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(54) Title: BARLEY WITH REDUCED LIPOXYGENASE ACTIVITY

(57) Abstract: According to the invention, there is provided barley with total loss of functional lipoxygenase (LOX)-1 and LOX-2 enzymes, and plant products produced thereof, such as malt manufactured by using barley kernels defective in the synthesis of the fatty acid-dioxygenating enzymes LOX-1 and LOX-2. Said enzymes account for the principal activities related to dioxygenation of linoleic acid into 9- and 13-hydroperoxy octadecadienoic acid, respectively. 9-Hydroperoxy octadecadienoic acid represents a LOX pathway metabolite, which - through further enzymatic or spontaneous reactions - may lead to the appearance of trans-2-nonenal (T2N). The invention enables brewers to produce a beer having insignificant levels of stale, T2N-specific off-flavors, even after prolonged storage of the beverage.



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## Barley with reduced lipoxygenase activity

5 All patent and non-patent references cited in the application are hereby incorporated by reference in their entirety.

### FIELD OF INVENTION

10 The present invention relates to advances in plant biotechnology, enabling the disclosure of barley plants, and products thereof, which are defective in synthesis of two lipoxygenase (LOX) enzymes, LOX-1 and LOX-2 – thus offering new raw materials for a variety of usages. Said raw materials, when for example exploited in beverage production, facilitate the fielding of new manufacture capabilities for more distinctive,  
15 flavor-stable beers that accumulate remarkably low levels of the off-flavor compound trans-2-nonenal (T2N) – with an additional benefit of only containing low levels of T2N potential.

### 20 BACKGROUND OF INVENTION

A large fraction of beer is produced on the basis of barley (*Hordeum vulgare*, L.), which is a monocotyledonous crop plant grown in many parts of the world. It is propagated not only due to its economic importance as a source of industrial products, such as  
25 beer, but also as a source of animal feed.

Methods are unfortunately not available for preparing transgenic barley plants that completely lack expression of a given gene and corresponding protein. In general for barley, application of antisense techniques may be utilized to generate transgenic  
30 plants that still express some of the protein (see, for example, Robbins et al., 1998; Stahl et al., 2004; Hansen et al., 2007). Also, effective methods have not been developed for preparing specific mutations using chimeric RNA/DNA or site-directed mutagenesis in barley plants. In fact, not a single example of successful oligonucleotide-directed gene targeting in barley has been published. Iida and Terada  
35 (2005) noted that oligonucleotide-directed gene targeting has been pursued in maize,

tobacco and rice, but not in barley – and in all cases with the ALS gene as a target. Part of the research conclusion was that it remains to be seen whether the strategy with appropriate modifications can be applicable to genes other than those directly selectable, such as the ALS genes. Targeted mutagenesis using zinc-finger nucleases  
5 represents another tool that could be used in the future to investigate basic plant biology or to modify crop plants (Durai et al., 2005; Tzfira and White, 2005; Kumar et al., 2006). Also in this case, mutagenesis has not been pursued or successfully applied in barley.

10 Barley mutants, however, may be prepared by random mutagenesis using chemical treatment or irradiation, such as by treatment with sodium azide ( $\text{NaN}_3$ ; FIG. 1). An example is barley kernels mutagenized with  $\text{NaN}_3$ , and screened for high levels of free phosphate, with the aim to identify low-phytate mutants (Rasmussen and Hatzack, 1998); a total of 10 mutants out of 2,000 screened kernels were identified. However,  
15 identification of a particular mutant after  $\text{NaN}_3$  treatment requires an effective screening method and is far from always successful.

In 1970, the molecule conferring the cardboard-like flavor in beer was isolated and identified as T2N, a volatile  $\text{C}_9$  alkenal (Jamieson and Gheluwe, 1970). Since the taste-  
20 threshold level for T2N in humans is extremely low, previously determined to be around 0.7 nM or 0.1 ppb (Meilgaard, 1975), products with even minute levels of the aldehyde are regarded as being aged due to the off-flavor taste of the product. However, the T2N level is generally very low in fresh beer (Lermusieau et al., 1999), so it has been speculated that during storage, free T2N may be liberated from T2N adducts (Nyborg  
25 et al., 1999). This notion was supported by a subsequent observation that the T2N potential in wort correlates with formation of T2N after product storage (Kuroda et al., 2005).

The barley kernel contains three LOX enzymes known as LOX-1, LOX-2, and LOX-3  
30 (van Mechelen et al., 1999). LOX-1 catalyzes the formation of 9-hydroperoxy octadecadienoic acid (9-HPODE; see FIG. 2 for a partial overview of LOX pathway) – a precursor of both T2N and trihydroxy octadecenoic acids (abbreviated THAs) – from linoleic acid. LOX-2 mainly catalyzes the conversion of linoleic acid to 13-HPODE, which is further metabolized to hexanal, a  $\text{C}_6$  aldehyde with a ~0.4-ppm-high taste  
35 threshold (Meilgaard, supra). LOX-3 action is probably not of relevance with respect to

the instant application for two reasons: the expression level of the corresponding gene in barley kernels is very low, and the product specificity of LOX-3 remains elusive.

5 In support of the aforementioned data, several reports have noted that T2N is produced via a biochemical pathway involving conversion of linoleic acid to 9-HPODE, initially catalyzed by LOX-1, and then cleavage of 9-HPODE through 9-hydroperoxide lyase action (see, for example, Kuroda et al., 2003, 2005; Noodermeer et al., 2001).

10 There appears to be no correlation between the overall LOX activity in malt and the wort nonenal potential. However, there have been speculations about a significant correlation between LOX-1 activity and wort T2N potential, primarily because LOX-2 activity was considered inferior with respect to formation of the T2N potential in wort (Kuroda et al., 2005).

15 In FIG. 2 is, as mentioned above, shown a part of the LOX pathway, here focusing on biochemical reactions from linoleic acid to T2N. The major activity of the LOX-1 enzyme concerns the conversion of linoleic acid to 9-HPODE, which is an upstream metabolite of the biochemical pathway leading to formation of T2N. In contrast, the major activity of LOX-2 relates to the conversion of linoleic acid into 13-HPODE, which  
20 is separate from the aforementioned biochemical pathway to T2N. It is notable that LOX-1 and LOX-2 enzymes may utilize linolenic acid as substrate, but this activity is outside the scope of the instant application as the corresponding pathways do not lead to T2N formation.

25 LOX-1 has been thought to contribute with the major LOX activity in malt (see, for example, Kuroda et al., 2003).

Several different barley plants have been developed, which are characterized by reductions in, or lack of, LOX-1 activity. For example, barley kernels and barley plants  
30 having a low LOX-1 activity were disclosed in PCT application WO 02/053721 to Douma, A.C. et al. And in WO 2005/087934 to Breddam, K. et al., attention was on two different barley mutants deficient in LOX-1 activity – a splice mutant and a mutant with a premature translational stop codon. These were identified following propagation and screening of mutated plants, as illustrated in FIG. 1. While the above-mentioned  
35 mutants were identified by screening NaN<sub>3</sub>-mutagenized barley, Hirota, N. et al.

described in EP 1609866 a barley plant with no LOX-1 activity, which was identified by screening a collection of barley landraces.

Several examples on mutated plants that synthesize low levels of LOX are known.

5 However, no barley plant deficient in several lipoxygenase activities, for example no barley plant deficient in activities of both LOX-1 and LOX-2 has been described.

Methods to enable genetic manipulation of plants are frequently specific to a specific kind of plant, and thus – despite that few rice plants, soy plants, or Arabidopsis plants are known to comprise low levels of LOX – the methods for preparing such plants

10 cannot be used in the generation of barley plants with low, or no, LOX activity. In addition, LOX mutants of one plant species may have different properties in comparison with LOX mutants of another plant species.

## 15 SUMMARY OF INVENTION

Wort is a complex key liquid in the production of malt based beverages such as beer (cf. FIG. 3 that provides an overview on the entire process of making beer). Wort made from barley plants deficient in LOX-1 activity is actually characterized by comprising

20 significant levels of T2N potential, although said level is lower than that in wort prepared from wild-type barley. Since said potential in wort correlates with T2N formed in beer after storage (Kuroda et al., 2005), there is accordingly a need for barley plants useful in the production of wort with a substantially reduced level of T2N potential.

25 Methods for reducing LOX activity by heat treatment have been described. However, the heat treatment was generally undertaken during malting and/or preparation of wort, and products of LOX activity were thus allowed to accumulate in the barley until undertaking of the heat treatment. Analysis of barley revealed that significant amounts of products of LOX activity were present in barley, even prior to malting (Wackerbauer

30 and Meyna, 2002).

The present invention provides barley plants deficient in LOX-1 and LOX-2 activities and discloses that said barley plants have several profound, surprising advantages. As mentioned herein above, Kuroda et al. (supra) described a lack in correlation between

35 the LOX activity in malt and the T2N potential in wort, and attributed this to the

presence of LOX-2 activity. Kuroda et al. (2005) accordingly proposed an insignificant role of LOX-2 with respect to the formation of T2N potential.

5 Brewing researchers have wrestled with understanding what dictates the formation of free T2N and T2N potential. It is therefore unexpected, perhaps perplexing, when the present invention provides experimental evidence to disclose the beneficial effect of using barley plants deficient in both LOX-1 and LOX-2 activities for production of wort that exhibits very low levels of T2N potential. And also the level of free T2N in wort prepared from barley plants deficient in both LOX-1 and LOX-2 activities was found to  
10 be lower than that prepared from null-LOX-1 barley.

It is one objective of the present invention to provide beverages prepared from a barley plant, or a part thereof, wherein said beverage comprises very low levels of T2N potential – and wherein said barley plant, or part thereof, comprises a first mutation that  
15 results in a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of function of LOX-2 activity, such as total loss of active LOX-2 enzyme.

The invention also is aimed at providing barley plants useful in the preparation of the  
20 beverages of the invention. Thus, the invention describes the generation of barley plants, or parts thereof, comprising a first mutation resulting in a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of function of LOX-2 activity, such as total loss of functional LOX-2 enzyme.

25 In addition, the invention provides plant products comprising a processed barley plant, or part thereof, wherein said plant comprises a first mutation that results in a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of function of LOX-2 activity, such as a total  
30 loss of functional LOX-2 enzyme. Without limitations, the aforementioned plant products may, for example, be a malt composition, or a wort composition, or a beverage (such as a malt-based beverage, e.g. beer or non-alcoholic malt based beverages), or a barley-based beverage, or a beverage based on a mixture of malt and barley and optionally other ingredients. The plant products may also be barley syrups,  
35 malt syrups, barley extracts, and malt extracts.

Moreover, the invention also relates to methods to produce a beverage characterized by a very low level of T2N potential, said methods comprising the steps of:

- 5 (i) preparing a composition comprising a barley plant, or parts thereof, comprising a first mutation that results in a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of functional LOX-2, such as total loss of active LOX-2 enzyme;
- 10 (ii) processing the composition of (i) into a beverage;

thereby obtaining a beverage characterized by a very low level of T2N potential.

The invention also concerns barley plants, which – in addition to comprising a first  
15 mutation resulting in a total loss of function of LOX-1 activity, and a second mutation resulting in a total loss of function of LOX-2 activity – comprise one or more additional, useful mutations.

## 20 DESCRIPTION OF DRAWINGS

FIG. 1 shows one example on how  $\text{NaN}_3$ -mutagenized barley kernels may be propagated. Kernels of generation M0 grow into plants that develop kernels of generation M1. These may be sown for development into M1 plants, which produce  
25 new kernels of generation M2. Next, M2 plants grow and set kernels of generation M3. Kernels of generation M3 may be allowed to germinate, for example for analysis of coleoptiles of the germinated M3 plants. Additionally, flowers derived from kernels of M3 plants may be used in crossings with barley lines or cultivars to obtain plants of generation M4. A similar figure is presented as FIG. 1A in PCT patent application  
30 WO 2005/087934 to Breddam, K. et al.

FIG. 2 shows a schematic illustration of a biochemical pathway for conversion and degradation of linoleic acid to T2N. LOX-1 primarily catalyzes the conversion of linoleic acid to 9-HPODE, which is enzymatically degraded to cis-nonenal. This compound  
35 undergoes a spontaneous, chemical isomerization to T2N. LOX-2 primarily catalyzes

the conversion of linoleic acid into 13-HPODE, which may be converted into 2-E-hexanal (not shown).

FIG. 3 shows a simplified, schematic overview of a preferred beer production process, including steeping of barley grain (1), malting (2), kiln drying (3), milling of the dried malt (4), mashing (5), filtration (6), wort boiling in the presence of added hops (7), fermentation in the presence of yeast (8), beer maturation (9), beer filtration (10), packaging, such as the packaging into bottles, cans, or the like (11), and labeling (12). The individual processes can be grouped into sections comprising malt production (1-3), wort production (4-7), fermentation (8-9), and the preparation of the finished beer (10-12). Although a preferred method is illustrated, other methods may be envisaged that omit some of the depicted steps (filtration may, for example, be omitted, or hops may not be added – or additional steps may be added, such as addition of adjuncts, sugars, syrups, or carbonate).

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FIG. 4 shows the results of comparisons of total LOX activities in germinated kernels of generation M3 (A), generation M4 (B), and generation M5 (C). Said activities were measured in extracts of embryos that were isolated from germinated kernels of double null-LOX mutant A689, null-LOX-1 mutant D112 and null-LOX-1 breeding line Ca211901. Heat-inactivated extract aliquots of the same samples served as experimental controls [in C: null-LOX-1 raw mutant D112 (\*), null-LOX-1 breeding line Ca211901 (\*\*)]. Shown are also the LOX-1 and LOX-2 genotypes of the plant samples analyzed (wt: wild-type).

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FIG. 5 shows the chromatograms of HPLC analyses used to assay for the formation of 9- and 13-HPODEs in 48-h germinated barley embryos. The HPODEs were detected by measuring the absorbance at 234 nm, with the results given in relative absorbance units (AU). Peaks of the elution profiles that correspond to 9- and 13-HPODEs are indicated by arrows. (A) Chromatogram of 9- and 13-HPODE standards. (B) Chromatogram of HPODEs formed in extracts prepared from germinated embryos of null-LOX-1 mutant D112. (C) Chromatogram of HPODEs formed in extracts prepared from germinated embryos of double null-LOX mutant A689, generation M4.

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FIG. 6 shows the chromatograms of HPLC analyses used to assay for the formation of 9- and 13-HPODEs in embryos of 72-h micro-malted kernels. HPODE were detected

35



by measuring the absorbance at 234 nm, with the results given in relative absorbance units (AU). Peaks of the elution profiles that correspond to 9- and 13-HPODEs are indicated by arrows. **(A)** Chromatogram of 9- and 13-HPODEs formed in wild-type cv. Barke. **(B)** Chromatogram of HPODEs formed in extracts of embryos of null-LOX-1 mutant D112. **(C)** Chromatogram of HPODEs formed in extracts of double null-LOX mutant A689, generation M5.

FIG. 7 shows the levels of free T2N formed in micro-malted samples of wild-type barley cv. Power, null-LOX-1 mutant D112, and three potential double null-LOX lines of mutant A689, generation M5. Included are also the LOX-1 and LOX-2 genotypes of the barley samples analyzed (wt: wild-type).

FIG. 8 shows the levels of free T2N formed in samples of boiled wort produced from micro-malted kernels of wild-type cv. Power, null-LOX-1 mutant D112, and double null-LOX mutant A689, generation M5. Shown are also the LOX-1 and LOX-2 genotypes of the barley samples analyzed (wt: wild-type).

FIG. 9 shows the levels of the T2N precursors in samples of boiled wort produced from micro-malted kernels of wild-type cv. Power, null-LOX-1 mutant D112 and double null-LOX mutant A689, generation M5.

FIG. 10 shows the levels of T2N precursors in beers produced in a 200-l-large brewing experiment using malt of cv. Power, null-LOX-1 mutant D112, and double null-LOX mutant A689. Shown are the LOX-1 and LOX-2 genotypes of the malts used (wt: wild-type).

FIG. 11 shows a comparison of the free T2N levels in aliquots of the indicated samples taken from 200-l-large boiled worts, produced by barley brewing or by normal malting and mashing. Shown are the LOX-1 and LOX-2 genotypes of the raw materials used (wt: wild-type).

FIG. 12 shows a comparison of the levels of T2N precursors in the indicated sample aliquots taken from 200-l-large boiled worts (cf. FIG. 11), either produced by barley brewing or using normal malting and mashing. Shown are also the LOX-1 and LOX-2 genotypes of the raw materials used (wt: wild-type).

FIG. 13 shows aspects of T2N development in beers from 200-l-large brewing trials, using the indicated barley cultivar and mutants as raw materials. (A) Analyses of barley-brewed beer only, focusing on the development of free T2N during forced aging. The taste threshold level of T2N at 50 ppt is indicated by a horizontal, dashed line. In a separate experiment was compared levels of T2N precursors in fresh beers of barley brews and normal brews (B); in (C) is shown the levels of free T2N in fresh beers (white bars) compared with levels after two weeks at 37°C (black bars). Shown are also the LOX-1 and LOX-2 genotypes of the raw materials used (wt: wild-type).

FIG. 14 shows a comparison of THA levels and ratios in both barley-brewed beers (A), and in beers produced using normal malt (B). 9,12,13- and 9,10,13-THAs are generated by partially unknown reactions of the LOX-1 and LOX-2 pathway, respectively.

FIG. 15 shows an illustration on the development over time of the off-flavors "papery" (A) and "aged" (B) in 200-l-scale barley-brewed beer, using the raw materials cv. Power (black bars), null-LOX-1 (grey bars), and double null-LOX (white bars). A specialized beer taste panel judged the beers, using a scale from 0 (no off-flavor) to 5 (extreme off-flavor).

FIG. 16 shows how foam develops in barley-brewed beers (A), and in malt-based beers (B). In both experiments, a commercial lager beer served as reference (thick dashed line), in comparison with beer produced using raw materials of cv. Power (thin dashed line), null-LOX-1 (thin unbroken line), and double null-LOX (thick unbroken line).

FIG. 17 shows a map on the organization of the genomic barley gene for LOX-2, focusing on the region spanning the start codon (ATG) and the stop codon (TAA). The 3229-bp sequence was found to consist of 6 exons (filled boxes), and 5 introns (lines). The mutation identified in the gene for LOX-2 of double null-LOX mutant A689 is indicated by a vertical arrow. Notably, double null-LOX mutant A689 also contains a premature translational stop codon in the gene for LOX-1 (cf. mutant D112 as illustrated in FIG. 15 of U.S. Patent No. 7,420,105).

FIG. 18 shows how genotype analysis can be used to identify null-LOX mutants. Focus concerns SNP-assisted detection of a double null-LOX barley plant, i.e. a way to achieve the combined detection of a G→A mutation at position 3474 of the LOX-1 gene, and a G→A mutation at position 2689 of the LOX-2 gene. (A) Diagram of agarose gel electrophoresis of PCR fragments amplified from the indicated template DNA using the indicated primer pairs, i.e. the wild-type LOX-2 gene can be amplified by using primers FL1035 and FL1039, while the null-LOX-2 mutant gene can be amplified using primers FL1034 and FL1039. (B) Diagram to illustrate how PCRs with primer pair FL820 and FL823 (containing a 3' base complementary to the null-LOX-1-specific mutation as shown by an asterisk), and primer pair FL1034 (containing a 3' base complementary to the null-LOX-2 mutation as illustrated by an asterisk) and FL1039 can generate PCR products of 166 bp and 200 bp, respectively, provided the presence of said null-LOX mutations. (C) Depiction of the result following agarose gel electrophoresis of marker DNA (lanes 1 and 4), and that of PCR-amplified DNA of double null-LOX mutant A689 using the aforementioned primer combinations for detection of the null-LOX-1 and null-LOX-2 mutations (lane 2). PCR reactions of wild-type barley yielded no corresponding PCR products (lane 3).

FIG. 19 shows an example on SNP-assisted analysis of off-spring plants from crosses with double null-LOX barley. (A) A schematic representation using the primer combinations detailed in the legend to FIG. 18 herein above, i.e. primers for PCR-based detection of null-LOX-1 and null-LOX-2 mutations. The first lane from left – with band pattern "a", as noted below the lane – contains no specific PCR product for the presence of the two mutations [i.e. both template regions had wild-type (W) sequences], while the next lane with band pattern "b" highlights a PCR product derived from a null-LOX-1 mutant plant (M). The third lane with band pattern "c" is derived from a null-LOX-2 plant, and that in the fourth lane with band pattern "d" is of a double null-LOX plant. (B) Agarose gel electrophoresis of wild-type, null-LOX-1, null-LOX-2, and double null-LOX barley genotypes. Analysis of the banding patterns, according to that described above in (A), revealed that lanes 2, 6, 10, 12, 18, and 22 contained amplified products from plants carrying the null-LOX-1 mutation (marked with "b" below the lanes), while lanes 1, 3, 9, 11, 19, 21, and 23 contained products from plants carrying the null-LOX-2 mutation (marked with "c" below the lanes). Lanes 7, 15, and 17 contained PCR products from double null-LOX plants carrying the combined LOX-1 and LOX-2 gene mutations (marked with "d" below the lanes). And lanes 4, 5, 8, 13,

14, 16, and 20 contained no amplification products from plants carrying either of the null-LOX alleles (marked with "a" below the lanes), i.e. the plants were wild-type with respect to the tested sequences. Marker DNA is separated in lane marked with "MW".

5

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

10 In the description, figures, and tables that follow, a number of terms are used. In order to provide the specifications and claims, including the scope to be given such terms, the following definitions are provided:

15 As used herein, "a" can mean one or more, depending on the context in which it is used.

The term "agronomic trait" describes a phenotypic or genetic trait of a plant that contributes to the performance or economic value of said plant. Such traits include disease resistance, insect resistance, virus resistance, nematode resistance, drought  
20 tolerance, high salinity tolerance, yield, plant height, days to maturity, kernel grading (i.e. kernel size fractionation), kernel nitrogen content and the like.

By "antisense nucleotide sequence" is intended a sequence that is in inverse orientation to the normal coding 5'-to-3' orientation of that nucleotide sequence. When  
25 present in a plant cell, the antisense DNA sequence preferably reduces normal expression of the nucleotide sequence for the endogenous gene, and may disrupt production of the corresponding, native protein. In barley plants expression of antisense nucleotides in general only reduces expression but does not prevent expression of said native protein.

30

The term "barley" in reference to the process of making beer and barley-based beverages, particularly when used to describe the malting process, means barley kernels. In all other cases, unless otherwise specified, "barley" means the barley plant (*Hordeum vulgare*, L.), including any breeding line or cultivar or variety, whereas part of  
35 a barley plant may be any part of a barley plant, for example any tissue or cells.

By "disease resistance" is intended that the plants avoid disease symptoms, which are the outcome of plant-pathogen interactions. In this way, pathogens are prevented from causing plant diseases and the associated disease symptoms. Alternatively, the disease symptoms caused by the pathogen are minimized or reduced, or even prevented.

As used herein the term "double null-LOX" refers to a total loss of function of LOX-1 activity and a total loss of function of LOX-2 activity. Thus, "double null-LOX" may be characterized by a total loss of functional LOX-1 enzyme and a total loss of functional LOX-2 enzyme. Thus, a "double null-LOX barley plant", is a barley plant comprising a first mutation resulting in a total loss of function of LOX-1 activity and a second mutation resulting in a total loss of function of LOX-2 activity. A "double null-LOX barley plant" may thus be characterized by a total loss of functional LOX-1 enzyme and a total loss of functional LOX-2 enzyme. Similarly, "double null-LOX kernels", are kernels comprising a first mutation resulting in a total loss-of-function of LOX-1 activity and a second mutation resulting in a total loss of function of LOX-2 activity, and so forth. "Double null-LOX kernels" may thus be characterized by a total loss of functional LOX-1 enzyme and a total loss of functional LOX-2 enzyme.

20

A "cereal" plant, as defined herein, is a member of the Graminae plant family, cultivated primarily for their starch-containing seeds or kernels. Cereal plants include, but are not limited to barley (*Hordeum*), wheat (*Triticum*), rice (*Oryza*), maize (*Zea*), rye (*Secale*), oat (*Avena*), sorghum (*Sorghum*), and Triticale, a rye-wheat hybrid.

25

By "encoding" or "encoded", in the context of a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid or polynucleotide encoding a protein may comprise non-translated sequences, e.g. introns, within translated regions of the nucleic acid, or may lack such intervening non-translated sequences, e.g. in cDNA. The information by which a protein is encoded is specified by the use of codons.

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As used herein, "expression" in the context of nucleic acids is to be understood as the transcription and accumulation of sense mRNA or antisense RNA derived from a

nucleic acid fragment. "Expression" used in the context of proteins refers to translation of mRNA into a polypeptide.

5 The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (promoter and terminator). Furthermore, plant genes generally consist of exons interrupted by introns. After transcription into RNA, the introns are removed by splicing to generate a mature messenger RNA (mRNA). The "splice sites" between exons and introns are typically determined by consensus sequences acting as splice signals for the splicing process,  
10 consisting of a deletion of the intron from the primary RNA transcript and a joining or fusion of the ends of the remaining RNA on either side of the excised intron. In some cases, alternate or different patterns of splicing can generate different proteins from the same single stretch of DNA. A native gene may be referred to as an "endogenous gene".

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As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention.

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The term "germination" as used herein means the beginning or resumption of growth by a barley kernel in various compositions, such as normal soil as found in nature. Thus, a germinating embryo is an embryo undergoing germination. Germination can also take place in the soil of pots placed in growth chambers and the like, or for example  
25 take place on wet filter paper placed in standard laboratory Petri dishes or during malting (for example, in steep tanks or germination boxes of the malting factory). Germination is generally understood to include hydration of the kernels, swelling of the kernels and inducing growth of the embryo. Environmental factors affecting germination include moisture, temperature and oxygen level. Root and shoot development is  
30 observed.

As used herein, the term "isolated" means that the material is removed from its original environment. For example, a naturally occurring polynucleotide or polypeptide present in a living organism is not isolated, but the same polynucleotide or polypeptide,  
35 separated from some or all of the coexisting materials in the natural system, is isolated.

Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated because such vector or composition is not part of its natural environment.

5 The term "kernel" is defined to comprise the cereal caryopsis, also denoted internal seed, the lemma and palea. In most barley varieties, the lemma and palea adhere to the caryopsis and are a part of the kernel following threshing. However, naked barley varieties also occur. In these, the caryopsis is free of the lemma and palea and threshes out free as in wheat. The terms "kernel" and "grain" are used interchangeably  
10 herein.

"Grain development" refers to the period starting with fertilization of the egg cell by a pollen cell. During fertilization metabolic reserves – e.g. sugars, oligosaccharides, starch, phenolics, amino acids, and proteins – are deposited, with and without vacuole  
15 targeting, to various tissues in the kernel (grain) endosperm, testa, aleurone, and scutellum, thus leading to kernel (grain) enlargement, kernel (grain) filling, and ending with kernel (grain) desiccation.

The term "total loss of function" refers to the lack of the given enzymatic activity. Thus  
20 a barley plant with a "total loss of function" of LOX-1 and LOX-2 activity is a barley plant with no detectable LOX-1 and LOX-2 activities. In the context of the present invention, LOX-1 and LOX-2 activities are determined by an assay procedure determining the formation of 9-HPODE and 13-HPODE from linoleic acid, even though LOX-1 and LOX-2 may have other activities. Preferably, formation of 9-HPODE and 13-  
25 HPODE from linoleic acid is determined as described in Example 4 herein below. The activity should be determined using protein extracts of germinated embryos. In the context of the present invention, generation of a chromatogram peak corresponding to less than 5%, preferably less than 3% of the 9-HPODE peak of the standard shown in FIG. 5A, and/or a peak corresponding to less than 5%, preferably less than 3% of the  
30 13-HPODE peak of the standard shown in FIG. 5A, when using linoleic acid as substrate, is considered as no detectable LOX-1 and LOX-2 activity, when using the assay described in Example 4. Molecular approaches to obtain a total loss of function of LOX activity comprise generation of mutations that either cause a total absence of transcripts for said enzyme, total absence of the corresponding encoded enzyme, or  
35 mutations that totally inactivate the encoded enzyme.

The term "LOX-1 activity" refers to the enzymatic activity of the barley LOX-1 enzyme. Particularly, in the context of the present invention, "LOX-1 activity" is the enzyme-catalyzed dioxygenation of linoleic acid to 9-HPODE, and to a much lesser extent 13-HPODE. Even though the LOX-1 enzyme is capable of catalyzing other reactions, for the purpose of determining the activity of LOX-1 according to the present invention, only the 9- and 13-HPODE forming activities should be considered. FIG. 2 outlines the biochemical pathway wherein linoleic acid is converted to 9-HPODE.

The term "LOX-2 activity" refers to the enzymatic activity of the barley LOX-2 enzyme. Particularly, in the context of the present invention, "LOX-2 activity" is the enzyme-catalyzed dioxygenation of linoleic acid to 13-HPODE, and to a much lesser extent 9-HPODE. Even though the LOX-2 enzyme is capable of catalyzing other reactions, for the purpose of determining the activity of LOX-2 according to the present invention, only the 13- and 9-HPODE forming activities should be considered. FIG. 2 outlines the biochemical pathway wherein linoleic acid is converted to 13-HPODE.

The term "malt beverage" or the term "malt based beverage" refer to beverages prepared using malt, preferably beverages prepared by a method including a step of incubating malt with hot water. Malt beverage may, for example, be beer or maltinas.

The term "fermented malt beverage" refers to malt beverages, which have been fermented, i.e. incubated with yeast.

"Malting" is a special form of germination of barley kernels taking place under controlled environmental conditions – including, but not limited to steep tanks and germination boxes of the malting factory. In accordance with the process of the present invention, malting begins to occur during and/or after the barley kernels have been steeped. The malting process may be stopped by drying of the barley kernels, for example, in a kiln drying process. In case that the malt has not been kiln dried, it is denoted "green malt". A malt composition prepared from double null-LOX barley is understood to comprise double null-LOX malt, such as pure double null-LOX malt, or any blend of malt comprising double null-LOX malt. Malt may be processed, for example, by milling and thus referred to as "milled malt" or "flour".



"Mashing" is the incubation of milled malt in water. Mashing is preferably performed at a specific temperature, and in a specific volume of water. The temperature and volume of water are of importance, as these affect the rate of decrease of enzyme activity derived from the malt, and hence especially the amount of starch hydrolysis that can occur; protease action may also be of importance. Mashing can occur in the presence of adjuncts, which is understood to comprise any carbohydrate source other than malt, such as, but not limited to, barley (including double null-LOX barley), barley syrups, or maize, or rice – either as whole kernels or processed products like grits or starch. All of the aforementioned adjuncts may be used principally as an additional source of extract (syrups are typically dosed during wort boiling). The requirements for processing of the adjunct in the brewery depend on the state and type of adjunct used, and in particular on the starch gelatinization or liquefaction temperatures. If the gelatinization temperature is above that for normal malt saccharification, then starch is gelatinized and liquefied before added to the mash.

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"Mutations" include deletions, insertions, substitutions, transversions, and point mutations in the coding and noncoding regions of a gene. Deletions may be of the entire gene, or of only a portion of the gene, wherein the noncoding region preferably is either the promoter region, or the terminator region, or introns. Point mutations may concern changes of one base or one base pair, and may result in stop codons, frameshift mutations or amino acid substitutions. Somatic mutations are those which occur only in certain cells or tissues of the plant and are not inherited to the next generation. Germline mutations can be found in any cell of the plant and are inherited. With reference to FIG. 1 herein – which presents an overview on how grains of mutated barley may be propagated in a breeding program – grains of generation M3, and directly propagated grains thereof, or of any subsequent generation, including the plants thereof, may be termed "raw mutants". Further, still with reference to FIG. 1 herein, the term "breeding line" refers to grains of generation M4, and any subsequent generation, including the plants thereof, which may be the result of a cross to a cultivar plant, or the result of a cross to another breeding line with a separate, specific trait.

30

The term "null-LOX" refers to the presence of a mutation in a LOX-encoding gene, causing a total loss of function of the encoded LOX enzyme (either LOX-1 or LOX-2). Mutations that generate premature termination (nonsense) codons in a gene encoding LOX represent only one mechanism by which total loss of function of LOX activity can

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be obtained. Molecular approaches to obtain total loss of function of a LOX enzyme comprise the generation of mutations that cause a total absence of transcripts for said enzyme, or mutations that cause total inactivation of the encoded enzyme. "null-LOX" with reference to a plant refers to a plant having a total loss of function of the specified  
5 LOX enzyme.

"Operably linked" is a term used to refer to the association of two or more nucleic acid fragments on a single polynucleotide, so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is  
10 capable of affecting the expression of that coding sequence, i.e. that the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

"PCR" or "polymerase chain reaction" is well known by those skilled in the art as a  
15 technique used for the amplification of specific DNA segments (U.S. Pat. Nos. 4,683,195 and 4,800,159 to Mullis, K.B. et al.).

"Plant" or "plant material" includes plant cells, plant protoplasts, and plant cell tissue cultures from which barley plants can be regenerated – including plant calli, and plant  
20 cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, kernels, leaves, roots, root tips, anthers, or any part or product of a plant.

By the term "plant product" is meant a product resulting from the processing of a plant or plant portion. Said plant product may thus, for example, be malt, wort, a fermented  
25 or non-fermented beverage, a food, or a feed product.

As used herein, "recombinant" in reference to a protein is a protein that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition by deliberate human intervention.  
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A "specialist beer taste panel" within the meaning of the present application is a panel of specialists extensively trained in tasting and describing beer flavors, with special focus on aldehydes, papery taste, and old taste. Although a number of analytical tools exist for evaluating flavor components, the relative significance of flavor-active  
35 components are difficult to assess analytically. However, such complex properties can

be evaluated by taste specialists. Their continuous training includes tasting and evaluation of standard beer samples.

By the term "splice site" is meant the boundaries between exons and introns of a gene. Thus, a splice site may be the border going from exon to intron (called a "donor site"), or the border separating intron from exon (denoted "acceptor site"). A splice site in plants typically comprises consensus sequences. The 5' end of an intron, in general, consists of a conserved GT dinucleotide (GU in the mRNA), and the 3' end of an intron usually consists of a conserved dinucleotide AG. The 5' splice site of an intron thus comprises the 5' end of an intron, and the 3' splice site comprises the 3' end of an intron. Preferably, within the context of the present invention, the splice site of an intron is either the 5' splice site consisting of the most 5' dinucleotides of the intron (which in general is GT), or the 3' splice site consisting of the most 3' dinucleotides of the intron (which in general is AG).

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Unless otherwise noted, "T2N" means trans-2-nonenal (T2N) in the free form. T2N is sometimes also referred to as 2-E-nonenal.

By the term "T2N potential" is described the chemical substances which have the capacity to release T2N, or be converted into T2N, in one or more reactions. In the present context, the T2N potential is defined as the concentration of T2N released into a solution, e.g. wort or beer, during incubation for 2 h at 100°C, pH 4.0. In practical terms, the starting T2N concentration is determined, after which the solution is incubated for 2 h at 100°C, pH 4.0, followed by determination of the T2N concentration. The difference between the starting and the end T2N concentration is denoted the T2N potential. The thermal, acidic treatment causes liberation of T2N from the T2N potential, e.g. from "T2N adducts", the latter term used to describe T2N conjugated to one or more substances, including, but not limited to protein(s), sulfite, cellular debris, cell walls, or the like. In general, T2N adducts per se are not sensed by humans as off-flavors. However, T2N released from said T2N adducts may give rise to an off-flavor.

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"Tissue culture" indicates a composition comprising isolated cells of the same or different types, or a collection of such cells organized into parts of a plant – including, for example, protoplasts, calli, embryos, pollen, anthers, and the like.

"Transformation" means introducing DNA into an organism so that the DNA is maintained, either as an extrachromosomal element (without integration and stable inheritance), or as a chromosomal integrant (genetically stable inheritance). Unless otherwise stated, the method used herein for transformation of *E. coli* was the  $\text{CaCl}_2$ -based method (Sambrook and Russel, *supra*). For transformation of barley, Agrobacterium-mediated transformation may be performed – preferentially as described by either Tingay et al. (1997), or Wang et al. (2001), except that alternative cultivars may be used as host.

10 A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein, "transgenic" includes reference to a cell that has been modified by the introduction of a heterologous nucleic acid, or that the cell is derived from a cell so modified. Thus, for example, transgenic cells express genes that are not found in an identical form within the native form of the cell, or express native genes that are otherwise abnormally expressed, under-expressed, or not expressed at all as a result of deliberate human intervention. The term "transgenic" as used herein in reference to plants, particularly barley plants, does not encompass the alteration of the cell by methods of traditional plant breeding – e.g.  $\text{NaN}_3$ -based mutagenesis, or by naturally occurring events without deliberate human invention.

"Wild barley", *Hordeum vulgare* ssp. *spontaneum*, is considered the progenitor of contemporary cultivated forms of barley. The transition of barley from a wild to a cultivated state is thought to have coincided with domestication of the plant into "barley landraces". These are genetically more closely related to modern cultivars than wild barley.

The term "wild-type" barley refers to a conventionally generated barley plant. Preferably, the term refers to the barley plant from which the barley plants of the instant invention have been derived, i.e. the parental plants. Wild-type barley kernels are generally available from, for example, seed companies as "cultivars" or "varieties" – i.e. those genetically similar kernels that are listed by national plant breeding organizations. Despite the availability of several null-LOX-1 barley cultivars (e.g. cvs. Chamonix and Charmay), but for the purpose of a better understanding of the instant

invention, all null-LOX-1, null-LOX-2, and double null-LOX plants are herein considered mutant plants, and not wild-type plants. The notations "cultivar" and "variety" are used interchangeably herein.

5 By the term "wort" is meant a liquid extract of malt, such as milled malt, or green malt, or milled green malt. In barley brewing, wort may also be prepared by incubating an extract of un-malted barley with an enzyme mixture that hydrolyzes the barley components. In addition to said malt or barley-derived extracts, the liquid extract may be prepared from malt and additional components, such as additional starch-containing  
10 material partly converted into fermentable sugars. The wort is in general obtained by mashing, optionally followed by "sparging", in a process of extracting residual sugars and other compounds from spent grains after mashing with hot water. Sparging is typically conducted in a lauter tun, a mash filter, or another apparatus to allow separation of the extracted water from spent grains. The wort obtained after mashing is  
15 generally referred to as "first wort", while the wort obtained after sparging is generally referred to as the "second wort". If not specified, the term wort may be first wort, second wort, or a combination of both. During beer production, wort is generally boiled together with hops. Wort, which has not been boiled with hops, may also be referred to as "sweet wort", whereas wort boiled with hops may be referred to as "boiled wort".

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### **Barley plant**

Barley is a family of plants. "Wild barley", *Hordeum vulgare* ssp. *spontaneum*, is  
25 considered the progenitor of today's cultivated forms of barley. The transition of barley from a wild to a cultivated state is thought to have coincided with a radical change of allele frequencies at numerous loci. Rare alleles and new mutational events were positively selected for by the farmers who quickly established the new traits in the domesticated plant populations, denoted "barley landraces". These are genetically  
30 more closely related to modern cultivars than wild barley. Until the late nineteenth century, barley landraces existed as highly heterogeneous mixtures of inbred lines and hybrid segregates, including few plants derived from random crossings in earlier generations. Most of the landraces have been displaced in advanced agricultures by pure line cultivars. Intermediate or high levels of genetic diversity characterize the  
35 remaining landraces. Initially, "modern barley" cultivars represented selections from

landraces. These were later derived from successive cycles of crosses between established pure lines, such as those of diverse geographical origins. Eventually, the result was a marked narrowing of the genetic base in many, probably all, advanced agricultures. Compared with landraces, modern barley cultivars have numerous improved properties (Nevo, 1992; von Bothmer et al., 1992), for example one or more, but not limited to the following:

- (i) Covered and naked kernels;
- (ii) Seed dormancy;
- 10 (iii) Disease resistance;
- (iv) Environmental tolerance (for example to drought or soil pH);
- (v) Proportions of lysine and other amino acids;
- (vi) Protein content;
- (vii) Nitrogen content;
- 15 (viii) Carbohydrate composition;
- (ix) Hordein content and composition;
- (x) (1-3,1-4)- $\beta$ -Glucan and arabinoxylan content;
- (xi) Yield
- (xii) Straw stiffness;
- 20 (xiii) Plant height.

Within the present invention, the term "barley plant" comprises any barley plant. Thus, the invention relates to any barley plant comprising a first mutation resulting in a total loss of function of LOX-1 activity, and a second mutation resulting in a total loss of function of LOX-2 activity. Thus the invention relates to any barley plant comprising a first mutation that results in a total loss of functional LOX-1 enzyme and a second mutation resulting in a total loss of functional LOX-2 enzyme.

However, preferred barley plants for use with the present invention are modern barley cultivars or pure lines. The barley cultivar to be used with the present invention may, for example, be selected from the group consisting of Sebastian, Celeste, Lux, Prestige, Saloon, Neruda, Harrington, Klages, Manley, Schooner, Stirling, Clipper, Franklin, Alexis, Blenheim, Ariel, Lenka, Maresi, Steffi, Gimpel, Cheri, Krona, Camargue, Chariot, Derkado, Prisma, Union, Beka, Kym, Asahi 5, KOU A, Swan Hals, Kanto Nakate Gold, Hakata No. 2, Kirin – choku No. 1, Kanto late Variety Gold, Fuji Nijo, New

Golden, Satukio Nijo, Seijo No. 17, Akagi Nijo, Azuma Golden, Amagi Nijpo, Nishino Gold, Misato golden, Haruna Nijo, Scarlett and Jersey preferably from the group consisting of Haruna Nijo, Sebastian, Celeste, Lux, Prestige, Saloon, Neruda and Power, preferably from the group consisting of Harrington, Klages, Manley, Schooner, 5 Stirling, Clipper, Franklin, Alexis, Blenheim, Ariel, Lenka, Maresi, Steffi, Gimpel, Cheri, Krona, Camargue, Chariot, Derkado, Prisma, Union, Beka, Kym, Asahi 5, KOU A, Swan Hals, Kanto Nakate Gold, Hakata No. 2, Kirin – choku No. 1, Kanto late Variety Gold, Fuji Nijo, New Golden, Satukio Nijo, Seijo No. 17, Akagi Nijo, Azuma Golden, Amagi Nijpo, Nishino Gold, Misato golden, Haruna Nijo, Scarlett and Jersey preferably 10 from the group consisting of Haruna Nijo, Sebastian, Tangent, Lux, Prestige, Saloon, Neruda, Power, Quench, NFC Tipple, Barke, Class and Vintage.

In one embodiment of the invention, the barley plant is accordingly a modern barley cultivar (preferably a cultivar selected from the group of barley cultivars described 15 herein above) comprising a first mutation resulting in a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of function of LOX-2 activity, such as total loss of functional LOX-2 enzyme. In this embodiment, it is thus preferred that the barley plant is not a barley landrace.

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The barley plant may be in any suitable form. For example, the barley plant according to the invention may be a viable barley plant, a dried plant, a homogenized plant, or a milled barley kernel. The plant may be a mature plant, an embryo, a germinated kernel, a malted kernel, a milled malted kernel, or the like.

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Parts of barley plants may be any suitable part of the plant, such as kernels, embryos, leaves, stems, roots, flowers, or fractions thereof. A fraction may, for example, be a section of a kernel, embryo, leaf, stem, root, or flower. Parts of barley plants may also be a fraction of a homogenate, or a fraction of a milled barley plant or kernel.

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In one embodiment of the invention, parts of barley plants may be cells of said barley plant, preferably viable cells that may be propagated in vitro in tissue cultures. In particular, said cells in one embodiment may be cells that are not capable of maturing into an entire barley plant, i.e. cells that are not a reproductive material.

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**Loss of function of LOX activity**

The present invention relates to barley plants – or part thereof, or plant products thereof – having a first and a second mutation, wherein the first mutation leads to a total loss of function of LOX-1 activity, and the second mutation leads to a total loss of function of LOX-2 activity. Thus, for example, the first mutation leads to a total loss of functional LOX-1 enzyme, and the second mutation leads to a total loss of functional LOX-2 enzyme.

The total loss of function of LOX-1 activity (such as the total loss of functional LOX-1 enzyme, and the total loss of function of LOX-2 activity (such as the total loss of functional LOX-2 enzyme), may independently be based on different mechanisms. For example, the total loss of function of one or both of LOX-1 activity and LOX-2 activity may be caused by malfunctioning proteins in the barley plant, i.e. a malfunctioning LOX-1 and/or LOX-2 protein, such as a mutated LOX-1 protein with no detectable 9-HPODE-forming activity (for example determined as described in Example 4), and/or a mutated LOX-2 protein with no detectable 13-HPODE forming activity (for example determined as described in Example 4).

The total loss of function of one or both of LOX-1 activity and LOX-2 activity may be caused by the lack of LOX-1 and/or LOX-2 protein. It is apparent that lack of LOX-1 protein will lead to loss of functional LOX-1 enzyme, and that lack of LOX-2 protein will lead to total loss of functional LOX-2 enzyme. Thus, the barley plant may preferably comprise no – or only very little, more preferably no detectable – LOX-1 and/or LOX-2 protein. The LOX-1 and/or LOX-2 protein may be detected by any suitable means known to the person skilled in the art. Preferably, however, the protein(s) is detected by techniques wherein LOX-1 protein is detected by specific antibodies to LOX-1 and LOX-2, such as polyclonal antibodies to LOX-1 and LOX-2. Said techniques may, for example, be Western blotting or ELISA. Said antibodies may be monoclonal or polyclonal. Preferably, however, said antibodies are of a polyclonal nature, recognizing several different epitopes within the LOX-1 and LOX-2 protein, respectively. LOX-1 and/or LOX-2 protein may also be detected indirectly, for example, by methods determining LOX-1 activity, or by methods determining LOX-2 activity. In one preferred embodiment of the invention, LOX-1 protein is detected using the methods outlined in



Example 4 of the international patent application WO 2005/087934. LOX-2 protein may be detected in a similar manner, using antibodies binding to LOX-2.

5 The total loss of function of one or both of LOX-1 activity and LOX-2 activity may also be a result of no, or very little, preferably no expression of a LOX-1 transcript and/or a LOX-2 transcript. The skilled person will acknowledge that the absence of a LOX-1 or a LOX-2 transcript also will result in the absence of translated LOX-1 or LOX-2 protein, respectively. Alternatively, the total loss of function of one or both of LOX-1 activity and LOX-2 activity (e.g. the total loss of functional LOX-1 and LOX-2 enzymes), may also  
10 be a result expression of an aberrant LOX-1 transcript and/or an aberrant LOX-2 transcript. An aberrant LOX-1 and/or LOX-2 transcript may be caused by aberrant splicing of the transcript, for example, due to a mutation in a splice site. Thus, the barley plants of the invention may carry a mutation in the a splice site, such as a 5' splice site or a 3' splice site, for example in one or the two most 5' nucleotides of an  
15 intron, or in one of the most 3' nucleotides of an intron. An example of a mutant with an aberrant splicing of the LOX-1 transcript is described as mutant A618 in WO 2005/087934. Expression of transcripts encoding LOX-1 or LOX-2 may, for example, be detected by Northern blotting or RT-PCR experiments.

20 The total loss of functional LOX-1 and LOX-2 enzymes of the barley plants of the present invention is caused by mutations. Thus, the barley plants of the present invention in general carry a mutation in the LOX-1 gene. Said mutation may be in the regulatory regions, for example within the promoter or introns, or said mutation may be in the coding region. Similarly, the barley plants of the present invention in general  
25 carry a mutation in the LOX-2 gene. Said mutation may be in the regulatory regions, for example within the promoter or introns, or said mutation may be in the coding region. Thus, the cause of the total loss of functional LOX-1 and/or LOX-2 enzymes may also be detected by the identification of mutations in the gene encoding LOX-1, or in the gene encoding LOX-2. Mutations in the gene encoding LOX-1 may, for example, be  
30 detected by sequencing said gene. Preferably, after identifying a mutation, the total loss of function is confirmed by testing for LOX-1 and/or LOX-2 activities.

The term "LOX-1 protein" is meant to cover the full-length LOX-1 protein of barley as set forth in SEQ ID NO:3 of WO 2005/087934, or in SEQ ID NO:7 of WO 2005/087934,  
35 or a functional homolog thereof. The active site of LOX-1 is situated in the C-terminal

part of the enzyme. In particular, it is anticipated that the region spanning amino acid residues 520-862, or parts thereof, are relevant for LOX-1 activity. Accordingly, in one embodiment, null-LOX-1 barley preferably comprises a gene that encodes a mutated form of LOX-1, lacking some or all of amino acids 520-862 of LOX-1. Said mutated  
5 LOX-1 may also lack other amino acid residues, which are present in wild-type LOX-1.

Accordingly, double null-LOX barley of the invention may comprise a truncated form of LOX-1, which is not functional – such as an N- or a C-terminal truncated form. Preferably, said truncated form comprises no more than 800, more preferably no more  
10 than 750, even more preferably no more than 700, yet more preferably no more than 690, even more preferably no more than 680, yet more preferably no more than 670 consecutive amino acids of LOX-1, such as no more than 665, for example no more than 650, such as no more than 600, for example no more than 550, such as no more than 500, for example no more than 450, such as no more than 425, for example no  
15 more than 399 consecutive amino acids of LOX-1 of SEQ ID NO:3 of WO 2005/087934. Preferably, said truncated form comprises only an N-terminal fragment of LOX-1, preferably at the most the 800, more preferably at the most the 750, even more preferably at the most the 700, yet more preferably at the most the 690, even more preferably at the most the 680, yet more preferably at the most the  
20 670, even more preferably at the most the 665 N-terminal amino acids of SEQ ID NO:3 of WO 2005/087934, such as no more than 665, for example no more than 650, such as no more than 600, for example at the most the 550, such as at the most the 500, for example at the most the 450, such as at the most the 425, for example at the most the 399 N-terminal amino acids of SEQ ID NO:3 of WO 2005/087934.

25 In one very preferred embodiment, the truncated form may consist of amino acids 1–665 of SEQ ID NO:3 of WO 2005/087934.

In a preferred embodiment of the invention, the barley plant of the invention comprises  
30 a LOX-1–encoding gene that is transcribed into mRNA, which comprises a nonsense codon or a stop codon upstream of the stop codon of wild-type LOX-1 mRNA. Such a nonsense codon is herein denoted a premature nonsense codon. Preferably, all LOX-1–encoding genes transcribed into mRNA of said plant comprise a premature nonsense codon or a stop codon. The nonsense codon or stop codon is preferably  
35 situated at the most 800, more preferably at the most the 750, even more preferably at

the most the 700, yet more preferably at the most the 690, even more preferably at the most the 680, yet more preferably at the most the 670, even more preferably at the most the 665 codons downstream of the start codon. The sequence of wild-type genomic DNA encoding LOX-1 is given in SEQ ID NO:1 of WO 2005/087934 or SEQ ID NO:5 of WO 2005/087934.

In one preferred embodiment, the barley plant of the invention comprises a gene encoding LOX-1, wherein the pre-mRNA transcribed from said gene comprises the sequence corresponding to SEQ ID NO:2 of WO 2005/087934.

In a very preferred embodiment of the invention, the gene encoding mutant LOX-1 of the double null-LOX barley plant according to the invention comprises a nonsense mutation, said mutation corresponding to a G→A substitution at position 3574 of SEQ ID NO:1 of WO 2005/087934.

The term "LOX-2 protein" is meant to cover the full-length LOX-2 protein of barley as set forth in SEQ ID NO:5 of the instant publication, or a functional homolog thereof. The active site of LOX-2 is situated in the C-terminal part of LOX-2. In particular, it is anticipated that the region spanning amino acid residues 515-717, or parts thereof, are relevant for LOX-2 activity. Based on an examination of the soybean LOX-1 crystal structure, anticipated sequence stretches of the active site cleft of the LOX-2 enzyme of barley are represented by amino acid residues 515-525 and 707-717. A translated, mutated LOX-2 protein, i.e. a C-terminally truncated form of LOX-2 of barley double null-LOX mutant A689 contains max. 684 residues, and will therefore lack the second sequence stretch of the active site cleft – making it inactive. According to one embodiment of the invention, double null-LOX barley of the invention preferably comprises a gene encoding a mutant form of LOX-2 that lacks some, or all, of amino acids 515-717 of LOX-2, preferably lacking some or all of amino acids 707 to 717, even more preferably lacking all of amino acids 707-717. Said mutant LOX-2 may also lack other amino acid residues, which are present in wild-type LOX-2.

Accordingly, double null-LOX barley may comprise a truncated form of LOX-2, which is not functional, such as an N-terminal or a C-terminal truncated form. Preferably, said truncated form comprises no more than 800, more preferably no more than 750, even more preferably no more than 725, yet more preferably no more than 700, even more

preferably no more than 690, yet more preferably no more than 684 consecutive amino acids of LOX-2 of SEQ ID NO:5 of the instant publication. Preferably, said truncated form comprises only an N-terminal fragment of LOX-2. Hence, preferably said truncated form comprises at the most the 800, more preferably at the most the 750, even more preferably at the most the 725, yet more preferably at the most the 700, even more preferably at the most the 690, yet more preferably at the most the 684 N-terminal amino acids of SEQ ID NO:5 of the instant publication.

In one very preferred embodiment, the truncated form may consist of amino acids 1–684 of SEQ ID NO:5 of the instant publication.

In a preferred embodiment of the invention, the barley plant comprises a gene transcribed into mRNA for LOX-2, wherein said mRNA comprises a nonsense codon or a stop codon upstream of the stop codon of wild-type LOX-2 mRNA. Such a nonsense codon is herein designated a premature nonsense codon. Preferably all genes transcribed into mRNA encoding LOX-2 of said plant comprise a premature nonsense codon or a stop codon. The nonsense codon or stop codon is preferably situated at the most 800, more preferably at the most the 750, even more preferably at the most the 725, yet more preferably at the most the 700, even more preferably at the most the 690, yet more preferably at the most the 684 codons downstream of the start codon. The sequence of wild-type genomic DNA encoding LOX-2 is given in SEQ ID NO:1 of the instant publication.

In a very preferred embodiment of the invention, the gene encoding mutated LOX-2 of the double null-LOX barley plant comprises a nonsense mutation, said mutation corresponding to a G→A substitution at position 2689 of SEQ ID NO:1.

The barley plant according to the invention may be prepared by any suitable method known to the person skilled in the art, preferably by one of the methods outlined herein below in the section "Preparing double null-LOX barley".

In one embodiment of the invention, it is preferred that the double null-LOX barley plant according to the present invention has plant growth physiology and grain development similar to that of wild-type barley. It is hence preferred that the null-LOX-1 barley plant

is similar to wild-type barley with respect to plant height, number of tillers per plant, onset of flowering, and/or number of grains per spike.

Also, it is preferred that the double null-LOX barley plant according to the present invention is similar to wild-type barley, in particular similar to cv. Barke with respect to plant height, heading date, disease resistance, lodging, ear-breakage, maturation time, and yield. In the present context, "similar" is to be understood as the same  $\pm 10\%$  in case of numbers. These parameters may be determined as described hereinafter in Example 5.

In a very preferred embodiment of the invention, the barley plant is the barley plant wherein seeds thereof have been deposited 4 December 2008 under the name "Barley, *Hordeum vulgare* L.; Line A689" with American Type Culture Collection (ATCC), Patent Depository, 10801 University Blvd., Manassas, VA 20110, United States (deposit number PTA-9640). Thus, the barley plant of the invention may be barley Line A689 (ATCC Patent Deposit Designation: PTA-9640), or any progeny barley plant thereof.

## **Preparing barley mutants**

The barley plant according to the invention may be prepared by any suitable method known to the person skilled in the art. Preferably, the barley plant of the invention is prepared by a method comprising the steps of mutagenizing barley plants or parts thereof, for example barley kernels, followed by screening and selecting barley plants characterized by a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme and a total loss of function of LOX-2 activity, such as total loss of functional LOX-2 enzyme. In one preferred embodiment, the barley plant may be prepared by a method involving mutagenizing barley plants, or parts thereof – for example barley kernels, wherein said barley plants already carry a mutation causing a total loss of functional LOX-1 enzyme. Such barley plants are, for example, described in international patent application WO 2005/087934.

In one interesting aspect, the present invention relates to a new and very efficient screening method that allows the identification of a barley plant carrying a mutation,

which causes total loss of LOX-2 activity. The new, reproducible screening method allows the identification of barley plants with no or very little LOX-2 activity. This new screening method includes the use of germinated embryos as starting material.

5 Interestingly, the present inventors have found that the use of mature embryos as starting material for a screening for LOX-2 activity is less preferable, based on the screening of as many as 21,000 mature embryos, which did not reveal a single null-LOX-2 barley mutant.

10 Thus, one important feature of the new screening method concerns the utilization of germinated embryos as starting material for detection of LOX-2 activity.

Accordingly, it is an objective of the present invention to provide methods of preparing a double null-LOX barley plant comprising the steps of:

- 15 (i) Providing a barley plant, or parts thereof, with a total loss of function of LOX-1 activity, such as total loss of functional LOX-1 enzyme; and
- (ii) Mutagenizing said barley plant, and/or barley cells, and/or barley tissue, and/or barley kernels, and/or barley embryos from said barley plant, thereby obtaining generation M0 barley; and
- 20 (iii) Breeding said mutagenized barley plants, kernels, and/or embryos for at least 2 generations, thereby obtaining generation Mx barley plants, wherein x is an integer  $\geq 2$ ; and
- (iv) Obtaining embryos from said Mx barley plants; and
- (v) Germinating said embryos; and
- 25 (vi) Determining the LOX-1 and LOX-2 activities in said germinated embryos, or parts thereof; and
- (vii) Selecting plants with a total loss of LOX-1 activity and LOX-2 activity in the germinated embryos; and
- (viii) Analyzing for a mutation in the LOX-1 gene and in the LOX-2 gene; and
- 30 (ix) Selecting plants carrying a mutation in the LOX-1 gene and the LOX-2 gene;

thereby obtaining a barley plant carrying mutations in the genes for LOX-1 and LOX-2, causing a total loss of LOX-1 activity and a total loss of LOX-2 activity.

The aforementioned barley plant with a total loss of LOX-1 activity may, for example, be any of the barley plants with a total loss of LOX-1 activity described in WO 2005/087934, preferably mutant D112, or progeny plants thereof.

- 5        Step (ii) in the aforementioned list may involve mutagenizing living material selected from the group consisting of barley plants, barley cells, barley tissue, barley kernels, and barley embryos – preferably selected from the group consisting of barley plants, barley kernels, and barley embryos, more preferably barley kernels.
- 10       Mutagenesis may be performed by any suitable method. In one embodiment, mutagenesis is performed by incubating a barley plant, or a part thereof – for example barley kernels or individual cells from barley – with a mutagenizing agent. Said agent is known to the person skilled in the art, including, for example, but not limited to, sodium azide ( $\text{NaN}_3$ ), ethyl methanesulfonate (EMS), azidoglycerol (AG, 3-azido-1,2-
- 15       propanediol), methyl nitrosourea (MNU), and maleic hydrazide (MH).

In another embodiment, mutagenesis is performed by irradiating, for example by UV, a barley plant or a part thereof, such as the kernel. In preferred embodiments of the invention, the mutagenesis is performed according to any of the methods outlined

20       herein below in the section "Chemical mutagenesis." A non-limiting example of a suitable mutagenesis protocol is given in Example 2.

It is preferred that the mutagenesis is performed in a manner such that the expected frequency of desired mutants is at least 0.5, such as in the range of 0.5 to 5, for

25       example in the range of 0.9 to 2.3 per 10,000 grains, when screening barley of generation M3. In a preferred embodiment, mutagenesis is performed on barley kernels. The kernels applied to the mutagen are designated as generation M0 (see also FIG. 1).

30       The LOX activity may be determined in a sample from a germinating barley embryo, preferably in a liquid extract from a germinating barley embryo. Said sample, such as said extract may be prepared from any suitable part of said germinating embryo. In general, the barley sample must be homogenized using any suitable method prior to preparation of an extract of said sample and determination of LOX-2 activity. In

35       particular, it is preferred that a protein extract is prepared from the germinating embryo,

or part thereof, and that the LOX activity is determined using said protein extract.

Homogenization may, for example, be performed using mechanical forces, for example by shaking or stirring, such as by shaking in the presence of a bead, such as a glass or a sand bead.

5

In a preferred embodiment, the germinating embryo is of generation Mx, wherein x is an integer  $\geq 2$ ; preferably x is an integer in the range of 2 to 10, more preferably in the range of 3 to 8. In a very preferred embodiment, LOX activity is determined in germinating embryos of generation M3, or a sample derived from such embryos. In that  
10 embodiment, it is preferred that mutagenized barley kernels of generation M0 are grown to obtain barley plants, which are crossed to obtain kernels of generation M1. The procedure is repeated until kernels of generation M3 are available (see also FIG. 1).

15 Determination of LOX activity may be carried out using any suitable assay, preferably by one of the methods outlined hereinafter. In particular, it is preferred that the assay provides data on the dioxygenation of linoleic acid to 9-HPODE and 13-HPODE by LOX-1 and LOX-2. In general, assaying will therefore involve the steps of:

- 20
- (i) Providing a protein extract prepared from a germinated barley embryo or part thereof; and
  - (ii) Providing linoleic acid; and
  - (iii) Incubating said protein extract with said linoleic acid; and
  - (iv) Detecting dioxygenation of linoleic acid to 9-HPODE and 13-HPODE.

25

Step (iv) of the method preferably comprises determining the level of 9-HPODE and 13-HPODE in said germinating embryos, preferably in a protein extract prepared from said germinating embryos. The step may comprise a direct or an indirect determination of the levels of 9-HPODE and 13-HPODE. The total level of all HPODEs may be  
30 determined, in which case it is preferred that specific measurements of 9-HPODE and 13-HPODE are performed for confirmation. One method could, for example, be a method wherein protein extracts from germinating embryos are incubated with linoleic acid as substrate for formation of 9-HPODE and 13-HPODE. Said HPODEs can then be detected by various methods. One method may involve generation of a detectable  
35 compound, such as a dye. For example the method may be the oxidative coupling of



3-dimethylaminobenzoic acid and 3-methyl-2-benzothiazolinone hydrazone in the presence of hemoglobin, catalyzed by the formed HPODEs to form the indamine dye, which can be measured at  $A_{595}$  using a spectrophotometer. An example of such a method is described in Examples 1 and 2 hereinafter. Using this assay, an absorption reading of less than 0.2  $A_{595}$  unit is considered as indicative of the absence of LOX-1 and the absence of LOX-2 activities. However, a more precise method for determining LOX-1 and LOX-2 activities is to incubate a protein extract from germinating embryos with linoleic acid, followed by determination of 9-HPODE and 13-HPODE contents. 9-HPODE and 13-HPODE contents may, for example, be determined using HPLC-based analysis.

Dioxygenation of linoleic acid to 9-HPODE and 13-HPODEs may be measured directly or indirectly. Any suitable detection method may be used with the present invention. In one embodiment of the invention, linoleic acid hydroperoxides are detected. 9-HPODE and 13-HPODE may be detected directly, for example, by chromatographic methods, such as HPLC as described in Example 4.

The present invention discloses that certain aspects of the procedure for extraction of protein from the germinating embryo is of great importance. Thus, it is preferred that the protein is extracted using an acidic buffer, preferably a buffer with a pH in the range of 2 to 6, more preferably in the range of 3 to 5, even more preferably in the range of 3.5 to 5, yet more preferably in the range of 4 to 5, even more preferably a pH of 4.5. The buffer used for extraction is preferably based on an organic acid, more preferably a lactic acid buffer. Most preferably, the protein extract is prepared using a 100-mM lactic acid buffer, pH 4.5.

Certain embodiments of the present invention disclose methods for detection of null-LOX-1 and null-LOX-2 plants that involve reaction of 9-HPODE and 13-HPODE with a dye, e.g. 3-methyl-2-benzothiazolinone hydrazone. Preferably, said dye, e.g. 3-methyl-2-benzothiazolinone hydrazone, is added to the protein extract after addition of linoleic acid. Preferably, the dye is added at least 1 min, more preferably at least 5 min, even more preferably at least 10 min, such as in the range of 1 to 60 min, for example in the range of 5 to 30 min, such as in the range of 10 to 20 min after contacting the protein extract with the linoleic acid.

Preferred methods for selecting barley plants according to the invention are detailed hereinafter in Example 2.

5 The selection procedure may be adjusted for microtitre plate-based assay procedures, or other known repetitive, high-throughput assay formats to allow rapid screening of many samples. It is preferred that at least 5000, such as at least 7500, for example at least 10,000, such as at least 15,000, for example at least 20,000, such as at least 25,000 mutagenized barley plants are analyzed for LOX-1 and LOX-2 activities.

10 Determination of a mutation in the gene encoding LOX-1 may be performed by several different methods. For example, the LOX-1 gene may be sequenced completely or partly, and the sequence compared to SEQ ID NO:1 of WO 2005/087934 or SEQ ID NO:5 of WO 2005/087934. If searching for a specific mutation, SNP analysis may be applied. The skilled person will be able to design useful primers for detection of a given  
15 specific mutation, such as one leading to a premature stop codon in the coding sequence for LOX-1 (e.g. any of the premature stop codons described hereinabove). One example of how to perform a SNP analysis is described in Example 10 hereinbelow, with primers that are useful for detecting a G→A mutation at nucleotide position 3474 of the LOX-1 gene.

20 Determination of a mutation in the gene encoding LOX-2 may be performed by several different methods. For example, the LOX-2 gene may be sequenced completely or partly, and the sequence compared to SEQ ID NO:1 of the instant publication. If searching for a specific mutation, SNP analysis may be used. The skilled person will be  
25 able to design useful primers for detection of a given specific mutation, such as one leading to a premature stop codon in the LOX-2 coding sequence (e.g. any of the premature stop codons described hereinabove). An example of how to perform a SNP analysis is described in Example 10 herein below, as are primers useful for detecting a G→A mutation at nucleotide position 2689 of the gene for LOX-2.

30 It is also comprised within the present invention that steps (viii) and (ix) of the method of preparing a double null-LOX barley plant, as detailed in this section hereinabove, may be performed prior to steps (vi) and (vii), in which case the method will comprise the steps (i), (ii), (iii), (iv), (v), (viii), (ix), (vi), and (vii) in that order. In particular, this

could be the case when searching for a specific mutation, for example in progeny plants of already identified double null-LOX barley plants.

Once a double null-LOX barley plant has been identified, which contains a particular mutation in a LOX-1 gene and a particular mutation in the LOX-2 gene (such as any of the above-mentioned mutations), additional barley plants with the identical mutations may be generated by conventional breeding methods, such as those well known to the skilled person. For example, said double null-LOX barley plant may be backcrossed with another barley cultivar.

Subsequent to the selection of useful barley plants with total loss of LOX-1 and LOX-2 functions, one or more additional screenings may optionally be performed. For example, selected mutants may be further propagated, and plants of new generations may be tested for the total loss of functional LOX-1 and LOX-2 enzymes.

Subsequent to the selection of useful barley plants, these may be subjected to breeding, such as conventional breeding. Methods of breeding are described herein below in the section "Plant breeding".

### **Plant products**

The present invention relates to beverages, or other plant products, with low levels of T2N potential, prepared from double null-LOX barley plants, or parts thereof.

The present invention thus relates to plant products, which may be compositions comprising the above-described barley plants, or parts thereof, or compositions prepared from said barley plants, or parts thereof, such as plant products prepared from said barley plants, or parts thereof. Because said barley plants lack LOX-1 and LOX-2 activities, the compositions in general comprise very low levels of T2N potential. Examples of useful compositions comprising, or prepared from, barley plants having a first mutation resulting in a total loss of function of LOX-1 activity, such as a total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of function of LOX-2 activity, such as total loss of functional LOX-2 enzyme, are described herein below.

It is preferred that said compositions comprise:

- 5 (i) Less than 60%, even more preferably less than 50%, yet more preferably less than 40%, such as less than 30%, preferably less than 20%, more preferably less than 10%, free T2N; and/or
- (ii) Less than 60%, even more preferably less than 50%, yet more preferably less than 40%, such as less than 30%, preferably less than 25% T2N potential;

10

compared to a similar composition prepared in the same manner from wild-type barley plants, preferably from cv. Power. The specific reduction in T2N may differ depending on the type of composition. Aforementioned reductions in T2N are particularly relevant for compositions, wherein the composition is selected from the group consisting of malt, wort and aged beverages.

15

In addition, it is preferred that said compositions comprise:

- 20 (i) less than 80%, preferably less than 70%, even more preferably less than 60%, such as less than 50% free T2N; and/or
- (ii) less than 80%, preferably less than 70%, even more preferably less than 60%, such as less than 50% T2N potential;

25 compared to a similar composition prepared in the same manner from the barley mutant D112 described in WO 2005/087934.

30 The present invention relates in one aspect to barley kernels having a first mutation that results in a total loss of function of LOX-1 activity (such as total loss of functional LOX-1 enzyme), and a second mutation resulting in a total loss of function of LOX-2 activity (such as total loss of functional LOX-2 enzyme). The present invention also relates to compositions comprising said kernels, and compositions prepared from said kernels, as well as to plant products prepared from said kernels.

It has been described that lipoxygenase activity in barley kernels may be reduced by a soaking process, wherein barley may be subjected to high temperatures and/or lactic acid treatment. Such treatment may have other adverse effects, such as reducing desirable enzymatic activities, e.g. phytase activity. In addition, such treatment only  
5 reduces lipoxygenase activity from the point when the heat treatment is undertaken and thus it does not affect the prior accumulation of lipoxygenase products.

Accordingly, in one embodiment the plant products according to the invention are prepared using a method, wherein the barley kernels are not subjected to soaking at a  
10 temperature of at least 70°C. It is also preferred that the plant products according to the invention are prepared using a method, wherein the barley kernels are not subjected to soaking at a temperature of at least 57°C in the presence of lactic acid.

In one aspect, the invention relates to malt compositions prepared from double null-  
15 LOX kernels by malting. By the term "malting" is to be understood germination of steeped barley kernels taking place under controlled environmental conditions (for example, as illustrated in FIG. 3, steps 2 and 3).

Said malt compositions preferably comprise less than 30%, more preferably less than  
20 20%, even more preferably less than 10% free T2N compared to a malt composition prepared in the same manner from a wild-type barley, preferably from cv. Power. More preferably, said malt compositions comprise less than 60%, more preferably less than 50% free T2N compared to a malt composition prepared in the same manner from the barley null-LOX-1 mutant D112 described in WO 2005/087934. It is furthermore  
25 preferred that said malt compositions comprise less than 60%, preferably less than 50%, more preferably less than 40%, even more preferably less than 30%, more preferably less than 20%, even more preferably less than 10% T2N potential compared to a malt composition prepared in the same manner from a wild-type barley, preferably from cv. Power. More preferably, said malt compositions comprise less than 60%, more  
30 preferably 50% T2N potential compared to a malt composition prepared in the same manner from the barley null-LOX-1 mutant D112 described in WO 2005/087934.

Malting is a process of controlled steeping and germination, followed by drying of the barley grain, preferably kiln drying of the barley grain. Prior to drying, the steeped and germinated barley grains are referred to as "green malt", which may also represent a  
35 plant product according to the present invention. This sequence of events is important

for the synthesis of numerous enzymes that cause grain modification, processes that principally depolymerize cell walls of the dead endosperm to mobilize the grain nutrients and activate other depolymerases. In the subsequent drying process, flavor and color are produced due to chemical browning reactions. Although the primary use  
5 of malt is for beverage production, it can also be utilized in other industrial processes, for example as an enzyme source in the baking industry, or as a flavoring and coloring agent in the food industry, such as malt or malt flour, or indirectly as a malt syrup, etc.

10 In one aspect, the present invention relates to methods of producing said malt composition. The methods preferably comprise the steps of:

- (i) Providing double null-LOX barley kernels;
- (ii) Steeping said kernels;
- (iii) Germinating the steeped kernels under predetermined conditions;
- 15 (iv) Drying said germinated kernels;

thereby producing a malt composition with a total loss of LOX-1 activity and LOX-2 activity. For example, the malt may be produced by any of the methods described by Briggs et al. (1981) and by Hough et al. (1982). However, any other suitable method for  
20 producing malt may also be used with the present invention, such as methods for production of specialty malts, including, but not limited to, methods of roasting the malt. Non-limiting examples are described in Examples 6 and 8.

Malt may be further processed, for example by milling. Thus, the plant product  
25 according to the invention may be any kind of malt, such as unprocessed malt or milled malt, or flour thereof. Milled malt and flour thereof comprise chemical components of the malt and dead cells that lack the capacity to re-germinate.

30 In another aspect, the plant products according to the invention comprise or even consist of syrup, such as a barley syrup, or a barley malt syrup. The plant product may also be an extract of barley or malt.

In another aspect, the invention relates to types of plant products, which are wort compositions prepared from malt compositions derived from double null-LOX kernels.  
35 Said malts may be prepared from only double null-LOX kernels, or mixtures comprising

other kernels as well. The invention also relates to wort compositions prepared using double null-LOX barley, or parts thereof, alone or mixed with other components.

5 Said wort compositions preferably comprise less than 30%, more preferably less than 20%, even more preferably less than 10% free T2N compared to a wort composition prepared in the same manner from a wild-type barley, preferably from cv. Power. More preferably, said wort compositions comprise less than 60%, more preferably less than 50% free T2N compared to a wort composition prepared in the same manner from  
10 barley mutant D112 described in WO 2005/087934. It is furthermore preferred that said wort compositions preferably comprise less than 40%, more preferably less than 30%, even more preferably less than 25% T2N potential compared to a wort composition prepared in the same manner from a wild-type barley, preferably from cv. Power. More preferably, said wort compositions comprise less than 60%, more preferably less than 50% T2N potential compared to a wort composition prepared in the same manner from  
15 barley mutant D112 described in WO 2005/087934.

Said wort may be the first, and/or the second, and/or further worts. The wort composition may be sweet wort, boiled wort, or a mixture thereof. The wort composition may also be barley wort. In general, a wort composition contains a high content of  
20 amino nitrogen and fermentable carbohydrates, the latter mainly being maltose. In FIG. 3, steps 4 to 6 illustrate the common method for preparation of wort from malt. In general, wort is prepared by combining and incubating malt and water, i.e. in a mashing process. During mashing, the malt/liquid composition may be supplemented with additional carbohydrate-rich adjunct compositions, for example milled barley,  
25 maize, or rice adjuncts. Unmalted cereal adjuncts usually contain little or no active enzymes, making it important to supplement with malt or exogenous enzymes to provide enzymes necessary for sugar conversion.

In general, wort production is initiated by the milling of malt such that water may gain  
30 access to grain particles in the mashing phase. Said mashing is basically an extension of the malting process, with enzymatic depolymerization of substrates. During mashing, milled malt is incubated with a liquid fraction, such as water. The incubation temperature is in general either kept constant (isothermal mashing), or gradually increased. In either case, soluble substances produced in malting and mashing are  
35 liberated into said liquid fraction. A subsequent filtration confers separation of wort and

residual solid particles, the latter also denoted "spent grain". Said wort may also be denoted "first wort". After sparging and filtration, a "second wort" may be obtained. Further worts may be prepared by repeating the procedure. Non-limiting examples of suitable procedures for preparation of wort is described by Briggs et al. (supra) and  
5 Hough et al. (supra).

It has been described that lipoxygenase activity may be reduced by heat treatment of the enzymes. It has been described that wort may be heat treated to reduced lipxogenase activity and/or that mashing is performed at high temperatures. However,  
10 in addition to reducing lipoxygenase activity, heat treatment may have other adverse effects, such as reducing other enzymatic activities. In addition, heat treatment only reduces lipoxygenase activity from the point when the heat treatment is undertaken and thus it does not affect the prior accumulation of lipoxygenase products.

15 Accordingly, in one embodiment wort according to the invention is prepared using a method wherein the initial mashing temperature does not exceed 70°C, preferably does not exceed 69°C, thus for example the initial mashing temperature may be in the range of 50°C to 69°C, such as in the range of 55°C to 69°C, for example in the range of 55°C to 65°C. It is also preferred that the wort according to the invention has not been  
20 subjected to temperatures of 70°C or higher for more than 25 min, preferably not for more than 20 min, more preferably not for more than 15 min. during mashing. If the mashing temperatures are too high, it will affect the enzymatic activity in the mash and may reduce, or even abolish, desirable enzymatic activities, which will result in an altered quality of the wort.

25 First, second and further worts may be combined, and thereafter subjected to boiling. The non-boiled wort, either a pure first wort or a combined wort, is also referred to as "sweet wort"; after boiling it may be referred to as "boiled wort". If the wort is to be used in production of beer, hops are frequently added prior to boiling.

30 The wort composition may also be prepared by incubating double null-LOX barley plants, or parts thereof, such as unmalted double null-LOX kernels, in particular milled, unmalted double null-LOX kernels, or parts thereof, with one or more suitable enzymes, such as enzyme compositions or enzyme mixture compositions, for example Cereflo, Ultraflo, or Ondea Pro (Novozymes). A method for producing a beverage from  
35 wort thus prepared may also be referred to as "barley brewing", and a wort composition



thereof as "barley wort", or "barley-brewed" wort. The wort composition may also be prepared using a mixture of malt and unmalted barley plants, or parts thereof, or unmalted barley only, optionally adding one or more suitable enzymes during said preparation, in particular amylases, glucanases [preferably (1-4)- and/or (1-3,1-4)- $\beta$ -glucanase], and/or xylanase (such as arabinoxylanase), and/or proteases, or enzyme mixtures comprising one or more of the aforementioned enzymes, e.g. adding the enzyme mixture Onda Pro (Novozymes).

In one specific embodiment of the invention the wort composition according to the present invention is a barley wort, such as boiled barley wort, i.e. wort prepared by incubating unmalted (and preferably milled) double null-LOX kernels with water, preferably by mashing and sparging. Such barley wort is characterized by extremely low levels of T2N and T2N potential. Thus, said barley wort preferably comprises less than 50%, more preferably less than 40%, even more preferably less than 30% free T2N compared to a barley wort composition prepared in the same manner from a wild-type barley, preferably from cv. Power. More preferably, said wort compositions comprise less than 70%, more preferably less than 60% free T2N compared to a wort composition prepared in the same manner from barley mutant D112 described in WO 2005/087934. Moreover, it is preferred that said barley wort comprises at the most 0.15 ppb free T2N, when said wort is adjusted to a °p in the range of 13 to 16, preferably in the range of 14 to 15, more preferably to 14.5°p. It is furthermore preferred that said barley wort preferably comprises less than 50%, more preferably less than 40%, even more preferably less than 30% T2N potential compared to a barley wort composition prepared in the same manner from a wild-type barley, preferably from cv. Power. It is also preferred that said barley wort preferably comprises less than 50%, more preferably less than 40%, even more preferably less than 30% T2N precursor compared to a barley wort composition prepared in the same manner from a wild-type barley, preferably from cv. Power.

The present invention also relates to plant products, which may be food compositions, feed compositions, and fragrance raw material compositions that comprise double null-LOX barley plants, or parts thereof. Food compositions, for example, may be, but are not limited to, malted and unmalted barley kernels, barley meals, bread, porridge, cereal mixes comprising barley, health products, such as beverages comprising barley, barley syrups, and flaked, milled or extruded barley compositions. Feed compositions,

for example, include compositions comprising barley kernels, and/or meals. Fragrance raw material compositions are described herein below.

The invention also relates to mixtures of the compositions of the invention. For  
5 example, the invention in one aspect relates to a composition prepared by a mixture of:

- (i) a composition comprising a barley plant, or a part thereof, comprising a first mutation that results in a total loss of function of LOX-1 activity, such as a total loss of functional LOX-1 enzyme, and a second mutation resulting in a  
10 total loss of function of LOX-2 activity, such as total loss of functional LOX-2 enzyme; and
- (ii) a malt composition prepared from double null-LOX kernels.

In a preferred aspect, the present invention relates to beverages, more preferred malt  
15 beverages, even more preferred fermented beverages, such as fermented malt beverages, preferably alcoholic beverages, such as beer having stable organoleptic qualities, wherein said beverage is prepared using double null-LOX barley, or parts thereof. Hence, in one preferred embodiment of the invention, the beverage is preferably prepared by fermentation of double null-LOX barley, or parts thereof, or  
20 extracts thereof, for example by fermentation of wort from double null-LOX malt, alone or in combination with other ingredients.

In other embodiments of the invention, however, the beverage is a non-fermented beverage, for example wort, preferably wort prepared from double null-LOX malt. It is  
25 also comprised within the present invention that said beverage may be prepared from unmalted barley plants, or parts thereof.

The beverage may be a non-alcoholic beverage, such as non-alcoholic beer or other kinds of non-alcoholic beverages, such as non-alcoholic malt beverages, such as  
30 maltina.

Preferably, however, said beverage is prepared from a malt composition prepared from double null-LOX barley kernels. More preferably, said beverage is beer. This may be any kind of beer known to the person skilled in the art. In one embodiment, the beer is,  
35 for example, a lager beer. The beer is preferably brewed using a malt composition

comprising germinated double null-LOX barley, more preferably said beer is brewed using a malt composition prepared exclusively from germinated double null-LOX barley. The malt composition may, however, also comprise other components, for example other germinated or non-germinated cereals, such as wild-type barley, null-  
5 LOX-1 barley, wheat and/or rye, or non-germinated raw materials that comprise sugars, or compositions derived from malted or unmalted raw materials, including syrup compositions. However, preferably all barley, such as all malted and/or unmalted barley and/or germinated and/or non-germinated barley used for preparation of said beer is preferably double null-LOX barley.

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In a preferred embodiment, the beverage according to the invention is beer that has been produced from wort prepared from kilned malt, preferably by mashing and optionally sparging. Such beer may also be referred to as "malted" herein. However, the beverage according to the invention may also be beer prepared from barley wort.  
15 Such beer is also referred to as "barley beer".

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In a preferred embodiment, the beverage according to the invention comprises less than 50%, preferably less than 40%, more preferably less than 35%, such as less 30% T2N potential compared to the T2N potential of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power. It is also preferred that the beverage according to the invention comprises less than 70%, preferably less than 60%, such as less than 50% T2N potential compared to a beverage prepared in the same manner from a null-LOX-1 barley mutant, preferably from barley mutant D112  
25 described in WO 2005/087934. It is also preferred that the beverages according to the invention comprise at the most 2 ppb, more preferably at the most 1.5 ppb, such as at the most 1 ppb T2N potential if the °p in the original extract upon which the beverage is based is adjusted to in the range of 10 to 12°p, more preferably to 11 °p.

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In a preferred embodiment, the beverage according to the invention comprises less than 50%, preferably less than 40%, more preferably less than 35%, such as less 30% T2N precursor compared to the T2N precursor of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power. It is also preferred that the beverage according to the invention comprises less than 70%, preferably less than  
35 60%, such as less than 50% T2N precursor compared to a beverage prepared in the

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same manner from barley mutant D112 described in WO 2005/087934. It is also preferred that the beverages according to the invention comprise at the most 2 ppb, more preferably at the most 1.5 ppb, such as at the most 1 ppb T2N precursor if the °p in the original extract upon which the beverage is based is adjusted to in the range of 10 to 12°p, more preferably to 11 °p.

In one specific embodiment of the invention, the beverage is barley beer, which comprises less than 50%, preferably less than 40%, more preferably less than 35% T2N potential compared to the T2N potential of a barley beer prepared in the same manner from wild-type barley, preferably from cv. Power. In this embodiment, it is also preferred that said barley beer comprises less than 50%, preferably less than 40%, more preferably less than 35% T2N precursor compared to the T2N precursors of a barley beer prepared in the same manner from wild-type barley, preferably from cv. Power. In this embodiment, it is also preferred that the barley beer according to the invention comprises at the most 2 ppb, more preferably at the most 1.5 ppb, even more preferably at the most 1.2 ppb T2N potential. In this embodiment, it is furthermore preferred that the barley beer according to the invention comprises at the most 2 ppb, more preferably at the most 1.5 ppb, even more preferably at the most 1.2 ppb T2N precursor if the °p in the original extract upon which the beverage is based is adjusted to in the range of 10 to 12°p, more preferably to 11 °p.

In another specific embodiment of the invention the beverage is beer prepared from malt, wherein said beer comprises less than 50%, preferably less than 40%, more preferably less than 30% T2N potential compared to the T2N potential of a beer prepared in the same manner from wild-type barley, preferably from cv. Power. In this embodiment, it is also preferred that said beer comprises less than 50%, preferably less than 40%, more preferably less than 30% T2N precursor compared to the T2N precursors of a beer prepared in the same manner from wild-type barley, preferably from cv. Power. In this embodiment, it is also preferred that the beer according to the invention comprises at the most 2 ppb, more preferably at the most 1.5 ppb, even more preferably at the most 1 ppb T2N potential. In this embodiment, it is furthermore preferred that the beer according to the invention comprises at the most 2 ppb, more preferably at the most 1.5 ppb, even more preferably at the most 1 ppb T2N precursor if the °p in the original extract upon which the beverage is based is adjusted to in the range of 10 to 12°p, more preferably to 11 °p.

"Organoleptic qualities" means qualities appealing to the human olfactory and taste senses. These are analyzed, for example, by a trained, specialized taste panel. Preferably, said taste panel is trained specifically to recognize aldehyde off-flavors, such as T2N. In general, the taste panel will consist of in the range of 3 to 30 members, for example in the range of 5 to 15 members, preferably in the range of 8 to 12 members. The taste panel may evaluate the presence of various flavors, such as papery, oxidized, aged, and bready off-flavors. In relation to the present invention, it is preferred that papery and/or aged off-flavors are in particular reduced. A method of determining the "organoleptic qualities" of a beverage is described in Example 6 in international patent application WO 2005/087934. Another preferred method of determining "organoleptic qualities" of a beverage is described in Examples 8 and 9 hereinafter. In preferred embodiments, the stable organoleptic qualities are, at least partly, a result of low levels of T2N or T2N potential.

It is preferred that the beverages according to the present invention are characterized by having a less papery taste compared to a similar beverage prepared in the same manner from a barley plant containing LOX-1 and LOX-2 activity after storage for at least 10 months at in the range of 15 to 25°C, such as around 20°C. Preferably, said papery taste is less than 90%, more preferably less than 80%, such as less than 70% as evaluated by a trained taste panel.

It is also preferred that the beverages according to the present invention have reduced papery taste as compared to a similar beverage prepared from wild-type barley after storage at elevated temperatures. When the property "papery taste" is determined by a trained, specialized taste panel – as described above, and scored on a scale from 0 to 5, where 0 is absent and 5 is extreme – then it is preferred that the beverages of the invention have one or more, preferably at least two, such as at least three, for example all of the following scores for papery taste:

- (i) A score for papery taste at least 0.5, preferably at least 0.7, more preferably at least 1.0 lower than the score for papery taste of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power after incubation at 37°C for one week;
- (ii) A score for papery taste at least 0.5, preferably at least 0.7, more preferably at least 1.0 lower than the score for papery taste of a beverage

prepared in the same manner from wild-type barley, preferably from cv.  
Power after incubation at 37°C for two weeks;

- 5 (iii) A score for papery taste at least 0.5, preferably at least 0.7, such as at least 1.0 lower than the score for papery taste of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power after incubation at 37°C for three weeks;
- 10 (iv) A score for papery taste at the most 90%, preferably at the most 80%, more preferably at the most 70%, even more preferably at the most 60%, yet more preferably at the most 50% of the score for papery taste of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power after incubation at 37°C for one week;
- 15 (v) A score for papery taste at the most 90%, preferably at the most 80%, more preferably at the most 70%, even more preferably at the most 60% of the score for papery taste of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power after incubation at 37°C for two weeks;
- 20 (vi) A score for papery taste at the most 90%, preferably at the most 80% of the score for papery taste of a beverage prepared in the same manner from wild-type barley, preferably from cv. Power after incubation at 37°C for three weeks;

A beverage is said to have "stable organoleptic qualities", when said beverage comprises very low levels of free T2N, even after storage. Accordingly, it is an objective of the present invention to provide beverages (such as beer with stable organoleptic

25 qualities), manufactured using a double null-LOX barley plant. Such beverages preferably comprise very low levels of T2N potential – preferably less than 50%, preferably less than 40%, more preferably less than 35%, such as less than 30%, for example less than 20%, such as less than 10% T2N potential compared to a beverage prepared in the same manner from wild-type barley, preferably from cv. Power – after

30 storage for at least 1 week, preferably at least 2 weeks, more preferably at least 3 weeks, even more preferably for at least 4 weeks, such as in the range of 1 to 3 months, for example in the range of 3 to 6 months, such as in the range of 6 to 12 months, for example for more than one year.

Moreover, it is preferred that the beverages according to the invention comprise very low levels of T2N – preferably less than 50%, preferably less than 40%, more preferably less than 35%, even more preferably less than 30%, for example less than 25% free T2N compared to a beverage prepared in the same manner from wild-type barley, preferably from cv. Power – after storage for at least 1 week, preferably at least 2 weeks, more preferably at least 3 weeks, even more preferably for at least 4 weeks at a temperature in the range of 30 to 40°C, preferably at 37°C, such as in the range of 1 to 3 months, for example in the range of 3 to 6 months, such as in the range of 6 to 12 months, for example for more than one year at a temperature in the range of 20-30°.

In particular, it is preferred that the beverages according to the invention comprise very low levels of T2N – preferably less than 50%, preferably less than 40%, more preferably less than 35% free T2N compared to a beverage prepared in the same manner from wild-type barley, preferably from cv. Power – after storage for 2 weeks at 37°C. It is also preferred that the beverages according to the invention comprises less than 50 ppt, even more preferably less than 40 ppt, even more preferably less than 30 ppt free T2N after storage for 2 weeks at 37°C. Preferably said storage is performed in the presence of a level of sulfite not exceeding 10 ppm, preferably a level of sulfite in the range of 1 to 10 ppm, more preferably in the range of 1 to 8 ppm, more preferably in the range of 2 to 6 ppm, yet more preferably in the range of 3 to 5 ppm, such as 4 ppm sulfite.

It is also preferred that the beverages according to the invention comprise less than 80%, preferably less than 75%, such as less than 60% free T2N compared to a beverage prepared in the same manner from null-LOX-1 barley, preferably from barley mutant D112 described in WO 2005/087934 – after storage for 2 weeks at 37°C. Preferably said storage is performed in the presence of a level of sulfite not exceeding 10 ppm, preferably a level of sulfite in the range of 1 to 10 ppm, more preferably in the range of 1 to 8 ppm, more preferably in the range of 2 to 6 ppm, yet more preferably in the range of 2 to 4 ppm.

It is also particularly preferred that the beverages (such as beer, for example barley beer) according to the invention comprise very low levels of T2N – preferably less than 50%, preferably less than 40%, more preferably less than 35%, even more preferably less than 30%, yet more preferably less than 25% free T2N compared to a beverage

prepared in the same manner from wild-type barley, preferably from cv. Power – after storage for 8 weeks at 37°C. It is furthermore preferred that the beverages (such as beer, for example barley beer) comprises at the most 50 ppt, even more preferably at the most 40 ppt, yet more preferably at the most 30 ppt, even more preferably at the most 20 ppt free T2N after storage for 8 weeks at 37°C. Preferably said storage is performed in the presence of a level of sulfite not exceeding 10 ppm, preferably a level of sulfite in the range of 1 to 10 ppm, more preferably in the range of 1 to 8 ppm, more preferably in the range of 1 to 6 ppm, yet more preferably in the range of 2 to 4 ppm, such as 3 ppm sulfite.

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It is also preferred that the beverages according to the invention comprise less than 70%, preferably less than 60%, even more preferably less than 55% free T2N compared to a beverage prepared in the same manner from null-LOX-1 barley, preferably from barley mutant D112 described in WO 2005/087934 – after storage for 8 weeks at 37°C. Preferably said storage is performed in the presence of a level of sulfite not exceeding 10 ppm, preferably a level of sulfite in the range of 1 to 10 ppm, more preferably in the range of 1 to 8 ppm, more preferably in the range of 2 to 6 ppm, yet more preferably in the range of 2 to 4 ppm.

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Furthermore, it is an objective of the present invention to provide beverages, such as beer manufactured using a double null-LOX barley plant – preferably comprising less than 70%, preferably less than 60%, such as less than 50% T2N and/or T2N potential, more preferably less than 70%, preferably less than 60%, such as less than 50% free T2N – compared to a beverage prepared in the same manner from barley mutant D112 as described in WO 2005/087934, after storage for at least 1 week, preferably at least 2 weeks, more preferably at least 3 weeks, even more preferably for at least 4 weeks at a temperature in the range of 30 to 40°C, preferably at 37°C, such as in the range of 1 to 3 months, for example in the range of 3 to 6 months, such as in the range of 6 to 12 months, for example for more than one year at a temperature in the range 20 to 30°C.

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In particular, it is preferred that the beverages (such as beer, for example barley beer) according to the invention comprise very low levels of T2N – preferably less than 70%, preferably less than 60%, more preferably less than 55%, even more preferably less than 52% free T2N – compared to a beverage prepared in the same manner from wild-

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type barley, preferably from cv. Power, after storage for 8 weeks at 37°C. Preferably said storage is performed in the presence of a level of sulfite not exceeding 10 ppm, preferably a level of sulfite in the range of 1 to 10 ppm, more preferably in the range of 1 to 8 ppm, more preferably in the range of 1 to 6 ppm, yet more preferably in the  
5 range of 2 to 4 ppm, such as 3 ppm sulfite.

Preferably, the beverage according to the invention also comprises in the range of 1 to 10 parts per million (ppm) sulfite, more preferably in the range of 2 to 8 ppm, more preferably in the range of 3 to 7 ppm, yet more preferably in the range of 4 to 6 ppm  
10 sulfite. The beverages of the invention preferably comprise at the most 0.07, preferably at the most 0.06, more preferably at the most 0.05, even more preferably at the most 0.04, such as at the most 0.03 parts per billion (ppb) free T2N after storage for at least 1 week, preferably at least 2 weeks, more preferably at least 3 weeks, even more  
15 preferably for at least 4 weeks, such as in the range of 1 to 3 months, for example in the range of 3 to 6 months, such as in the range of 6 to 12 months, for example for more than one year after storage at a temperature in the range of 15°C to 40°C, preferably in the range of 30°C to 37°C, more preferably at 37°C. In one preferred  
embodiment of the invention, the beverages according to the invention comprise at the most 0.03 ppb, preferably at the most 0.025 ppb, more preferably at the most 0.02 ppb  
20 (parts per billion) free T2N after storage for 4 weeks at 37°C in the presence of in the range of 4 to 6 ppm sulfite.

The beverages with stable organoleptic qualities according to the invention preferably comprise low levels of T2N potential, preferably less than 40%, more preferably less  
25 than 30%, even more preferably less than 25% T2N potential compared to a similar beverage prepared in the same manner from a wild-type barley plant, preferably from cv. Power.

In one embodiment, the invention relates to beverages, such as beer, with low levels of  
30 certain trihydroxy octadecenoic acids (also denoted THAs), in particular to beverages with low levels of 9,12,13-THA and 9,10,13-THA. THAs are characterized by a bitter taste (Baur and Grosch, 1977; Baur et al., 1977), and are therefore considered undesirable.

It is thus desirable that the level of 9,12,13-THA and 9,10,13-THA is as low as possible, preferably lower than 1.3 ppm, such as lower than 1 ppm. However, the overall concentration of 9,12,13-THA and 9,10,13-THA in a malt-derived beverage – such as beer – is also dependent on the amount of malt used for preparation of said specific beverage. Thus, in general, a strong beer will comprise more 9,12,13-THA and 9,10,13-THA than a lighter beer, making a higher over-all level of 9,12,13-THA and 9,10,13-THA acceptable in a stronger beer. Accordingly, it is preferred that the beverage according to the invention comprises a lower level of 9,12,13-THA and 9,10,13-THA than a beverage prepared in the same manner from wild-type barley, preferably from cv. Power. In particular, a beverage according to the invention preferably has a level of 9,12,13 THA, which is at the most 50%, preferably at the most 40%, more preferably at the most 30% compared to the level in a beverage prepared in the same manner from a wild-type barley, preferably from cv. Power. It is furthermore preferred that a beverage according to the invention has a level of 9,10,13 THA, which is at the most 70%, preferably at the most 60% compared to the level in a beverage prepared in the same manner from a wild-type barley, preferably from cv. Power. Such beverages may be prepared by using double null-LOX barley.

In one embodiment of the invention, the beverages have improved foam quality. This is in particular relevant when the beverage is a beer. Accordingly, it is an objective of the invention to provide beverages, such as beer, with superior foam quality. Preferably, the beverages of the invention produce at least 10% more, preferably at least 20% more, yet more preferably at least 25% more foam in 60 to 80 min, preferably in 80 min. compared to a beverage prepared in the same manner from wild-type barley, preferably from cv. Power. Said foam production is determined as described in Example 9 herein below.

The invention also relates to methods of producing said beverage. The methods preferably comprise the steps of:

- (i) Providing a malt composition comprising germinated double null-LOX kernels;
  - (ii) Processing said malt composition into a beverage;
- thereby obtaining a beverage with more stable organoleptic qualities.

In one preferred embodiment, the beverage is beer. In this case, the processing step preferably comprises preparing wort from said malt composition, for example by any of the methods described hereinabove, and fermenting said wort.

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In general terms, alcoholic beverages – such as beer – may be manufactured from malted and/or unmalted barley grains. Malt, in addition to hops and yeast, contributes to flavor and color of the beer. Furthermore, malt functions as a source of fermentable sugar and enzymes. A schematic representation of a general process of beer production is shown in FIG. 3, while detailed descriptions of examples of suitable methods for malting and brewing can be found, for example, in publications by Briggs et al. (1981) and Hough et al. (1982). Numerous, regularly updated methods for analyses of barley, malt and beer products are available, for example, but not limited to, American Association of Cereal Chemists (1995), American Society of Brewing Chemists (1992), European Brewery Convention (1998), and Institute of Brewing (1997). It is recognized that many specific procedures are employed for a given brewery, with the most significant variations relating to local consumer preferences. Any such method of producing beer may be used with the present invention. Non-limiting example are described in Example 8 and Example 9.

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The malt composition of the aforementioned beverage – such as beer, malt drinks, or non-fermented wort – may, for example, be obtained by any of the methods described herein above. Wort may be prepared from said malt composition as described hereinabove.

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The first step of producing beer from wort preferably involves boiling said wort. During boiling, other ingredients may be added – for example hops that provide the typical bitter and aromatic beer characteristics. Boiling of wort also causes aggregation between polyphenols and denatured proteins, which mainly precipitate during the subsequent phase of wort cooling. After being cooled, the wort is transferred to fermentation tanks containing yeast. Preferably, said yeast is brewer's yeast, *Saccharomyces carlsbergensis*. The wort will be fermented for any suitable time period, in general in the range of 1 to 100 days. During the several-day-long fermentation process, sugar is converted to alcohol and CO<sub>2</sub> concomitantly with the development of some flavor substances.

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Subsequently, the beer may be further processed, for example chilled. It may also be filtered and/or lagered – a process that develops a pleasant aroma and a less yeasty flavor. Also additives may be added. Furthermore, CO<sub>2</sub> may be added. Finally, the beer  
5 may be pasteurized and/or filtered, before it is packaged (e.g. bottled or canned).

Despite advances in the area of beer production, it would be beneficial to reduce the levels of T2N and T2N potential in beer. Accordingly, there remains a need for new raw materials, particularly barley and malt that contribute with less off-flavors to the finished  
10 beer. It is therefore an objective of the present invention to provide such barley plants and malt.

Various methods are available to determine whether a barley plant, or a plant product, is prepared from a barley plant carrying mutations in the genes for LOX-1 and LOX-2, causing a total loss of functional LOX-1 enzyme and a total loss of functional LOX-2  
15 enzyme. Plant products will, in general, comprise at least some genomic DNA from the plant utilized for its production. Thus, malt will contain large amounts of genomic DNA, but even barley or malt extracts, such as wort, may comprise genomic DNA or fragments thereof from said barley or malt. Also barley-based beverages, such as beer,  
20 may comprise genomic DNA or fragments thereof from said plant. By analysis of DNA in a plant product, it may be established whether the plant, from which the plant product is prepared, carries mutations in the LOX-1 and LOX-2 genes, causing a total loss of functional LOX-1 enzyme and a total loss of functional LOX-2 enzyme. Said mutations could, for example, be any of the mutations in the LOX-1 and LOX-2 genes  
25 described hereinabove in the section "Loss of function of LOX activity". The genomic DNA may be analyzed by any useful method, such as sequencing or by amplification-based methods, including PCR-based methods. If particular mutations in the LOX-1 gene and/or the LOX-2 gene are assumed, then polymorphism analysis may be employed, for example SNP analysis. Such analysis may be performed as described  
30 hereinafter in Example 10. The skilled person will be able to adapt the specific SNP analysis described in these examples for use with other mutations or other starting material.

If the above-mentioned plant products only are prepared from barley plants carrying  
35 mutations in the genes for LOX-1 and LOX-2, causing a total loss of functional LOX-1

enzyme and a total loss of functional LOX-2 enzyme, then presence vs. absence of barley LOX-1 mRNA and LOX-2 mRNA and/or LOX-1 protein and LOX-2 protein may also be indicative of whether said plant product is prepared from a double null-LOX barley plant. Examination of the plant product may also be accomplished by western blot analysis, or other protein analysis, or by RT-PCR, or by Northern blot analysis, or by other mRNA analyses. Such analyses are particularly useful when the plant product is malt.

## 10      **Chemical mutagenesis**

In order to generate double null-LOX barley plants according to the present invention – i.e. plants comprising a first mutation that results in a total loss of functional LOX-1 enzyme and a second mutation resulting in a total loss of functional LOX-2 enzyme – a very large number of barley mutants are prepared by any suitable mutagenesis method, for example by the use of chemical mutagenesis of barley kernels. This method is known to introduce mutations at random. Mutagenesis of barley may be performed using any mutagenizing chemical. However, it is preferably performed by treating kernels with  $\text{NaN}_3$ , letting the surviving kernels germinate, followed by analysis of off-spring plants. The plant generation growing from the mutagenized kernels, referred to as M0, contains heterozygote chimeras for any given mutation. Progeny plants collected after self-pollination are referred to as the M1 generation, in which a given mutation segregates into the corresponding heterozygotes and homozygotes (cf. FIG. 1).

Treating kernels with  $\text{NaN}_3$  is not equivalent to treating a single cell, because the kernels after the treatment will contain some non-mutant cells and a variety of cells having DNA mutations. Since mutations in cell lineages that do not lead to the germ line will be lost, the goal is to target the mutagen to the few cells that develop into reproductive tissues which contribute to development of the M1 generation.

To assess the overall mutation efficiency, albino chimeras and albino plants may be counted in the generations M0 and M1. Scoring mutant number as a function of surviving plants gives an estimate for the mutation efficiency, while scoring mutant

number as a function of treated seeds measures the combination of both mutation efficiency and kernel kill.

5 It is notable that cells have quality assurance mechanisms at virtually every step of gene expression, possibly to moderate the effects of damaging mutations. One well-studied example in eukaryotes is nonsense-mediated mRNA decay, denoted NMD, which prevents the synthesis of potentially deleterious, prematurely truncated proteins (Maquat and Carmichael, 2001; Wu et al., 2007). In NMD, a termination codon is identified as premature by its position relative to downstream destabilizing elements.  
10 Mutations that generate premature termination (nonsense) codons (PTCs) sometimes increase the levels of alternatively spliced transcripts that skip the offending mutations, thereby potentially saving protein function (Mendell and Dietz, 2001).

## 15 **Plant breeding**

In one embodiment of the invention, the objective is to provide agronomical useful barley plants comprising the double null-LOX trait. Crop development is often a lengthy and difficult process that begins with the introduction of the new trait. From the  
20 perspective of a plant breeder, however, this step almost always results in a plant that has a less desirable overall profile of agronomic traits than do current commercial varieties.

In addition to the double null-LOX trait, there are additional factors which also may be  
25 considered in the art of generating a commercial malting barley variety, for example kernel yield and size, and other parameters that relate to malting performance or brewing performance. Since many – if not all – relevant traits have been shown to be under genetic control, the present invention also provides modern, homozygous, high-yielding malting cultivars, which may be prepared from crosses with the double null-  
30 LOX barley plants that are disclosed in the present publication. Kernels of such barley plants provide a new raw material having low capacity for generation of T2N potential, i.e. malt produced from such kernels preferably have less than 50% T2N potential compared to malt produced in the same manner from barley null-LOX-1 mutant D112 described in WO 2005/087934. The skilled barley breeder will be able to select and  
35 develop barley plants, which – following crossings with double null-LOX barley – will

result in superior cultivars. Alternatively, the barley breeder may utilize plants of the present invention for further mutagenesis to generate new cultivars derived from double null-LOX barley.

- 5 One method to ensure that the double null-LOX trait is maintained in progeny lines concerns SNP analysis of the LOX-1 gene and of the LOX-2 gene. Preferably, LOX-1 and LOX-2 activities are also determined.

10 The barley plants according to the present invention may be introduced into any suitable breeding scheme.

Another objective of the present invention is to provide agronomical elite barley plants comprising the double null-LOX trait. Accordingly, this invention also is directed to methods for producing a new double null-LOX barley plant by crossing a first parental  
15 barley plant with a second parental barley plant, wherein the first or second plant is a double null-LOX barley. Additionally, both first and second parental barley plants can come from a double null-LOX barley variety. Thus, any such methods using the double null-LOX barley variety are part of this invention: selfing, backcrossing, crossing to populations, and the like. All plants produced using a double null-LOX barley variety as  
20 a parent are within the scope of this invention, including those plants developed from varieties derived from a double null-LOX barley variety. The double null-LOX barley can also be used for genetic transformation in such cases where exogenous DNA is introduced and expressed in the double null-LOX plant or plant tissue.

25 Backcrossing methods can be used with the present invention to introduce into another cultivar the double null-LOX trait of a mutated barley plant, for example cv. Scarlett or cv. Jersey, both of which are contemporary, high-yielding malting barley cultivars. In a standard backcross protocol, the original variety of interest, i.e. the recurrent parental plant, is crossed to a second variety (non-recurrent parental plant), carrying the mutant  
30 LOX genes of interest to be transferred. The resulting double null-LOX progeny plants from this cross are subsequently crossed to the recurrent parental plant, with the process being repeated until a barley plant is obtained wherein essentially all of the characteristics specified by the recurrent parent are recovered in the generated plant – in addition to the double null-LOX trait of the nonrecurrent parental plant. Eventually,

the last-generated, backcrossed plant is selfed to yield a pure double null-LOX breeding progeny plant.

A way to accelerate the process of plant breeding comprises the initial multiplication of generated mutants by application of tissue culture and regeneration techniques. Thus, another aspect of the present invention is to provide cells, which upon growth and differentiation produce barley plants having the double null-LOX trait. For example, breeding may involve traditional crossings, preparing fertile anther-derived plants or using microspore culture.

### LOX pathway products

In various embodiments, the present invention relates to barley plants, and products thereof, comprising low levels of T2N potential. LOX enzymes catalyze dioxygenation of polyunsaturated fatty acids with a cis-1,cis-4 pentadiene system. In barley, the C<sub>18</sub> polyunsaturated fatty acids linoleic acid (18:2<sup>Δ<sup>9,12</sup></sup>) and α-linolenic acid (18:3<sup>Δ<sup>9,12,15</sup></sup>) are major LOX substrates. The lipoxygenase pathway of fatty acid metabolism is initiated by the addition of molecular oxygen at the C-9 position (mostly catalyzed by LOX-1) or C-13 position (mostly catalyzed by LOX-2) of the acyl chain, yielding the corresponding 9- and 13-HPODEs [9- and 13-hydroperoxy octadecatrienoic acids (HPOTEs) are products when the substrate is α-linolenic acid, but HPOTEs do not function as precursors for T2N]. In the hydroperoxide lyase branch of the LOX pathway, both 9- and 13-HPODEs may be cleaved to short-chain oxoacids and aldehydes (cf. FIG. 2). In particular, 9-HPODE may be cleaved to form cis-nonenal that is converted to T2N, whereas 13-HPODE is the precursor of 2-E-hexenal. Thus, 13-HPODE, the major product of LOX-2-catalyzed dioxygenation of linoleic acid was not anticipated to be an upstream component in the pathway leading to formation of the stale flavor T2N.

It is recognized that the present invention encompasses influencing production of downstream metabolites of LOX-1 and LOX-2 catalysis, which are not produced as a direct product of a LOX-1 or LOX-2-catalyzed reaction, but as a result of a subsequent series of reactions. These include spontaneous, factor-induced, or enzyme-catalyzed isomerizations and conversions. Thus, the production of these downstream metabolites



could be influenced by modulating the expression of other components of the pathway, for example hydroperoxide lyase (HPL).

## 5      **T2N potential**

An important objective of the present invention is to reduce or eliminate the T2N potential. Thus, it is an objective of the present invention to reduce the formation of T2N precursors and aldehyde adducts. Although several chemical reactions related to beer staling remain elusive, generation of free T2N from T2N potential is recognized as a major cause of the development of stale flavor in beer products (Kuroda et al., supra). Accordingly, it is an objective of the present invention to provide beverages with low level of T2N potential as well as beverages with low level of T2N precursors.

Most of the T2N potential is transferred from wort to the finished beer, in which free T2N may be liberated (Liégeois et al., 2002), with the conditions of acidity and temperature being important factors in this process. With reference to the present invention, T2N potential is defined as described hereinabove in the definitions. Other methods for determining the level of T2N potential are also available. In order to avoid confusion, the meaning of "T2N potential" in the present context is as described herein above in the definitions. The chemical substances which have the capacity to release T2N or be converted into T2N are denoted "T2N precursors" herein, and T2N precursors determined or measured by alternative methods other than the method for determining T2N potential are referred to as "T2N precursors". T2N precursors may in particular be determined by first treating a sample such that essentially all (preferably all) of its chemical substances, which have the capacity to release T2N or be converted into T2N actually do release T2N and/or convert to T2N, respectively. Thereafter, the level of T2N is determined.

Barley kernels of the instant invention comprise no LOX-1 and LOX-2 activities. Interestingly, such barley kernels contain very little T2N potential.

Beers produced using double null-LOX barley kernels will therefore not only possess a very low level of T2N, but also a very low level of T2N potential. Within the scope of the present invention are double null-LOX barley kernels, which yield beer products that

contain very low levels of T2N potential, preferably less than 40%, more preferably less than 30%, even more preferably less than 25% of the level of T2N potential of a similar beer product produced in the same manner from wild-type barley (preferably cv. Power). Thus, said beer product preferably comprises less than 60%, more preferably less than 50% of the T2N potential of a similar beer product produced in the same manner from barley mutant D112 described on WO 2005/087934.

Also, it is preferred that plant products derived from double null-LOX barley kernels possess a very low level of T2N precursors. Within the scope of the present invention are plant products prepared from double null-LOX barley kernels, said plant products containing less than 40%, more preferably less than 30%, even more preferably less than 25% of the level of T2N precursors of a similar plant product produced in the same manner from wild-type barley (preferably cv. Power). Thus, said plant product preferably comprises less than 60%, more preferably less than 50% of the T2N precursors of a similar plant product produced in the same manner from barley mutant D112 described on WO 2005/087934.

It is notable that measured T2N values often are higher in samples of, and in products from, a micro-malted raw material than that from a raw material produced in larger scale, for example from a 30-kg-large pilot-malted sample. However, the relative, experimental values of T2N between large- and small-scale experiments are in general similar.

Similarly, it is notable that measured T2N potentials (and T2N precursors) often are higher in samples of, and in products from, a micro-malted raw material than that from a a raw material produced in larger scale, for example from a 30-kg-large pilot-malted sample. However, the relative, experimental values of T2N potentials between large- and small-scale experiments are in general similar.

30

### Examples

The examples herein illustrate preferred embodiments of the invention and should not be considered as limiting for the invention.

35

Unless otherwise indicated, basic molecular biological techniques were performed for manipulating nucleic acids and bacteria as described in Sambrook and Russel (2001).

## 5      **Example 1**

### **Screening for low LOX-2 activity in mutated, mature embryos of a mutated null-LOX-1 population, generation M3**

10      LOX-1 and LOX-2 are both synthesized in the maturing barley embryo (with LOX-1 being the predominant enzyme), and also in the germinating embryo (with equal levels of both enzymes). For determination of LOX activities in thousands of samples, assays utilizing extracts of barley embryos would be most convenient. And in assays for LOX-2 levels, it would be highly advantageous to screen kernels of mutagenized null-LOX-1  
15      barley, cf. WO 2005/087934.

Kernels were collected from barley plants of null-LOX-1 mutant D112 (described in WO 2005/087934 and deposited with ATCC under the number PTA-5487), and incubated with the mutagen  $\text{NaN}_3$  to induce point mutations in the barley genomic  
20      DNA. The experimental set-ups followed the recommendations provided by Kleinhofs et al. (supra).

Next, mutated grains of generation M1 were propagated in field plots through two subsequent generations, eventually yielding a high proportion of homozygous plants of  
25      generation M3 for screening purposes (cf. FIG. 1 for a procedure overview). Mutated grains of generation M3 were expected to contain gene mutations at a frequency of 0.9-2.3 per 10,000 grains (Kleinhofs et al., supra).

LOX-2 activity in mature embryos of the null-LOX-1 mutant was found to be low, as  
30      measured by the conventional LOX-1 assay. Here, the stock solutions of the reaction mixture are combined simultaneous with the extract (as described in WO 2005/087934). In the application is detailed a method that improves assay sensitivity, wherein both the enzyme extraction conditions as well as the mixing of the reaction mixture stock solutions were altered.

35

For LOX extraction, the present inventors found interestingly that using a 100-mM lactic acid buffer, pH 4.5, conferred a significantly reduced background level of HPODE-consuming activity, permitting accumulation of HPODEs in the assay buffer.

5 Additionally, the inventors found that the assay could be further improved by allowing a minor fraction of the formed HPODEs to activate LOX-2 through  $\text{Fe}^{++} \rightarrow \text{Fe}^{+++}$  oxidation of its bound iron atoms prior to addition of the remaining assay reactants, eventually forming a blue indamine dye.

10 In short, LOX-2 enzyme was extracted from isolated, mature embryos using 200  $\mu\text{l}$  extraction buffer (100 mM lactic acid, pH 4.5). Extraction was performed in 96-well plates in which each 1.2-ml well contained a circular 5-mm glass bead. The plates were incubated on ice for 10 min before transfer to a MM 300 laboratory mill (Retsch), electronically adjusted to shake at a frequency of  $27 \text{ sec}^{-1}$  for 35 sec. Subsequently, the plate was centrifuged at 4,000 rpm in an Allegra 6R Centrifuge (Beckman-Coulter) for  
15 10 min at  $4^\circ\text{C}$  to precipitate insoluble material, and thereafter kept on ice for max. 2 h before further processing. The 96-well plate was transferred to a Biomek 2000 Laboratory Automation Workstation (Beckman-Coulter) for further pipetting. Initially,  $96 \times 40 \mu\text{l}$  embryo extracts were transferred to a standard 96-well microtitre plate (Nunc), followed by addition of 90  $\mu\text{l}$  of Reagent A [12.5 mM 3-(dimethylamino)benzoic acid, 0.625 mM linoleic acid], which was made by first mixing 155  $\mu\text{l}$  of linoleic acid, corresponding to 134 mg free acid (Sigma, L-1376) and 257  $\mu\text{l}$  Tween-20; then  $\text{H}_2\text{O}$   
20 was added to a volume of 5 ml, followed by addition of 600  $\mu\text{l}$  of 1 M NaOH. The cleared solution was adjusted to 20 ml with  $\text{H}_2\text{O}$ . The mixtures were incubated for 10 min before adding 90  $\mu\text{l}$  Reagent B (0.25 mM 3-methyl-2-benzothiazolinehydrazone, 0.125 mg/ml hemoglobin). After further incubation for 5 min,  $A_{595}$  was measured in  
25 each of the 96 wells of the plate using a Flouorstar Galaxy spectrophotometer (BMG Labtechnologies), with the color formation reflecting the presence of HPODEs generated by LOX-2-catalyzed dioxygenation (activities are accordingly given in  $A_{595}$  units).

30 Using the above-described procedure, a total of 21,000 barley mutant lines were assayed for LOX-2 activity, and 1,550 lines thereof selected based on low LOX-2 activity. These lines were further propagated in the field, with the LOX-2 activity thereafter measured in the mature grains. However, none of the lines were found to  
35 transmit the low LOX-2 trait to the next generation.

## Example 2

### 5 Screening for low LOX-2 activity in germinating barley embryos

Improved screening material. Kernels collected from barley plants of null-LOX-1 line Ca211901 – generated by the crosses (null-LOX-1 mutant D112 × Jersey) × Sebastian – were incubated with the mutagen  $\text{NaN}_3$  according to the details provided by Kleinhofs et al. (*supra*). This procedure was chosen since it is known to induce point mutations in the genomic DNA of barley, eventually conferring amino acid residue substitutions or truncations in proteins encoded by the mutagenized DNA. In the mutagenesis experiments of the instant publication, it was chosen to propagate mutated grains of generation M1 in field plots through two subsequent generations, eventually yielding a high proportion of homozygous plants for screening purposes (cf. FIG. 1). While grains of generation M2 were not screened, primarily because these were expected to contain a relatively high proportion of heterozygous point mutations, mutant grains of generation M3 were used as screening material, expecting 0.9–2.3 mutations per 10,000 grains (Kleinhofs et al., *supra*).

20

Surprisingly, the instant inventors found that analysis of germinating embryos provided much-improved assay results as compared to analysis of extracts of mature embryos (as described above in Example 1). A high-throughput screening procedure was therefore established to measure LOX-2 activity in the germinating embryo, including its scutellum tissue.

25

Two embryos were isolated from mature grains of 35,125 barley ears (20,977 lines of generation M4 of null-LOX-1 mutant D112, and 14,148 lines of generation M3 of null-LOX-1 line Ca211901 lines), and transferred to 96-well storage plates (ABgene).

30

Embryo germination was initiated following addition of 20  $\mu\text{l}$  water to each well, which was covered with a wet Kimnett tissue and a plastic lid. The plates were incubated in plastic bags at 20°C for 48 h. After incubation, LOX-2 enzyme was extracted; to each well was first added a 5-mm glass bead and 200  $\mu\text{l}$  of extraction buffer (100 mM lactic acid solution, pH 4.5), followed by milling for 35 sec at a frequency of 27  $\text{sec}^{-1}$  in an MM

300 laboratory mill (Retsch). Subsequently, the plate was centrifuged at 4,000 rpm for 10 min at 4°C in an Allegra 6R centrifuge (Beckman-Coulter), to precipitate insoluble material. LOX-2 activity was determined basically as described for analysis of LOX-2 activity of mature embryo extracts (Example 1), only differing in the usage of only 30 µl extract per assay instead of 40 µl.

Identification of potential mutants. As described above, two grains each of the above-mentioned 35,125 barley lines were analyzed for LOX-2 activity, with the aim to identify grains highly reduced in said activity when compared with null-LOX-1 and wild-type grains. A total of 7 potential raw mutants were identified in the M3 generation of line Ca211901. These were further propagated in the greenhouse, harvested, and then re-screened for the trait related to very low LOX activity. Eventually, only one mutant of line Ca211901, denoted mutant A689 (and herein also denoted double null-LOX mutant A689), was shown to exhibit essentially no LOX-2 activity. Detailed measurements of total LOX activity were performed with extracts of germinated embryos in which the LOX activity was conferred almost exclusively by LOX-2 (Schmitt and van Mechelen, 1997). For germinated embryos of M3 grains of mutant A689, the total LOX activity – as determined by the colorimetric LOX assay (Example 1; Table 1) – was  $0.163 \pm 5.5\% A_{595}$  U/germinated embryo, while that for the null-LOX-1 mother variety Ca211901 was  $1.224 \pm 3.8\% A_{595}$  U/germinated embryo (the corresponding value for null-LOX-1 raw mutant D112 was  $1.215 \pm 6.0\% A_{595}$  U/germinated embryo).

### Example 3

#### Barley mutant A689

Analyses were conducted to establish whether null-LOX-2 plants of generations M4 and M5 were homozygous for the corresponding mutant phenotype. This type of analysis would help to determine whether the mutation was genetically recessive or dominant. Additionally, if plants of generation M3 were heterozygous for a dominant mutation, then individuals of subsequent generations would segregate for that phenotype.

Total LOX activity was measured in barley embryos of generations M3, M4, and M5 of mutant A689, and activities were not only compared with those of embryos of the mother line Ca211901, but also with null-LOX-1 mutant D112. Determination of LOX activity was as described in Example 1 of the instant publication. In all of the experiments, control samples included standard extracts from germinating embryos of the mother line, as well as heat inactivated, standard extracts from embryos of null-LOX-1 mutant line Ca211901 and mutant D112. For embryos of generation M4 grains of mutant A689, the average total LOX activity was  $0.151 \pm 2.6\% A_{595} \text{ U}$  (n=24), and that for germinating embryos of generation M5 of mutant A689 was  $0.16 \pm 2.3\% A_{595} \text{ U}$  (n=90). For comparison, germinating embryos of null-LOX-1 line Ca211901 and mutant D112 yielded  $1.210 \pm 3.0\% A_{595} \text{ U}$  (n=90; generation M4) and  $1.199 \pm 4.6\% A_{595} \text{ U}$  (n=90; generation M5), respectively. The results are summarized in Table 1, and in FIG. 4A–C. In summary, the experimental data confirmed that grains of generations M4 and M5 of mutant D112 were homozygous for the genetic trait specifying a very low, total LOX activity, reflecting a double null-LOX phenotype.

**Table 1.** Total LOX activity (average) in extracts prepared from germinated barley embryos of raw mutants (generation M3), and progenies (generations M4 and M5).

Extracts	Total LOX activity	Lines assayed	Standard deviation
	<i>A<sub>595</sub> units</i>	<i>No.</i>	<i>%</i>
<b>Generation M3</b>			
Mutant A689	0.163	8	5.5
Ca211901	1.224	6	3.8
D112	1.215	6	6.0
Ca211901 (heat-inactivated)	0.099	10	1.3
D112 (heat-inactivated)	0.096	10	1.1
<b>Generation M4 (progeny)</b>			
Mutant A689	0.151	24	2.6
Ca211901	1.223	6	2.3
D112	1.209	6	5.6
Ca211901 (heat-inactivated)	0.096	10	1.7

D112 (heat-inactivated)	0.101	10	1.0
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**Generation M5 (progeny)**

Mutant A689	0.160	90	2.3
Ca211901	1.210	90	3.0
D112	1.199	90	4.6
Ca211901 (heat-inactivated)	0.103	10	2.0
D112 (heat-inactivated)	0.097	10	1.2

---

**Example 4**

**5 HPLC-based analysis of HPODEs in germinated and micro-malted barley**

Analysis for barley HPODEs was carried out essentially as described in International Patent Application WO 2005/087934, except utilizing germinating instead of mature embryos. Grains of generation M4 were germinated for 48 h as described in

10 Example 2. Grains of generation M5 were subjected to a micro-malting procedure performed essentially as described in Example 6 hereinafter, but the specific HPODE levels were determined after 72 h of germination, and the kilning procedure was omitted. Levels of 9- and 13-HPODEs were determined by letting crude protein extracts from germinating embryos of generations M4 and M5 of mutant A689 and control

15 samples incubate with the substrate linoleic acid. Reaction products were analyzed by HPLC.

The germinated embryos were dissected from the barley grains using a scalpel to cut between the scutellum and the endosperm. Each 4-embryo-large sample was then

20 placed between two pieces of weighing paper, and hammered gently to produce a homogenous flour. This was transferred to a 1.5-ml microcentrifuge tube; 600 µl of a 200-mM lactic acid buffer, pH 4.5, was added, and the tube was placed on ice for 10 min before further homogenization using a plastic pestle (Kontes). Subsequently, 600 µl H<sub>2</sub>O was added to each tube, and the samples were centrifuged for 2 min at

25 20.000×g. A 100-µl aliquot of the resulting supernatant was transferred to a 15-ml centrifuge tube (Cellstar; cat. no.188271), to prepare for analysis of the reaction products following LOX action. 2 ml of a 100-mM Na-phosphate buffer, pH 6.5,



containing 260  $\mu$ M linoleic acid [this substrate was prepared by mixing 10 ml of a 100-mM Na-phosphate buffer, pH 6.5, with 100  $\mu$ l of a 24-mM linoleic acid stock solution. The latter was made by first mixing 155  $\mu$ l of linoleic acid (corresponding to 134 mg free acid; L-1376, Sigma) and 257  $\mu$ l Tween-20, then adding H<sub>2</sub>O to a volume of 5 ml, followed by addition of 600  $\mu$ l 1 M NaOH, and when the solution turned clear, adjusting the volume to 20 ml with H<sub>2</sub>O. After a 15-min incubation at ~30 rpm on a blood tube rotator, 2 ml ethyl acetate was added, and the sample content mixed by vigorous shaking for 5 sec in order to extract 9- and 13-HPODEs. The sample was then centrifuged for 10 min at 800 $\times$ g, and 1 ml ethyl acetate was transferred to a 1.5-ml microcentrifuge tube, in which ethyl acetate was evaporated under a stream of nitrogen gas. Subsequently, the HPODEs were resuspended in 300  $\mu$ l methanol, and filtered through a 0.45- $\mu$ m membrane (Millex-HN filter, Millipore). Analysis of the HPODE content was performed by HPLC. A total of 15  $\mu$ l from each sample was injected into a HPLC apparatus (HP 1100 Series, Hewlett Packard), equipped with a 4.6 $\times$ 250 mm Symmetry C18 column (Waters). The mobile phase used was a 16:12:12:10:0.5 (v:v:v:v:v) mixture of water:methanol:acetonitrile:tetrahydrofuran:trifluoroacetic acid. The flow of the mobile phase was 1 ml min<sup>-1</sup>, and the pressure measured in front of the column was 140 bars. The separation was performed at 30°C. Detection of linoleic acid hydroperoxides with conjugated double bonds was performed at 234 nm.

20

In FIG. 5 is shown a comparison of HPODE profiles obtained. A chromatogram of a standard sample comprising a mixture of 9- and 13-HPODEs is shown in FIG. 5A. Comparisons of the chromatogram of an extract prepared from 48-h germinated grains of generation M4 of null-LOX mutant D112 (FIG. 5B), with that of double null-LOX mutant A689 (FIG. 5C), revealed pronounced 13-HPODE formation by LOXs extracted from germinated embryos of null-LOX-1 mutant D112, whereas only no or extremely little of 9- and 13-HPODEs were observed in extracts of double null-LOX mutant A689.

Similar characteristics were observed when analyzing 72-h micro-malted embryos of wild-type cv. Barke (FIG. 6A), null-LOX-1 mutant D112 (FIG. 6B), and double null-LOX mutant A689 (FIG. 6C). Again, significant levels of both 9- and 13-HPODEs were formed in extracts of mature embryos of the wild-type cv. Barke (FIG. 6A). Extracts of null-LOX-1 mutant D112 embryos formed very low amounts of 9-HPODE, but high amounts of 13-HPODE (FIG. 6B), thus verifying the lack of LOX-1 activity. And again, embryo extracts of double null-LOX mutant A689 formed no significant levels of 9- and

35

13-HPODEs (FIG. 6C), confirming the total absence of both LOX-1 and LOX-2 activities.

## 5 Example 5

### Properties of barley double null-LOX mutant A689, its null-LOX-1 mother variety Ca211901, and null-LOX-1 mutant D112

10 Plant propagation in the greenhouse. Generation M4 and M5 grains of double null-LOX mutant A689 and its mother line Ca211901 were germinated in the greenhouse, and grown in daily cycles under 20 h of light at 12°C and 65% relative humidity. The growth characteristics of line Ca211901 and double null-LOX mutant A689 plants were similar with respect to plant height, number of tillers per plant, the onset of flowering, and the  
15 number of grains per spike – emphasizing that grain development and growth physiology of mutant A689 is wild-type-like.

Agronomic performance of mutant A689 under field conditions. Plants of double null-LOX mutant A689 and null-LOX-1 mutant D112, as well as plants of line Ca211901,  
20 were compared in standard field trials – aiming to identify possible differences with respect to plant height, heading date, disease resistance, lodging, ear breakage, maturation time, and yield (cf. Table 2). Accordingly, equal amounts of the aforementioned kernels were sown in 7.88-m<sup>2</sup> plots at one location, each comprising two replications. Agronomic properties, again with emphasis on the aforementioned  
25 properties, were carefully observed throughout the entire growth season. No differences with respect to agronomic traits were observed for any of the tested plants.

**Table 2.** Comparison of agronomic performance.

Property	Mutant A689	Mother line Ca211901	Mutant D112
LOX genotype	null-LOX-1, null-LOX-2	null LOX-1, wt LOX-2	null-LOX-1, wt LOX-2
Date of sowing 2007	29 March	29 March	29 March
Length at maturity (cm)	68	69	74

Heading date 2007	14 June	14 June	12 June
Powdery mildew*	0	0	0
Spot blotch*	3	3	2
Net blotch*	1	1	1
Ramularia*	3	3	2
Rust*	1	1	2
Lodging*	2	2	4
Date of maturity 2007	1 August	1 August	1 August
Relative yield 2007**	107	108	100
Relative yield 2008**	104	103	99

\* On a scale from 0 to 9, where 0 represents no infection or lodging, and 9 represents extreme infection or lodging.

\*\* Relative yield performance compared to cv. Barke, the mother variety of null-LOX-1 mutant D112.

5

## Example 6

### Analysis of micro-malts and worts thereof

10

Micro-malting was performed with 100-g samples of barley mutants A689 and D112, as well as cvs. Barke and Power. The mutant lines were propagated in the field for several seasons in order to obtain sufficient grain material for malting and brewing trials.

15 Steeping. The conditions were: 8 h wet; 14 h dry; 8 h wet; 10 h dry; 4 h wet in steeping water at 16°C.

20 Malting. The conditions were: 12 h at 18°C; 24 h at 16°C; 24 h at 14°C; 60 h at 12°C. Kiln drying conditions were: 12 h at 60°C; 3 h at 68°C; 4 h at 74°C; 3 h at 80°C. The finish malts were subjected to T2N measurements.

In addition, barley and malt samples of mutants A689 and D112, as well as cvs. Barke and Power, barley and malt samples were subjected to analyses by standard EBC methods (see Table 3).

25

Wort preparation. To test the properties of mutants A689 and D112 in comparison with cv. Power, malt samples of 25-225 g were produced, and used in a laboratory mashing system that comprised an external stirrer and a water bath equipped with a thermostat capable of ramping the temperature in a defined pattern. Mashing was performed in small-scale, with the finished mash separated through a paper filter to obtain the sweet wort. Wort boiling was performed in laboratory-scale using a heating mantel and a round-bottomed flask connected to a reflux cooler.

Both sweet and boiled worts were subjected to T2N measurements (cf. FIGs. 7–9). For samples derived from generation M5 grains of double null-LOX mutant A689, there was observed a pronounced ~75% decrease in levels of T2N (average of three A689 mutant lines). Additionally, notable reductions were registered for levels of both free T2N and T2N precursors in wort produced from the micro-malted samples (FIGs. 8–9).

T2N levels were determined by gas chromatography with mass spectrometric detection (GC-MS), following derivatization of carbonyls with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine, essentially as described by Groenqvist et al. (1993).

**Table 3.** Micro-malt analysis.

Sample		cv. Power	Mutant A689	Mutant D112
LOX genotype		wt LOX-1, wt LOX-2	null LOX-1, null-LOX-2	null-LOX-1, wt LOX-2
<b>Barley</b>				
Moisture content	%*	9.3	9.5	10.5
Protein	%*	12	10.6	10.9
Starch	%*	61.7	63.0	62.4
Germination, 72 h	%	100	95	95
Germination Index		9.9	6.6	8.3
Screening	>2.5 mm	97	98.8	91.6
TKW	G	48.6	55.8	37.5
β-Glucan	%, dry	4.0	3.6	3.3
β-Amylase**	U/g	1039	1458	1027

Predicted DP	WK	385	538	381
<b>Malt</b>				
Malting time	H	124	148	124
Malt yield	%	95.1	95.9	95.3
Moisture content	%*	4.8	5.6	4.6
Protein, dry	%*	11.2	10.3	9.9
N, dry	%*	1.72	1.65	1.58
Soluble N	%*	0.67	0.62	0.64
<b>Wort</b>				
Extract	%*	81.0	81.0	80.9
$\beta$ -Glucan	%*, dry	0.2	0.3	0.26
Malt modification	%	95	95	96
Malt homogeneity	%	81	70	71
$\beta$ -Amylase**	U/g	956	1235	937
Predicted DP	WK	385	478	379
$\alpha$ -Amylase***	U/g	242	234	264

\* Measured by standard near infrared transmittance spectroscopy.

\*\*  $\beta$ -Amylase activity was measured using a Megazyme kit (product code K-BETA).

\*\*\*  $\alpha$ -Amylase activity was measured using a Megazyme kit (product code K-CERA).

5

## Example 7

### THAs in beer produced from malt of double null-LOX mutant A689

10

Beer-specific THAs are likely to be derived from linoleic acid (Drost et al., 1974).

Several reports have verified that the total content of THAs in beer ranges from 5.7 to 11.4  $\mu\text{g/ml}$  (Hamberg, 1991; and references therein). While 9,12,13-THA normally constitutes 75-85% of the THAs in beer, that of 9,10,13-THA is normally only 15-25%.

15

Other isomers are found in trace amounts.

In beer produced from wort prepared from malt of barley double null-LOX mutant A689 (cf. Example 8, except that a mashing in temperature of 60°C was used), the concentration of 9,12,13-THA was reduced by 75% compared to the control beer made from malt of cv. Power, and reduced by 45% compared to the null-LOX-1 raw mutant D112 (Table 4). Similar differences were also observed for the 9,10,13-THA isomer. These measurements were carried out using standard HPLC-mass spectrometry analyses (Hamberg, supra).

**Table 4.** THAs in beers produced from malt of cv. Power, null-LOX-1 mutants D112, and double null-LOX mutant A689 (average of 3 measurements).

Malt type	THA		
	9,12,13-	9,10,13-	9,12,13-:9,10,13-
	<i>ppm</i>		<i>ratio</i>
cv. Power	3.76	1.12	3.35
Mutant D112	1.72	0.94	1.80
Mutant A689	0.94	0.50	1.88

### Example 8

#### Pilot malting and brewing

Malting. The experiments were carried out with kernels of double null-LOX mutant A689, null-LOX-1 mutant D112, and cv. Power (in all cases of harvest 2007). Maltings in 30-kg-large scales were performed in a malt house as follows:

- (i) Steeping at 16°C: 8 h wet; 14 h dry; 8 h wet; 10 h dry; 4 h wet;
- (ii) Malting: 12 h at 18°C; 24 h at 16°C; 24 h at 14°C; 60 h at 12°C;
- (iii) Drying: 12 h at 60°C; 3 h at 68°C; 4 h at 74°C; 3 h at 80°C.

Properties of the barley and the malt are listed in Table 5. Examination of the results revealed that the samples fulfilled malt specifications for use of the raw materials in brewing.

**Table 5.** Experimental data.

Sample		cv. Power	Mutant A689	Mutant D112	cv. Barke
LOX genotype		wt LOX-1 wt LOX-2	null LOX-1 null LOX-2	null LOX-1 wt LOX-2	wt LOX-1 wt LOX-2
<b>Barley</b>					
Protein	%*	12.0	10.6	10.9	10.6
Starch	%*	61.7	63.0	62.5	64.2
Germination, 72 h	%	100	95	85	100.0
Germination Index	1–10	9.9	6.6	8.3	9.6
Water sensitivity	%	1	78	45	14
Grading >2.5 mm	%	97.0	98.8	91.6	88.3
TKW	g	48.6	55.8	37.5	49.5
$\beta$ -Glucan	%, dry	4.0	3.6	3.3	3.6
$\beta$ -Amylase**	U/g	1039	1458	1027	1093
<b>Malt</b>					
Steeping and germination time	h	124	148	124	124.0
Extract	%*	81.0	80.7	80.9	82.5
Kolbach Index	%	37.5	37.1	40.2	42.0
Modification	%	95	94	97	96
Homogeneity	%	81	61	81	83
$\beta$ -Glucan	%, dry	0.2	0.3	0.2	0.1
$\beta$ -Amylase**	U/g	956	1261	928	985
$\alpha$ -Amylase***	U/g	242	222	262	201

\* Measured by standard near infrared transmittance spectroscopy.

5 \*\*  $\beta$ -Amylase activity was measured using a Megazyme kit (product code K-BETA).

\*\*\*  $\alpha$ -Amylase activity was measured using a Megazyme kit (product code K-CERA).

Pilot brewing with malt. An initial 200-l trial, with key results thereof illustrated in FIG. 10A,B, involved the following steps:

- (i) Wort preparation;  
(ii) Wort separation;  
(iii) Wort boiling;  
5 (iv) Fermentation;  
(v) Lagering;  
(vi) Bright beer filtration; and  
(vii) Bottling.

10 Worts were prepared using 30-kg-large malt samples of wild-type cv. Power, null-LOX-1 mutant D112, and double null-LOX. Mashing-in was at 48°C for 20 min, followed by 18 min of heating in which the temperature was raised from 48°C to 67°C. The saccharification pause at 67°C was for 30 min, followed by a heat-up step to 72°C for 5 min, and a rest for 15 min before mashing-off at 78°C. The brewing steps as referred to  
15 herein above – i.e. wort boiling and filtration, whirlpool separation, fermentation, lagering, and packaging in green glass bottles – were according to specifications for standard brewing practice .

The T2N precursors of the finished beers were determined (FIG. 10), using the same  
20 experimental set-up as described in Example 6. Results of the instant large-scale experiment showed the same trend in relative levels of the T2N precursors as that in boiled wort of micro-malted kernels (compare FIGs. 9 and 10), again revealing notably lower values for both null-LOX-1 mutant D112 and double null-LOX mutant A876 in comparison with that of cv. Power. More specifically, the T2N precursors were reduced  
25 by ~40% and ~70% in beer samples of null-LOX-1 and double null-LOX, respectively, when compared to beer of wild-type cv. Power malt.

Also, levels of free T2N were measured in the aforementioned pilot-brewed beers, again showing the same trend as observed for the T2N potential – namely that beers  
30 made of null-LOX-1 and double null-LOX malt were notably lower in T2N than that brewed with malt of cv. Power. With respect to T2N levels, beer of double null-LOX malt was accordingly again demonstrated to be superior to that of null-LOX-1.

A separate aim was to establish whether beers were superior in taste qualities after  
35 forced aging when these were produced in 200-l-scale using a double null-LOX raw



material. The specialized beer taste panel as described in Example 9 was therefore asked to evaluate the pilot-brewed, normal beers for papery off-flavor taste after forced-aging at 37°C for one week, using a score from 0 (absent) to 5 (extreme). While beer of cv. Power was found to yield a papery score of 1.6, those of null-LOX-1 and double null-LOX were 1.2. and 0.6, respectively. This result substantiated the superiority of the double null-LOX raw material for production of flavor-stable beer.

### Example 9

#### Comparisons of normal and barley-brewed beers

Kernels of unmalted double null-LOX mutant A689, null-LOX-1 barley mutant D112, and wild-type cv. Power were applied to identical processes of barley brewings, each utilizing 25 kg milled, unmalted barley as the raw material for production of 200 l beer (for comparative purposes, 30 kg of milled malt was used to produce 200 l of normal beer in a parallel running experiment). The aim was not only to compare properties of wort and beer from barley and malt-derived brewings, but also to determine whether the above-mentioned mutants could confer improved off-flavor characteristics in both barley-brewed and normal, finished beer. The 200-l-large brewing trials comprised the same production steps as listed above in Example 8, with specific details described herein below.

Mashing and brewing with barley. In the instant experiment, wort was prepared in the presence of the enzyme mixture Onda Pro (Novozymes; batch no. NFNG0005), added at mashing-in according the recommendations provided by manufacturer (i.e. 87.5 g enzyme mix per 80 l H<sub>2</sub>O). The mashing conditions were: mashing-in at 54°C for 30 min; 10 min of heating to raise the temperature to 64°C; incubation for 45 min 64°C; heating up to 78°C for 13 min; 10 min pause at 78°C. The brewing steps of wort filtration and boiling, whirlpool separation, fermentation, lagering, and packaging in green glass bottles were according to specifications for standard brewing practice.

Standard malting, mashing, and brewing. Malting utilized barley kernels of double null-LOX mutant A689, null-LOX-1 mutant D112, and wild-type cv. Power (all from harvest 2009). Steeping incubation times at 16°C were: 8 h wet; 14 h dry; 8 h wet; 10 h dry; 4 h

wet. Malting conditions were: 12 h at 18°C; 24 h at 16°C; 24 h at 14°C; 60 h at 12°C. Kiln drying conditions were: 12 h at 60°C; 3 h at 68°C; 4 h at 74°C; 3 h at 80°C. Barley and malt analyses of the aforementioned raw materials were performed according to standard EBC methods, with the results listed in Table 6. All of the malts were  
5 considered suitable for brewing.

Mashing conditions were: initial incubation at 48°C for 20 min; a 18-min-long heating-up to 67°C; 30 min incubation at 67°C; then heating-up to 72°C for 5 min; 15 min of incubation at 72°C; heating-up to 78°C for 6 min; ending with a 5-min incubation at  
10 78°C. The brewing steps of wort filtration and boiling, whirlpool separation, fermentation, lagering, and packaging in green glass bottles were according to specifications for standard brewing practice.

Analysis of boiled wort – Free T2N. T2N levels in the extracts were determined by gas  
15 chromatography with mass spectrometric (GC-MS) detection following solid phase extraction on a C18 column, and derivatization of carbonyls with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine, essentially as described by Groenqvist et al. (1993); see also Example 5.

20 In a direct comparison with samples of cv. Power and null-LOX-1 mutant D112, there was observed a notable reduction in free T2N in boiled wort produced from both barley-brewed and normal malt of double null-LOX mutant A689 (FIG. 11). When compared with barley-brewed, boiled wort produced from null-LOX-1 mutant D112, that of double null-LOX mutant A689 exhibited a ~45% reduction in the level of free T2N (for the  
25 corresponding malted samples, the reduction was ~15%).

Similarly, a ~72% reduction was noted for free T2N in beer of double null-LOX mutant A689 in comparison with that of cv. Power (for the corresponding malted samples, the reduction was ~45%).

30 Analysis of boiled wort – T2N precursor. T2N precursors in boiled, barley-brewed and normal wort samples of cv. Power, null-LOX-1 mutant D112, and double null-LOX mutant A689 were determined by GC-MS following derivatization of carbonyls with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine, essentially as described by Gronqvist et al. (supra).  
35

As illustrated in FIG. 12, the T2N precursors of boiled, barley-brewed and normal worts were markedly lowest in samples from double null-LOX mutant A689, amounting to a reduction of ~70% in comparison with that of wild-type cv. Power (reductions were also registered in comparisons with samples from null-LOX mutant D112).

Analysis of barley-brewed beer only – Free T2N, forced aging. Bottled beer derived from fermentations of barley-brewed wort samples – and comprising similar levels of sulfite – were analyzed within one month for production of T2N over time.

The aforementioned barley-brewed beers were forced-aged at 37°C to follow the development of free T2N, as described herein above.

As illustrated in FIG. 13A, the three types of barley-brewed beers were easily distinguished as a result of pronounced differences in the kinetics of T2N development. While the reference beer of cv. Power barley performed as expected (64 ppt T2N after 8 weeks at 37°C) – with T2N levels 10-20% higher than a similar malt-based beer (not shown) – an unexpected and remarkably low development of T2N was observed in the barley beers derived from double null-LOX mutant A689 (16 ppt T2N after 8 weeks at 37°C), corresponding to a 75% reduction in free T2N of the final beer. For the barley-brewed beer of null-LOX-1 mutant D112 as compared with the same type of beer of wild-type kernels, there was 52% less free T2N over 8 weeks.

The forced-aging experiment emphasized the notable differences among the beers analyzed. Already after 1.5 weeks, the T2N level of the reference, barley-brewed beer of cv. Power exceeded the taste threshold level of ~50 ppt, while that of double null-LOX mutant 689 levelled-off at ~16 ppt T2N, thus far better than 32 ppt T2N for the barley-brewed beer of null-LOX-1 mutant D112.

Comparisons of barley-brewed and normal beer – T2N precursor. In FIG. 13B is provided a summary on data specifying levels of T2N precursor in fresh beer of barley-brewed and malted raw materials, both produced in a 200-l volume. Again, double null-LOX raw materials were superior in measured properties, actually with lower T2N potentials in barley-brewed beers (1.2 ppb), than in beers produced with null-LOX-1 malt (1.5 ppb).

Comparisons of barley-brewed and normal beer – Forced development of T2N. Also in this case, i.e. forced aging of beer at 37°C, there was a marked difference between beers made of wild-type and mutant raw materials (FIG. 13C). While both barley-brewed and normal beer of wild-type cv. Barke kernels exhibited ~50 ppt T2N after two weeks, the corresponding values were reduced by ~50% and ~75% for raw materials of null-LOX-1 mutant D112 and double null-LOX mutant A876, respectively. The same trend was observed after three weeks of forced-aging. Accordingly, it was substantiated that the use in beer production of raw materials deficient in LOX enzymes represents a superior way to drastically reduce the development of T2N during aging. And further in this respect, the raw materials of double null-LOX mutant A876 are superior to those of null-LOX-1 mutant D112.

Comparisons of barley-brewed and normal beer – THAs. Beer-specific THAs derived from linoleic acid were already described several decades back in time (Drost et al., 1974). Since then, various reports have verified that the total content of THAs in beer ranges from ~5-12 ppm (Hamberg, 1991; and references therein). While 9,12,13-THA normally constitutes 75-85% of the THAs in beer, that of 9,10,13-THA amounts to 15-25%; other isomers are found in trace amounts.

In fresh barley-brewed beers of a 200-l-scale experiment with raw materials of null-LOX-1 mutant D112 and double null-LOX mutant A689, the levels of LOX-1 and LOX-2 pathway-derived 9,12,13- and 9,10,13-THA were found to be reduced by ~60% and ~80%, respectively, in comparison with that of the control of cv. Power (FIG. 14A). A surprisingly high level of 9,10,13-THA, the main THA product from the LOX-2 branch of the LOX pathway, was measured in barley-brewed beer of null-LOX-1 mutant D112, namely +47% in comparison with that of beer from cv. Power. The result contrasted that obtained with double null-LOX mutant A689, for which there was measured a 60% decrease, again in comparison with cv. Power. Results of a separate experiment confirmed the aforementioned conclusion. The molecular basis for the observation remains elusive, but some cellular mechanism(s) may be speculated to be activated to compensate for the absence of LOX-1 in mutant D112 by enhancing the synthesis of enzymes involved in 9,10,13-THA formation.

For that reason, barley-brewed beer produced from double null-LOX mutant A689 generated a significantly lower 9,12,13-THA:9,10,13-THA ratio in comparison with cv. Power.

5 Determination of the 9,12,13-THA and 9,10,13-THA levels, combined with determination of their ratio, represents a convenient, initial tool to indicate whether a beer is produced using barley of double null-LOX mutant A689. However, a firmer assessment on that issue may involve additional examinations as described in the instant application.

10

In beer produced from malt of double null-LOX mutant A689, the concentration of LOX pathway-derived 9,12,13- and 9,10,13-THA was found to be reduced by ~75% and ~40%, respectively, as compared to the control beer of malted cv. Power (FIG. 14B). In said beer, there can also be calculated a very low 9,12,13:THA:9,10,13-THA ratio  
15 when compared to beer of cv. Power. In general, but not in all instances, THA levels are slightly lower in malt-based beers than in barley-brewed beers (compare FIG. 14A and B).

Analysis of barley-brewed beer only – Taste and flavor stability. A panel of flavor  
20 specialists evaluated the aforementioned, forced-aged barley-brewed beers of cv. Power, null-LOX-1 mutant D112, and double null-LOX mutant A689.

In general, the taste panel found satisfactory flavor profiles for all types of the fresh and the forced-aged beers after 1 week at 37°C. However, the "papery" taste scores were  
25 significantly higher for the reference beer than for that produced using malt of double null-LOX mutant A689 and null-LOX-1 mutant D112 (FIG. 15A), i.e. the reference beer was characterized by a more intense taste of said off-flavor. In general, the taste panel preferred the barley-brewed beer produced from double null-LOX barley of mutant A689.

30

The trained taste panel also evaluated the more general "aged" taste after beer storage for 1 month and 3 months at 30°C, again demonstrating that said flavor scores were significantly higher for the beers of cv. Power and null-LOX-1 than that of double null-LOX barley mutant A689 (FIG. 15B).

35

In summary, the improved flavor stability of barley-brewed and normal beer of double null-LOX mutant 689 is remarkable – simply because of the low T2N levels following storage, particularly at high temperatures. Accordingly, the actions of LOX-1 and LOX-2 in brewing set-ups represent key determinants for the appearance of T2N, a principal off-flavor compound in aged beer.

Comparisons of barley-brewed and normal beer – Beer foam. Barley-brewed and control beers were degassed for 20 min in an ultrasonic bath before 50 ml H<sub>2</sub>O was added to 150 ml beer. The mixture was slowly poured into a foam tower, consisting of a 16-cm-long, 7-cm-wide glass tube (with a glass filter and connector at the bottom and top, respectively). N<sub>2</sub> gas, at a flow rate of 400 ml min<sup>-1</sup>, was bubbled through said mixture from the bottom to generate beer foam. This was led through a tube, and collected in a graded sedimentation cone positioned on a weight.

The total foam weight was recorded at time intervals of 5 min until foam development ceased, both for barley-brewed beer (FIG. 16A), and for malt-brewed beer (FIG. 16B). In either case, beer of raw materials from double null-LOX mutant A689 generated the most foam. Actually, foam development was better in barley-brewed beer than in the comparable malt-based beer.

**Table 6.** Barley, malt, wort, and beer analyses.

Sample		cv. Power	Mutant D112	Mutant A689
<b>Barley analyses</b>				
Protein	%*	11.7	11.2	10.1
Starch	%*	63.3	62.9	62.7
Germination at 72 h	%	98	99	99
Germination Index	1–10	8.4	7.8	7.8
Water sensitivity	%	9	17	24
Grading >2.5 mm	%	96.2	98.9	98.0
TKW	g	48.2	54.2	47.7
β-Amylase (barley)*	U/g	1039	979	1491

trans-2-Nonenal <sup>†</sup>	ppb	270	190	150
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**Malt, wort, and beer analyses**

Steeping and germination	h	120	120	120
Extract	%*	81.5	81.2	81.4
Soluble N	%, NIT*	0.67	0.63	0.64
Modification	%	96	97	97
Homogeneity	%	88	86	81
β-Amylase**	U/g	768	865	1007
α-Amylase***	U/g	175	172	200
trans-2-Nonenal <sup>†</sup>	ppb	1100	470	390
Alcohol	%	3.94	3.87	3.80
Real extract	% Plato	3.76	3.80	3.30
Ph		4.3	4.2	4.2
Color	EBC U	7.1	8.2	5.2
Original extract	% Plato	11.42	11.33	10.72
Real degree of fermentation	%	68.4	67.8	70.4
Alcohol	% (vol.)	4	3	4
CO <sub>2</sub>	g/l	5.6	6.1	5.9
SO <sub>2</sub>	ppm	4	4	5
Diacetyl	ppm	23	21	10

\* Measured by standard near infrared transmittance spectroscopy.

\*\* β-Amylase activity was measured using a Megazyme kit (product code K-BETA).

\*\*\* α-Amylase activity was measured using a Megazyme kit (product code K-CERA).

<sup>†</sup> Measured basically as described by Groenqvist et al. (1993).

**Example 10****The gene for LOX-2 in barley mutant A689 is mutated**

5 The genomic nucleotide sequence encoding LOX-2 of cv. Barke (SEQ ID NO:1), and that of double null-LOX mutant A689 (SEQ ID NO:2), were obtained as described hereinafter. The sequences obtained were subsequently compared in order to determine the molecular basis for the null-LOX-2 genotype of mutant A689, the phenotype of which is characterized by the absence of LOX-2 activity in the  
10 germinating barley grain.

For said comparative purposes, barley genomic DNAs from mutant A689 and wild-type cv. Barke were isolated from leaflets of seedlings using the Plant DNA Isolation Kit (Roche). The two 3331-bp sequences covering the protein coding regions for LOX-2 in  
15 the genomic DNAs of mutant A689 and cv. Barke were amplified by PCR using the primers 5'-CGCAGCGAGCTAACTTAGAAGCGTGCCACA-3' (SEQ ID NO:3) and 5'-CCTCATGCCTTTGTGCTATCCTTGCTTGCT-3' (SEQ ID NO:4); basis for the primer sequences comprised the genomic sequence of the gene for LOX-2 (i.e. SEQ ID NO:1).

20 The PCR reactions consisted of 100 ng genomic DNA resuspended in 20 µl reaction buffer containing 5 pmol of each primer and 3.0 U FailSafe polymerase (Epicentre). The PCR amplifications were carried out in an MJ cycler, using the following parameters: 30 sec at 98°C for 1 cycle; 15 sec at 98°C; 30 sec at 65°C; and 60 sec at  
25 72°C for 30 cycles; 10 min at 72°C for 1 cycle. The resulting PCR products were separated on 1% agarose gels, and DNA fragments corresponding in length to the amplicons were purified using the Qiaex II gel extraction kit (Qiagen), and inserted into the plasmid vector pCR Blunt II TOPO Blunt (Invitrogen). The coding regions were applied to dideoxynucleotide chain termination reactions with specific oligonucleotide  
30 primers, followed by sequence determination on a MegaBACE 1000 DNA sequencer (GE Biosystems). In FIG. 17 is shown a schematic illustration of the genomic sequence spanning the start and stop codons of the region encoding LOX-2. Sequence comparisons were performed using the Lasergene sequence analysis software package ver. 5.2 (DNASTAR), revealing one point mutation in the form of a G→A  
35 substitution at position 2689 of SEQ ID NO: 2 in exon 6 (FIG. 17).



The wild-type sequence for LOX-2 encodes a 864-residue-long protein (SEQ ID NO:5), with a predicted mass of 96.7 kDa. While the mutation at position 684 in the LOX-1–encoding sequence of mutant D112 caused the introduction of a premature stop codon, that of the LOX-2–encoding gene of mutant A689 resulted in a C-terminal truncation of 180 amino acids, thus encoding a 76.8-kDa protein (SEQ ID NO:6).

Table 7 provides a summary on the molecular differences related to the gene of LOX-2 of the wild-type barley, null-LOX-1 barley mutant D112 (described in WO 2005/087934), and barley mutant A689 (double null-LOX).

**Table 7.** Molecular data on wild-type and mutant barley.

Property	Wild-type barley	Mutant D112*	Mutant A689
<b>LOX genotype</b>	wt LOX-1, wt LOX-2	null-LOX-1, wt LOX-2	null-LOX-1, null-LOX-2
<b>Base mutation**</b>			
LOX-1	–	G3474→A	G3474→A
LOX-2	–	–	G2689→A
<b>Protein data***</b>			
LOX-1	862 aa (96.4 kDa)	665 aa (74.2 kDa)	665 aa (74.2 kDa)
LOX-2	864 aa (96.7 kDa)	864 aa (96.7 kDa)	684 aa (76.8 kDa)

\* Mutant A689's mother line, Ca211901, exhibits identical properties.

\*\* Sequence numbering according to SEQ ID NO:1 of WO 2005/087934 for the LOX-1 gene, and for the LOX-2 gene according to SEQ ID NO:1 of the present publication.

\*\*\* Predicted protein lengths in amino acids (aa), with the corresponding, predicted masses in parentheses.

**Example 11****Genetic detection of barley double null-LOX mutant A689**

5 The single nucleotide polymorphism (SNP) assay represents a convenient way to identify plant mutants. By SNP is here meant a mutation point with at least two different nucleotides at one locus. Said assay is based on the combined use of two sets of PCR reactions using genomic DNA as template. Both reactions contain a locus-specific primer, and one of the two SNP primers (one for each allele of the sequence). Two sets  
10 of PCR reactions are performed per plant line, with the result of a PCR reaction being either that the SNP primer binds to sequences of the mutant or to the wild-type allele (FIG. 18A). In one of several methods, SNP analysis can be based on the identification of mutant lines by evaluating the banding pattern following gel electrophoresis of PCR products.

15

Genomic barley DNA from a barley breeding line and from the wild-type cv. Quench were isolated from leaf tissues of seedlings, using the Plant DNA isolation kit (Roche) according to the manufacturer's recommendations. The oligonucleotide primers used to amplify the SNP of the wild-type LOX-2 gene were  
20 5'-ACCTCAAGGACGCGGCGTGG-3' (SEQ ID NO:7) and 5'-GAGCGAGGAGTACGCGGAG-3' (SEQ ID NO:8), while those for the corresponding mutant gene were 5'-ACCTCAAGGACGCGGCGTGA-3' (SEQ ID NO:9) and 5'-GAGCGAGGAGTACGCGGAG-3' (SEQ ID NO:8). These primer combinations were used in PCR reactions to amplify DNA fragments of 200 bp comprising parts of the  
25 coding regions for LOX-2 of double null-LOX mutant A689 and cv. Quench, respectively (FIG. 18A). The PCR reactions consisted of 100 ng genomic DNA in 20 µl reaction buffer containing 25 pmol primer and 7 µl REDTaq mix (Sigma), used according to the manufacturer's instructions. The 29-cycles PCR amplifications were carried out in an MJ cycler: 2 min at 96°C for 1 cycle; 1 min at 95°C; 1 min at 68°C; 1  
30 min at 72°C; ending with a 10-min extension at 72°C.

Cross pollination between a homozygous barley plant carrying the LOX-1 mutation and a homozygous plant carrying the LOX-2 mutation may result in 4 different events. Using two sets of primers, one SNP primer set – FL820 (SEQ ID NO:10 and FL823  
35 (SEQ ID NO:11) – for identification of the LOX-1 mutation (G→A mutation at position

3474 of the LOX-1 gene), and one SNP primer set – FL1034 (SEQ ID NO:9) and FL1039 (SEQ ID NO:8) – for identification of a LOX-2 gene mutation [G→A substitution at position 2689 of the LOX-2 gene (SEQ ID NO:1)], it should be possible to identify one of the four aforementioned crossing events (outlined in FIG. 18B). In other words, a single combined PCR reaction can be used to generate PCR products specific for both of the aforementioned LOX-1 and LOX-2 mutations. The oligonucleotide primers used to amplify the SNP PCR product for the G→A mutation at position 3474 in the LOX-1 gene were 5'-CAAGGTGCGGTTGCTGGTGTC-3' (SEQ ID NO:10) and 5'-CTCGCGCGTCTCCTTCCAT-3' (SEQ ID NO:11), generating a 166-bp DNA fragment comprising a part of the coding region for LOX-1. The oligonucleotide primers used to amplify the SNP PCR product for detection of the G→A mutation at position 2689 in the gene for LOX-2 were 5'-ACCTCAAGGACGCGGCGTGA-3' (SEQ ID NO:9) and 5'-GAGCGAGGAGTACGCGGAG-3' (SEQ ID NO:8), generating a 200-bp DNA fragment comprising part of the coding region of LOX-2. FIG. 18C, lane 2, details that double null-LOX mutant A689 carried both of the aforementioned mutations, whereas the wild-type control carried neither (FIG. 18C, lane 3).

In another experiment, using the REDEExtract-N-Amp Plant PCR Kit (Sigma) according to the manufacturer's instructions, genomic barley DNA was isolated from leaf tissues of 23 breeding line seedlings, and subsequently used in PCR reactions that consisted of 100 ng genomic DNA in 20 µl reaction buffer containing 25 pmol primer. Amplifications were carried out in a DNA Engine cycler (MJ Research), according to the manufacturer's instructions: 2 min at 96°C for 1 cycle; 1 min at 95°C, 1 min at 68°C, 1 min at 72°C for 29 cycles; and finally 10 min at 72°C for 1 cycle. FIG. 19 shows the banding pattern following electrophoresis of the PCR products. Analysis revealed that the procedure is useful for selection of the desired combination null-LOX-1 and null-LOX-2 mutations.

### Sequence listing

SEQ ID NO:1	The sequence of wild-type genomic DNA encoding LOX-2 from cv. Barke.
SEQ ID NO:2	The sequence of mutant LOX-2 genomic DNA from barley

	mutant A689.
SEQ ID NO:3	Primer for amplification of protein coding region for LOX-2 (also designated FL960).
SEQ ID NO:4	Primer for amplification of protein coding region of genomic LOX-2 DNA (also designated FL961).
SEQ ID NO:5	The sequence of full length LOX-2 protein of wild type barley, cv. Barke.
SEQ ID NO:6	The sequence of mutant LOX-2 protein lacking LOX-2 activity from barley mutant A689.
SEQ ID NO:7	Primer for amplification of wild type LOX-2 DNA (also designated FL1035).
SEQ ID NO:8	Primer for amplification of LOX-2 DNA (also designated FL1039).
SEQ ID NO:9	Primer for amplification of mutant LOX-2 DNA of mutant A689 (also designated FL1034).
SEQ ID NO:10	Primer for amplification of mutant LOX-1 DNA of mutant D112 (also designated FL820).
SEQ ID NO:11	Primer for amplification of LOX-1 DNA (also designated FL823).

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**CLAIMS**

1. A beverage prepared from a barley plant, or a part thereof, wherein said beverage comprises a very low level of T2N potential, and wherein said barley plant, or part thereof, comprises a first mutation that results in a total loss of functional lipoxygenase (LOX)-1 enzyme, and a second mutation resulting in a total loss of functional LOX-2 enzyme.  
5
2. The beverage according to claim 1, wherein said beverage is a malt beverage.  
10
3. The beverage according to any one of claims 1 and 2, wherein the beverage is a fermented malt beverage.
4. The beverage according to any one of the preceding claims, wherein the beverage is beer.  
15
5. The beverage according to any one of claims 1 and 4, wherein said beverage is barley beer.
6. The beverage according to any one of the preceding claims 1 to 3, wherein the beverage is a non-alcoholic malt beverage.  
20
7. The beverage according to any one of the preceding claims, wherein the beverage comprises less than 50%, preferably less than 40%, more preferably less than 35%, T2N potential compared to the T2N potential of a beverage prepared in the same manner from barley cv. Power.  
25
8. The beverage according to any one of the preceding claims, wherein the beverage comprises less than 70%, preferably less than 60% T2N potential compared to a beverage prepared in the same manner from a null-LOX-1 barley mutant.  
30
9. The beverage according to any one of the preceding claims, wherein the beverage comprises less than 50%, preferably less than 40%, more preferably less than 35%, even more preferably less than 30%, yet more preferably less than 25% free T2N

compared to a beverage prepared in the same manner from barley cv. Power, after storage for 8 weeks at 37°C.

5 10. The beverage according to any one of the preceding claims, wherein the beverage comprises less than 50%, preferably less than 40%, more preferably less than 35%, free T2N compared to a beverage prepared in the same manner from barley cv. Power, after storage for 2 weeks at 37°C. in the presence of in the range of 2 to 4 ppm sulfite.

10 11. The beverages according to any one of the preceding claims, wherein the beverage comprises less than 70%, preferably less than 60%, even more preferably less than 55% free T2N compared to a beverage prepared in the same manner from null-LOX-1 barley after storage for 8 weeks at 37°C.

15 12. The beverages according to any one of the preceding claims, wherein the beverage comprises less than 80%, preferably less than 75% free T2N compared to a beverage prepared in the same manner from null-LOX-1 barley after storage for 2 weeks at 37°C.

20 13. The beverage according to any one of the preceding claims, wherein the beverage has a score for papery taste at least 0.5, preferably at least 0.7, more preferably at least 1.0 lower than the score for papery taste of a beverage prepared in the same manner from cv. Power after storage at 37°C for two weeks, when evaluated by a trained, specialized taste panel and scored on a scale from 0 to 5, where 0 is absent and 5 is extreme.

25 14. The beverage according to any one of claims 9 to 13, wherein said storage is performed in the presence of in the range of 2 to 6 ppm sulfite.

30 15. A barley plant, or part thereof, comprising a first mutation resulting in a total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of functional LOX-2 enzyme.

16. The barley plant, or a part thereof, according to claim 15, wherein the gene encoding LOX-1 of said plant comprises a premature stop codon.

17. The barley plant, or a part thereof, according to any one of claims 15 to 16 ,  
wherein the gene encoding LOX-1 of said plant comprises a premature stop codon  
situated at the most 705, preferably at the most 665 codons downstream of the start  
codon.
- 5 18. The barley plant, or a part thereof, according to claim 16, wherein the gene  
encoding LOX-1 of said plant comprises a nonsense codon, said codon corresponding  
to base nos. 3572–3574 of SEQ ID NO:2 of WO 2005/087934.
- 10 19. The barley plant, or a part thereof, according to any one of claims 15 to 18,  
wherein the gene encoding LOX-2 of said plant comprises a premature stop codon.
20. The barley plant, or a part thereof, according to any one of claims 15 to 19,  
wherein the gene encoding LOX-2 of said plant comprises a premature stop codon  
15 situated at the most 707, preferably at the most 684 codons downstream of the start  
codon.
21. The barley plant, or a part thereof, according to claim 20, wherein the gene  
encoding LOX-2 of said plant comprises a mutation at nucleotide position 2689 of SEQ  
20 ID NO:1, leading to formation of a stop codon.
22. The barley plant, or a part thereof, according to any one of claims 15 to 21,  
wherein said plant is selected from the group consisting of plants designated "Barley,  
Hordeum vulgare L.: Line A689", deposited with ATCC with the deposit number PTA-  
25 9640, and progeny plants thereof.
23. The barley plant, or a part thereof, according to any one of claims 15 to 22,  
wherein said part of said barley plant is kernel(s).
- 30 24. The barley plant, or a part thereof, according to any of claims 15 to 23, wherein  
said plant is produced by a method comprising the steps of; or said plant is progeny of  
a plant produced by a method comprising the steps of:
- (i) Providing a barley plant, or parts thereof, with a total loss of LOX-1 activity;  
and

- (ii) Mutagenizing said barley plant, and/or barley cells, and/or barley tissue, and/or barley kernels, and/or barley embryos from said barley plant, thereby obtaining generation M0 barley; and
- (iii) Breeding said mutagenized barley plants, kernels, and/or embryos for at least 2 generations, thereby obtaining generation Mx barley plants, wherein x is an integer  $\geq 2$ ; and
- (iv) Obtaining embryos from said Mx barley plants; and
- (v) Germinating said embryos;
- (vi) Determining the LOX-1 and LOX-2 activities in said germinated embryos, or parts thereof; and
- (vii) Selecting plants with a total loss of LOX-1 and LOX-2 activities in the germinated embryos;
- (viii) Determining the presence or absence of a mutation in the gene encoding LOX-1, and in the gene encoding LOX-2; and
- (ix) Selecting plants carrying a mutation in the gene encoding LOX-1, and in the gene encoding LOX-2;

thereby obtaining a barley plant carrying a mutation in the gene encoding LOX-1- and in the gene encoding LOX-2 causing a total loss of LOX-1 activity and a total loss of LOX-2 activity.

25. A beverage according to any one of claims 1 to 14, wherein the beverage is prepared from a barley plant, or a part thereof, according to any one of claims 15 to 24.
26. A composition comprising the barley plant, or part thereof, according to any of claims 15 to 24.
27. A plant product comprising a processed barley plant, or part thereof, wherein said barley plant is the barley plant according to any of claims 15 to 24 .
28. The plant product according to claim 27, wherein the plant product is a malt composition comprising a processed barley plant, or part thereof, wherein said barley plant is the barley plant according to any of claims 15 to 24.
29. The malt composition according to claim 28, wherein said part of said barley plant is kernel(s).

30. The malt composition according to any of claims 28 to 29, wherein the malt composition comprises at the most 60% free T2N compared to a malt composition prepared in the same manner from barley mutant D112 having American Type Culture  
5 Collection (ATCC) deposit accession No. PTA-5487.

31. The malt composition according to any of claims 20 to 22, wherein the malt composition comprises less than 30%, more preferably less than 20%, even more preferably less than 10% free T2N compared to a malt composition prepared in the  
10 same manner from cv. Power.

32. The plant product according to claim 27, wherein the plant product is a wort composition prepared using the barley plant, or part thereof, according to any of claims 15 to 24, or using a malt composition prepared from said barley plant, or part thereof,  
15 or mixtures thereof.

33. The wort composition according to claim 32, wherein said part of said plant is kernel(s).

20 34. The wort composition according to any one of claims 32 and 33, wherein said wort composition is barley wort.

35. The wort composition according to any one of claims 32 to 34, wherein said wort composition comprises less than 50%, more preferably less than 40%, even more preferably less than 30% free T2N compared to a wort composition prepared in the  
25 same manner from a wild-type barley.

36. The wort composition according to any one of claims 32 to 35, wherein said wort composition comprises less than 50%, more preferably less than 40%, even more preferably less than 30% T2N potential compared to a wort composition prepared in  
30 the same manner from a wild-type barley,

37. The wort composition according to any one of claims 32 to 36, wherein said malt composition is a malt composition according to any of claims 28 to 31.  
35

38. The wort composition according to any one of claims 32 to 37, wherein the wort composition has been produced using a mashing in temperature not exceeding 70°C, preferably not exceeding 69°C.

5        39. The wort composition according to any one of claims 32 to 38, wherein the wort composition has been produced by a method wherein the temperature has not been higher than 70°C for more than 25 min during mashing.

40. The plant product according to claim 27, wherein the plant product is a composition prepared from a mixture of:

- 10            (i)    A composition comprising a barley plant, or a part thereof, according to any of claims 15 to 24; and
- (ii)    A malt composition according to any of claims 28 to 31.

41. The plant product according to claim 27, wherein the plant product is a wort  
15        composition, or a beverage, prepared from the composition of claim 40.

42. The beverage according to any of claims 1 to 14, wherein said beverage is prepared from a malt composition according to any one of claims 28 to 31, or a wort composition according to any one of claims 32 to 39.

20

43. The plant product according to claim 27, wherein the plant product is selected from the group consisting of barley syrups, malt syrups, barley extracts, and malt extracts.

44. A method of producing a beverage with a very low level of T2N potential, said  
25        method comprising the steps of:

- (i)    Preparing a composition comprising a barley plant, or parts thereof, according to any of claims 15 to 24; and
- (ii)    Processing the composition of (i) into a beverage;
- thereby obtaining a beverage with a very low level of T2N potential.

30

45. The method according to claim 44, wherein said very low level of T2N potential is at the most 50%, preferably at the most 40%, more preferably at the most 35% of the T2N potential of a beverage prepared in the same manner from barley cv. Power.

46. The method according to any one of claims 42 to 43, wherein said very low level of T2N potential is less than 70%, preferably less than 60% compared to a beverage prepared in the same manner from a null-LOX-1 barley mutant.

5 47. The method according to any one of claims 44 to 46, wherein step (i) comprises preparing a malt composition from kernels of said barley plant, or part thereof.

48. The method according to any of claims 44 to 47, wherein processing the composition into a beverage comprises a mashing step, thereby producing a wort  
10 composition.

49. The method according to any of claims 44 to 48, wherein processing the composition into a beverages comprises a mashing step and a sparging step, thereby producing a wort composition.  
15

50. The method according to any one of claims 48 and 49, wherein said mashing step involves mashing of milled malt or mashing of milled barley or mashing of a mixture of milled malt and barley.

20 51. The method according to any of claims 48 to 50, wherein processing the composition into a beverages comprises fermentation of said wort.

52. A method of producing a malt composition comprising very low levels of free T2N, said method comprising the steps of:

- 25 (i) Providing kernels according to claim 23;  
(ii) Steeping said kernels;  
(iii) Germinating the steeped kernels under predetermined conditions;  
(iv) Treating germinated kernels with heat;

thereby producing a malt composition comprising a very low level of free T2N.

30 53. The method according to claim 52, wherein the very low levels of free T2N is at the most 50% T2N compared to the free T2N in a malt composition prepared in the same manner from barley mutant D112 having ATCC deposit accession No. PTA-5487.

54. A method of preparing a barley plant comprising a first mutation resulting in a total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of functional LOX-2 enzyme, comprising the steps of:

- 5 (i) Providing a barley plant, or parts thereof, with a total loss of functional LOX-1 enzyme; and
- (ii) Mutagenizing said barley plant, and/or barley cells, and/or barley tissue, and/or barley kernels, and/or barley embryos from said barley plant. thereby obtaining generation M0 barley; and
- 10 (iii) Breeding said mutagenized barley plants, kernels, and/or embryos for at least 2 generations, thereby obtaining generation Mx barley plants, wherein x is an integer  $\geq 2$ ; and
- (iv) Obtaining embryos from said Mx barley plants; and
- (v) Germinating said embryos;
- 15 (vi) Determining the LOX-1 and LOX-2 activities in said germinated embryos, or parts thereof; and
- (vii) Selecting plants with a total loss of LOX-1 and LOX-2 activities in the germinated embryos;
- (viii) Determining the presence or absence of a mutation in the gene encoding LOX-1, and in the gene encoding LOX-2; and
- 20 (ix) Selecting plants carrying a mutation in the gene encoding LOX-1, and in the gene encoding LOX-2;

thereby obtaining a barley plant comprising a first mutation resulting in a total loss of functional LOX-1 enzyme, and a second mutation resulting in a total loss of functional LOX-2 enzyme.



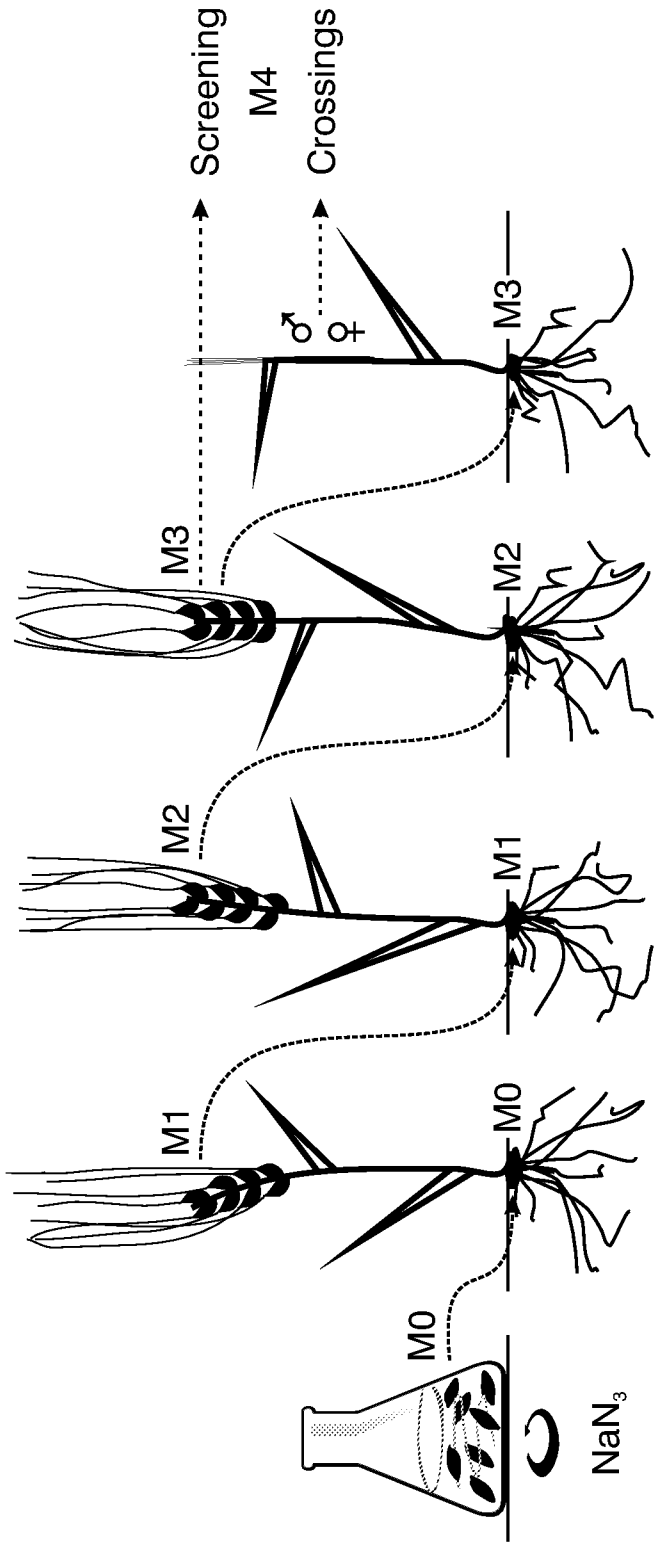


FIG. 1

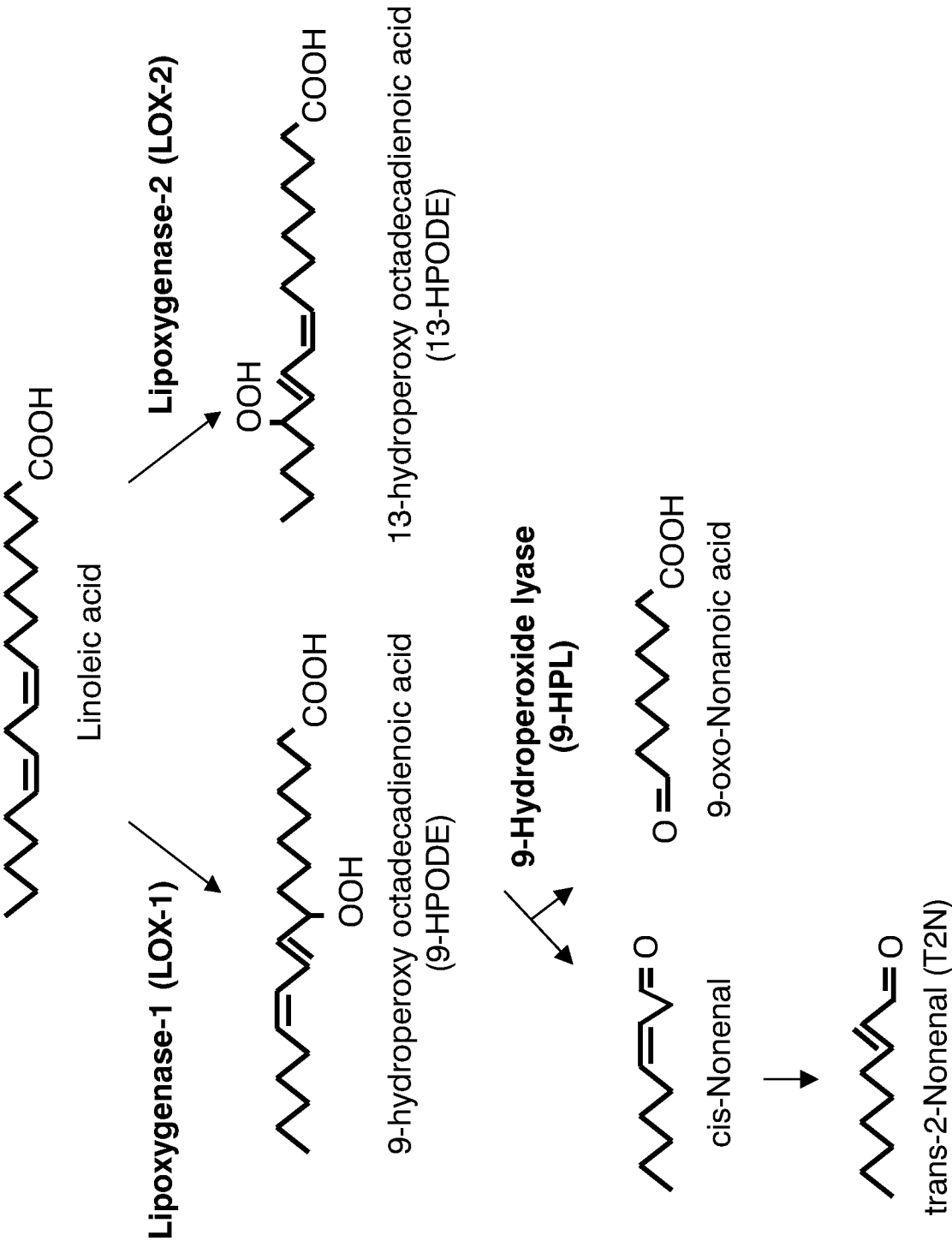


FIG. 2

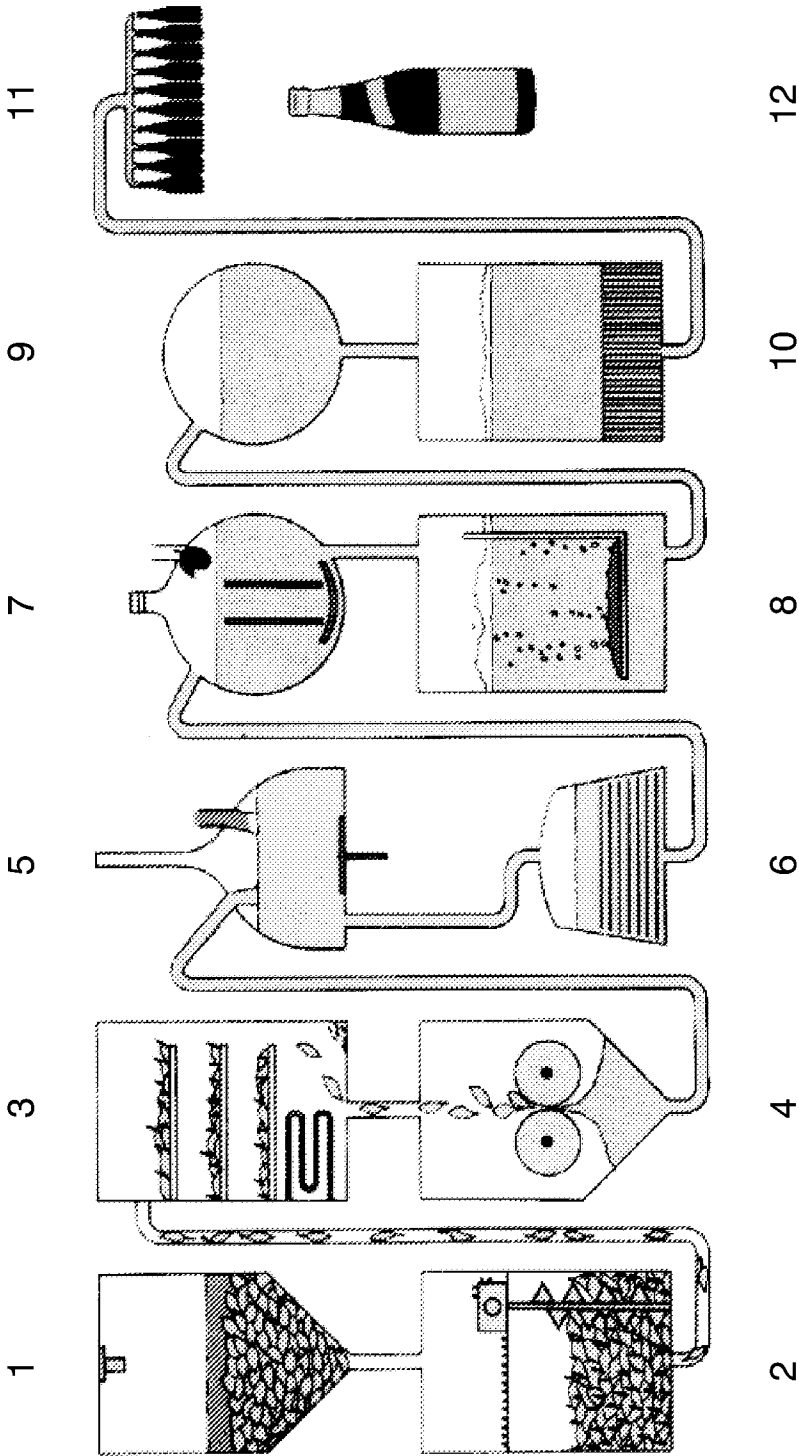
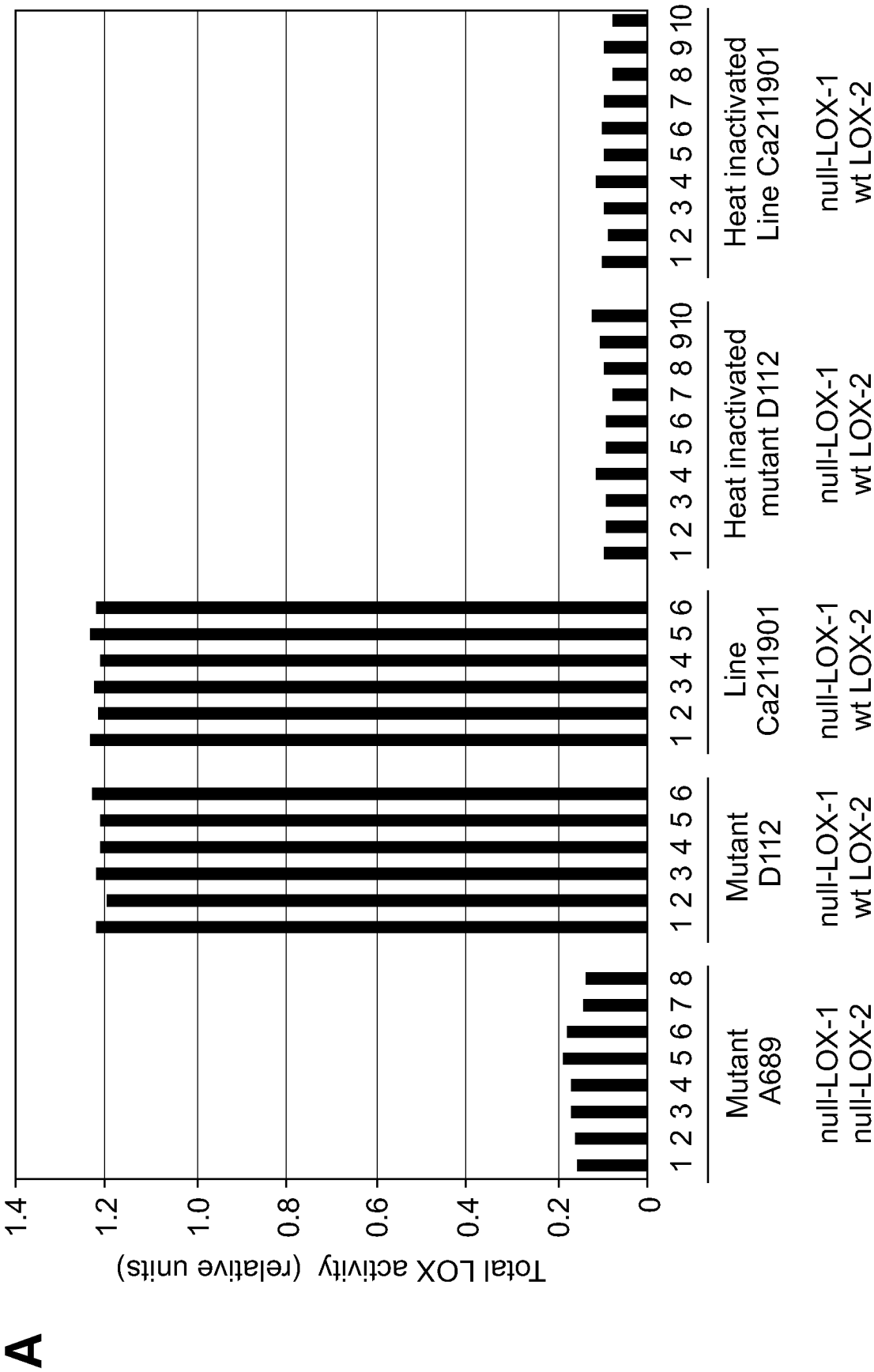


FIG. 3



**FIG. 4**

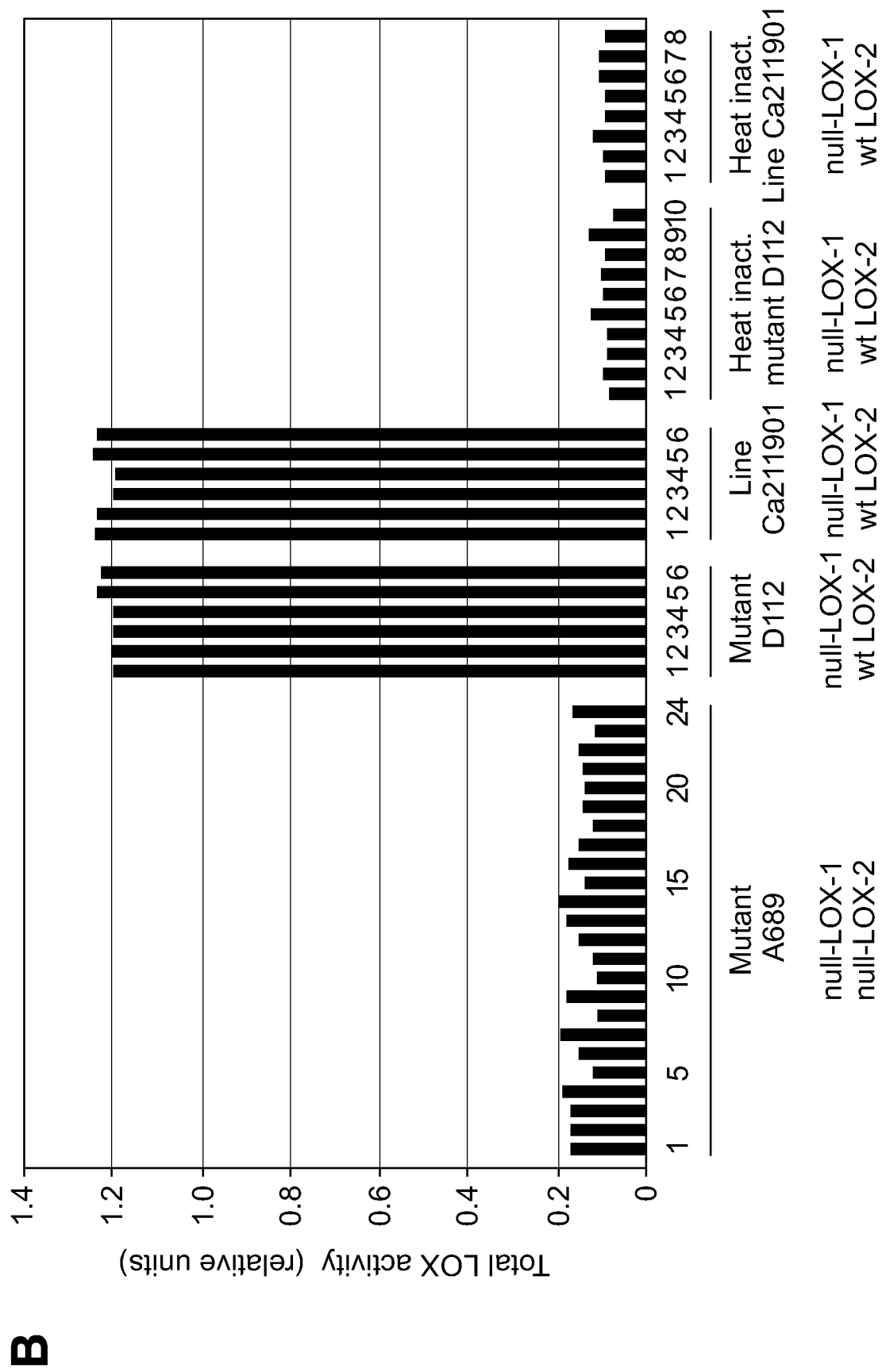


FIG. 4

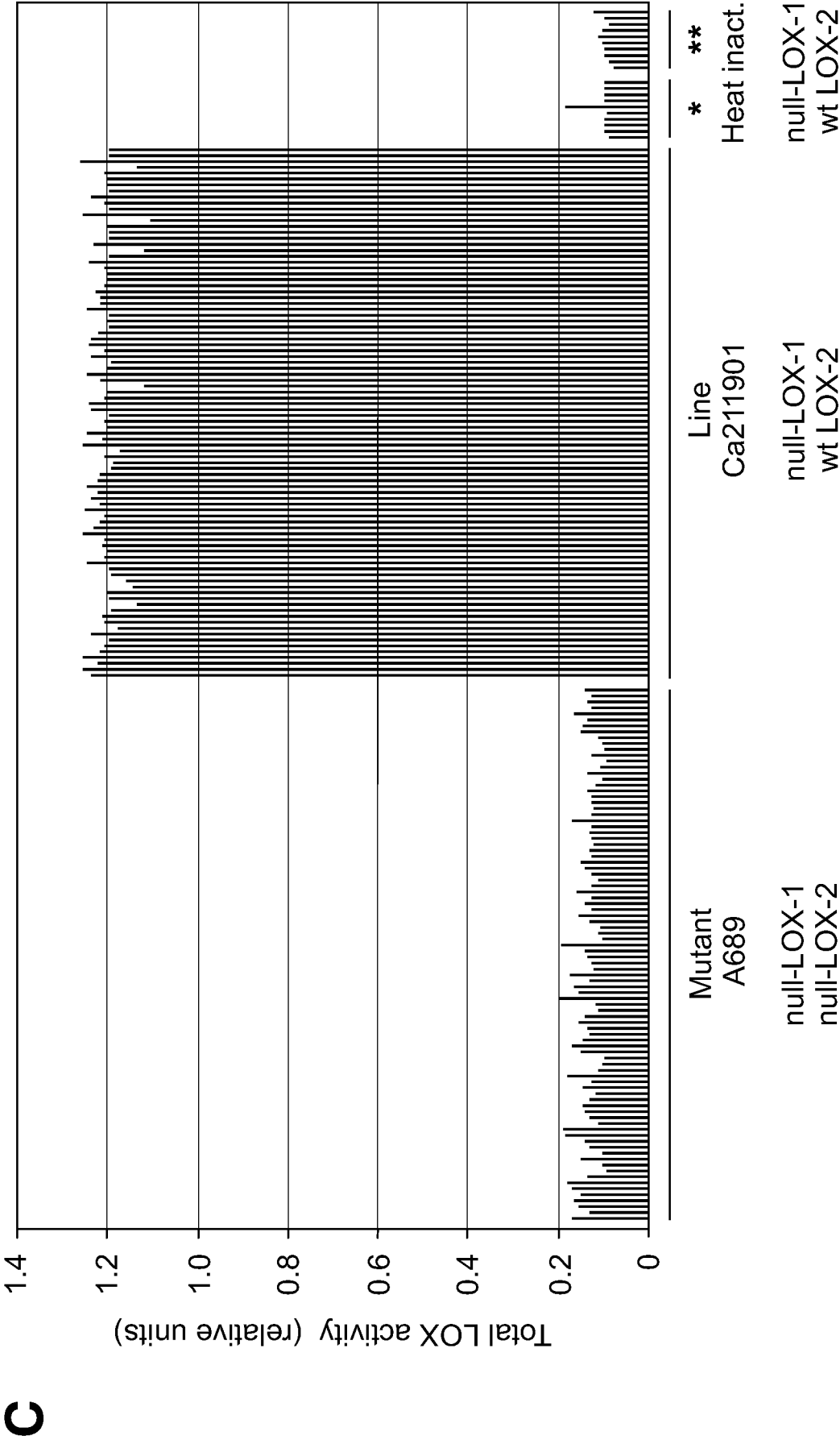


FIG. 4

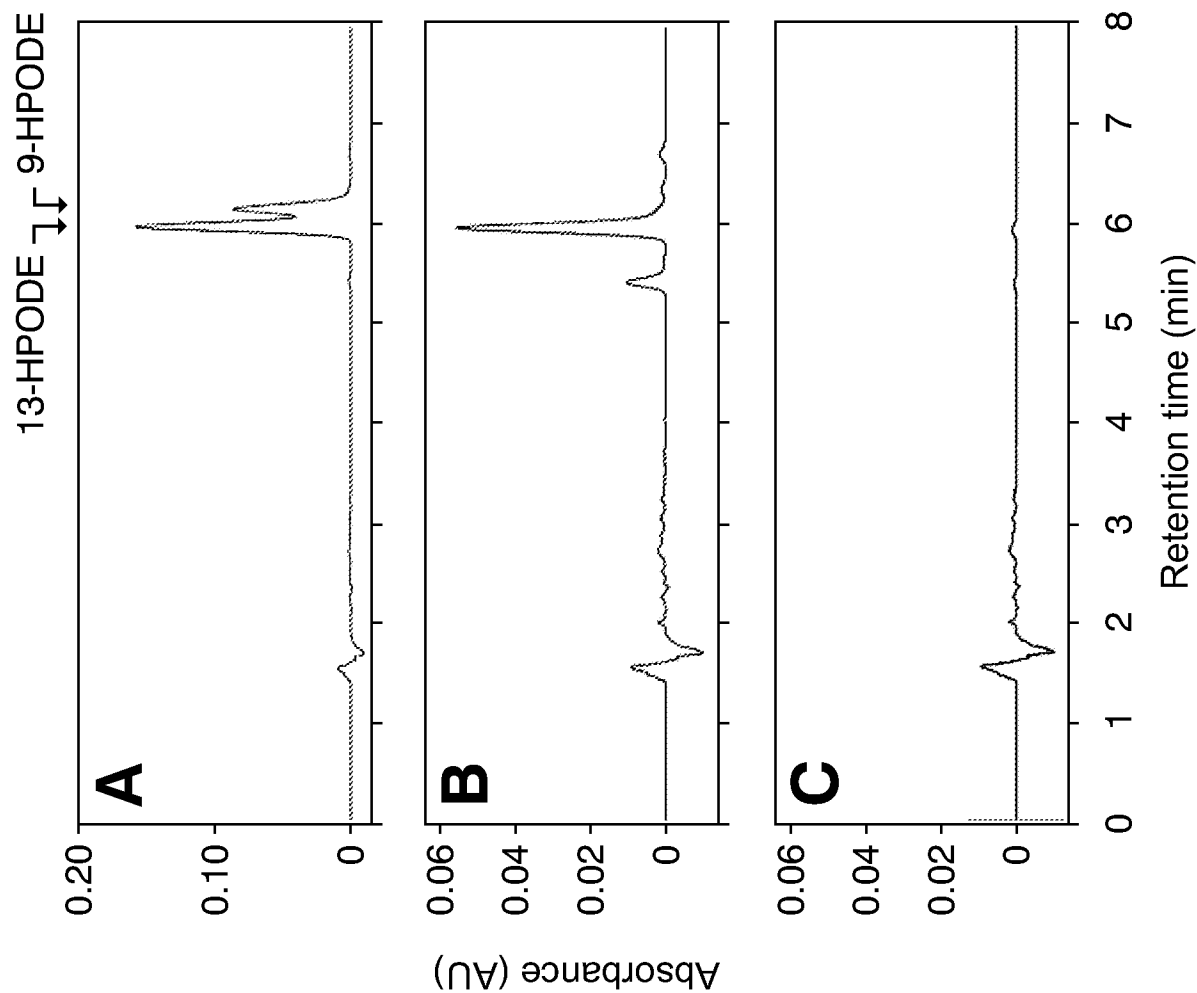


FIG. 5

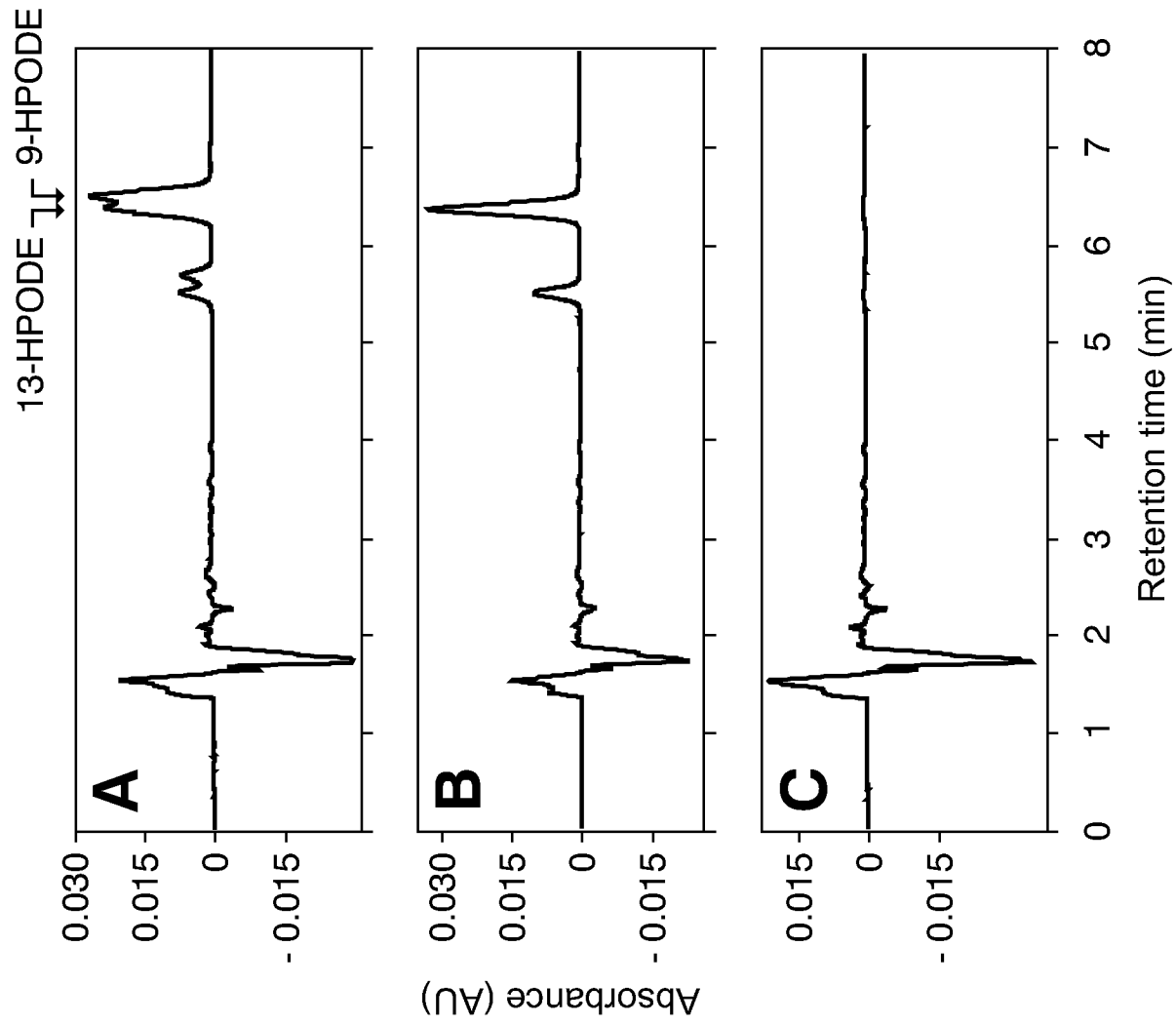


FIG. 6



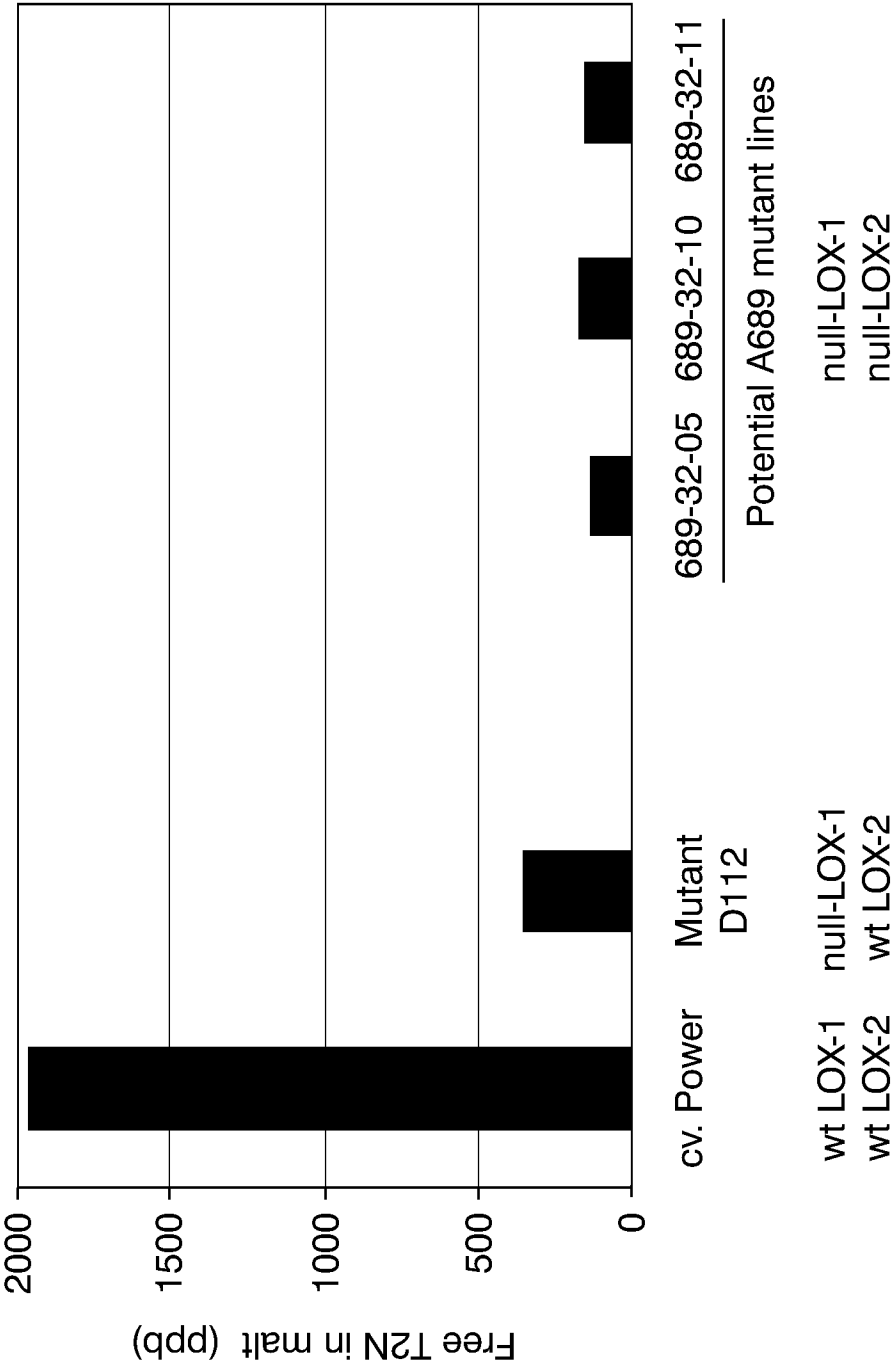


FIG. 7

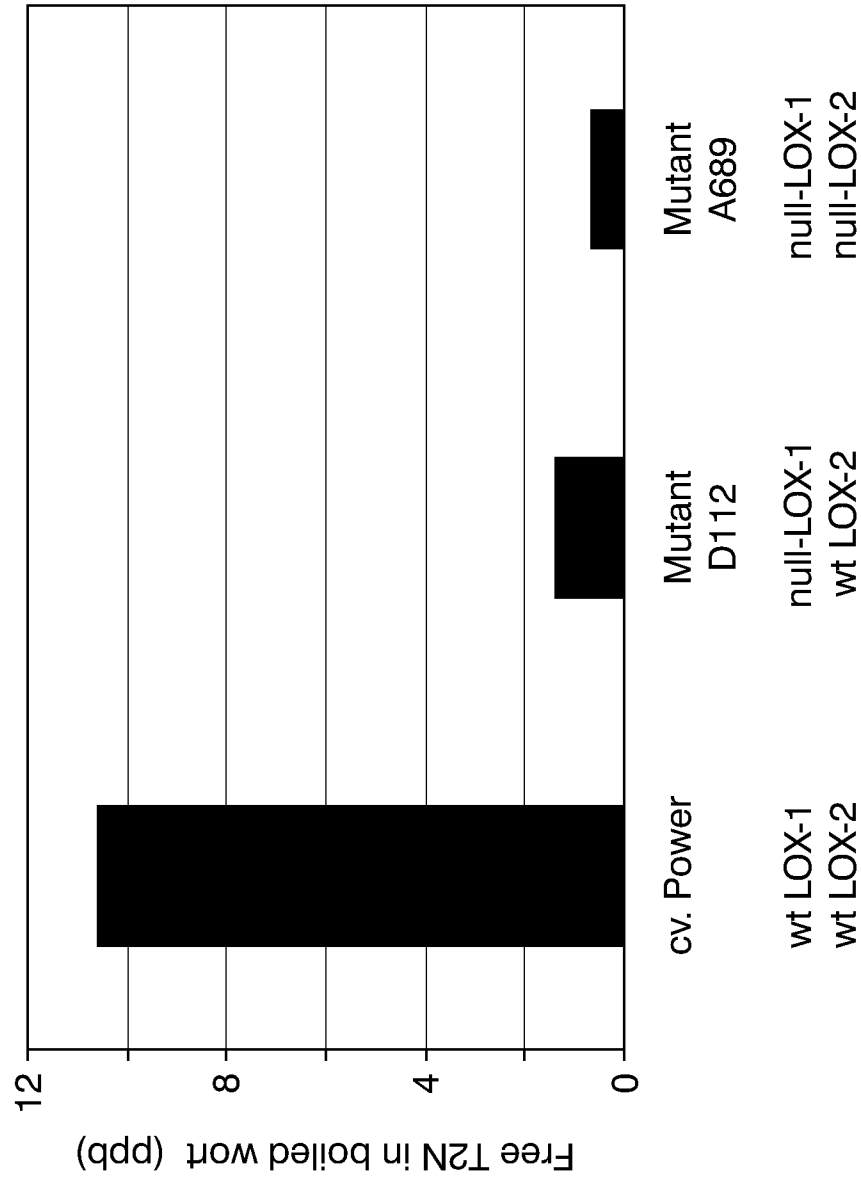


FIG. 8

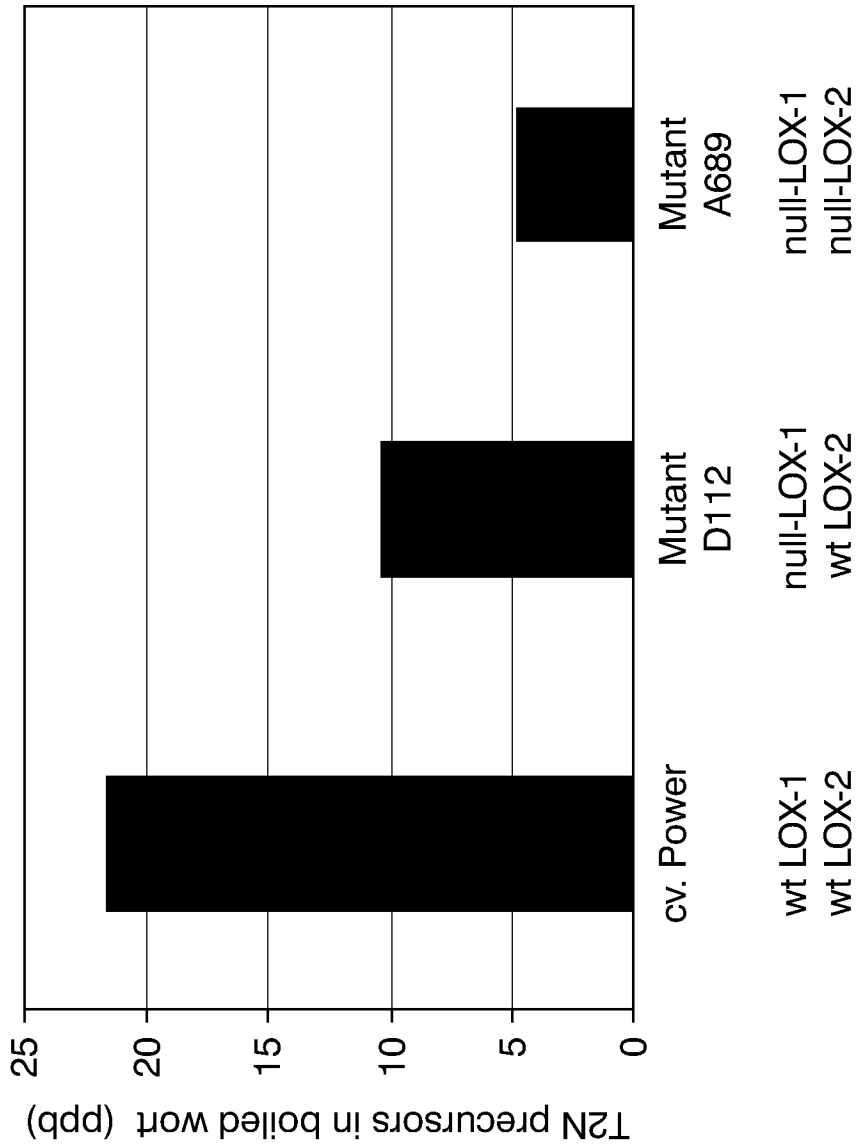


FIG. 9

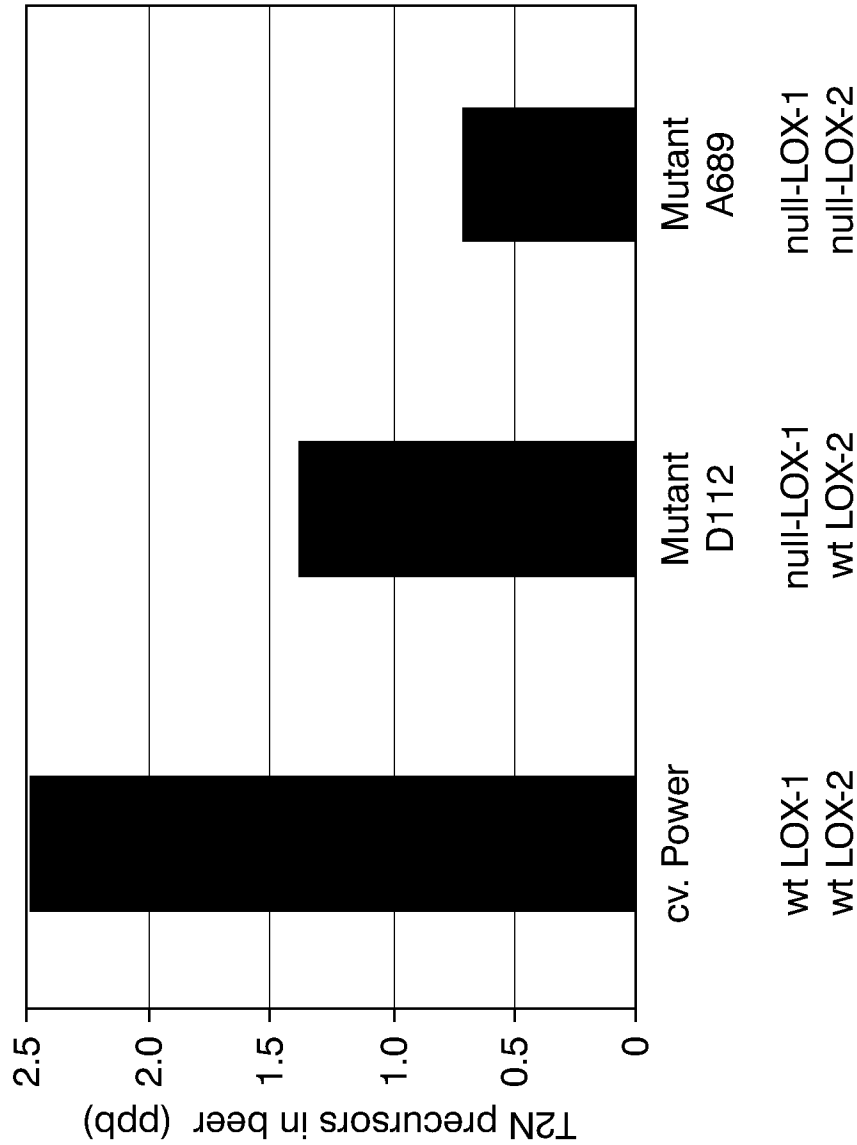


FIG. 10

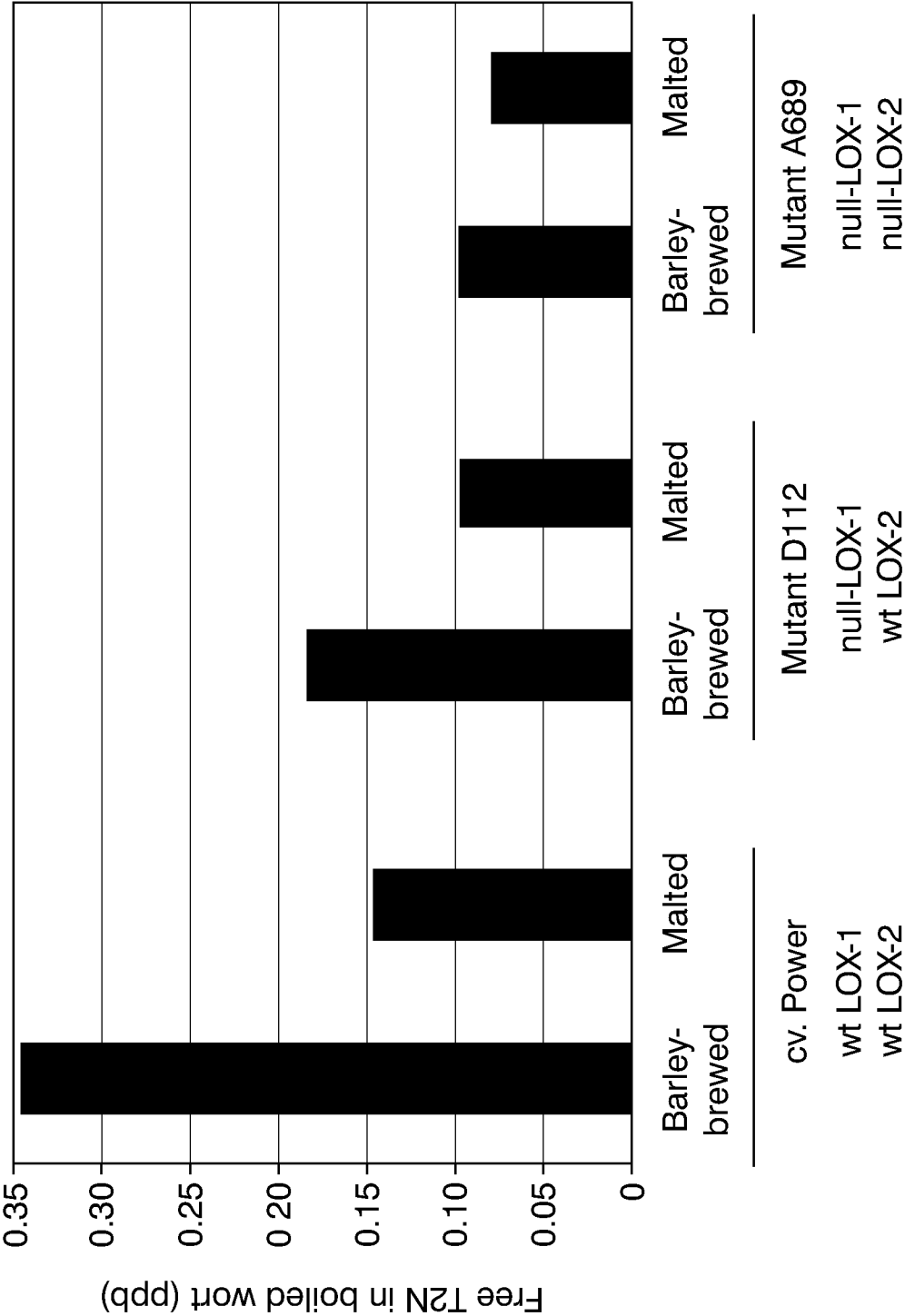


FIG. 11

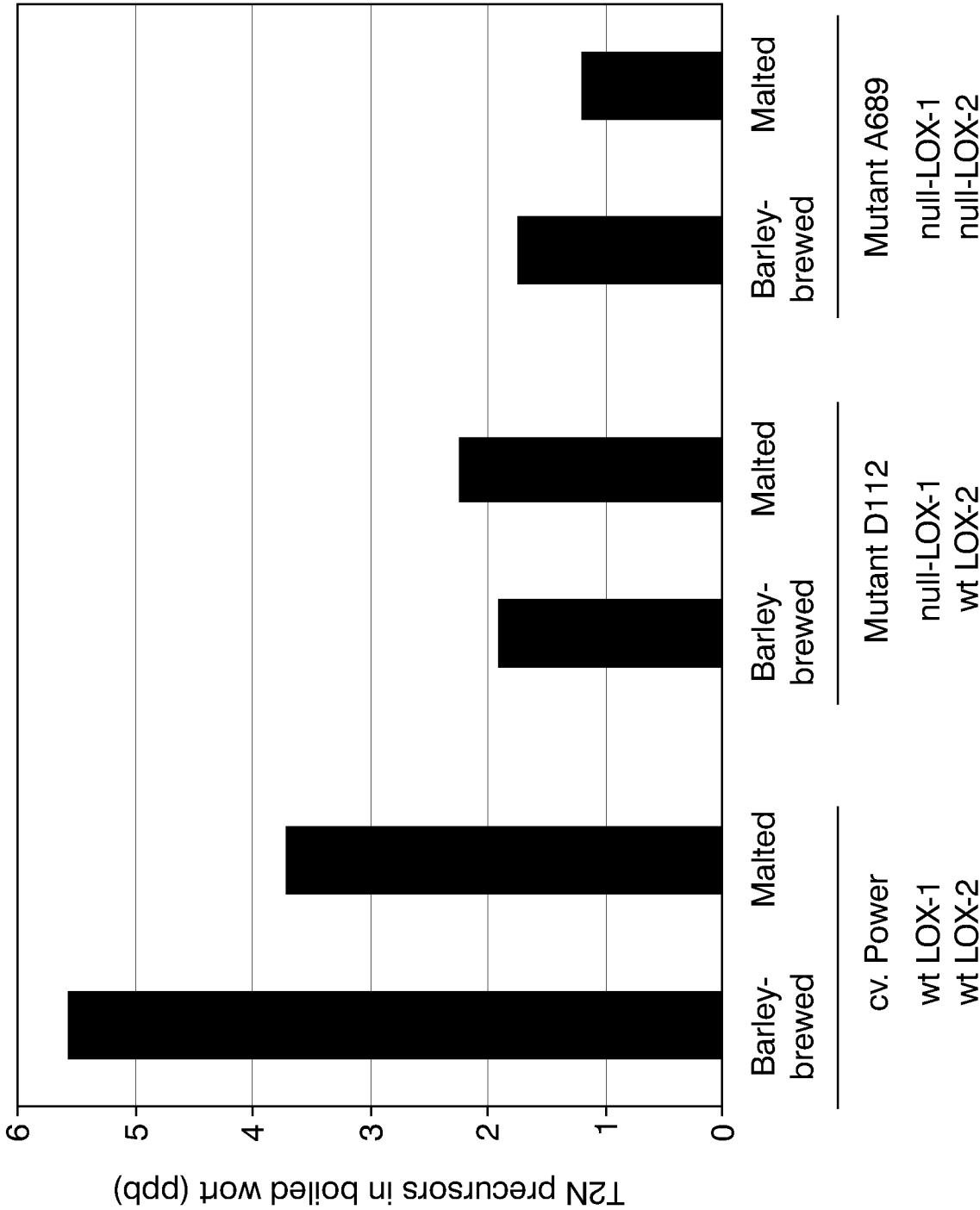


FIG. 12

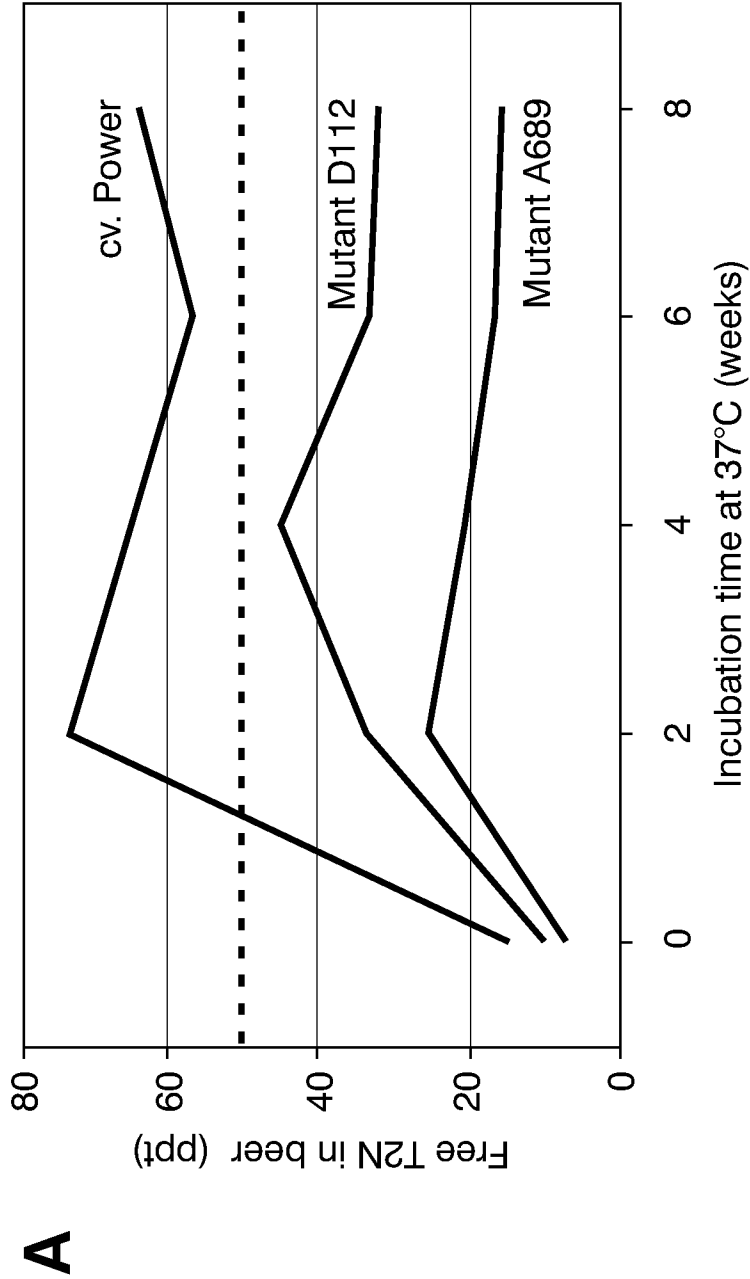


FIG. 13

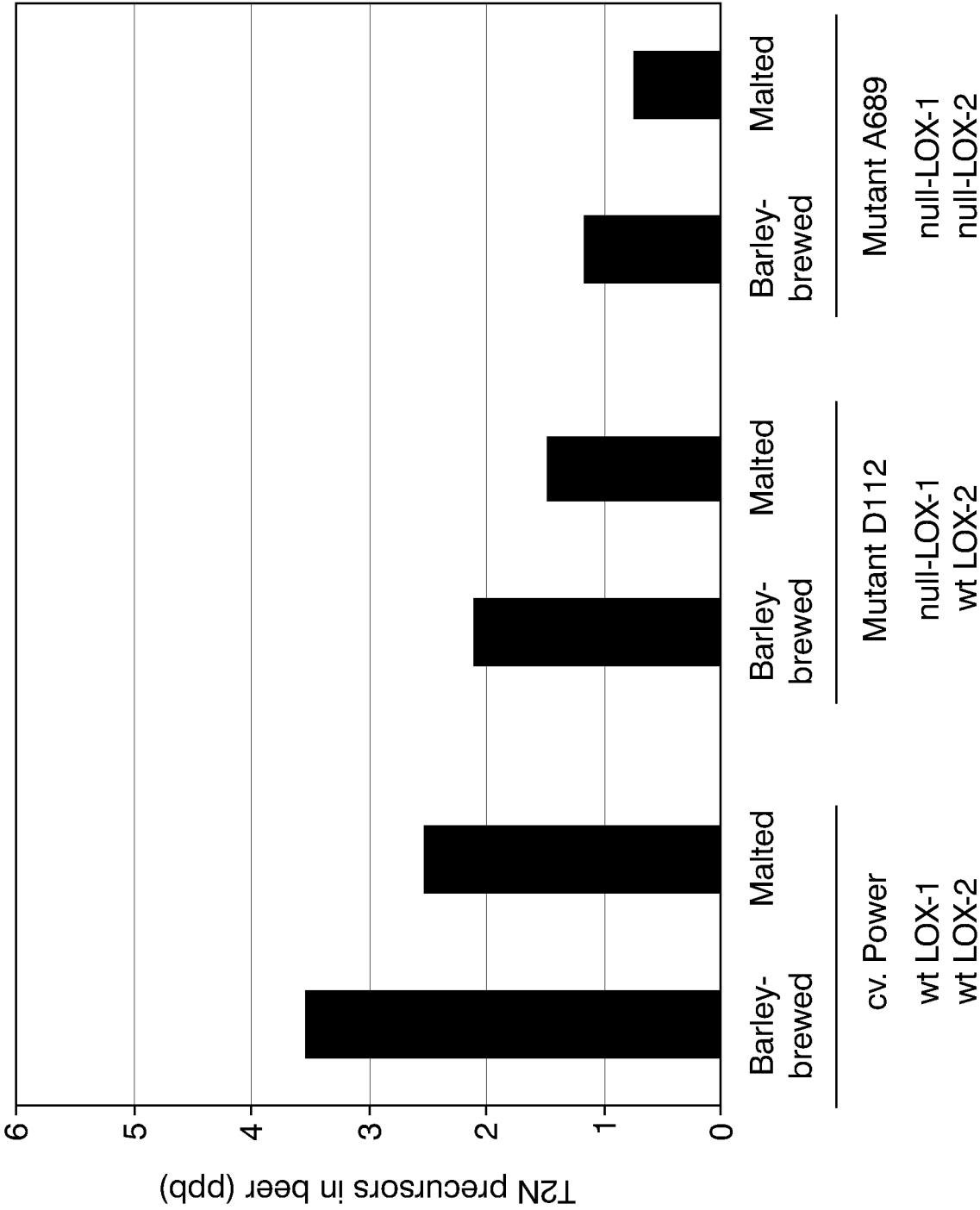


FIG. 13

B



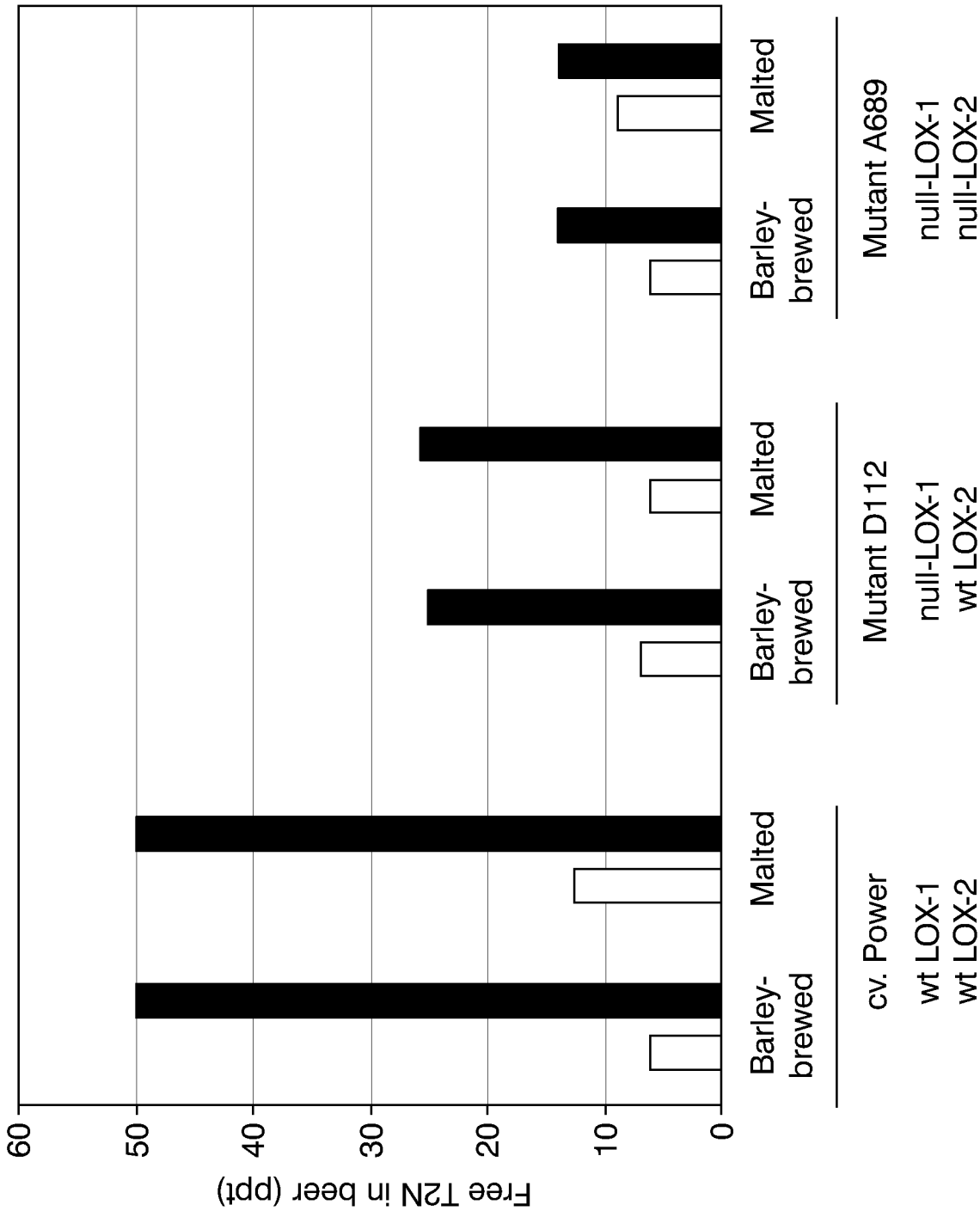
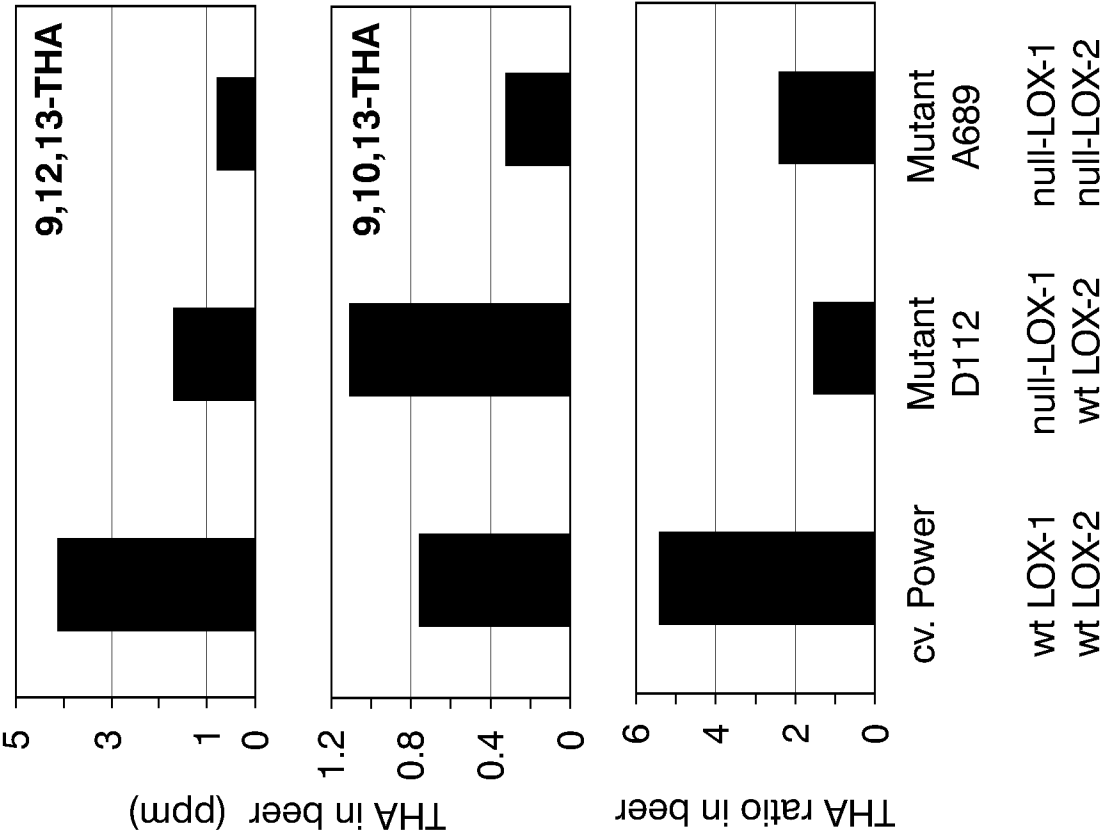


FIG. 13

**A**



**B**

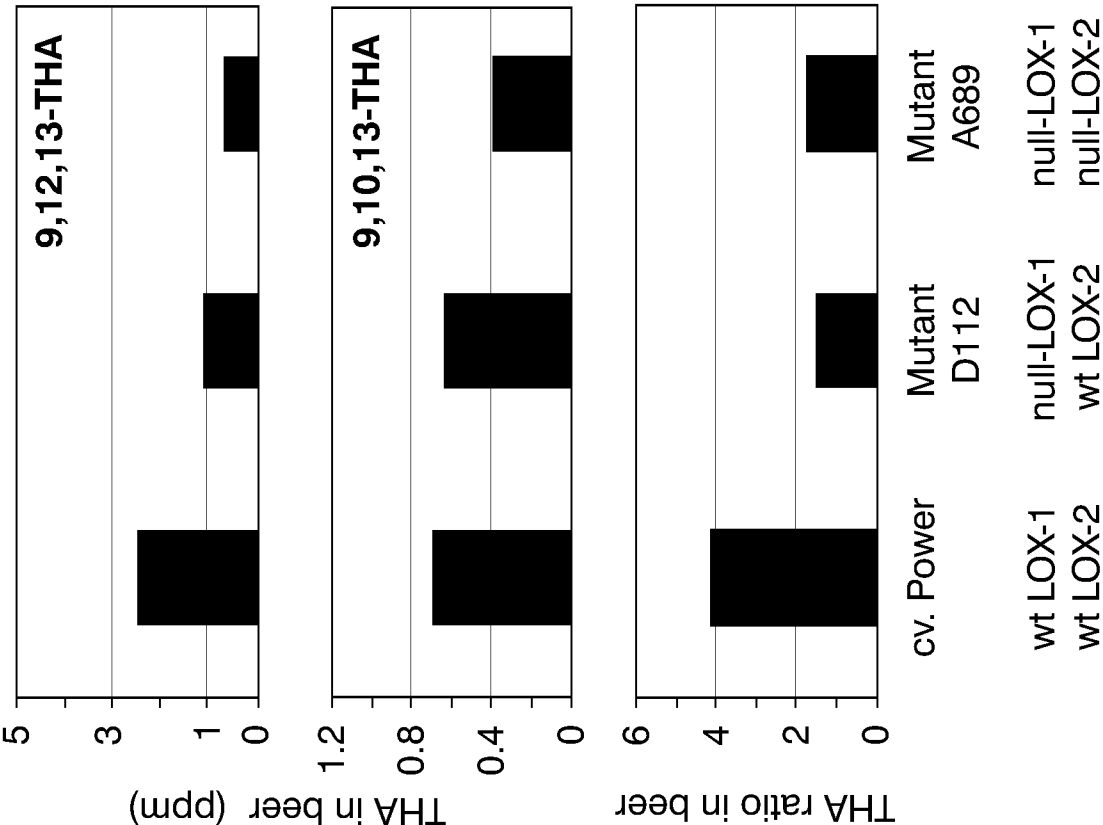


FIG. 14

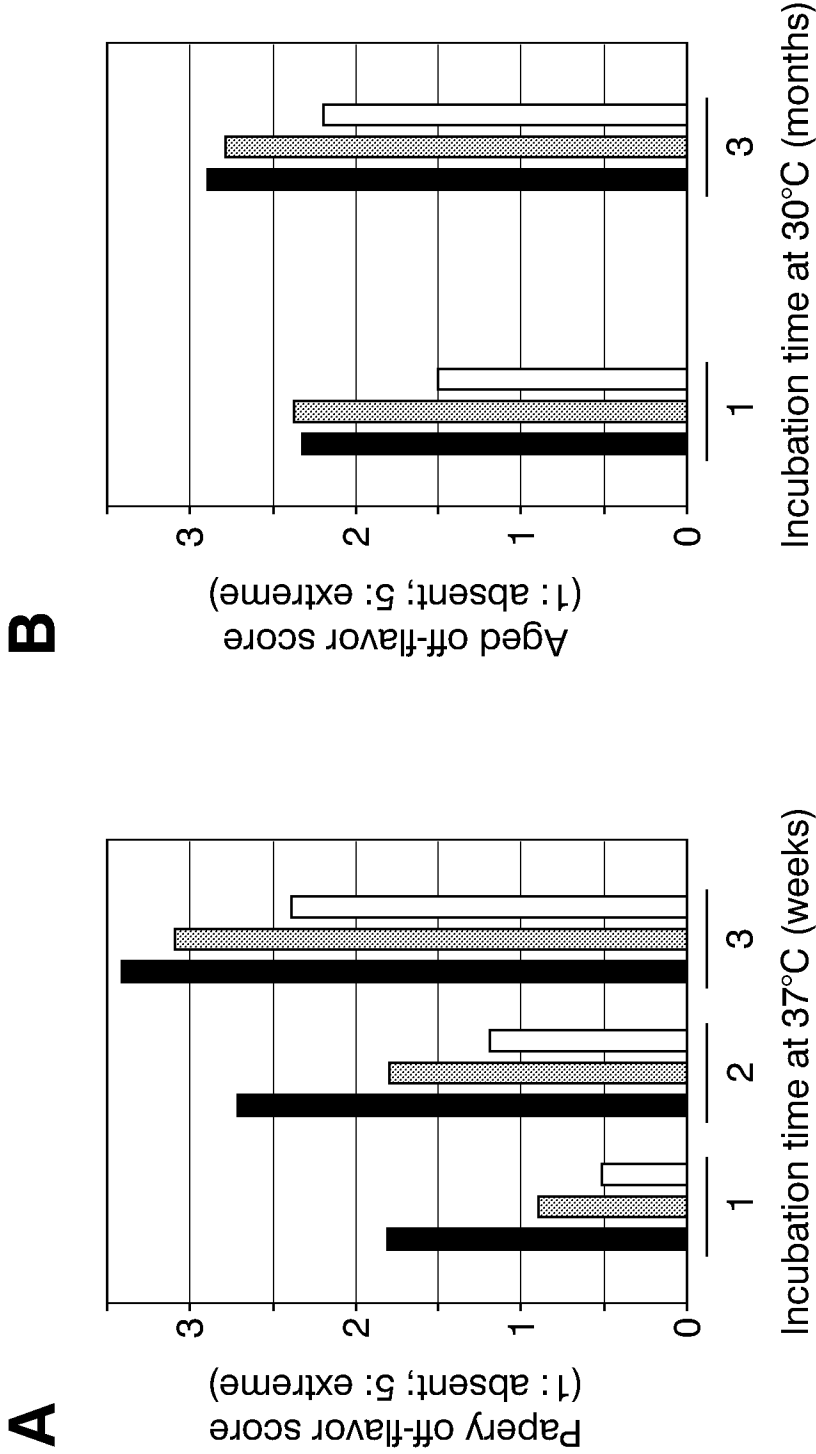


FIG. 15

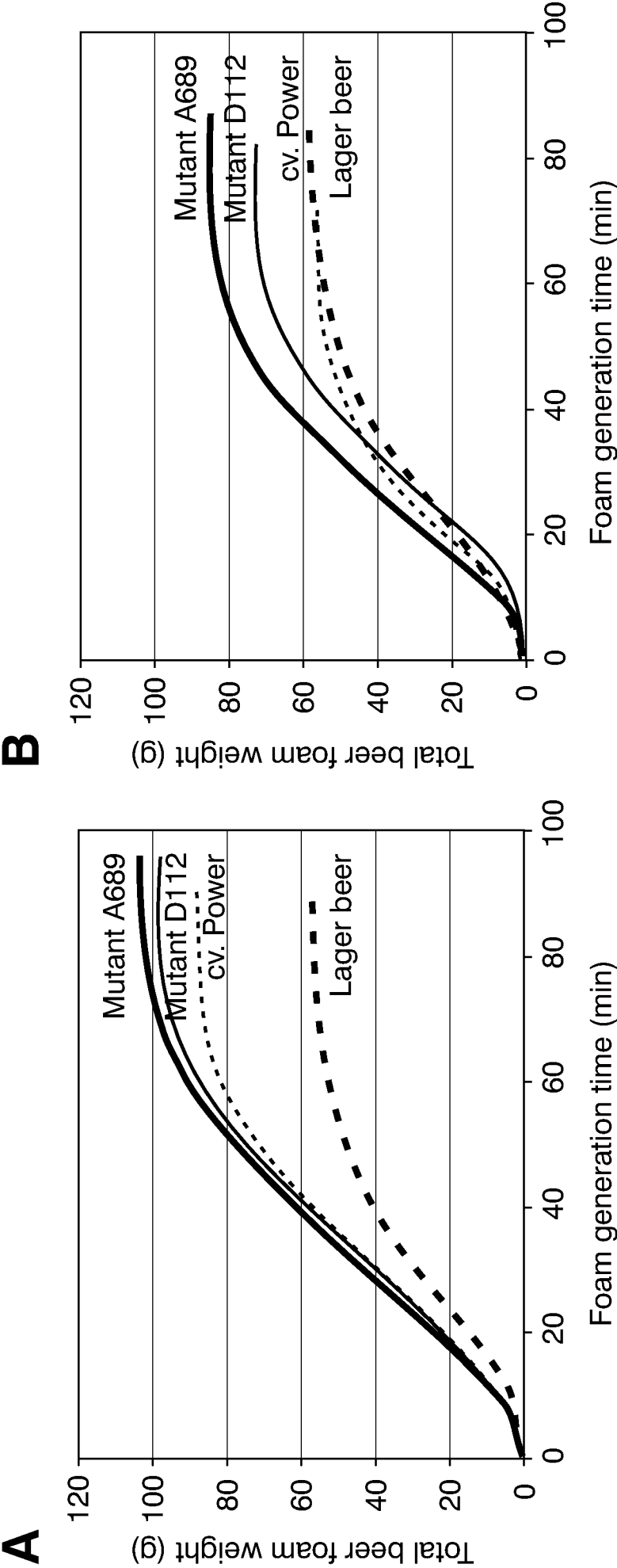


FIG. 16

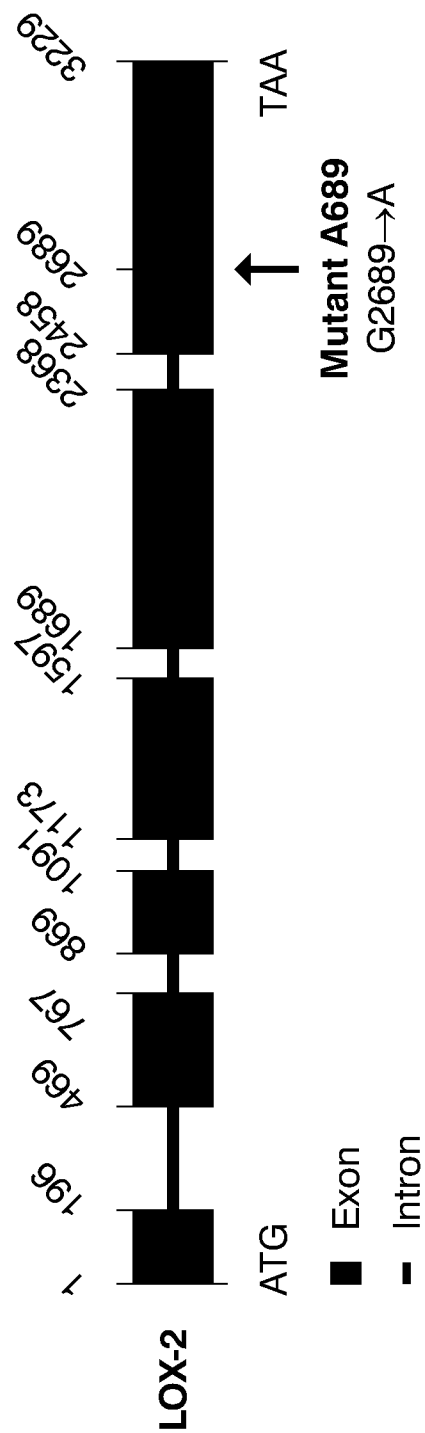


FIG. 17

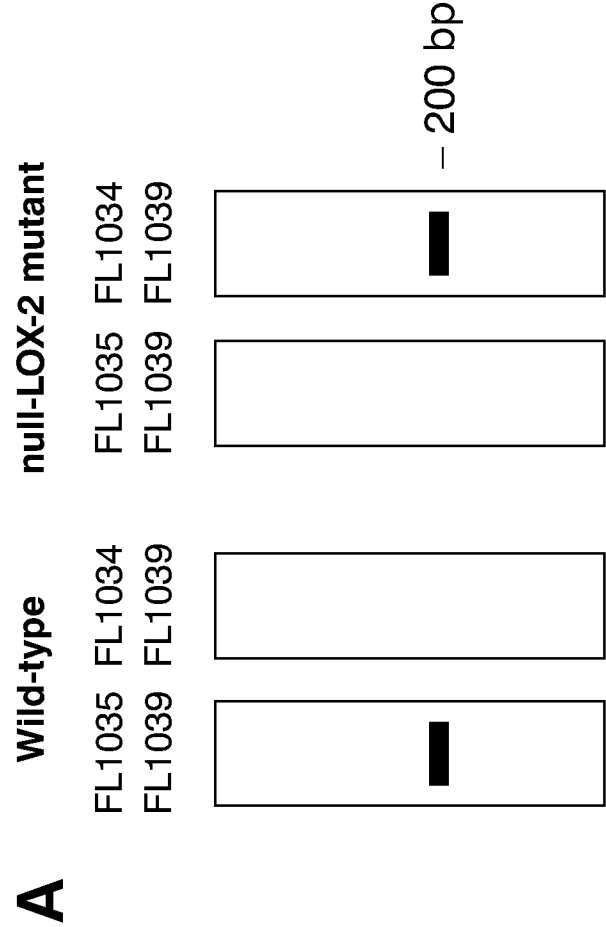


FIG. 18

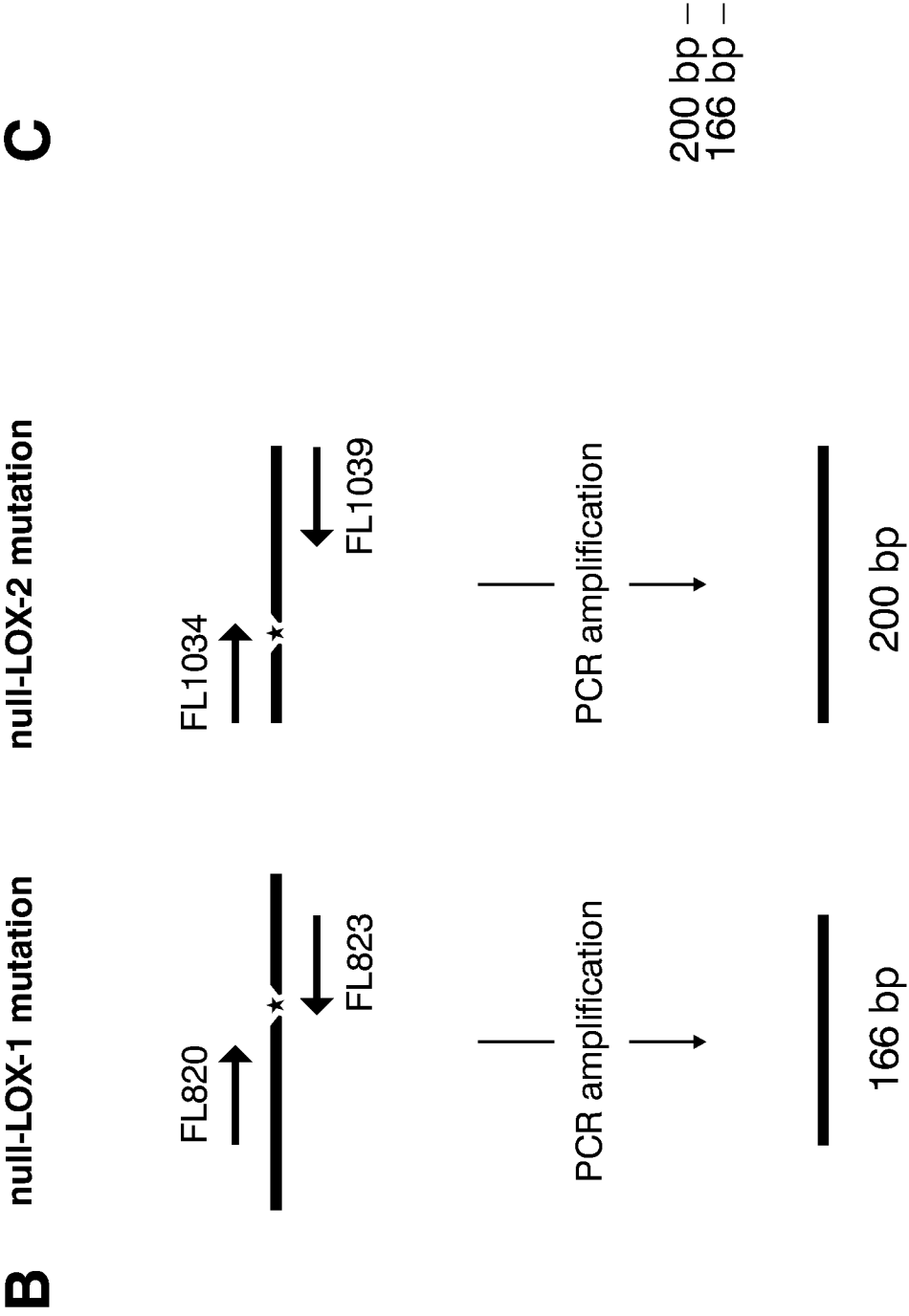
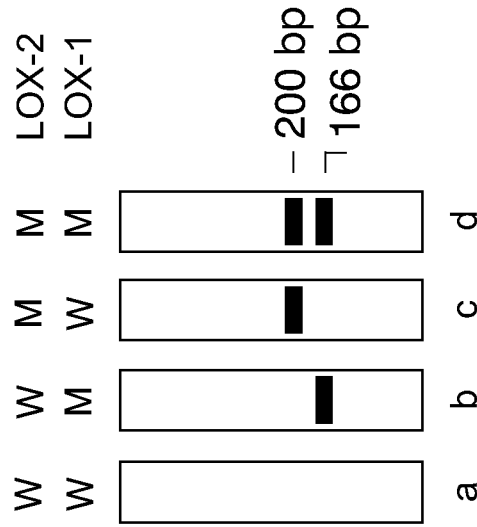


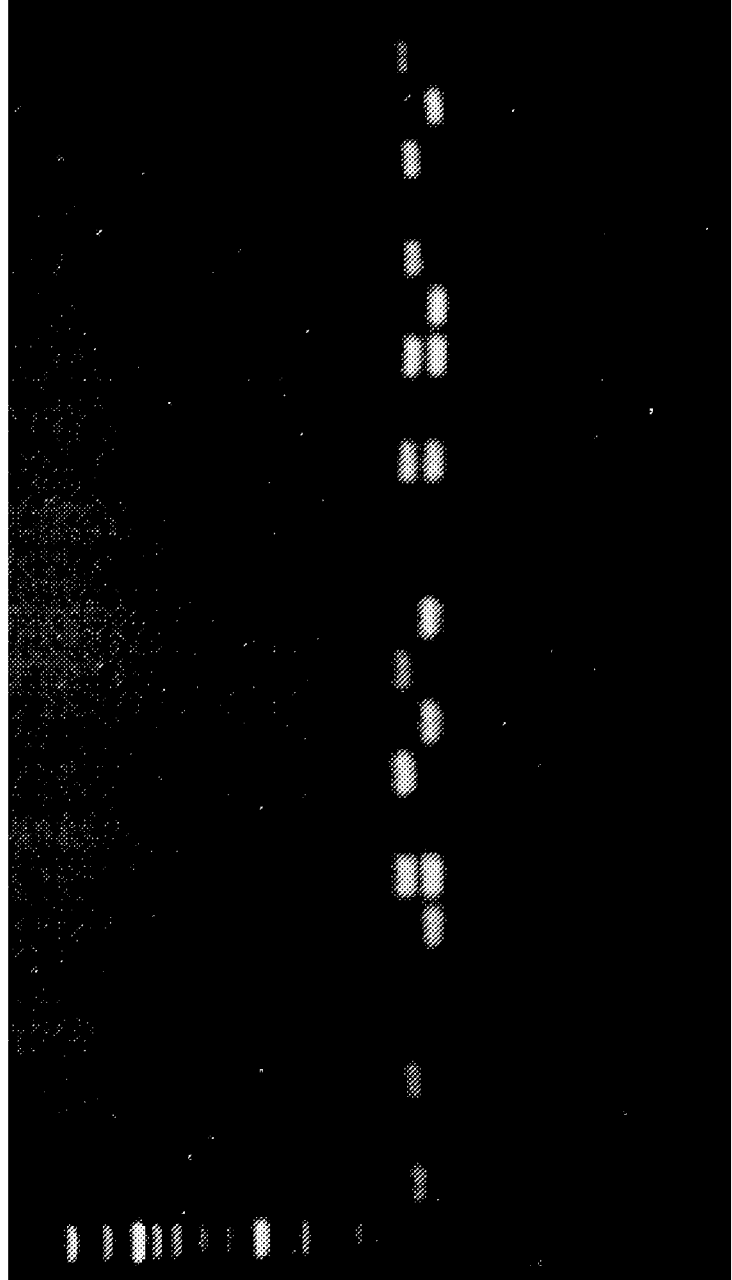
FIG. 18

**A**



**B**

MW 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23



c b c a a b d a c b c b a a d a d b c a c b c

FIG. 19