCATCGTGAA CGGCATCGAC
MOREX     TCATACGGCA GAACGACTGG AAGACCCGCG GCATCGTGAA CGGCATCGAC
292       TCATACGGCA GAACGACTGG AAGACCCGCG GCATCGTGAA CGGCATCGAC
HIMALAYA  TCATACGGCA GAACGACTGG AAGACCCGCG GCATCGTGAA CGGCATCGAC

          1851         &                       1900
MK6827    AACATGGAGT GGAACCCTGA GGTGGACGTC CACCTGAAGT CGGACGGCTA
MOREX     AACATGGAGT GGAACCCTGA GGTGGACGTC CACCTGAAGT CGGACGGCTA
292       AACATGGAGT GAAACCCTGA GGTGGACGTC CACCTGAAGT CGGACGGCTA
HIMALAYA  AACATGGAGT GGAACCCTGA GGTGGACGTC CACCTGAAGT CGGACGGCTA

          1901                                                 1950
MK6827    CACCAACTTC TCCCTGAAGA CGCTGGACTC CGGCAAGCGG CAGTGCAAGG
MOREX     CACCAACTTC TCCCTGAAGA CGCTGGACTC CGGCAAGCGG CAGTGCAAGG
292       CACCAACTTC TCCCTGAAGA CGCTGGACTC CGGCAAGCGG CAGTGCAAGG
HIMALAYA  CACCAACTTC TCCCTGAAGA CGCTGGACTC CGGCAAGCGG CAGTGCAAGG

          1951                                                 2000
MK6827    AGGCCCTGCA GCGCGAGCTG GGGCTGCAGG TCCGCGGCGA CGTGCCGCTG
MOREX     AGGCCCTGCA GCGCGAGCTG GGGCTGCAGG TCCGCGGCGA CGTGCCGCTG
292       AGGCCCTGCA GCGCGAGCTG GGGCTGCAGG TCCGCGGCGA CGTGCCGCTG
HIMALAYA  AGGCCCTGCA GCGCGAGCTG GGGCTGCAGG TCCGCGGCGA CGTGCCGCTG
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          2001                                                2050
MK6827    CTCGGGTTCA TCGGGCGGCT GGACGGGCAG AAGGGCGTGG AGATCATCGC
MOREX     CTCGGGTTCA TCGGGCGGCT GGACGGGCAG AAGGGCGTGG AGATCATCGC
292       CTCGGGTTCA TCGGGCGGCT GGACGGGCAG AAGGGCGTGG AGATCATCGC
HIMALAYA  CTCGGGTTCA TCGGGCGGCT GGACGGGCAG AAGGGCGTGG AGATCATCGC

          2051                                                2100
MK6827    GGACGCGATG CCCTGGATCG TGAGCCAGGA CGTGCAGCTG GTGATGCTGG
MOREX     GGACGCGATG CCCTGGATCG TGAGCCAGGA CGTGCAGCTG GTGATGCTGG
292       GGACGCGATG CCCTGGATCG TGAGCCAGGA CGTGCAGCTG GTGATGCTGG
HIMALAYA  GGACGCGATG CCCTGGATCG TGAGCCAGGA CGTGCAGCTG GTGATGCTGG

          2101                                                2150
MK6827    GCACGGGGCG CCACGACCTG GAGAGCATGC TGCAGCACTT CGAGCGGGAG
MOREX     GCACGGGGCG CCACGACCTG GAGAGCATGC TGCAGCACTT CGAGCGGGAG
292       GCACGGGGCG CCACGACCTG GAGAGCATGC TGCAGCACTT CGAGCGGGAG
HIMALAYA  GCACGGGGCG CCACGACCTG GAGAGCATGC TGCAGCACTT CGAGCGGGAG

          2151                                                2200
MK6827    CACCACGACA AGGTGCGCGG GTGGGTGGGG TTCTCCGTGC GCCTGGCGCA
MOREX     CACCACGACA AGGTGCGCGG GTGGGTGGGG TTCTCCGTGC GCCTGGCGCA
292       CACCACGACA AGGTGCGCGG GTGGGTGGGG TTCTCCGTGC GCCTGGCGCA
HIMALAYA  CACCACGACA AGGTGCGCGG GTGGGTGGGG TTCTCCGTGC GCCTGGCGCA

          2201                                                2250
MK6827    CCGGATCACG GCGGGCGCCG ACGCGCTCCT CATGCCCTCC CGGTTCGAGC
MOREX     CCGGATCACG GCGGGCGCCG ACGCGCTCCT CATGCCCTCC CGGTTCGAGC
292       CCGGATCACG GCGGGCGCCG ACGCGCTCCT CATGCCCTCC CGGTTCGAGC
HIMALAYA  CCGGATCACG GCGGGCGCCG ACGCGCTCCT CATGCCCTCC CGGTTCGAGC

          2251                                                2300
MK6827    CGTGCGGGCT GAACCAGCTC TACGCGATGG CCTACGGCAC CATCCCTGTC
MOREX     CGTGCGGGCT GAACCAGCTC TACGCGATGG CCTACGGCAC CATCCCTGTC
292       CGTGCGGGCT GAACCAGCTC TACGCGATGG CCTACGGCAC CATCCCTGTC
HIMALAYA  CGTGCGGGCT GAACCAGCTC TACGCGATGG CCTACGGCAC CGTCCCCGTC

          2301                                                2350
MK6827    GTGCACGCCG TCGGCGGCCT GAGGGATACC GTGCCGCCGT TCGACCCCTT
MOREX     GTGCACGCCG TCGGCGGCCT GAGGGATACC GTGCCGCCGT TCGACCCCTT
292       GTGCACGCCG TCGGCGGCCT GAGGGATACC GTGCCGCCGT TCGACCCCTT
HIMALAYA  GTGCACGCCG TCGGCGGCTT GAGGGATACC GTGCCGCCGT TCGACCCCTT

          2351                                                2400
MK6827    CAACCACTCC GGGCTCGGGT GGACGTTCGA CCGCGCCGAG GCGCACAAGC
MOREX     CAACCACTCC GGGCTCGGGT GGACGTTCGA CCGCGCCGAG GCGCACAAGC
292       CAACCACTCC GGGCTCGGGT GGACGTTCGA CCGCGCCGAG GCGCACAAGC
HIMALAYA  CAACCACTCC GGGCTCGGGT GGACGTTCGA CCGCGCCGAG GCGCACAAGC

          2401                                                2450
MK6827    TGATCGAGGC GCTCGGGCAC TGCCTCCGCA CCTACCGGGA CCACAAGGAG
MOREX     TGATCGAGGC GCTCGGGCAC TGCCTCCGCA CCTACCGGGA CCACAAGGAG
292       TGATCGAGGC GCTCGGGCAC TGCCTCCGCA CCTACCGGGA CCACAAGGAG
HIMALAYA  TGATCGAGGC GCTCGGGCAC TGCCTCCGCA CCTACCGGGA CCACAAGGAG

          2451                                                2500
MK6827    AGCTGGAGGG GCCTCCAGGA GCGCGGCATG TCGCAGGACT TCAGCTGGGA
MOREX     AGCTGGAGGG GCCTCCAGGA GCGCGGCATG TCGCAGGACT TCAGCTGGGA
292       AGCTGGAGGG GCCTCCAGGA GCGCGGCATG TCGCAGGACT TCAGCTGGGA
HIMALAYA  AGCTGGAGGG GCCTCCAGGA GCGCGGCATG TCGCAGGACT TCAGCTGGGA
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              2501                                                    2550
MK6827        ACATGCCGCC AAGCTCTACG AGGACGTCCT CGTCCAGGCC AAGTACCAGT
MOREX         ACATGCCGCC AAGCTCTACG AGGACGTCCT CGTCCAGGCC AAGTACCAGT
292           ACATGCCGCC AAGCTCTACG AGGACGTCCT CGTCCAGGCC AAGTACCAGT
HIMALAYA      ACATGCCGCC AAGCTCTACG AGGACGTCCT CGTCCAGGCC AAGTACCAGT

              2551                                                    2600
                *** stop codon
MK6827        GGTGAACGCT GCTACCCGGT CCAGCCCCGC ATGCGTGCAT GAGAGGATGG
MOREX         GGTGAACGCT GCTACCCGGT CCAGCCCCGC ATGCGTGCAT GAGAGGATGG
292           GGTGAACGCT GCTACCCGGT CCAGCCCCGC ATGCGTGCAT GAGAGGATGG
HIMALAYA      GGTGAACGCT GCTACCCGGT CCAGCCCCGC ATGCGTGCAT GAGAGGATGG

              2601                                                    2650
MK6827        AAATGCGCAT TGCGCACTTG CAGATTTGGC GCATGCAGGA ACGTGCCGTC
MOREX         AAATGCGCAT TGCGCACTTG CAGATTTGGC GCATGCAGGA ACGTGCCGTC
292           AAATGCGCAT TGCGCACTTG CAGATTTGGC GCACGCAGGA ACGTGCCGTC
HIMALAYA      AAATGCGCAT TGCGCACTTG CAGATTTGGC GCACGCAGGA ACGTGCCGTC

              2651                                                    2700
MK6827        CTTCTTGATG GGAACGCCGG CATCCGCGAG GTTGAGACGC TGATTCCGAT
MOREX         CTTCTTGATG GGAACGCCGG CATCCGCGAG GTTGAGACGC TGATTCCGAT
292           CTTCTTGATG AGAACGCCGG CATCCGCGAG GTTGAGACGC TGATTCCGAT
HIMALAYA      CTTCTTGATG AGAACGCCGG CATCCGCGAG GTTGAGACGC TGATTCCGAT

              2701                                                    2750
MK6827        CTGGTCCGTC GCAGAGTAGA GTGAAACGCT CCTTGTTGCA GGTATATGGG
MOREX         CTGGTCCGTC GCAGAGTAGA GTGAAACGCT CCTTGTTGCA GGTATATGGG
292           CTGGTCCGTC GCAGAGTAGA GTGAAACGCT CCTTGTTGCA GGTATATGGG
HIMALAYA      CTGGTCCGTC GCAGAGTAGA GTGAAACGCT CCTTGTTGCA GGTATATGGG

              2751                                                    2800
MK6827        AATGTTTTTT TTTTCC.TTT TTTTTTTTGC GAGGGAGGTA TATGGGAATG
MOREX         AATGTTTTTT TTTTCCTTTT TTTTTTTTGC GAGGGAGGTA TATGGGAATG
292           AATGTTTTTT TT..CC...T TTTTTTTTGC GAGGGAGGTA TATGGGAATG
HIMALAYA      AATGTTTTTT TT..CC...T TTTTTTTTGC GAGGGAGGTA TATGGGAATG

              2801                                                    2850
MK6827        TTAACTTGGT ATTGTAATGT GGTATGCTGT GTGCATTATT ACATCGGTTG
MOREX         TTAACTTGGT ATTGTAATGT GGTATGCTGT GTGCATTATT ACATCGGTTG
292           TTAACTTGGT ATTGTAATGT GGTATGCTGT GTGCATTATT ACATCGGTTG
HIMALAYA      TTAACTTGGT ATTGTAATGT GGTATGCTGT GTGCATTATT ACATCGGTTG

              2851                                                    2900
MK6827        TTGTTGCTTA TTCTTGCTAG CTAAGTCGGA GGCCAAGAGC GAAAGCTAGC
MOREX         TTGTTGCTTA TTCTTGCTAG CTAAGTCGGA GGCCAAGAGC GAAAGCTAGC
292           TTGTTGCTTA TTCTTGCTAG CTAAGTCGGA GGCCAAGAGC GAAAGCTAGC
HIMALAYA      TTGTTGCTTA TTCTTGCTAG CTAAGTCGGA GGCCAAGAGC GAAAGCTAGC

              2901                                                    2950
MK6827        TCACATGTCT GATGTATGCA AGTGACATGG TTGGTTTGGT TGTGCAGTGC
MOREX         TCACATGTCT GATGTATGCA AGTGACATGG TTGGTTTGGT TGTGCAGTGC
292           TCACATGTCT GATGTATGCA AGTGACATGG TTGGTTTGGT TGTGCAGTGC
HIMALAYA      TCACATGTCT GATGTATGCA AGTGACATGG TTGGTTTGAA AAAAAAAAAA

              2951
MK6827        AAACGGCA
MOREX         AAACGGCA
292           AAACGGCA
HIMALAYA      AAAAAAAA
```

Comparison of SSII Amino Acid Sequences

	1			**MK6827 mutation #**	
Morex	MSSAVASPAS	FLALASASPG	RSSRRRARVG	ASPTRAGAGR	LQWRPSPLQR
Himalaya	MSSAVASPAS	FLALASASPG	RSSRRRARVG	ASPTRAGAGR	LQWRPSPLQR
292	MSSAVASPAS	FLALASASPG	RSSRRRARVG	ASPTRAGAGR	LQWRPSPLQR
MK6827	MSSAVASPAS	FLALASASPG	RSSRRRARVG	ASPTRAGAGR	LQ*RPSPLQR
	51				100
Morex	TARDGAVAAR	AAGIDDAAPG	RQPRARRYGA	ATKVADPVKT	LDRDAAEGGG
Himalaya	TARDGAVAAR	AAGIDDAAPG	RQPRARRYGA	ATKVADPVKT	LDRDAAEGGG
292	TARDGAVAAR	AAGIDDAAPG	RQPRARRYGA	ATKVADPVKT	LDRDAAEGGG
MK6827	TARDGAVAAR	AAGIDDAAPG	RQPRARRYGA	ATKVADPVKT	LDRDAAEGGG
	101				150
Morex	PSPPAPRQDA	ARLPSKNGTL	INGENKPTGG	GGATKDSGLP	TPARAPHLSI
Himalaya	PSPPAPRQDA	ARLPSKNGTL	INGENKPTGG	GGATKDSGLP	TPARAPHLSI
292	PSPPAPRQDA	ARLPSKNGTL	INGENKPTGG	GGATKDSGLP	TPARAPHLSI
MK6827	PSPPAPRQDA	ARLPSKNGTL	INGENKPTGG	GGATKDSGLP	TPARAPHLSI
	151				200
Morex	QNRVPVNGEN	KHKVASPPTS	IVDVASPGSA	ANISISNKVP	PSVVPAKKTP
Himalaya	QNRVPVNGEN	KHKVASPPTS	IVDVASPGSA	ANISISNKVP	PSVVPAKKTP
292	QNRVPVNGEN	KHKVASPPTS	IVDVASPGSA	ANISISNKVP	PSVVPAKKTP
MK6827	QNRVPVNGEN	KHKVASPPTS	IVDVASPGSA	ANISISNKVP	PSVVPAKKTP
	201				250
Morex	PSSVFPAKKT	LPSSGSNFVS	SASAPRLDTV	SDVELAQKKD	ALIVKEAPKP
Himalaya	PSSVFPAKKT	LPSSGSNFVS	SASAPRLDTV	SDVELAQKKD	ALIVKEAPKP
292	PSSVFPAKKT	LPSSGSNFVS	SASAPRLDTV	SDVELAQKKD	ALIVKEAPKP
MK6827	PSSVFPAKKT	LPSSGSNFVS	SASAPRLDTV	SDVELAQKKD	ALIVKEAPKP
	251				300
Morex	KALSAPAAPA	VQEDLWDFKK	YIGFEEPVEA	KDDGSAVADD	AGSFEHHQNH
Himalaya	KALSAPAAPA	VQEDLWDFKK	YIGFEEPVEA	KDDGSAVADD	AGSFEHHQNH
292	KALSAPAAPA	VQEDLWDFKK	YIGFEEPVEA	KDDGSAVADD	AGSFEHHQNH
MK6827	KALSAPAAPA	VQEDLWDFKK	YIGFEEPVEA	KDDGSAVADD	AGSFEHHQNH
	301				350
Morex	DSGPLAGENV	MNVVVVAAEC	SPWCKTGGLG	DVAGALPKAL	AKRGHRVMVV
Himalaya	DSGPLAGENV	MNVVVVAAEC	SPWCKTGGLG	DVAGALPKAL	AKRGHRVMVV
292	DSGPLAGENV	MNVVVVAAEC	SPWCKTGGLG	DVAGALPKAL	AKRGHRVMVV
MK6827	DSGPLAGENV	MNVVVVAAEC	SPWCKTGGLG	DIAGALPKAL	AKRGHRVMVV
	351				400
Morex	VPRYGDYEEA	YDVGVRKYYK	AAGQDMEVNY	FHAYIDGVDF	VFIDAPLFRH
Himalaya	VPRYGDYEEA	YDVGVRKYYK	AAGQDMEVNY	FHAYIDGVDF	VFIDAPLFRH
292	VPRYGDYEEA	YDVGVRKYYK	AAGQDMEVNY	FHAYIDGVDF	VFIDAPLFRH
MK6827	VPRYGDYEEA	YDVGVRKYYK	AAGQDMEVNY	FHAYIDGVDF	VFIDAPLFRH
	401				450
Morex	RQQDIYGGSR	QEIMKRMILF	CKAAVEVPWH	VPCGGVPYGD	GNLVFIANDW
Himalaya	RQQDIYGGSR	QEIMKRMILF	CKAAVEVPWH	VPCGGVPYGD	GNLVFIANDW
292	RQQDIYGGSR	QEIMKRMILF	CKAAVEVPWH	VPCGGVPYGD	GNLVFIANDW
MK6827	RQQDIYGGSR	QEIMKRMILF	CKAAVEVPWH	VPCGGVPYGD	GNLVFIANDW
	451				500
Morex	HTALLPVYLK	AYYRDHGLMQ	YSRSVMVIHN	IAHQGRGPVD	EFPFTELPEH
Himalaya	HTALLPVYLK	AYYRDHGLMQ	YSRSVMVIHN	IAHQGRGPVD	EFPFTELPEH
292	HTALLPVYLK	AYYRDHGLMQ	YSRSVMVIHN	IAHQGRGPVD	EFPFTELPEH
MK6827	HTALLPVYLK	AYYRDHGLMQ	YSRSVMVIHN	IAHQGRGPVD	EFPFTELPEH
	501				550
Morex	YLEHFRLYDP	VGGEHANYFA	AGLKMADQVV	VVSPGYLWEL	KTVEGGWGLH
Himalaya	YLEHFRLYDP	VGGEHANYFA	AGLKMADQVV	VVSPGYLWEL	KTVEGGWGLH
292	YLEHFRLYDP	VGGEHANYFA	AGLKMADQVV	VVSPGYLWEL	KTVEGGWGLH
MK6827	YLEHFRLYDP	VGGEHANYFA	AGLKMADQVV	VVSPGYLWEL	KTVEGGWGLH

FIGURE 12

```
          551                          $ 292 mutation                600
Morex     DIIRQNDWKT RGIVNGIDNM EWNPEVDVHL KSDGYTNFSL KTLDSGKRQC
Himalaya  DIIRQNDWKT RGIVNGIDNM EWNPEVDVHL KSDGYTNFSL KTLDSGKRQC
292       DIIRQNDWKT RGIVNGIDNM E*NPEVDVHL KSDGYTNFSL KTLDSGKRQC
MK6827    DIIRQNDWKT RGIVNGIDNM EWNPEVDVHL KSDGYTNFSL KTLDSGKRQC

          601                                                650
Morex     KEALQRELGL QVRGDVPLLG FIGRLDGQKG VEIIADAMPW IVSQDVQLVM
Himalaya  KEALQRELGL QVRGDVPLLG FIGRLDGQKG VEIIADAMPW IVSQDVQLVM
292       KEALQRELGL QVRGDVPLLG FIGRLDGQKG VEIIADAMPW IVSQDVQLVM
MK6827    KEALQRELGL QVRGDVPLLG FIGRLDGQKG VEIIADAMPW IVSQDVQLVM

          651                                                700
Morex     LGTGRHDLES MLQHFEREHH DKVRGWVGFS VRLAHRITAG ADALLMPSRF
Himalaya  LGTGRHDLES MLQHFEREHH DKVRGWVGFS VRLAHRITAG ADALLMPSRF
292       LGTGRHDLES MLQHFEREHH DKVRGWVGFS VRLAHRITAG ADALLMPSRF
MK6827    LGTGRHDLES MLQHFEREHH DKVRGWVGFS VRLAHRITAG ADALLMPSRF

          701                                                750
Morex     EPCGLNQLYA MAYGTIPVVH AVGGLRDTVP PFDPFNHSGL GWTFDRAEAH
Himalaya  EPCGLNQLYA MAYGTIPVVH AVGGLRDTVP PFDPFNHSGL GWTFDRAEAH
292       EPCGLNQLYA MAYGTIPVVH AVGGLRDTVP PFDPFNHSGL GWTFDRAEAH
MK6827    EPCGLNQLYA MAYGTIPVVH AVGGLRDTVP PFDPFNHSGL GWTFDRAEAH

          751                                                800
Morex     KLIEALGHCL RTYRDHKESW RGLQERGMSQ DFSWEHAAKL YEDVLVQAKY
Himalaya  KLIEALGHCL RTYRDHKESW RGLQERGMSQ DFSWEHAAKL YEDVLVQAKY
292       KLIEALGHCL RTYRDHKESW RGLQERGMSQ DFSWEHAAKL YEDVLVQAKY
MK6827    KLIEALGHCL RTYRDHKESW RGLQERGMSQ DFSWEHAAKL YEDVLVQAKY

          801
Morex     QW*
Himalaya  QW*
292       QW*
MK6827    QW*
```

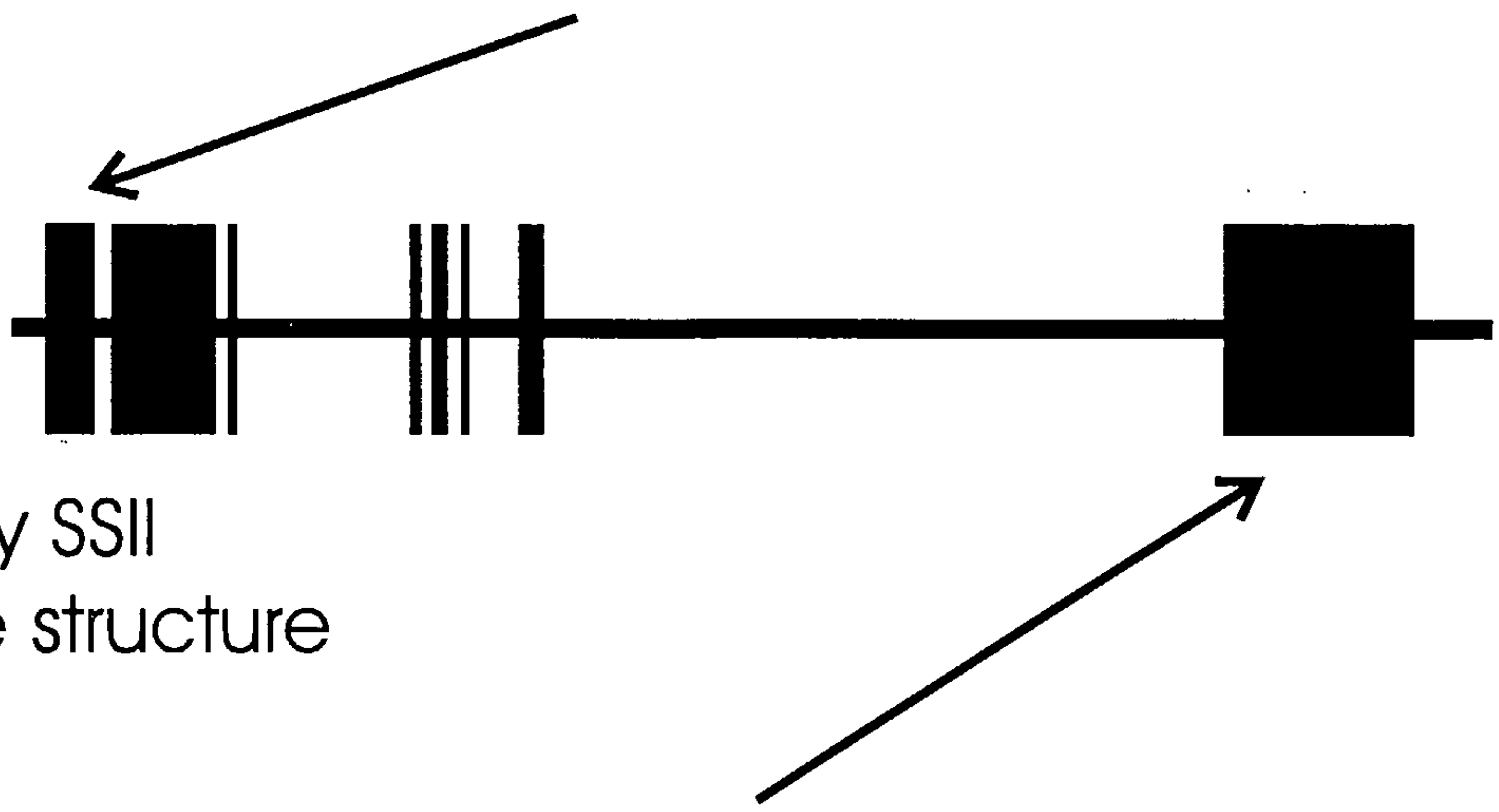
Position 242 of barley SSII
cDNA sequence

Himalaya ↓

.....CGGCAGGCTGCAATGGCGGCCGTCGCCGCT....

MK6827 ↓

.....CGGCAGGCTGCAATGACGGCCGTCGCCGCT....



Barley SSII
gene structure

Position 1829 of barley SSII
cDNA sequence

292 ↑

.....GACAACATGGAGTGAAACCCTGAGGTGGACGTCCA.....

Himalaya ↑

.....GACAACATGGAGTGGAACCCTGAGGTGGACGTCCA.....

FIGURE 13

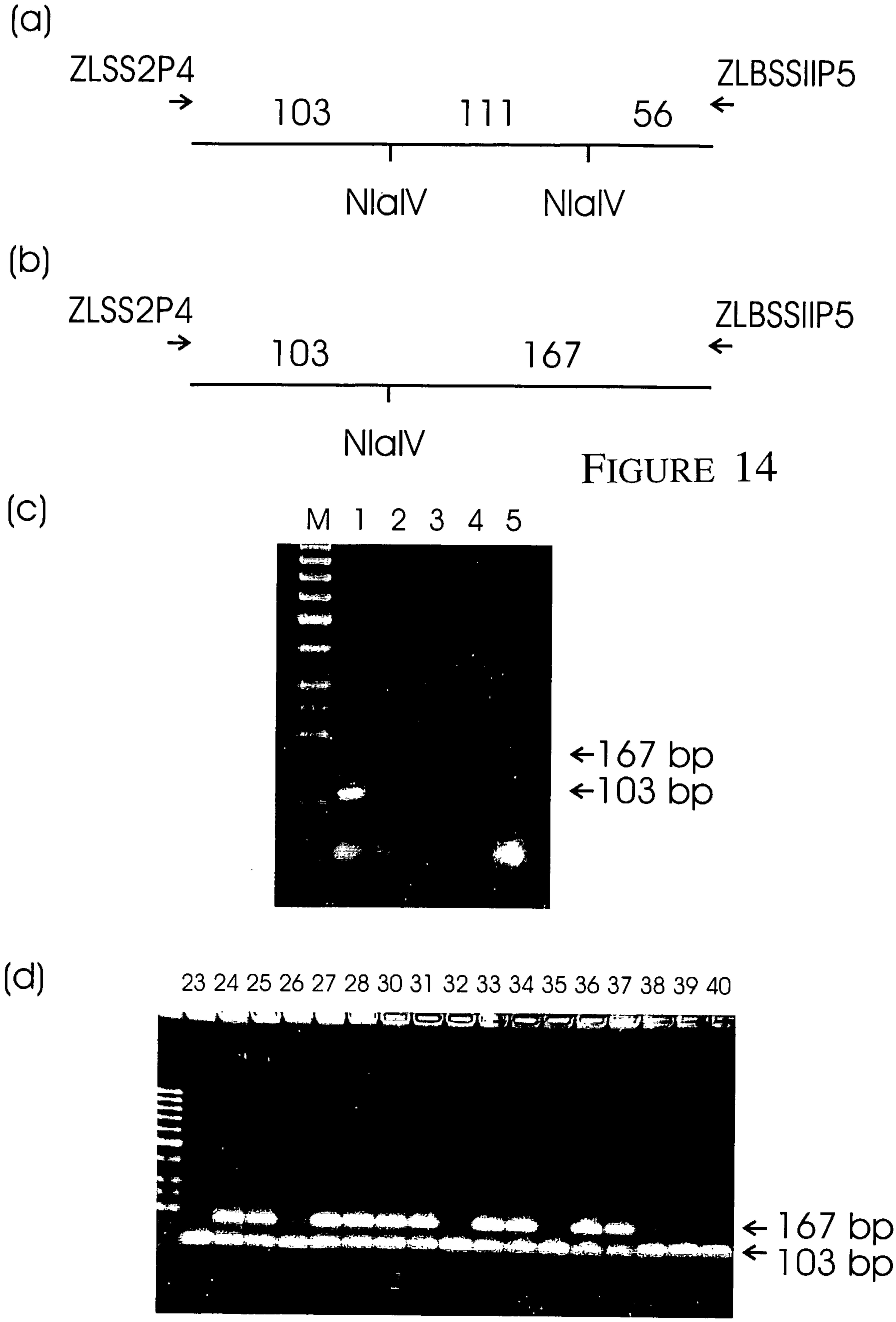

(a)
ZLSS2P4
ZLBSSIIP5
103
111
56
NlaIV
NlaIV
(b)
ZLSS2P4
ZLBSSIIP5
103
167
NlaIV
(c)
M 1 2 3 4 5
←167 bp
←103 bp
(d)
23 24 25 26 27 28 30 31 32 33 34 35 36 37 38 39 40
← 167 bp
← 103 bp

FIGURE 14

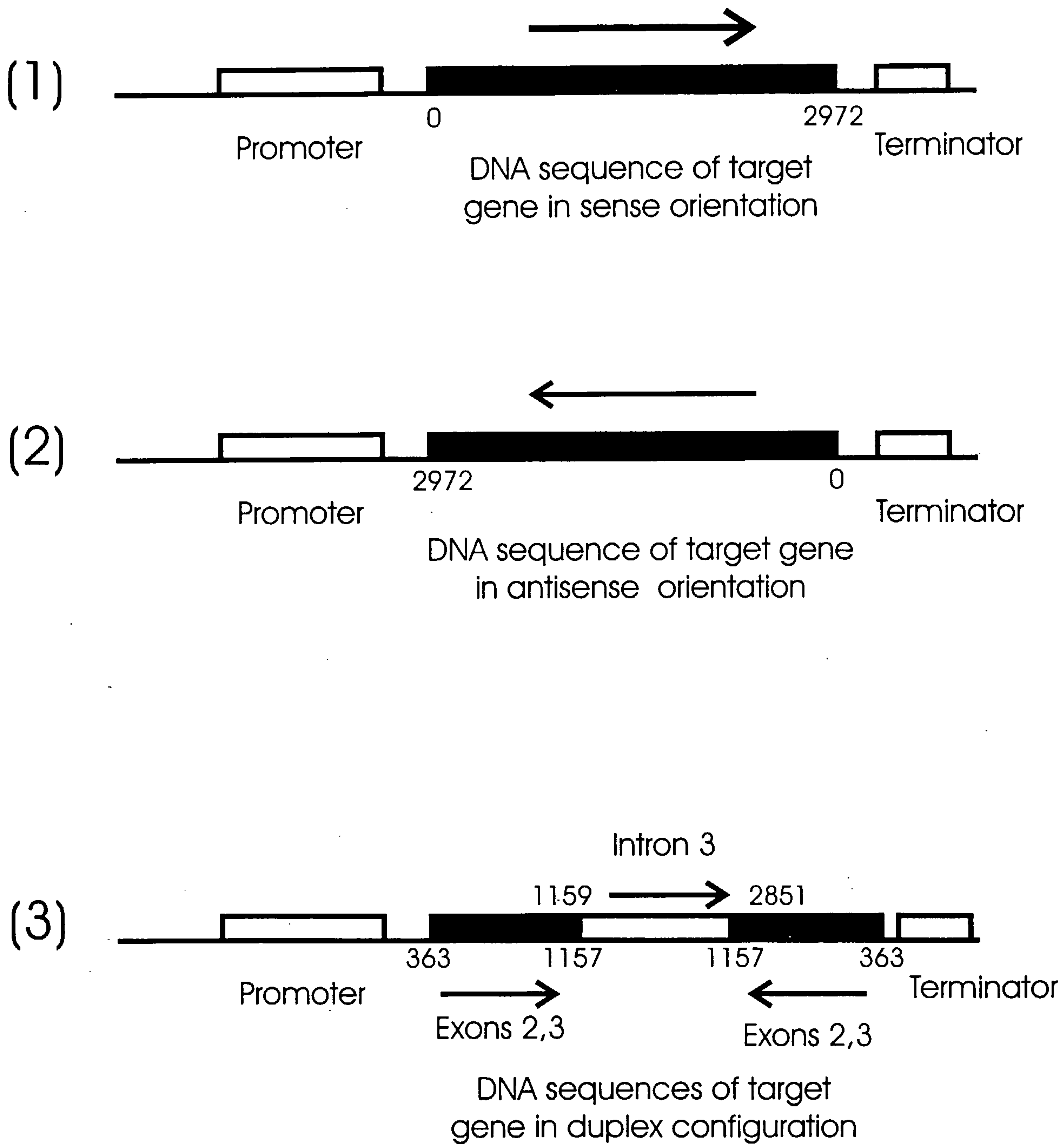

(1)
0
2972
Promoter
Terminator
DNA sequence of target
gene in sense orientation
(2)
2972
0
Promoter
Terminator
DNA sequence of target gene
in antisense orientation
(3)
Intron 3
1159
2851
363
1157
1157
363
Promoter
Terminator
Exons 2,3
Exons 2,3
DNA sequences of target
gene in duplex configuration

FIGURE 15

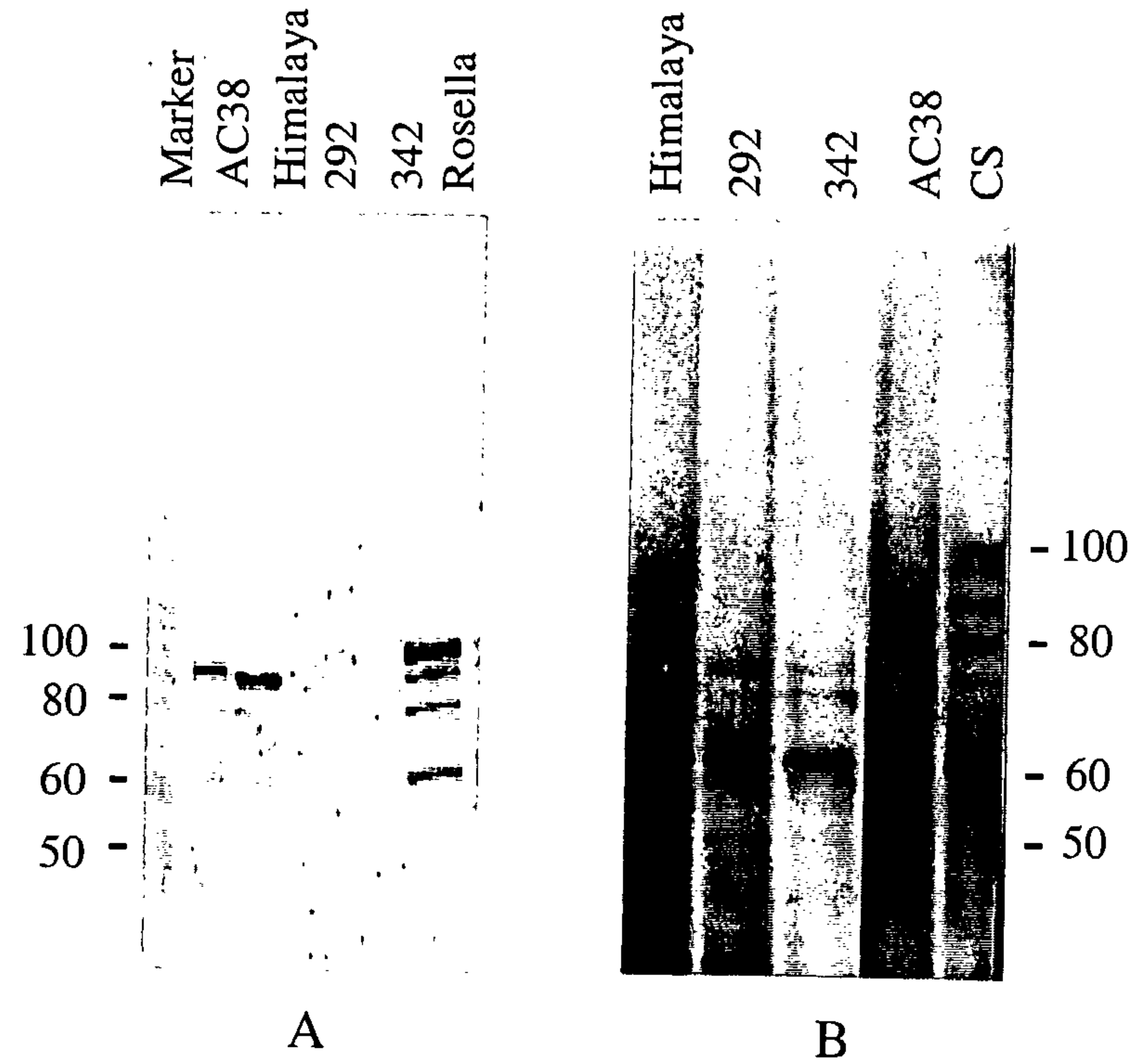

Marker
AC38
Himalaya
292
342
Rosella
100
80
60
50
A
Himalaya
292
342
AC38
CS
100
80
60
50
B

FIGURE 16