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(54) Title: HIGH EXPRESSION CEREAL PHYTASE GENE

(57) **Abrégé/Abstract:**

The present invention provides mutant cereal plants and mature grain thereof, characterised by enhanced levels of the enzyme phytase in the grain, and methods for inducing, detecting and selecting the mutant cereal plants. The invention further relates to animal feed comprising said grain having enhanced amounts of phytase.

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(54) Title: HIGH EXPRESSION CEREAL PHYTASE GENE

(57) Abstract: The present invention provides mutant cereal plants and mature grain thereof, characterised by enhanced levels of the enzyme phytase in the grain, and methods for inducing, detecting and selecting the mutant cereal plants. The invention further relates to animal feed comprising said grain having enhanced amounts of phytase.

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Title: High expression cereal phytase gene**Technical field of the invention**

The present invention relates to mutant cereal plants and mature grain thereof, characterised by an enhancer polynucleotide capable of directed enhanced expression of a operably-linked gene, in particular a operably-linked gene encoding the enzyme phytase, causing enhanced levels of phytase in the grain. The invention further relates to animal feed comprising said cereal grain having enhanced amounts of phytase.

Background description of the invention

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) [EC 3.1.3.26 and EC 3.1.3.8] are phosphatases that initiate the sequential liberation of orthophosphate groups from phytate (InsP₆, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate), providing phosphate, inositol phosphates and inositol required for a range of cellular activities (Brinch-Pedersen et al., 2002). A number of enzymes with phytase activity are known from plants, animals and microorganisms (Dvorakova, 1998).

Phytases are of particular importance during seed germination where they mobilize phosphate from phytate, the major reserve of phosphorus (P) in plant seeds accounting for ~70% of the total P (Lott, 1984). Different plant species have developed various strategies for phytase mediated degradation of phytate during germination. Among cereals, barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* and *durum* L.) and rye (*Secale cereale* L.) synthesize and accumulate phytase during grain development and the mature seed has a significant level of preformed phytase activity. Levels of phytase activity of 582, 1193 and 5130 U kg⁻¹ have been detected in mature grain of barley, wheat and rye respectively (Eeckhout and de Paepe, 1994). Preformed phytase catalyses the first wave of phytate hydrolysis during early germination. Other cereals possess little

(maize (*Zea mays* L.) $\sim 41 \text{ U kg}^{-1}$) or close to no (rice (*Oryza sativa* L.)) preformed phytase activity in the mature seed and depend entirely on *de novo* synthesis during germination (Eeckhout and de Paepe, 1994).

The spatial and temporal regulation of phytase biosynthesis in plant seeds has profound effects on phosphate bioavailability when dry grains are used as food and feed. Monogastric animals such as pigs, poultry and humans have little or no phytase activity in their digestive tracts and thus depend on either a phosphate supplement or on the presence of the enzyme phytase in their diet, in order to meet their nutritional phosphate requirements. In most cases the amount of preformed phytase activity in mature cereal grain is not sufficient to ensure sufficient phytate degradation when included in animal feed. As a consequence, most of the cereal grain phytate consumed by an animal is excreted, thereby adding to the phosphate load on the environment which can be massive in areas with intense livestock production. One current solution to this problem has been to supplement animal feed, on a large scale, with inorganic phosphate, in order to meet an animal's need for phosphate. However, this solution can only continue in the short term since phosphate is a non-renewable resource, which will be depleted within a few decades. An alternative solution relies on the addition of phytase enzyme, in particular microbial-derived phytase, to feed intended for intense pig and poultry production. It has become common practise to include the enzyme phytase in pre-mixes for addition to animal fodder, and animal fodder, which is an additional cost factor. Thus there exists a need for alternative cheaper methods for enhancing the bioavailability of phosphate in cereals used for animal feed.

A DNA sequence comprising a coding sequence for wheat phytase has been deposited in GenBank (AX298209). Patent application (WO2001/083763A2) describes said wheat phytase as a 66 kDa PAPhy with the same temperature and pH optima as PHYI (Rasmussen et al., 2004), and describes the production of transgenic wheat plants comprising said coding sequence.

Summary of the invention

According to a first embodiment, the present invention provides a mutant cereal plant comprising a polynucleotide selected from any one of:

- 5 a. ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2] wherein V is any nucleotide other than T, more preferably where V is C or G,
- b. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 14, 15 and 44,
- 10 c. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 14, 15 and 44, wherein said polynucleotide is operably linked to a second polynucleotide encoding a polypeptide,

wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant, and, and wherein said cereal is selected from *Avena* L species, *Hordeum* L species; *Oryza* L species; *Secale* L species; *Sorghum* L species; *Triticum aestivum*, *Triticum durum*; *Triticum spelta* and *Zea* species.

In a further embodiment, the polypeptide of the mutant cereal plant is phytase [EC 3.1.3.26 and EC 3.1.3.8] and has *myo*-inositol hexakisphosphate phosphohydrolase activity.

Further to the first embodiment according to (a), the genomic DNA of the mutant cereal plant comprises a first polynucleotide located 5' upstream of a second polynucleotide, and operably-linked to the second polynucleotide, wherein said first polynucleotide comprises the nucleotide sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], and wherein said second polynucleotide encodes a phytase polypeptide having *myo*-inositol hexakisphosphate phosphohydrolase activity.

Further to the above embodiments, the mutant cereal plant is selected from *Avena sativa*, (Oats); *Hordeum vulgare* (Barley); *Oryza sativa* (rice); *Secale cereale* (Rye); *Sorghum bicolor*; *Triticum aestivum*, *Triticum durum*;, *Triticum spelta* (wheat species); *Zea mays* (maize).

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Further to the above embodiments, the mutant cereal plant is a *Triticum spp.*, and the phytase polypeptide has an amino acid sequence having at least 70% sequence identity to a sequence selected from SEQ ID NO: 18, 20, and 22.

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Further to the above embodiments, the mutant cereal plant is a *Secale spp.*, and the phytase polypeptide has an amino acid sequence having at least 70% sequence identity to a sequence selected from SEQ ID NO: 26 or 28.

15 Further to the above embodiments, the mutant cereal plant is a *Hordeum spp.*, and the phytase polypeptide has an amino acid sequence having at least 70% sequence identity to a sequence selected from SEQ ID NO: 30.

20 Further to the above embodiments, the mutant cereal plant is selected from a mutant of *Triticum aestivum* having Deposit No: PTA-11732 [TaHighPhy 01], and PTA-11731 [TaHighPhy 02]; and a mutant of *Secale cereale* having Deposit No PTA-11730 [ScHighPhy 01], said plants being deposited with ATCC Patent Depository, 10801 University Blvd., Manassas, VA 20110,

25 Further to the above embodiments, the mutant cereal plant is a grain.

According to a second embodiment, the invention provides a plant part (e.g. grain or caryopsis) derived from a mutant cereal plant of the first embodiment or further embodiments of the invention.

30

According to a third embodiment, the invention teaches the use of grain derived from a mutant cereal plant according to the first or further embodiments of the invention, for the manufacture of a composition, wherein said composition is any one of: a milled grain composition; animal fodder;
5 and steam-pelleted animal fodder.

According to a fourth embodiment, the invention provides a composition comprising a mutant cereal plant according to the first or further embodiments of the invention, wherein said composition is any one of: a
10 milled grain composition; animal fodder and steam-pelleted animal fodder.

According to a fifth embodiment, the invention teaches a use of a composition, comprising a mutant cereal plant, according to the fourth embodiment as animal fodder.
15

According to a sixth embodiment, the invention teaches a method for detecting a mutant cereal plant, said plant comprising a polynucleotide selected from one of:

- a) ACA VGA GTC ATG CAT [SEQ ID NO: 1];
- 20 b) a polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 12, 14 and 15, and
- c) polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 12, 14 and 15, wherein said polynucleotide is operably linked to a second polynucleotide encoding a polypeptide, and
25 wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant,

comprising the steps of: (i) isolating genomic DNA from said plant, and (ii) detecting the presence of the nucleotide V at the 5' end of a polynucleotide having the nucleotide sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or
30 T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], wherein said polynucleotide is comprised in said genomic DNA.

According to a seventh embodiment, the invention teaches a method for inducing and selecting a mutant cereal plant, said plant comprising a polynucleotide selected from one of:

- 5 a) ACA VGA GTC ATG CAT [SEQ ID NO: 1];
 b) a polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 12, 14 and 15, and
 c) polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 12, 14 and 15, wherein said polynucleotide is operably
10 linked to a second polynucleotide encoding a polypeptide, and wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant,

comprising the steps of: (i) treating a cereal plant, or plant part thereof, with a chemical mutagen; (ii) growing and/or multiplying the treated plant, or plant
15 part; (iii) isolating genomic DNA from said plant or progeny thereof, and (d) detecting the presence of a polynucleotide having the nucleotide sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], wherein said polynucleotide is comprised in said genomic DNA.

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Further to the sixth or seventh embodiments, the genomic DNA comprises a first polynucleotide located 5' upstream of and operably-linked to a second polynucleotide, wherein the first polynucleotide comprises the nucleotide
25 sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], and wherein the second polynucleotide encodes a phytase polypeptide having *myo*-inositol hexakisphosphate phosphohydrolase activity.

Further to the sixth or seventh embodiments, the cereal is selected from
30 *Avena L spp*, *Hordeum L spp*; *Oryza L spp*; *Secale L spp*; *Sorghum L spp*;

Triticum aestivum, *Triticum durum*; *Triticum monococcum*; and *Zea spp.*

Further to the sixth or seventh embodiments, the cereal is selected from *Avena L spp*, *Hordeum L spp*; *Oryza L spp*; *Secale L spp*; *Sorghum L spp*;
 5 *Triticum aestivum*, *Triticum durum*; *Triticum monococcum*; and *Zea spp*, and the amino acid sequence of the phytase polypeptide has at least 70% sequence identity to a sequence selected from SEQ ID NO: 18, 20, 22, 24, 26, 28, and 30.

- 10 Further to the sixth or seventh embodiments, the cereal is selected from *Avena L spp*, *Hordeum L spp*; *Oryza L spp*; *Secale L spp*; *Sorghum L spp*;
Triticum aestivum, *Triticum durum*; *Triticum monococcum*; and *Zea spp*, and the amino acid sequence of the phytase polypeptide has at least 70%
 sequence identity to a sequence selected from SEQ ID NO: 18, 20, 22, 24,
 15 26, 28, and 30, wherein said polypeptide is encoded by a polynucleotide having a nucleotide sequence having at least 70% sequence identity to a sequence selected from SEQ ID NO: 17, 19, 21, 23, 25, 27, 29, and nucleotides 2091-4090 of 45, respectively.

20 **Detailed description of the invention**

Listing of the figures:

Figure 1. Phytase activity in mature whole grain derived from 52 individual lines of wheat (*Triticum aestivum*).

- Figure 2.** Phytase activity in mature whole grain, bran and endosperm
 25 fractions of grain derived from 52 individual lines of wheat (*Triticum aestivum*).

Figure 3. Cartoon showing the exon-intron structure of a TaPAPhy a1 gene isolated from *Triticum aestivum* cv Skagen (TaG2) corresponding to SEQ ID NO: 45.

- 30 **Figure 4.** Pair wise comparison of the nucleotide sequence of the 1000 bp 5'flanking promoter region of phytase genes amplified from 9 *T. aestivum*

cultivars and the corresponding promoter region from two HighPhy *T. aestivum* cultivars. The upper comparison counts differences, whereas the lower comparison shows the identity in percent.

Figure 5. Pair wise comparison of the nucleotide sequence of the 288 bp

5'flanking promoter region of phytase genes amplified from eight *T. aestivum* cultivars, two *T. tauschii* accession lines (NGB90403; NGB9855), and the corresponding promoter regions from two HighPhy *T. aestivum* cultivars. The upper comparison counts differences, whereas the lower comparison shows the identity in percent.

Figure 6. Multiple alignment of the start codon and 288 bp 5' flanking promoter region 7 *T. aestivum* cultivars (cv Skagen; cv Bob White; cv

Pentium; cv Flair; cv landrace 01; cv Landrace 02; cv Spelt, which share SEQ ID NO: 7), two *T. tauschii* accession lines (NGB90403; NGB9855, which share SEQ ID NO: 8), and the corresponding promoter regions from

two HighPhy *T. aestivum* lines (HighPhy 01; HighPhy 02 which share SEQ ID NO: 5). The 5'flanking promoter region of the *T. aestivum* cv Skagen is represented by both the lambda clone TaG2 and a PCR amplicon, with SEQ ID NO: 7. The enhancer sequence [ACA CGA GTC ATG CAT] in the HighPhy 01 and 02 cultivars is located a position: -247 to – 237 in the 5' flanking region. Note that SEQ ID NO: 5, 7 and 8 are identical to the corresponding sequences in Figure 6, but with the exception that the last 3 nucleotides (ATG) of each corresponding sequence in Figure 6 are excluded from the sequence given in the SEQ ID NO listing.

Figure 7. Multiple alignment of the start codon and 5'flanking promoter

region 5 wild type *T. aestivum* cultivars (TaPAPhy_a1: NormPhy [SEQ ID NO: 9]; TaPAPhy_a3 [SEQ ID NO: 10], TaPAPhy_a4 [SEQ ID NO: 11];

TaPAPhy_a5; TaPAPhy_a6) and the corresponding promoter region from a High Phytase *T. aestivum* line (TaPaPhy_a1: HighPhy [SEQ ID NO: 6]). The

5'flanking promoter region of the *T. monococcum* (TmPAPhy_a1 [SEQ ID

NO: 12]); *Hordeum vulgare* (HvPAPhy_a1 [SEQ ID NO: 13]) and high and

normal phytase *Secale cereale* (ScPAPhy_a1 HighPhy [SEQ ID NO: 15]) and

(ScPAPhy_a1 NormPhy [SEQ ID NO: 16]). Note that SEQ ID NO: 6, 9, 10, 11, 13, 13, 15 and 16 are identical to the corresponding sequences in Figure 7, but with the exception that the last 3 nucleotides (ATG) of each corresponding sequence in Figure 6 excluded from the sequence given in the SEQ ID NO listing.

The nucleotide sequence of the polynucleotides comprised within the NormalPhy element and the HighPhy enhancer element are included in the alignment.

Figure 8. Phytase activity in mature whole grain derived from 5 individual lines of rye (*Secale cereale*). One line (LPP03) has low phytase activity and 4 lines have medium to high phytase activity.

Figure 9. Phytase activities in Bob White wild type (BW) and HighPhy *T. aestivum* (HIGHPHY) wheat flour after 0, 10, 20 and 40 min of incubation at 80°C in 100% relative humidity.

Figure 10. Phytase activities in HighPhy *Secale cereale* (rye) flour after 0, 1, 2, 3, 4, 5, 10, 30, 45 and 60 min of incubation at 80°C in 100% relative humidity.

Figure 11. Phytate content in Bob White wild type (BW) and HighPhy *T. aestivum* (HIGHPHY) wheat dough during the fermentation

Figure 12. Percentage residual phytate in flour of Bob White wild type and HighPhy *T. aestivum* (HIGHPHY) wheat after 0.5, 1, 1.5, 2 and 3 hrs of fermentation.

Figure 13. UPGMA tree of the HIGHPHY and TaPAPhy_a1, _a2 and a_3 genes.

Abbreviations:

AS-PCR: Allele specific - polymerase chain reaction;

CTP: cytosine 5'-triphosphate;

dNTP: Deoxynucleotide Triphosphate;

PAPhy: Purple acid phosphates (PAP) with phytase activity; also called PAP phytases (PAPhy);

Pfu: plaque forming units;

SNP: single-nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide (A, T, C, or G) in the genome differs between members of a species or paired chromosomes in an individual

5 1x SSPE buffer: 150 mM Sodium Chloride, 10mM Sodium Hydrogen Phosphate, 1mM EDTA, pH 7.4);

SPP: species;

V: is the nucleotide A or C or G (not T), where B is the nucleotide in the complementary sequence.

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Definitions:

Cereal: A plant belonging to the *Poaceae* family, in particular a plant belonging to the Genus and species thereof: *Avena* L (e.g. *Avena sativa*, Oats); *Hordeum* L e.g. *Hordeum vulgare*, Barley); *Oryza* L (e.g. *Oryza sativa*, rice); *Secale* L (e.g. *Secale cereale*, Rye); *Sorghum* L (e.g. *Sorghum bicolor*);
15 *Triticum* (e.g. *Triticum aestivum*, *Triticum durum*; *Triticum monococcum*, *Triticum spelta*, wheat); *Zea* (e.g. *Zea mays*, maize).

Promoter operably-linked to a gene: a promoter is a DNA molecule that is located on the same DNA strand and upstream (towards the 5' region of the sense strand) of the transcriptional start site of a down-stream gene, where
20 the operational function of the promoter is to regulate the expression of the down-stream gene to which it is operably-linked. DNA molecules, whose function is to regulate expression of a down-stream gene, typically comprise a smaller DNA molecule that acts as an “enhancer”, the enhancer serving to
25 modulate expression levels of the down-stream gene. An “Enhancer” is characterised by a conserved nucleotide sequence, often comprising various conserved sequence motifs whose function is to modulate gene expression levels. A gene is defined to include a polynucleotide molecule comprising coding and optionally non-coding sequence(s), the coding sequence(s)
30 encoding a polypeptide, e.g. phytase.

Triticum aestivum: line of *T. aestivum*, cultivar of *T. aestivum* is a cultivated variety of *T. aestivum* that has been created or selected intentionally for specific desirable characteristics and maintained through cultivation.

Sequence identity: Identity can be measured as percent identity. The term "percent sequence identity" indicates a quantitative measure of the degree of homology between two nucleotide sequences of equal length. When the two sequences to be compared are not of equal length, they are aligned to give the best possible fit, by allowing the insertion of gaps or, alternatively, truncation at the ends of the nucleotide sequences. The $(N_{\text{ref}} - N_{\text{dif}}) / N_{\text{ref}} \times 100$ can be calculated as $\frac{N_{\text{ref}} - N_{\text{dif}}}{N_{\text{ref}}} \times 100$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. The percent sequence identity between one or more sequence may also be based on alignments using the clustalW software (<http://www.ebi.ac.uk/clustalW/index.html>).

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Detailed description of embodiments of the invention:

It is recognised that, in most cases, the amount of preformed phytase activity in mature cereal grain is not sufficient to ensure sufficient phytate degradation when included in animal feed. It is further recognised that bread dough having a low phytate content has superior mixing properties, and the resulting bread has a higher nutritional value, due to an enhanced availability of minerals, including inorganic phosphate. One solution to this problem has been to produce genetically modified cereal plants having higher levels of phytase in the grain, for example by expressing a transgene encoding a heterologous or homologous gene encoding phytase. Current agricultural policy in many parts of the world, in particular Europe, has restricted the growth of transgenic crop plants. Furthermore, organic farming, based on methods that are internationally regulated and legally enforced by many nations, are not certified to use genetically modified plants or feed enzymes derived from microbial phytases. Accordingly, there remains a need for non-

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transgenic plants producing grain having a high phytase phenotype. The present invention addresses this need.

I. A polynucleotide acting as an enhancer of grain-specific gene expression in mutant cereal plant

One embodiment of the invention provides a mutant cereal plant whose genome comprises an enhancer polynucleotide having the nucleotide sequence:

ACA VG A GTC ATG CAT [SEQ ID NO: 1],

10 or

T AGA ACA VG A GTC ATG CAT [SEQ ID NO: 2],

wherein said polynucleotide is capable of enhancing grain-specific gene expression. The enhancer polynucleotide comprises a mutation whereby the nucleotide V is any nucleotide other than T (i.e. C or G or A), as compared to the corresponding polynucleotide in a wild type cereal plant having normal wild type levels of phytase (e.g. the enhancer in the wild type normal phytase polynucleotide ACA TGA GTC ATG CAT [SEQ ID NO: 3] from wheat). In a preferred embodiment, the enhancer polynucleotide has SEQ ID NO: 1, wherein the nucleotide residue designated as V, is either G or C.

20

A series of four overlapping motifs have been identified in the polynucleotide having the sequence: AACATGAGTCATGCGATGGGA [SEQ ID NO: 4] which comprises the enhancer of the wild type wheat phytase gene. These motifs include an “odd base palindrome sequence” and a “GCN4 motif”, a “skn-1 motif” and a “palindromic RY-repeat”. The odd base palindrome and GCN4 motif have been shown to interact with Opaque2, a maize basic leucine zipper (bZIP) transcription factor that is involved in the regulation of seed storage protein expression [3,4], whereas the RY-repeat has been shown to interact with transcription factors containing the B3 domain.

30

Cereal plants comprising the mutant enhancer polynucleotide, show enhanced grain-specific expression of an operably-linked gene located downstream of the enhancer polynucleotide, indicating that the mutant polynucleotide acts to regulate enhanced gene expression in a tissue-specific manner. Proteins encoded by the operably-linked gene, whose expression in cereal plants is regulated by a structurally- and operably-linked upstream promoter polynucleotide molecule comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2, accumulate enhanced levels of the encoded protein in the grain when compared to wild-type cereal plants having the wild-type enhancer sequence (see Example 1 and 7).

The enhancer polynucleotide according to the invention is further characterised by an altered expression pattern of its operably-linked gene in the grain. The enhancer causes both increased gene expression throughout the grain, but also preferential expression in the endosperm tissue of the grain, which constitutes the majority of the grain as measured by weight (Example 1 and 13). The enhanced gene expression leads to enhanced levels of the gene-encoded proteins in the endosperm of the grain, having the advantage that downstream grain processing steps, such as dehusking/milling does not lead to a loss the protein as is the case for proteins expressed in the aleurone tissue, and outer layers/coat of the grain.

II. A mutant cereal plant, whose genome has a promoter polynucleotide comprising an enhancer of grain-specific gene expression

25

A further embodiment of the invention provides a mutant cereal plant, whose genome comprises a promoter polynucleotide molecule, said molecule comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2. The mutant cereal plant of the invention is a member of the Poaceae family, preferably belonging to the Genus *L*, and Species (spp) thereof of the following: *Avena L* (e.g. *Avena sativa*); *Hordeum L* (e.g. *Hordeum vulgare*);

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Oryza L (e.g. *Oryza sativa*); *Secale* L (e.g. *Secale cereale*); *Sorghum* L (e.g. *sorghum bicolor*); *Triticum* spp selected from *Triticum aestivum*, *Triticum durum*, and *Triticum spelta*; and *Zea* (e.g. *Zea mays*).

In one example, the mutant cereal plant is a mutant *Triticum aestivum*,
5 whose genome comprises the enhancer polynucleotide having SEQ ID NO: 1
or 2. The genome of the mutant *Triticum aestivum* may comprise a promoter
polynucleotide having SEQ ID NO: 5 or 6 or 44, this promoter itself
comprising the enhancer polynucleotide having SEQ ID NO: 2. Wheat plants
comprising a promoter having SEQ ID NO: 5 or 6 show enhanced
10 endosperm-specific expression of a operably-linked gene located down-
stream of this promoter, indicating that the promoter acts to regulate
enhanced gene expression in a tissue-specific manner. The corresponding
promoter polynucleotide in wild-type *Triticum aestivum* cv's and *Triticum*
tauschii that lack the enhancing properties of the mutant are provided as
15 SEQ ID NO: 7, 10 and 11 and in 8 respectively.

In one example, the mutant cereal plant is a mutant *Secale cereale* whose
genome comprises the enhancer polynucleotide having SEQ ID NO: 1 or 2.
The genome of the mutant *Secale cereale* may comprise a promoter
polynucleotide having SEQ ID NO: 15, this promoter itself comprising the
20 enhancer polynucleotide having SEQ ID NO: 2. The corresponding promoter
polynucleotide in wild-type *Secale cereale* that lacks the enhancing
properties of the mutant is provided as SEQ ID NO: 16.

In one example, the mutant cereal plant is a mutant *Hordeum vulgare* whose
genome comprises the enhancer polynucleotide having SEQ ID NO: 1 or 2.
25 The genome of the mutant *Hordeum vulgare* may comprise a promoter
polynucleotide having SEQ ID NO: 14, this promoter itself comprising the
enhancer polynucleotide having SEQ ID NO: 1. The corresponding promoter
polynucleotide in wild-type *Hordeum vulgare* that lacks the enhancing
properties of the mutant is provided as SEQ ID NO: 13.

30

III A mutant wheat plant with high phytase grain

A further embodiment of the invention provides a mutant cereal plant comprising a promoter polynucleotide, said promoter comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2, wherein said promoter polynucleotide lies upstream and is operably linked to a cognate phytase gene encoding a polypeptide having *myo*-inositol hexakisphosphate phosphohydrolase activity (phytase [EC 3.1.3.26 and EC 3.1.3.8]).

In one example, the cereal plant is a mutant *Triticum aestivum* plant comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2, where the promoter polynucleotide preferably has SEQ ID NO: 5 or 6, or 44, and the cognate phytase gene encodes a polypeptide having both *myo*-inositol hexakisphosphate phosphohydrolase activity and an amino acid sequence having at least 70, 75, 80, 85, 90, 95 or 98% sequence identity to a sequence selected from SEQ ID NO: 18, 20, and 22. In a further embodiment, said phytase polypeptide having an amino acid sequence selected from SEQ ID NO: 18, 20, and 22 is encoded by a polynucleotide having a nucleotide sequence that has at least 70, 75, 80, 85, 90, 95 or 98% sequence identity to a sequence selected from SEQ ID NO: 17, 19, 21, and 45 (nucleotides 2091-4090), respectively.

In one example, the cereal plant is a mutant *Secale cereale* plant comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2, where the promoter polynucleotide preferably has SEQ ID NO: 15, and the cognate phytase gene encodes a phytase polypeptide having both *myo*-inositol hexakisphosphate phosphohydrolase activity and an amino acid sequence having at least 70, 75, 80, 85, 90 or 95% sequence identity to a SEQ ID NO: 26 or 28. In a further embodiment, said phytase polypeptide having an amino acid sequence of SEQ ID NO: 26 or 28 is encoded by a polynucleotide having nucleotide sequence that has at least 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO: 25 or 27, respectively.

In one example, the cereal plant is a mutant *Hordeum vulgare* plant comprising the enhancer polynucleotide having SEQ ID NO: 1 or 2, where the promoter polynucleotide preferably has SEQ ID NO: 14, and the cognate

phytase gene encodes a phytase polypeptide having both *myo*-inositol hexakisphosphate phosphohydrolase activity and an amino acid sequence having at least 70, 75, 80, 85, 90 or 95% sequence identity to a SEQ ID NO: 30. In a further embodiment, said phytase polypeptide having an amino acid
 5 sequence of SEQ ID NO: 30 is encoded by a polynucleotide having nucleotide sequence that has at least 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO: 29.

10 **III Methods for detecting a cereal germplasm comprising the HighPhy SNP**

The polynucleotide: "ACA^VGAGTCCATGCATG" in the genomic DNA of a cereal plant e.g. *Triticum* spp, characteristic of the HighPhy SNP, can be detected using standard DNA analysis protocols (see Example 5). For example, amplification of genomic DNA comprising the HighPhy SNP with
 15 the forward TTTCAAGCTACACTTTGTAGAACAC [SEQ ID NO: 39] and reverse GCACTAGCCAAGTTTGGACG [SEQ ID NO: 40] primers will generate a 66 bp PCR product when using Taq polymerase, whereas amplification of genomic DNA comprising a "wildtype cereal" with wild-type levels of normal phytase will give a similar product with forward
 20 TTTCAAGCTACACTTTGTAGAACAT [SEQ ID NO: 41] and reverse GCACTAGCCAAGTTTGGACG [SEQ ID NO: 42] primers (where the second primer [SEQ ID NO: 42] is universal).

25 **IV Methods for inducing and selecting cereal germplasm comprising the HighPhy SNP**

HighPhy cereals (e.g. *Triticum* spp) can be generated by mutagenesis and subsequent screening for individuals where the polynucleotide:
 ACA IGA GTC ATG CAT, corresponding to the wild type (NormPhy) enhancer, in the cereal genome has been converted into the mutant
 30 (HighPhy) enhancer: ACA VGA GTC ATG CAT, where V can be A or C or G.

In one embodiment the mutagenesis is carried out with sodium azide which preferentially generates A:T to G:C substitutions in the cereal, barley (8). Screening mutagenized populations for the desired mutation could be done by allele specific polymerase chain reaction (AS-PCR), as described in
5 Example 6.

In an alternative embodiment, mutagenesis is carried out on cereal grain using methylene Methyl Sulphonate (MMS) to generate a population of M1 plants with random point mutations in their genome. MMS treatment leads to
10 errors during DNA replication and thus introduces mutations. Typically this means T/A nucleotides within a sequence are converted to G/C by transversion. The M1 plants are self-fertilised and the M2 seed harvested and sown. The M2 germplasm will allow recessive and lethal alleles to be recovered as heterozygotes. DNA is individually extracted from M2 plants
15 into 96 well plates and their seed stored for further propagation. To increase throughput of analysis, the M2 DNA samples are 8x pooled and amplified, using gene specific primers, located up- and down-stream of the mutant (HighPhy) enhancer: ACA VG A GTC ATG CAT that is to be detected. Preferably each primer carries a different fluorescent label. For example, the
20 forward strand may be labelled with FAM [5 - Carboxyfluorescein; 3',6'-Dihydroxy-3-oxospiro[2-benzofuran-1,9'-xanthene]-5-carboxylic acid, CAS #: 76823-03-5] and the reverse strand with HEX [HEX being a hexa-chloro derivative of FAM]. In the presence of a mutant, the amplification products when heated and cooled will form mismatched heteroduplexes between the
25 wild type and mutated DNA. To enable identification of the point mutations induced by EMS, the amplification products are incubated with a plant endonuclease called CEL I which preferentially cleaves at sites of heteroduplex mismatches that occur between wild-type and mutant DNA. The cleavage products are size-separated on a DNA sequencing instrument,
30 for example a capillary DNA sequencer and the fluorescently labelled traces are analysed. The differential end-labelling of the amplification products

permits the two cleavage fragments to be observed and to identify the position of the mismatch. When a mutation is detected in the pooled DNA, the DNA samples in the pool are individually sequenced to identify the specific plant carrying the mutation.

5

The amount of phytase enzyme in the grain of plants having the mutant (HighPhy) enhancer: ACA VGGA GTC ATG CAT in their genome, is then determined to confirm that the selected mutant has the high phytase phenotype, for example by employing the phytase assay described in

10 Example 1.

V Use of high-phytase cereal grain for producing a composition

Processing of cereal grain (for example wheat or rye grain) having phytase

15 activity in accordance with the present invention, is carried out using traditional processing steps including one or more of the following steps:

i. Cleaning/conditioning cereal grain: First the grain is cleaned. For example the grain may be passed through magnets and/or metal detectors to remove any metal contamination. Machines can be used to separate any other

20 seeds, stones or dust that may have got mixed with the wheat.

ii. Gristing grain: The cleaned and conditioned grain is blended with other types of grain in different proportions to make different kinds of flour.

The gristed grain passes through special rollers called break rolls. They break each grain into its three parts: cereal grain germ, bran and endosperm.

25 Sieves sift the three separated parts into different streams.

iii Mixing: The bran, germ and endosperm fractions, having been separated out, can optionally be blended, and can be milled to make different types of milled cereal grain composition, such as Wholemeal flour using all parts of the grain; Brown flour contains about 85% of the original grain, but with some

30 bran and germ removed; and White flour is made from the endosperm only.

A flour mix comprising flour prepared from high-phytase cereal grains of the

invention has particular value for bread making, due to the rapid degradation of phytate during dough fermentation (see Example 12) that confers both improved dough mixing properties and enhances the nutritional value (by increasing mineral uptake from the diet, in particular zinc, iron, calcium and
5 and inorganic phosphate ions) of the bread produced with the dough.

iv. Steam pelleting: Milled cereal grain composition may be combined with other fodder ingredients in a steam-pelleting machine, where the components are exposed to steam at a temperature of about 80°C – 90°C for a period of time sufficient to reduce the microbial population to levels safe for animal
10 consumption, and the product is converted to dried pellets. Steam pelleted animal feed prepared from HighPhy cereal grain of the invention retain sufficiently high levels of phytase activity following steam-treatment, that addition of supplementary phytase granules can be avoided.

15 **VI A composition comprising high-phytase cereal grain**

In a further embodiment, the present invention provides animal fodder comprising grain derived from the mutant cereal plant of the present invention (for example a wheat, rye or barley high-phytase mutant), where the grain are characterized by enhanced levels of phytase. Animal fodder
20 comprising grain from the mutant wheat plant have the advantage, that the need to supplement the fodder with phytase is considerably reduced and preferably avoided, and at the same time the added high phytase cereal grain has the advantage that it is not classified as genetically modified material.

25

VII A wild-type *Triticum aestivum* gene TaPAPhy_a1

In a further embodiment, the present invention provides an isolated full-length *Triticum aestivum* gene TaPAPhy_a1 (SEQ ID NO: 45), comprising a promoter sequence (SEQ ID NO: 7) and down-stream coding sequence
30 comprising 5 exons and 4 introns (figure 3). Part, or all, of the isolated

TaPAPhy_a1 gene can be used for the construction of gene constructs for transformation into wheat.

Example 1. Phytase activity of different *Triticum aestivum* cultivars:

5 1.1 *Comparative levels of total phytase enzymatic activity in mature wheat grain*

The phytase activity was measured in mature seeds of 52 individual cultivars or lines of wheat (*Triticum aestivum*). Mature seeds were milled and protein was extracted from 0.250 g of flour by adding 2.5 ml 220 mM Na-acetate buffer (pH 5.5) including 68 mM CaCl₂ and Tween 20 (100 mg/l). The suspension was vortexed for 1 hour at room temperature and subsequently centrifuged at 3000xg for 10 min. The supernatant was collected and assayed for phytase activity as described by Engelen et al., 1994 [1]. One phytase unit (U) is defined as 1 µmol of Pi released upon phytate hydrolysis at 37°C at the enzymes pH optimum.

Phytase activity ranged from ~650 to ~1900 FTU/kg in grain from 50 of the wheat lines (Figure 1). Similar levels of phytase activity have previously been reported [2], in 13 individual wheat lines. However in two lines, HIGHGPY01 and HIGHPHY02, the level of phytase activity was ~6000 FTU/kg and ~4300 FTU/kg respectively, exceeding all other wheat lines analysed.

2.2 *Distribution of phytase enzymatic activity in mature wheat grain*

The distribution of phytase activity between outer layers and endosperm tissues of wheat grain derived from HIGHPHY wheat lines and wild type wheat lines was determined. The wheat grain samples were milled and divided into outer bran and inner endosperm fractions. The phytase activities were measured in each fraction (Figure 2). In wild-type wheat grains with a total activity on ~1200 FTU/kg, phytase activity was mainly localised in the bran fraction with ~3500 FTU/kg, while phytase activity in endosperm tissue was about ~600 FTU/kg. A significantly different distribution was seen in

HIGHPHY01 grain, where the activity in the endosperm was 6900 FTU/kg, exceeding the level of phytase activity in both bran (5300 FTU/kg) and whole grains (6300 FTU/kg).

5 **Example 2. Isolation of a wheat phytase gene**

2.1 *Construction of a genomic library from genomic DNA from Triticum aestivum, cv Skagen:*

A genomic library of DNA extracted from Triticum aestivum, cultivar Skagen, was generated using the Lambda Fix II/ Xho I Partial Fill-In Vector Kit (Agilent Technologies - Stratagene Products) according to the manufacturer's instructions. The initial library was titered and the size found to be 5×10^6 pfu. Given the constraints of the vector, which will accommodate inserts of 9-23 kb, this corresponds to 45000 -115000 Mb or 2.8-7.2 times the size of the wheat genome. The library was amplified on 150 120×120 mm NZY agar plates according to the manufacturer's instructions to achieve a final titer of 3×10^6 pfu/μL.

2.2 *Screening a Triticum aestivum, cv Skagen genomic library for a phytase gene:*

The amplified library was plated out on 240×240 mm NZY agar plates at a density of 600 pfu/cm². Plaque lifts were performed with Hybond N+ membranes (GE Healthcare), and the DNA was fixed on the membrane by alkaline denaturation and UV cross linking. The membranes were prehybridized in 0.25 M sodium phosphate buffer, pH 7.2, with 7% SDS and 0.17 mg/mL salmon sperm DNA at 65°C for two hours in rolling tubes. The membranes were then hybridised in a solution comprising the radiolabelled Triticeae PAPhy specific Probe (20 microcuries), 0.25 M sodium phosphate buffer, pH 7.2, with 7% SDS at 65°C overnight. Preparation of the probe is set out below. Ten membranes were washed at a time for 15 min in the hybridization tubes at 65°C with 1x SSPE buffer followed by one wash for one hour at 65°C in 1L 1x SSPE buffer and 10 seconds in room temperature

1x SSPE. Finally the membranes were blot dried on filter paper for 10 min and sealed in plastic envelopes.

X-ray films were exposed with the membranes at -80°C, and the developed films subsequently analysed radiolabel signals. Positive clones were cut from
5 the original plate and isolated by successive rounds of plaque screenings.

2.3 A *Triticeae* PAPHy phytase gene specific Probe:

A 20µCi ³²P labelled probe was generated by PCR using [αP32]dCTP and the primers:

10 PAP ex3 Fw: CTTGAGCCTGGGACGAAGT (SEQ ID NO: 31) and
PAP ex3 Rv: GAGAAGGACCCGCTCTCC (SEQ ID NO: 32),
and a template consisting of a plasmid comprising a cDNA molecule whose nucleotide sequence encoded the wheat Purple Acid Phosphatase Phytase b (TaPAPHy_b). The primers amplified a portion of the cDNA molecule whose
15 nucleotide sequence corresponds to the highly conserved third exon of the *Triticeae* PAPHy_b gene. The amplified sequence generated a DNA probe of 479 nucleotides in length. Remaining unincorporated dNTPs were removed with an Illustra MicroSpin G-50 Column (GE Healthcare). The probe was denaturated by boiling followed by shock cooling in 500 µL of 10 µg/µL
20 sonicated salmon sperm DNA.

2.4 Isolation and characterisation of *Triticeae* PAPHy phytase gene:

Isolated lambda (λ) clones, selected by the *Triticeae* PAPHy specific probe, were amplified on five to twenty 82 mm diameter NZY agar plates and λ DNA
25 was isolated from the phage harvested from the plates using the Lambda midi kit (Qiagen) according to the manufacturer's instructions.

One isolated λ clone, comprising the genomic DNA molecule designated TaG2, was sequenced (SEQ ID NO: 45) and found to comprise a polynucleotide comprising a ~2000 bp promoter region having the sequence
30 (SEQ ID NO: 43).

Example 3. Amplification and characterization of phytase gene promoters from different *Triticum aestivum* and *Triticum tauschii* cultivars:

5 3.1 *Isolation of phytase gene promoters by PCR*

Genomic DNA was isolated from 10 cultivars of *T. aestivum* and 2 accessions of *T. tauschii* (also known as *Ae. Tauschii*), as shown in table 1.

10 Table 1. Cultivars and accessions from which the TaPAPhy_a1 promoter was amplified.

Cultivar/ accession	Notes
Bob White	<i>T. aestivum</i> model cultivar
Skagen	<i>T. aestivum</i> commercial cultivar
Flair	<i>T. aestivum</i> commercial cultivar
Spelt	<i>T. aestivum</i> spp <i>spelta</i> commercial sample
Pentium	<i>T. aestivum</i> commercial cultivar
Landrace 01	<i>T. aestivum</i> Landrace
Landrace 02	<i>T. aestivum</i> Landrace
HighPhy 01	Novel high phytase <i>T. aestivum</i> cultivar
HighPhy 02	Novel high phytase <i>T. aestivum</i> cultivar
NGB90403	<i>T. tauschii</i>
NGB9855	<i>T. tauschii</i>

15 TaPAPhy_a1 promoter region was amplified from genomic DNA isolated from each of the above cultivars using primer pairs based on the sequence of the high phytase gene, designated, λ clone: TaG2. The first primer pair was designed for amplifying the first exon and 2041 bp 5' upstream flanking region (promoter) of the TaPAPhy_a1 gene:

TaPAPhy_a1-pro-ex1 Fw: TTATGTGTCCGCGTGAAGTG (SEQ ID NO: 33)

and

TaPAPhy_a1-pro-ex1 Rv: ACCAAGAGTCAATGCCATCC (SEQ ID NO: 34)

An additional primer pair was designed to amplify a shorter sequence which includes 288 bp of the 5' flanking region (promoter) and 147 bp of the first exon of the TaPAPhy_a1 gene:.

5 TaPAPhy_a1 -311 cons Fw: TTTGGACGAGCCATAGCTGCATA (SEQ ID NO: 35) and

TaPAPhy_a1 167 Rv: CGCTGCACCCGGGGGTCCGT (SEQ ID NO: 36)

The latter primer pair was used with cultivars where the first primer pair failed to yield an amplification product.

10 PCR was performed with Herculase II (Agilent Technologies - Stratagene Products) according to the manufacturer's instructions, but with the modification that 6% DMSO was used in the reaction mixture.

Amplicons of the expected size were isolated from agarose gels and cloned in the pCR4Blunt TOPO vector (Invitrogen) and sequenced.

15

3.2 Characterization of the promoter region of isolated T. aestivum and T. tauschii phytase genes – alignment of 1000bp

The long PCR amplicon, (corresponding to 2041 bp 5' upstream flanking promoter region the first exon and of the TaPAPhy_a1 gene) was obtained
20 from 10 cultivars of *T. aestivum*, whereas only the short amplicon (corresponding to 147 bp of the first exon and 288 bp of the 5' flanking promoter region of the TaPAPhy_a1 gene) was obtained from two accessions of *T. tauschii*. The 1000 bp 5'flanking region and start codon of each of the *T. aestivum* genes were aligned, and used for a pair wise
25 comparison (see Figure 4).

The *T. aestivum* PAPhy phytase gene (in λ clone TaG2) and the PCR amplicon obtained from amplifying genomic DNA from the same cultivar, Skagen, had the same nucleotide sequence, and are included in Figure 4.

The nucleotide sequence of the 1000 bp 5'flanking promoter region of each
30 of the *T. aestivum* genes share at least 99.7% identity, whereas the nucleotide sequence of the corresponding promoter regions from two

HighPhy cultivars only share 97.7-98.1 % sequence identity to the other *T. aestivum* genes. The nucleotide sequence of the promoter regions of the two HighPhy cultivars [SEQ ID NO: 5 and 6], however, share 99.8% sequence identity with each other, differing in nucleotide sequence by only two base pairs. A polynucleotide comprising a ~2000 bp promoter region from the HighPhy cultivars, corresponding to the promoter region of the wild type *T. aestivum* PAPHy phytase gene, has the nucleotide sequence (SEQ ID NO: 44).

10 3.3 *Characterization of the promoter region of isolated T. aestivum and T. tauschii phytase genes – alignment of 288bp*

The 288 bp 5'flanking region and start codon of each of the *T. aestivum* genes together with the corresponding sequence from *T. tauschii* were aligned, and used for a pair wise comparison (Figure 5).

15 The amplified 288 bp 5'flanking promoter region from each of: wild-type *T. aestivum* cultivars; HighPhy *T. aestivum* cultivars; and *T. tauschii* cultivars shared nucleotide sequence identity within each of the three groups. However, the nucleotide sequence of the promoter regions of the two HighPhy *T. aestivum* cultivars differs from the wild-type 9 *T. aestivum* cultivars in two nucleotides, and differs from the two *T. tauschii* cultivars in 3 nucleotides. In turn the nucleotide sequence of the promoter regions from the two *T. tauschii* cultivars differs from wild-type *T. aestivum* cultivars in 3 nucleotides.

25 It can be seen from the alignment in Figure 6, that the 5'flanking promoter region of the two HighPhy *T. aestivum* cultivars [SEQ ID NO: 5] comprises a single nucleotide polymorphism (SNP) (-244 T→C), that is unique to these HighPhy cultivars, when compared to the wild type cultivars [SEQ ID NO: 7 and 8].

30 **Example 4. Genomic context of the SNP in HighPhy *T. aestivum* cultivars**

Sequence analyses of the immediate surroundings of the HighPhy SNP reveals sequence motifs known to be involved in gene regulation. Consider first the sequence found in wild type *T. aestivum* (wheat) cultivars (wt):

AACATGAGTCATGCATGGGA [SEQ ID NO: 4]

5 It consists of four overlapping motifs:

- In bold font, the odd base palindrome sequence reported by [3];
- In enlarged font, GCN4 motif, involved in endosperm specific gene expression [4];
- In italic font, the skn-1 motif reported by [5];
- 10 • In underlined font, the palindromic RY-repeat identical to that reported by [6].

Note that the skn-1 and GCN4 motifs are contained within the odd base palindrome. The odd base palindrome and GCN4 motif have been shown to interact with Opaque2, a maize basic leucine zipper (bZIP) transcription factor involved in the regulation of seed storage proteins [3,4], whereas the RY-repeat has been shown to interact with transcription factors containing the B3 domain. It is known to be an enhancer of seed-specific expression and a repressor of vegetative expression in *A. thaliana* [7].

Consider now the sequence from the HighPhy *T. aestivum* cultivars:

20 **AACA^CGAGT**CATGCATGGGA

The mutation, identified by the elevated "C", abolishes the odd base palindrome and the GCN4 motif, but leaves the skn-1 motif and the RY-repeat unchanged. A new motif, boxed, is thereby introduced. This motif shows similarity to the G-box CACGTG (1) but lacks the highly conserved palindromic nature of the G-box, and represents a completely novel motif, acting as a *cis*-acting regulatory element. This mutation, found in HighPhy *T. aestivum* cultivars, is either the result of the abolition of the odd base palindrome or the result of the introduction of the novel motif.

Example 5. Method for detecting the HighPhy SNP in the genome of cereal plants

The SNP in the genome of a cereal plant that is located in a polynucleotide comprising the enhancer element having the nucleotide sequence

5 “CGAGTCATGCATGGA” was detected using the technique of “High Resolution Amplicon Melting Analysis” [10]. PCR was performed in 10 μ L volumes in a LightCycler (Roche Applied Systems) with programmed transitions of 20 °C/s unless otherwise indicated. The amplification mixture included 50 ng genomic DNA as template, 200 μ M each deoxynucleotide
10 triphosphate (dNTP), 0.4 U KlenTaq1 polymerase (ABPeptides), 88 ng TaqStart antibody (ClonTech), 3 mM MgCl₂, 50 mM Tris (pH 8.3), 500 ng/ μ L bovine serum albumin, 0.5 μ M primers located upstream and downstream of the SNP and 1–10 μ M LCGreen, in order to amplify an polynucleotide of around 40-300 nucleotides in length. Melting analysis was performed on the
15 LightCycler. After amplification, the samples are heated momentarily in the LightCycler to 94 °C and cooled to 40 °C. The LightCycler capillary is then transferred to the high-resolution melting instrument and heated at 0.3 °C/s. Sample temperature and fluorescence signals are converted to 16-bit digital signals, which are then analysed to detect the SNP.

20

Example 6. Method for inducing and selecting wheat germplasm comprising the HighPhy SNP and grain with high levels of the enzyme phytase

HighPhy wheat can be generated by mutagenesis and subsequent screening
25 for individuals where the polynucleotide TGAGTCATGCATG, corresponding to the wild type (NormPhy) element, in the wheat genome has been converted into the mutant (HighPhy) element CGAGTCATGCATG. The mutagenesis is carried out with sodium azide which preferentially generates A:T to G:C substitutions in barley (8). Screening mutagenized populations for
30 the desired mutation could be done by allele specific polymerase chain reaction (AS-PCR).

Procedure: Wheat grains are presoaked for 15 hours in demineralised water at 5°C and then treated with an oxygenated solution of 1 mM sodium azide at pH 3 for 2 hours. The grains are washed and sown out, and grown to mature plants. Genomic DNA is isolated from leaves of each individual plant before the plant begins to senesce, using a standard DNA extraction procedure [9], whereas grains are harvested at maturity. Grains from individual plants are kept apart and labelled so they can be matched with the corresponding DNA isolates. The DNA isolates are screened by AS-PCR using the following primer pair:

HighPhy Fw: 5'CAAGCTACACTTTGTAGAACAC 3' [SEQ ID NO: 37]

PAPhy Rv: 5'CGCTGCACCCGGGGGTCCGT 3' [SEQ ID NO: 38]

The first 21 nucleotides of the HighPhy Fw primer anneal 5' to the HighPhy enhancer polynucleotide, whereas the 3'C nucleotide anneals to the actual SNP, this SNP being the distinguishing nucleotide between the HighPhy and NormPhy element polynucleotides. The PAPhy Rv primer anneals to a highly conserved part of the coding sequence of the PAPhy phytase gene, and can thus be expected to anneal to all known loci in the genome containing the wheat PAPhy phytase gene. The AS-PCR is performed using a non-proofreading polymerase to ensure specificity, and detection of the SNP. A series of replicate AS-PCR, using the HighPhy Fw primer and PAPhy Rv primer pair, are performed under conditions of increasing stringency (e.g. increasing PCR annealing temperature), on control genomic DNA samples isolated from HIGHPHY01 wheat grain of the invention and a wildtype NormPhy wheat plant. Under selected conditions of stringency, AS-PCR is then performed on DNA isolated from HighPhy mutation positive plants to amplify an amplicon of 300 to 700 bp in length, which can be identified by agarose gel electrophoresis, whereas plants lacking the HighPhy mutation will not produce an amplicon. The amplified product is then cloned and

sequenced to confirm that the presence of the mutant (HighPhy) element CGAGTCATGCATG in the genomic DNA isolate. AS-PCR conditions that are sufficiently stringent to selectively amplify HighPhy mutation positive plants, are then employed to screen genomic DNA isolated from each individual
5 mutagenized plant. Grain from HighPhy mutation positive plants can then be cultivated further to generate sufficient grain for subsequence breeding and crop production.

Example 7: Phytase activity of different *Secale cereale* cultivars:

10 7.1 *Comparative levels of total phytase enzymatic activity in mature Secale cereale (rye) grain*

The phytase activity was measured in mature seeds of 5 individual cultivars rye, as described for seeds of *Triticum aestivum*, detailed in Example 1.1. Phytase activity ranged from ~1600 to ~6000 FTU/kg in grain from the 5 rye
15 line (Figure 8). In one line, LPPO3, the level of phytase activity was ~1600 FTU/kg, which was lower than levels measured in the other 4 lines, of which one line had ~6000 FTU/kg.

**Example 8. Amplification and characterization of phytase gene
20 promoters from different *Secale cereale* and *Hordeum vulgare* cultivars:**

8.1 *Isolation of phytase gene promoters by PCR*

Genomic DNA was isolated from *Secale cereale* and *Hordeum vulgare* cultivars and the phytase gene promoter was amplified by PCR as described in Example 3.1.

25

Example 9. Structural characterisation of cereal phytase enzymes and the coding sequence of their cognate genes:

The promoter of the invention comprising an enhancer polynucleotide having SEQ ID NO: 1 or 2, is structurally and operably linked to a polynucleotide
30 molecule comprising a coding sequence encoding a phytase enzyme. The polynucleotide molecule in the genome of a cereal plant encoding a phytase

enzyme comprises a coding sequence (comprising one or more exon) and a non-coding sequence (comprising one or more intron). The amino sequence and the nucleotide sequence of the coding sequence encoding a phytase enzyme derived from the *Triticum aestivum* cv., (Ta); *Secale cereale* cv., (Sc); and *Hordeum vulgare* cv., (Hv) are as follows:

- TaPAPhy_a1 phytase (SEQ ID NO: 18) encoded by TaPAPhy_a1 cDNA (SEQ ID NO: 17);
- TaPAPhy_a2 phytase (SEQ ID NO: 20) encoded by TaPAPhy_a2 cDNA (SEQ ID NO: 19);
- TaPAPhy_a3 phytase (SEQ ID NO: 22) encoded by TaPAPhy_a3 cDNA (SEQ ID NO: 21);
- 10 • TmPAPhy_a4 phytase (SEQ ID NO: 24) encoded by TmPAPhy_a4 cDNA (SEQ ID NO: 23);
- ScPAPhy_a1 phytase (SEQ ID NO: 26) encoded by ScPAPhy_a1 cDNA (SEQ ID NO: 25);
- ScPAPhy_a2 phytase (SEQ ID NO: 28) encoded by ScPAPhy_a1 cDNA (SEQ ID NO: 26);
- HvPAPhy_a1 phytase (SEQ ID NO: 30) encoded by HvPAPhy_a1 cDNA (SEQ ID NO: 29);

15 The phytase enzyme in mutant HighPhy *Triticum aestivum* has an amino acid sequence similar to the phytase enzyme in wild type *Triticum aestivum* cv's Bobwhite and Skagen. Their amino acid sequences differ by the deletion of three amino acid residues and the substitution of three residues in the HighPhy cultivar when compared to cv's Bobwhite and Skagen, these
20 differences being located within the 120 residue long signal peptide region at the amino-terminus. The substitutions are conservative, G→A and S→T.

Example 10 Stability of phytase activity in wheat flour subjected to steam treatment

25 Animal feed comprising milled cereal grain, is commonly subjected to steam pelleting, which is a two-step process of conditioning followed by pelleting. During conditioning the milled feed is mixed and simultaneously heated to about 80°C and its moisture content is increased by exposure to steam. The experimental set up used in this example simulates the combination of heat
30 and moisture used during conditioning.

The experimental setup consisted of a GFL 1083 water bath with a plastic tray floating on the surface of the water and occupying approximately half of the surface area of the water. A thermometer, placed inside the tray, was

used to monitor the headspace temperature before and after incubation. The water bath was equipped with a thermostat and a lid so a constant temperature and steam-filled headspace with 100% relative humidity could be maintained. The water bath was set to 80°C, and once this temperature
5 was reached, it was allowed to equilibrate for one hour. The headspace temperature was found to be 80°C at this point.

Eight to nine grams of sample wheat grains were milled on a Retsch RM100 mortar grinder mill, and the resulting flour was distributed in weighing boats, 500 mg in each. The steam treatment consisted of incubating the weighing
10 boats with samples on the plastic tray for various periods of time. The lid of the water bath remained closed for the duration of the incubation and the temperature of 80°C in the headspace was verified before and after each incubation. Following steam treatment, the wheat flour samples were dessicated overnight in an exicator with silica gel.

15 Once dried, the phytase activity of the wheat flour samples was assayed using the assay described by (Engelen, Vanderheeft, Randerheeft & Smidt, 1994 [1])

Two measurements, (A) and (B), of phytase activities of wild type wheat (*T. aestivum* cv. Bobwhite (BW)) and HighPhy (HIGHPHY) wheat, after 0, 10,
20 20 and 40 min of incubation at 80°C at 100% relative humidity, are shown in figure 9. At t=0, the phytase activities of BW and HIGHPHY were 1286 and 4311 FTU/kg, respectively. After 10 min of incubation, HIGHPHY still exhibited a very substantial phytase activity of 3046 FTU/kg, while BW only exhibited 940 FTU/kg. After 20 min of incubation, HIGHPHY had 1801
25 FTU/kg residual phytase activity, still higher than the starting level in BW, while in BW the activity was only 667 FTU/kg. The most extreme incubation of 40 min reduced phytase activity in the HIGHPHY to 962 FTU/kg, while levels in BW were reduced to 476 FTU/kg.

The experimental conditions used to test phytate stability were more extreme
30 than those of commercial steam-pelleting, where the duration of steam treatment of normally around 1 minute. It is thus expected that the residual

phytase activity in pelleted feed made from HighPhy cereal grains of the invention will lie above the level at which supplementary phytase is required (circa 2500 FTU/kg).

5 Example 11. Stability of phytase activity in HighPhy *Secale cereale* (rye) subjected to steam treatment

Grain of HighPhy rye were milled and the resulting flour was subjected to simulated conditioning, using the same experimental set up as used for wheat flour in example 10.

- 10 A single measurement of phytase activity of HighPhy rye, after 0, 1, 2, 3, 4, 5, 10, 30, 45 and 60 min of incubation at 80°C at 100% relative humidity, are shown in figure 10. At t=0, the phytase activities of HighPhy rye was 4013 FTU/kg, and after 10 min still exhibited a very substantial phytase activity of 3818 FTU/kg. After 30 min of incubation phytase activities in the flour
15 dropped to 1746 FTU/kg.

Example 12. Enhanced phytate degradation in High-Phy wheat flour

- Phytase degradation during fermentation of dough improves its nutritional and bread-making quality, since phytase degradation enhances inorganic
20 phosphate levels and mineral content in bread, and is known to improve dough mixing quality. Dough made from HighPhy wheat is shown to provide these advantages.

- Wild type wheat (*T. aestivum* cv Bobwhite (BW)) and HighPhy wheat (HP)
25 grains were milled on a Retsch RM100 mortar grinder mill. The phytate content in the resulting flour was determined using the procedure described by (Vaintraub & Lapteva, 1988). Flour (250 mg) was mixed with 0.1 ml of bakers yeast water stock (dry yeast in 3 mg/ml water). An additional 0.2 ml of water was added to form a dough. The dough was fermented at 25°C for
30 0.5, 1, 3 and 3 hrs. After fermentation, the phytate content was determined again, using the same procedure (Vaintraub and Lapteva, 1988).

The initial phytate content of HP wheat flour was a little higher than that of BW wheat (Figure 11). However during fermentation, phytate levels were decreased to a lower level in flour from HP than BW wheat. Thus, phytate levels were reduced significantly more during fermentation in wheat dough from HP in comparison to dough from BW, both in terms of percentage and in the final phytate levels. Already after 0.5 hr, phytate was reduced more in HP than in BW wheat (Figure 12 and table 2). After 3 hrs, only ~44% of the initial phytate was left in the HP wheat, whereas ~68% was left in the wild type BW wheat.

Table 2. Percent residual phytate (IP6) in dough of wild-type (BW) and HighPhy (HP) wheat flour

Time	BW residual IP6 (%)	HP residual IP6 (%)
0	100	100
0.5	97.8	85.9
1.0	91.9	82.7
1.5	86.2	66.9
2.0	73.8	53.6
3.0	68.3	43.8

Example 13. The HighPhy enhancer in the barley phytase gene confers both aleurone and endosperm-specific expression in developing barley grain

13.1 Cloning of promoter-GUS constructs for examination of the HighPhy mutation: The HighPhy mutation was introduced in the pCLEAN-G185-*PAPhy_a* construct with the mutagenic primers:

HvPAPhy_a SDmut Fw 5'GTAGAACACGAGCCATGCATGAGAC3' (SEQ ID No. 46)

HvPAPhy_a SDmut Rv 5'TGGCTCGTGTTCTACAAAATGTAGC3' (SEQ ID No. 47)

This yielded the pCLEAN-G185-HP-*PAPhy_a* construct.

The two constructs pCLEAN-G185-*PAPhy_a* and pCLEAN-G185-HP-*PAPhy_a* were further modified to serve as promoter-reporter gene constructs. To achieve this, the *PAPhy_a* coding open reading frame and
5 terminator was replaced by the *UidA* open reading frame followed by the NOS terminator. The cloning was performed with the “In-Fusion” technology as described in (Zhu, Cai, Hall and Freeman, 2007). This approach ensured seamless joining of the promoter and reporter gene so the start codon context was preserved.

10 The vector backbone and promoter of both constructs were amplified using the primers:

Cis to GUS Fw 5' TCGAGTCGACGTTTCCTTGAC3' (SEQ ID No. 48)

Cis to GUS Rv 5' GTTGATGTTGTTGCTTGGCATTG3' (SEQ ID No. 49)

The *UidA* and NOS terminator were amplified from pGUSN which is a pUC18
15 plasmid comprising an *UidA* gene and a downstream NOS terminator, using the primers:

GUS Fw m. overhang 5'

AGCAACAACATCAACATGTTACGTCCTGTAGAAACC3' (SEQ ID No. 50)

GUS Rv m. overhang 5'

20 GGAACGTCGACTCGACTATGACCATGATTACGAATTCC3'(SEQ ID No. 52)

Performing the In-Fusion with the resulting amplicons gave the two GUS reporter constructs, pCLEAN-G185-wt-proGUS (SEQ ID No. 52) and pCLEAN-G185-HP-proGUS (SEQ ID No. 54).

25

13.2: Constructing randomized phytase gene enhancer element sequences to confirm the criticality of the promoter enhancer element comprising the HighPhy mutation. The enhancer element motifs surrounding the *HighPhy* mutation were removed by sequence randomization by taking the 20bp
30 corresponding to SEQ ID 4 in the pCLEAN-G185-*PAPhy_a* construct and subjecting the sequence to a nucleotide randomizer

(http://molbiol.ru/eng/scripts/01_16.html) using settings designed to preserve the nucleotide ratios of the original sequence. The resulting sequence, 5' gcatacgaagcatagtagca3', was only identical to the original in three nucleotide positions and did not contain any regulatory elements known by PlantCARE*.

- 5 The original 20 bp in pCLEAN-G185-wt-proGUS was replaced by the randomized sequence as described by Zhu and co-workers using the primers:

Kill triad Fw 5' gcatacgaagcatagtagcaCGTAGGCGTCCAAACTTTG3' (SEQ ID No. 56);

- 10 Kill triad Rv 5'

tcgtactatgcttcgtatgcCTACAAAATGTAGCTTGAAATTAAAGAG3' (SEQ ID No. 57)

The resulting construct was pCLEAN-G185-KOtriad-proGUS (SEQ ID No. 58).

15

13.3 Transient expression in developing barley endosperm and aleurone:

The three constructs were individually introduced into developing (from 14 to 35 days after pollination) barley endosperm and aleurone cells by particle gun bombardment. Immature barley seeds were sterilized, and cultured on media and bombarded in a DuPont PDS 1100 helium biolistic delivery system using the procedures described in (BrinchPedersen, Galili, Knudsen, & Holm, 1996). Expression of the *uidA* gene was assayed in the plant tissues two days after bombardment, using the gus reaction buffer, as described in Jefferson, Kavanagh, & Bevan, 1987. Gus expression was scored by localizing blue spots on the bombarded tissues.

25

In tissues bombarded with the pCLEAN-G185-wt-proGUS plasmid, blue spots were mainly identified in the aleurone layers, with very limited expression in the endosperm. In pCLEAN-G185-HP-proGUS bombarded tissues more expression could be observed in the endosperm tissue. No expression was detected in grain bombarded with the pCLEAN-G185-KOtriad-proGUS. These data confirm that the HighPhy mutation in the

30

context of the barley phytase gene enhancer confers both aleurone and endosperm-specific expression

Example 14. Identification of the wild type locus in the wheat genome corresponding to the HighPhy phytase gene

The mutant gene was aligned to the three homeologous *PAPhy_a* genes from the wild type cultivar “Chinese spring”. The alignment was adjusted to include only the exons and introns of the gene. An UPGMA tree was generated with 1000 bootstrap replications (Figure 13). The tree clearly points to *TaPAPhy_a1* as the wild type locus corresponding to the HighPhy gene.

14.1 Chromosomal mapping of the TaPAPhy_a1 gene: Wheat chromosomal mapping was performed using the Chinese Spring nullisomic-tetrasomic lines described by (Kimber & Sears, 1979). There are 42 possible nullisomic-tetrasomic lines, of which two were missing in the present set of lines (the nullisomic (N) 2A tetrasomic (T) 2B and the N4BT4D lines), but their absence did not compromise the mapping. The following primers were designed to specifically amplify a 522 basepair segment of the *TaPAPhy_a1* gene:

Forward 5'GAGATTCCGAGACCAACGAA3'

Reverse 5'TTTGCCTCCACTCTGCCTAC3'

The amplicon was exclusively absent from two lines nullisomic for chromosome 5D and tetrasomic for chromosome 5A and 5B respectively (N5DT5A and N5DT5B). Thus, *TaPAPhy_a1* maps to chromosome 5D.

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30

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PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences:

Magali Lescot, Patrice Déhais, Gert Thijs, Kathleen Marchal, Yves Moreau, Yves Van de Peer, Pierre Rouzé and Stephane Rombauts
Nucleic Acids Res. 2002 Jan 1;30(1):325-327

PCT

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.221 MT/FOP 20020701/0.20.5.9
0-2	International Application No.	
0-3	Applicant's or agent's file reference	P1275PC00
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	Paragraph number	4
1-3	Identification of deposit	
1-3-1	Name of depositary institution	ATCC American Type Culture Collection
1-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209 United States of America
1-3-3	Date of deposit	04 March 2011 (04.03.2011)
1-3-4	Accession Number	ATCC PTA-11730
1-4	Additional Indications	Rye grains: High Rye
1-5	Designated States for Which Indications are Made	All designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	Paragraph number	4
2-3	Identification of deposit	
2-3-1	Name of depositary institution	ATCC American Type Culture Collection
2-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209 United States of America
2-3-3	Date of deposit	04 March 2011 (04.03.2011)
2-3-4	Accession Number	ATCC PTA-11731
2-4	Additional Indications	Wheat grains: High Phy-2
2-5	Designated States for Which Indications are Made	All designations

PCT

3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
3-1	Paragraph number	4
3-3	Identification of deposit	
3-3-1	Name of depositary institution	ATCC American Type Culture Collection
3-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209United States of America
3-3-3	Date of deposit	04 March 2011 (04.03.2011)
3-3-4	Accession Number	ATCC PTA-11732
3-4	Additional Indications	Wheat grains: High Phy-1
3-5	Designated States for Which Indications are Made	All designations

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0-4	This form was received with the international application: (yes or no)	YES
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Claims

1. A mutant cereal plant comprising a polynucleotide selected from any one of:
 - 5 a. ACA VGA GTC ATG CAT [SEQ ID NO: 1],
 - b. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 14 and 15,
 - c. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 14 and 15, wherein said polynucleotide is
 - 10 operably linked to a second polynucleotide encoding a polypeptide,wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant, and
wherein said cereal is selected from *Avena L species*, *Hordeum L species*; *Oryza L species*; *Secale L species*; *Sorghum L species*;
15 *Triticum aestivum*, *Triticum durum*; *Triticum spelta* and *Zea species*.
2. The mutant cereal plant of claim 1, wherein said polypeptide is phytase and has *myo*-inositol hexakisphosphate phosphohydrolase
20 activity.
3. The mutant cereal plant of claim 1 or 2, wherein said cereal is selected from *Avena sativa*; *Hordeum vulgare*; *Oryza sativa*; *Secale cereale*; *Sorghum bicolor*; *Triticum aestivum*, *Triticum durum*; *Triticum spelta*
25 and *Zea mays*.
4. The mutant cereal plant of claim 2, wherein said plant is selected from *Triticum aestivum*, *Triticum durum* and *Triticum spelta*, and said polypeptide has an amino acid sequence having at least 70%
30 sequence identity to a sequence selected from SEQ ID NO: 18, 20,

and 22.

- 5 5. The mutant cereal plant of claim 2, wherein said plant is a *Secale*
 spp., and said polypeptide has an amino acid sequence having at
 least 70% sequence identity to a sequence selected from SEQ ID NO:
 26 or 28.
- 10 6. The mutant cereal plant of claim 2, wherein said plant is a *Hordeum*
 spp., and said polypeptide has an amino acid sequence having at
 least 70% sequence identity to a sequence selected from SEQ ID NO:
 30.
- 15 7. A mutant cereal plant according to any one of claims 1-6, wherein said
 plant is a grain.
8. Use of grain according to claim 7, for the manufacture of a
 composition, wherein said composition is any one of:
 a. a milled grain composition;
 b. animal fodder and
20 c. steam-pelleted animal fodder.
9. A composition comprising a mutant cereal plant according to any one
 of claims 1-7, wherein said composition is any one of:
 a. a milled grain composition;
25 b. animal fodder and
 c. steam-pelleted animal fodder.
10. Use of a composition according to claim 9 as animal fodder.
- 30 11. A method for detecting a mutant cereal plant comprising a
 polynucleotide selected from any one of:

- 5
- a. ACA VGA GTC ATG CAT [SEQ ID NO: 1],
 - b. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 12, 14 and 15,
 - c. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 12, 14 and 15, wherein said polynucleotide is operably linked to a second polynucleotide encoding a polypeptide,

wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant, comprising the steps of:

- 10
- i. isolating genomic DNA from said plant, and
 - ii. detecting the presence of the nucleotide V at the 5' end of a polynucleotide having the nucleotide sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], wherein said polynucleotide is comprised in
- 15
- said genomic DNA.

12. Method for inducing and selecting a mutant cereal plant comprising a polynucleotide selected from any one of:

- 20
- a. ACA VGA GTC ATG CAT [SEQ ID NO: 1],
 - b. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 5, 6, 12, 14 and 15,
 - c. polynucleotide comprising a nucleotide sequence selected from SEQ ID NO: 1, 5, 6, 12, 14 and 15, wherein said polynucleotide is operably linked to a second polynucleotide encoding a polypeptide,
- 25

wherein said polynucleotide is capable of enhancing gene expression in a grain of said plant, comprising the steps of:

- i. treating a cereal plant, or plant part thereof, with a chemical mutagen;
- 30
- ii. growing and/or multiplying the treated plant, or plant part;
- iii. isolating genomic DNA from said plant or progeny thereof, and

- iv. detecting the presence of a polynucleotide having the nucleotide sequence ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], wherein said polynucleotide is comprised in said genomic DNA.

5

13. The method according to claim 11 or 12, wherein said genomic DNA comprises a first polynucleotide located 5' upstream of and operably-linked to a second polynucleotide, wherein said first polynucleotide comprises the nucleotide sequence
10 ACA VGA GTC ATG CAT [SEQ ID NO: 1] or T AGA ACA VGA GTC ATG CAT [SEQ ID NO: 2], and wherein said second polynucleotide encodes a phytase polypeptide having *myo*-inositol hexakisphosphate phosphohydrolase activity.

- 15 14. The method according to claim 11 or 12, wherein said cereal is selected from a species of *Avena L*, *Hordeum L*; *Oryza L*; *Secale L*; *Sorghum L*; *Triticum*; and *Zea*.

- 20 15. The method according to claim 13, wherein the amino acid sequence of said phytase polypeptide has at least 70% sequence identity to a sequence selected from SEQ ID NO: 18, 20, 22, 24, 26, 28, and 30.

Figures

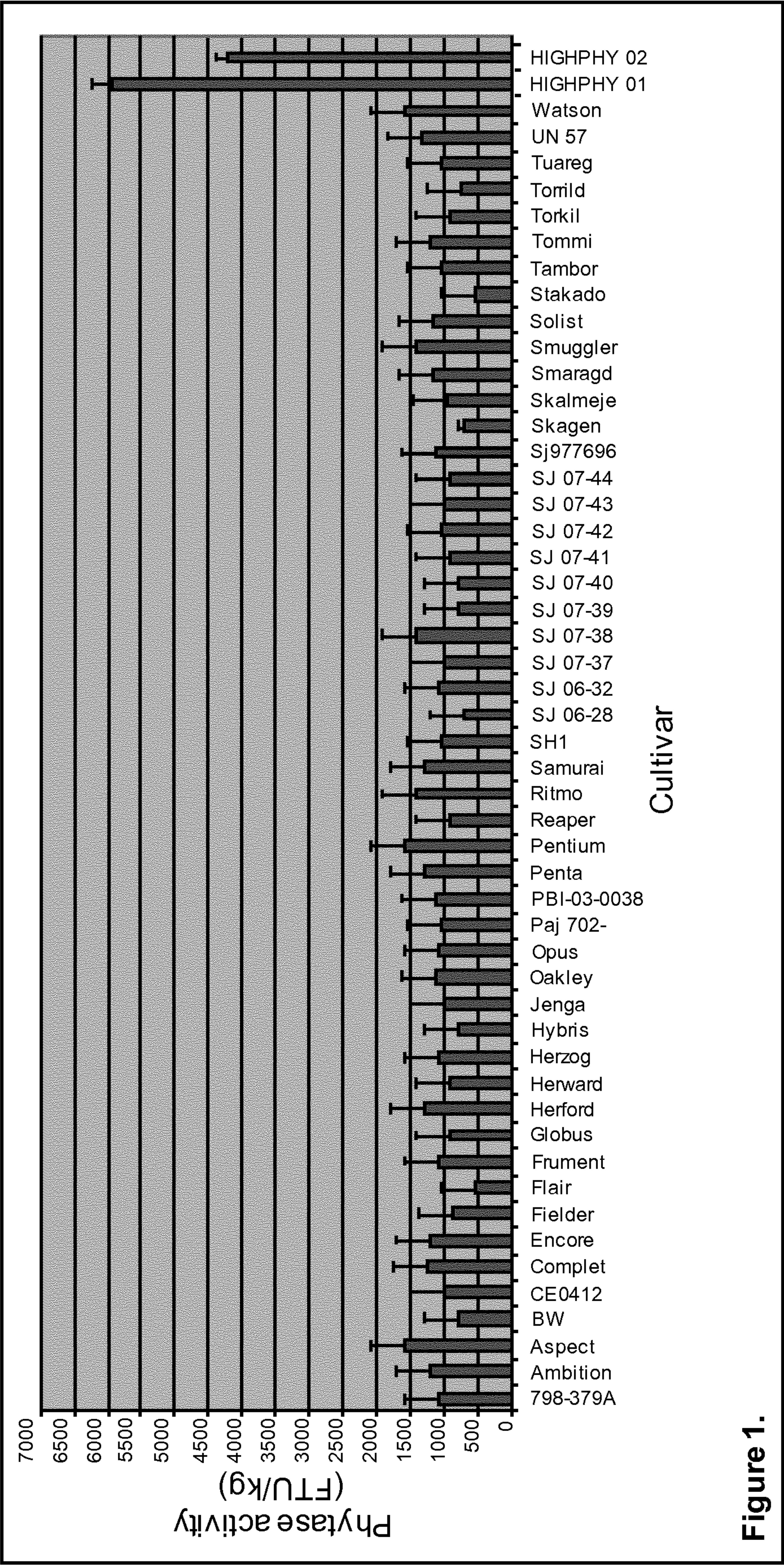
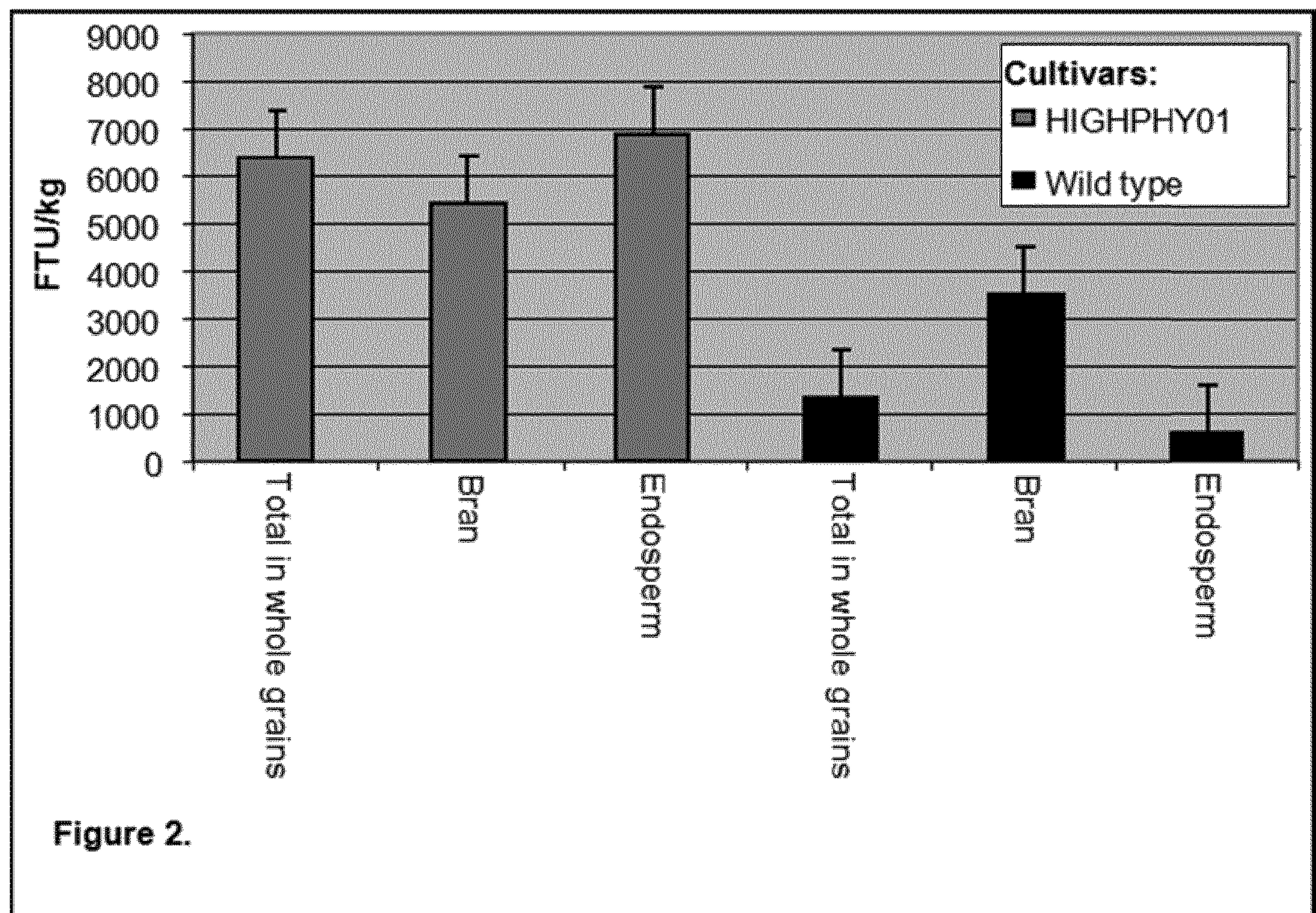


Figure 1.



5

10

15

Figure 3

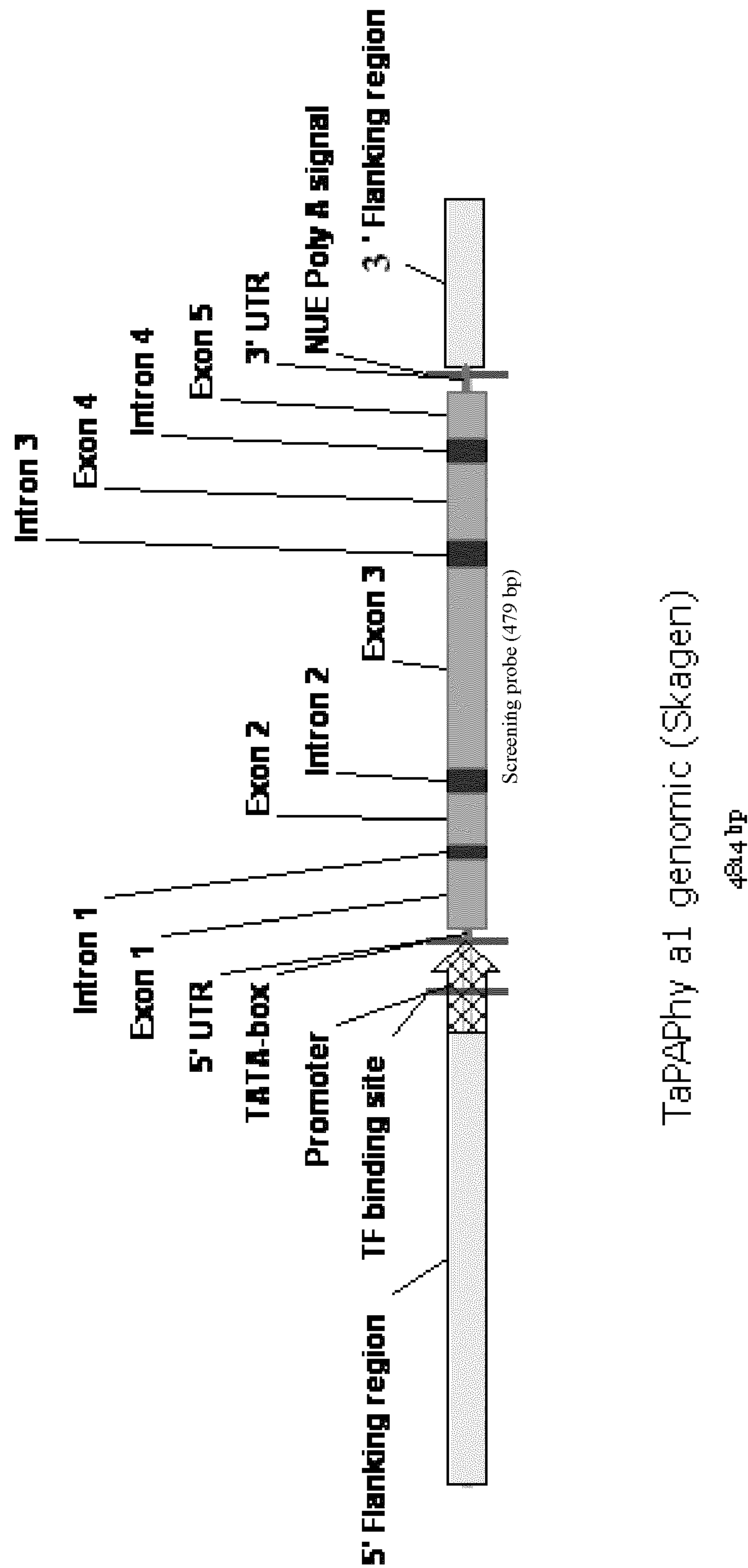


Figure 4.

	1	2	3	4	5	6	7	8	9	10
TaG2 lambda clone, Skagen cultivar		0	0	0	2	0	1	1	19	21
Skagen PCR clone	100,00		0	0	2	0	1	1	19	21
Bob White PCR clone	100,00	100,00		0	2	0	1	1	19	21
Pentium PCR clone	100,00	100,00	100,00		2	0	1	1	19	21
Flair PCR clone	99,80	99,80	99,80	99,80		2	3	3	21	23
Landrace 01 PCR clone	100,00	100,00	100,00	100,00	99,80		1	1	19	21
Landrace 02 PCR clone	99,90	99,90	99,90	99,90	99,70	99,90		2	20	22
Spelt PCR clone	99,90	99,90	99,90	99,90	99,70	99,90	99,80		20	22
HighPhy 01 PCR clone	98,10	98,10	98,10	98,10	97,90	98,10	98,00	98,01		2
HighPhy 02 PCR clone	97,90	97,90	97,90	97,90	97,70	97,90	97,80	97,81	99,80	

		-280		-260	
TaG2 lambda clone, Skagen cultivar	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Skagen PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Bob White PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Pentium PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Flair PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Landrace 01 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Landrace 02 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
Spelt PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACAT				45
HighPhy 01 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACA				45
HighPhy 02 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACACTTTGTAGAACA				45
NGB90403 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACA				45
NGB9855 PCR clone	TTTTGTTGCTTGCGCTTTAGTTTCAAGCTACA				45
		-240		-220	
TaG2 lambda clone, Skagen cultivar	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Skagen PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Bob White PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Pentium PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Flair PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Landrace 01 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Landrace 02 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
Spelt PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
HighPhy 01 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
HighPhy 02 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
NGB90403 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
NGB9855 PCR clone	GAGTCATGCATGGGACGAAGGCGTCCAAACTTGGCTAGTGCAGCT				90
		-180		-160	
TaG2 lambda clone, Skagen cultivar	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Skagen PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Bob White PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Pentium PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Flair PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Landrace 01 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Landrace 02 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
Spelt PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
HighPhy 01 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
HighPhy 02 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
NGB90403 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
NGB9855 PCR clone	GCCTGCGCGTTTACAAGGCACCAAAGCGCAGGCGGCAAAGTTTGC				135
		-140		-120	
TaG2 lambda clone, Skagen cultivar	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Skagen PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Bob White PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Pentium PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Flair PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Landrace 01 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Landrace 02 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
Spelt PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
HighPhy 01 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
HighPhy 02 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
NGB90403 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
NGB9855 PCR clone	TCGTTTATTATCTTGGCGGTCCAAGATGGGCGGCAGGTTCCAGAC				180
		-100		-80	
TaG2 lambda clone, Skagen cultivar	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Skagen PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Bob White PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Pentium PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Flair PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Landrace 01 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Landrace 02 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
Spelt PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
HighPhy 01 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
HighPhy 02 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
NGB90403 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
NGB9855 PCR clone	GATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCAACCTCCT				225
		-60		-40	
TaG2 lambda clone, Skagen cultivar	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Skagen PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Bob White PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Pentium PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Flair PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Landrace 01 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Landrace 02 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
Spelt PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
HighPhy 01 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
HighPhy 02 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
NGB90403 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
NGB9855 PCR clone	CTGCCCCGATTTCATATAAGTTTTCCTGCCAAAGGCATTCCAATTCTG				270
				-20	
TaG2 lambda clone, Skagen cultivar	TCAATGCCAAGCAACAACATG				291
Skagen PCR clone	TCAATGCCAAGCAACAACATG				291
Bob White PCR clone	TCAATGCCAAGCAACAACATG				291
Pentium PCR clone	TCAATGCCAAGCAACAACATG				291
Flair PCR clone	TCAATGCCAAGCAACAACATG				291
Landrace 01 PCR clone	TCAATGCCAAGCAACAACATG				291
Landrace 02 PCR clone	TCAATGCCAAGCAACAACATG				291
Spelt PCR clone	TCAATGCCAAGCAACAACATG				291
HighPhy 01 PCR clone	TCAATGCCAAGCAACAACATG				291
HighPhy 02 PCR clone	TCAATGCCAAGCAACAACATG				291
NGB90403 PCR clone	TCAATGCCAAGCAACAACATG				291
NGB9855 PCR clone	TCAATGCCAAGCAACAACATG				291

Figure 6

	-300	-280	-260	
NormPhy element				
TaPAPhy_a1 NormPhy	-----TGAGTCATGCATG-----	13		
TaPAPhy_a1 HighPhy	TAGTTTCAAGCTACACTTTGTAGAACATGAGTCATGCATGGGACG	45		
HighPhy element	-----CGAGTCATGCATG-----	13		
ScPAPhy_a1 HighPhy	TAGTTTCAAGCTACATTTTCTAGAACACGAGTCATGCATGGGACG	45		
ScPAPhy_a1 NormPhy	TAGTTTCAAGCTACATTTTGTAGAACATGAGTCATGCATGGGACG	45		
TaPAPhy_a3	TAGTTTCAAGCTACATTTTGTAGAACATGAGTCATGCATGGGACG	45		
TaPAPhy_a4	TATTTTCAAGCTACATTTTGTAGAACATGAGTCATGCATGGGACG	45		
TaPAPhy_a5	TAGTTTCAAGCTACATTTTGTAGAACAGGAGTCATGCATGG-ACG	44		
TaPAPhy_a6	AGTTATCAAGCTACATTTTGTAGAACATGAGTCATGCATTGGACG	45		
TmPAPhy_a1	TAGTTTCAAGCTACATTTTGTAGAACAGGAGTCATGCATGG-ACG	44		
HvPAPhy_a1	TAATTTCAAGCTACATTTTGTAGAACATGAGCCATGCATGAGACG	45		
	-240	-220		
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	AAGGCGTCCAAA-----CTTGGCTA--GTGCAGCTGCCTGC--	79		
TaPAPhy_a1 HighPhy	AAGGCGTCCAAA-----CTTGGCTA--GTGCAGCTGCCTGC--	79		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	AAGGTGTCCAAAGTCCAAACTTTGGCTTTTGTGCAGCTGCCTGC--	88		
ScPAPhy_a1 NormPhy	AAGGTGTCCAAAGTCCAAACTTTGGCTTTTGTGCAGCTGCCTGC--	88		
TaPAPhy_a3	AAGGTGTCCAAAGTCCAAACTCGGCTA--GTGCAGCTGCCTGCAC	88		
TaPAPhy_a4	AAGGTGGCCAAAGTCCAAACTTTGGC-----	70		
TaPAPhy_a5	AAGGTGTCCAAAGTCCAAACTTTGGCTA--GCGCAGCTGCCTGC--	85		
TaPAPhy_a6	AAGGTGTCCAAAGTCCAAACTT-----	67		
TmPAPhy_a1	AAGGTGTCCAAAGTCCAAACTTTGGCTA--GCGCAGCTGCCTGC--	85		
HvPAPhy_a1	TAGGCGTCCAAACT-----TTGGCTA--GCGCAGCTGCATGC--	80		
	-200	-180		
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	-----GCGTTCACAAGGCACCAAAG-GCGAGGCGGCAAAGTT--	115		
TaPAPhy_a1 HighPhy	-----GCGTTCACAAGGCACCAAAG-GCGAGGCGGCAAAGTT--	115		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	-----ACGTTTACAAGGCACCAAAG-GCGAGGCGGCAAACCTTAA	126		
ScPAPhy_a1 NormPhy	-----ACGTTTACAAGGCACCAAAG-GCGAGGCGGCAAACCTT--	124		
TaPAPhy_a3	GTTCCTGACGTTTACAAGGCACCAAAG-GCGAGGCGGCAAACCTT--	130		
TaPAPhy_a4	-----AGGCGGCAAAGTT--	83		
TaPAPhy_a5	-----ACGTTTACAAGGCACCAAAG-GCGAGGCGGCAAAGTT--	121		
TaPAPhy_a6	-----TCACAAGGCACCAAAG-GCGAGGCGGCAAAGTT--	99		
TmPAPhy_a1	-----ACGTTTACAAGGCACCAAAG-GCGAGGCGGCAAAGTT--	121		
HvPAPhy_a1	-----ACGTCACACAAGGCACCAAAGGCGCAGGCGGCAAC-TT--	116		
	-160	-140		
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	--TGCTCGTTTATTATCTTTGGCGGTCCAAGATGGGCGGCAGGTTT	158		
TaPAPhy_a1 HighPhy	--TGCTCGTTTATTATCTTTGGCGGTCCAAGATGGGCGGCAGGTTT	158		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	TTTGCTCGTTTATTATCTTTGCTGGTCCAAGATGGGCGGCAGGTTG	171		
ScPAPhy_a1 NormPhy	--TGCTCGTTTATTATCTTTGCTGGTCCAAGATGGGCGGCAGGTTG	167		
TaPAPhy_a3	--TGCTCGTTTATTATCTCGCGGTCCAAGATGGGCGGCAAGTTT	173		
TaPAPhy_a4	--TGCTCGTTTATCATCTTTGCCGGTCCAAGATGGGCGGCAGGTTT	126		
TaPAPhy_a5	--TGCTCGTTTATTATCTTTGCCGGTCCAAGACGGGCGGCAGGTTT	164		
TaPAPhy_a6	--TGCTCGTTTATCATCTTTGCCGGTCCAAGATGGGCGGTAGGTTT	142		
TmPAPhy_a1	--TGCTCGTTTATTATCTTTGCCGGTCCAAGACGGGCGGCAGGTTT	164		
HvPAPhy_a1	--TGCTCGTTTATTTTCTTTGCCGGTCCAAGATGAG-----TTC	152		
	-120	-100	-80	
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	CAGACGATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCC-AA	202		
TaPAPhy_a1 HighPhy	CAGACGATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCC-AA	202		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	CA-----CCCACCGAGTTCCACTTCCGGCTCCCAA	201		
ScPAPhy_a1 NormPhy	CAGAAGATGGACGAAGACCCACCGAGTTCCACTTCCGGCTCCCAA	212		
TaPAPhy_a3	TAGACGCTGGACGAAGACCCACCGAATTCCATTTCCGGCTCCCAA	218		
TaPAPhy_a4	CAGGCGATGGACGAAGACCCCGGAGTCCCACTTCCGGCTCCCAA	171		
TaPAPhy_a5	CAGACGATGGACGAAGACCCACCGAATTCCATTTCCGGCTCCCAA	209		
TaPAPhy_a6	CAGACGATGGACGAAGACCC-----A	163		
TmPAPhy_a1	CAGACGATGGACGAAGACCCACCGAATTCCATTTCCGGCTCCCAA	209		
HvPAPhy_a1	CAGACCATGGACGAA-----TTCCACTTCCGGCTCCCAA	186		
	-60	-40		
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	CCTCCTCTGCCCGATTTCATATAAGTTTCTGCCAA-AGGCATTCC	246		
TaPAPhy_a1 HighPhy	CCTCCTCTGCCCGATTTCATATAAGTTTCTGCCAA-AGGCATCCC	246		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	TCTCCTGTGCCTGATTTCCTATAAGTTTCTGCCAA-AAGCATCCC	245		
ScPAPhy_a1 NormPhy	TCGCCTCTGCCCGATTTCCTATAAGTTTCTGCCAA-AGGCATCCC	256		
TaPAPhy_a3	CCTCCTCTGCCCGATTTCCTGTAAGTTTCTGCCAA-AATCATCCC	262		
TaPAPhy_a4	CCTCCTCTGCCCGATTTCATATAAGTTTCTGCCAA-AGGCATCCT	215		
TaPAPhy_a5	CCTCCTCTGCCCGATTTCCTACAAGTTTCTGCCAA-AGGCATCCC	253		
TaPAPhy_a6	CCTCCTCTGTCCGATTTCATATAAGTTTCTGCCAA-AGGCATCCC	207		
TmPAPhy_a1	CCTCCTCTGCCCGATTTCCTACAAGTTTCTGCCAA-AGGCATCCC	253		
HvPAPhy_a1	TCTCCTCTGCCCGATTTCCTATAAGTTTCTGCCAAGAAGCATCCC	231		
	-20	1		
NormPhy element	-----	13		
TaPAPhy_a1 NormPhy	AATTCTGTCAATGCCAAGCAA-----CAACATG	274		
TaPAPhy_a1 HighPhy	AATTCTGTCAATGCCAAGCAA-----CAACATG	274		
HighPhy element	-----	13		
ScPAPhy_a1 HighPhy	AATTCTGTCAATGCCAAGCAA-----CAACATG	273		
ScPAPhy_a1 NormPhy	AATTCTGTCAATGCCAAGCAA-----CAACATG	284		
TaPAPhy_a3	AATTCTCTCAATGCCAAGCAA-----CACCATG	290		
TaPAPhy_a4	AATTCTGTCAATACCAAGCAA-----CAACATG	243		
TaPAPhy_a5	AATTCTGTCAATGCCAAGCAA-----CGCCATG	281		
TaPAPhy_a6	AATTCTGTCAATGCCAAGCAA-----CAACATG	235		
TmPAPhy_a1	AATTCTGTCAATGCCAAGCAA-----CGCCATG	281		
HvPAPhy_a1	AATCCCCTCAATGCCAAGCAACAACATCAACATG	265		

Figure 7

Figure 8

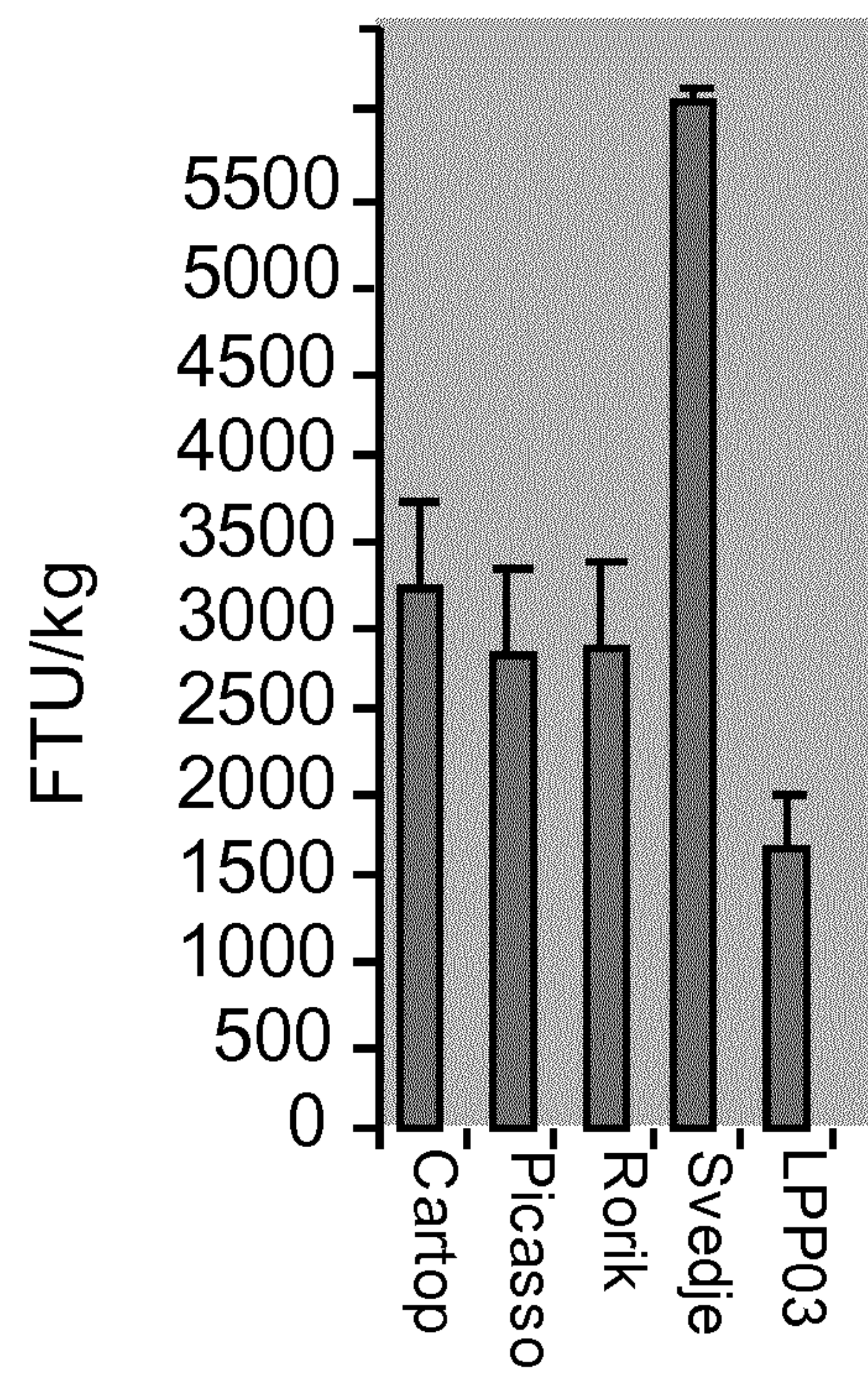


Figure 9

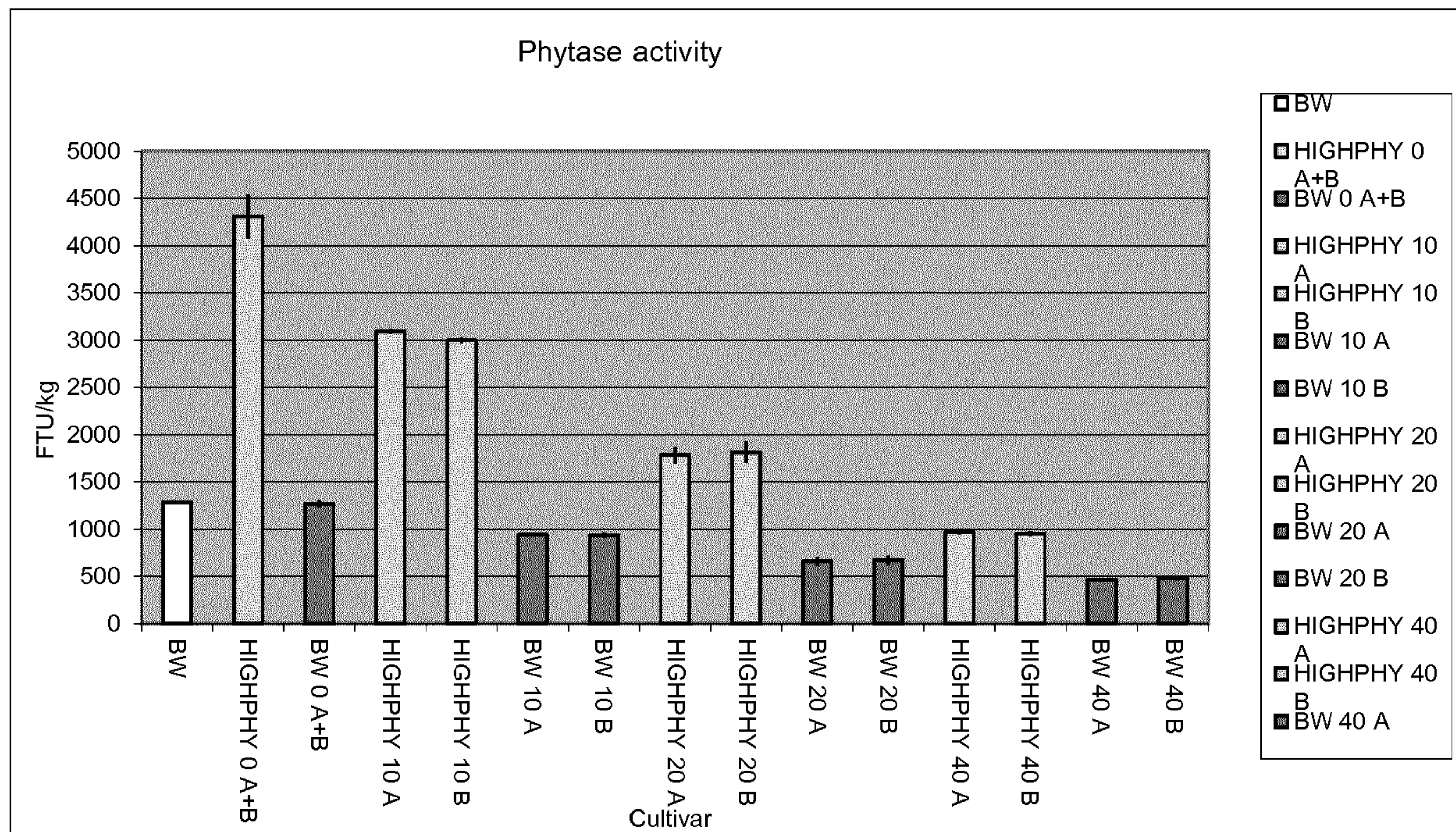


Figure 10

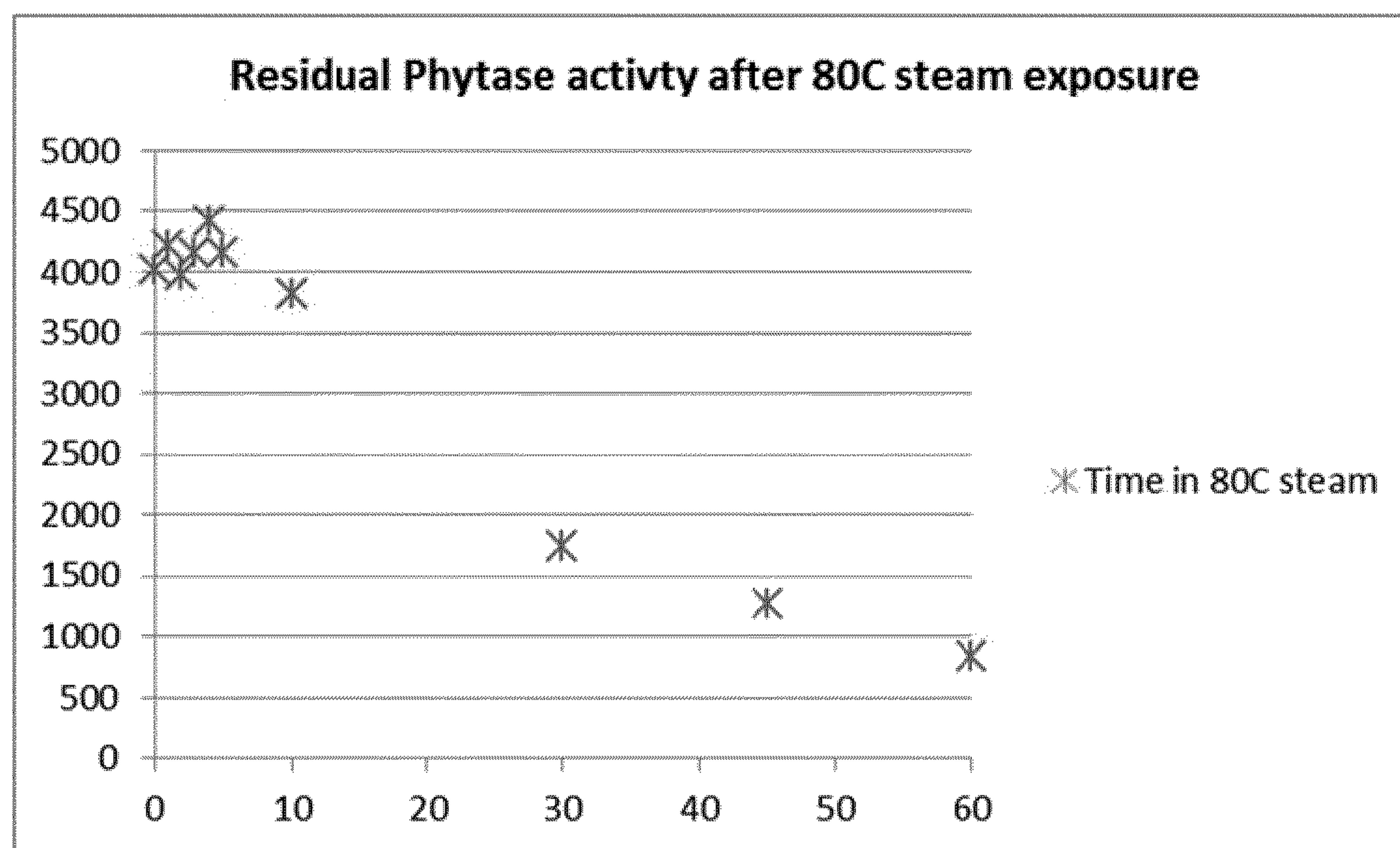
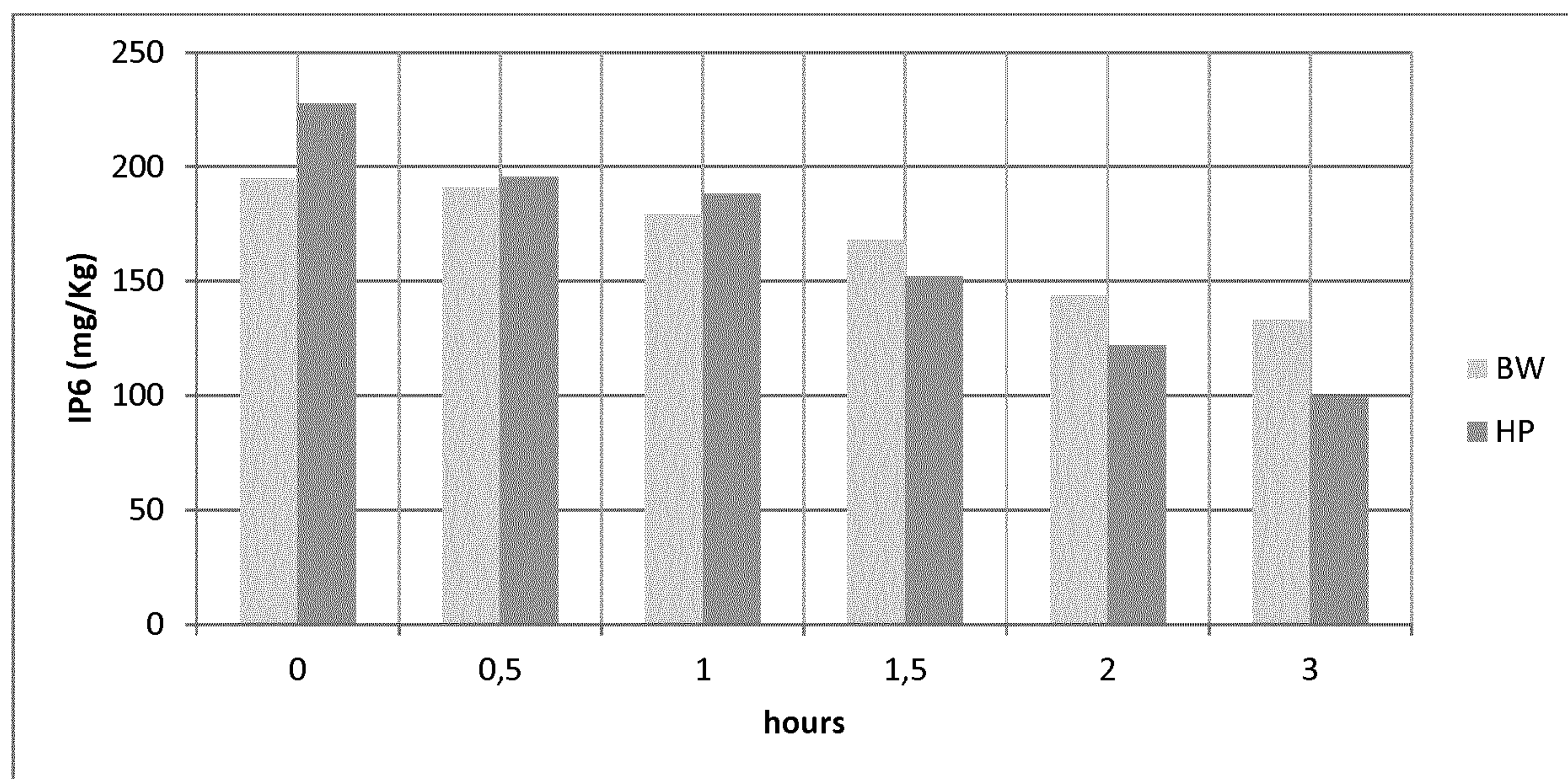


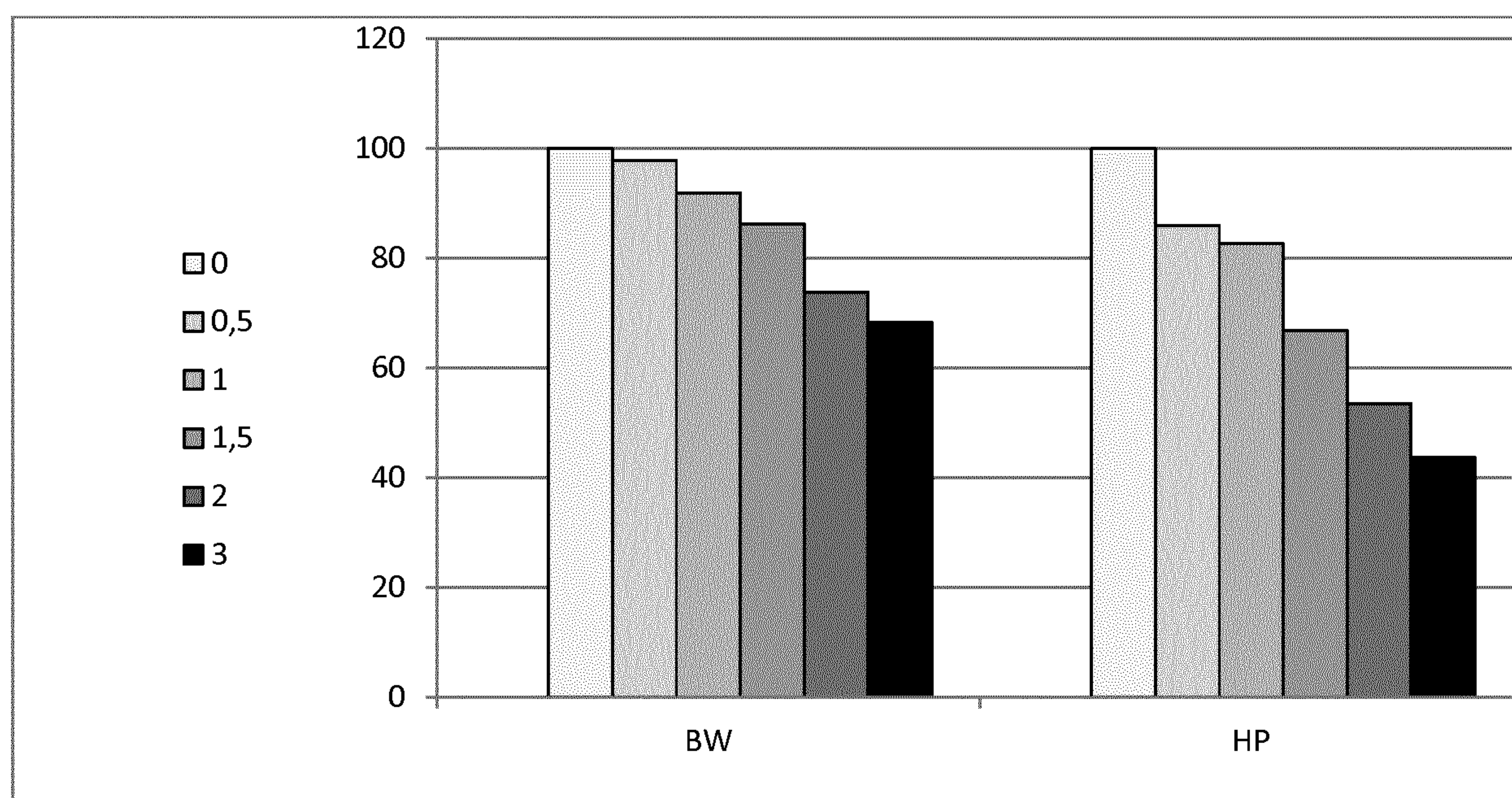
Figure 11



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Figure 12



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Figure 13

