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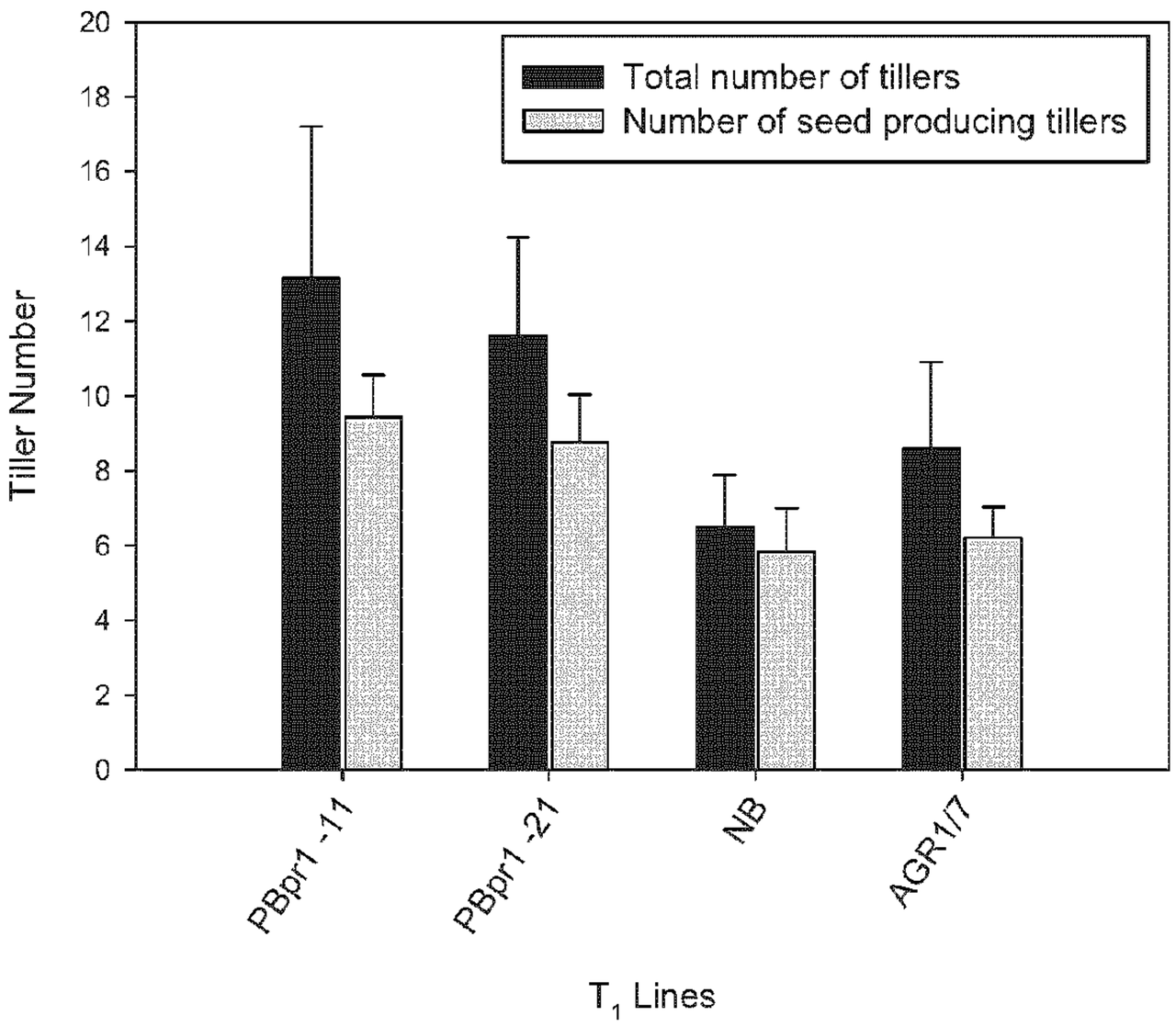

FIG. 3

(57) Abrégé/Abstract:
A transgenic plant comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter is provided. The transgenic plant exhibits increased nitrogen use efficiency, increased biomass and/or increased seed yield. Seeds from such transgenic plants, genetic constructs to prepare such plants, methods of generating and growing transgenic plants are also provided.

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[Continued on next page]

(54) Title: PLANTS HAVING ENHANCED NITROGEN EFFICIENCY

(57) Abstract: A transgenic plant comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter is provided. The transgenic plant exhibits increased nitrogen use efficiency, increased biomass and/or increased seed yield. Seeds from such transgenic plants, genetic constructs to prepare such plants, methods of generating and growing transgenic plants are also provided.

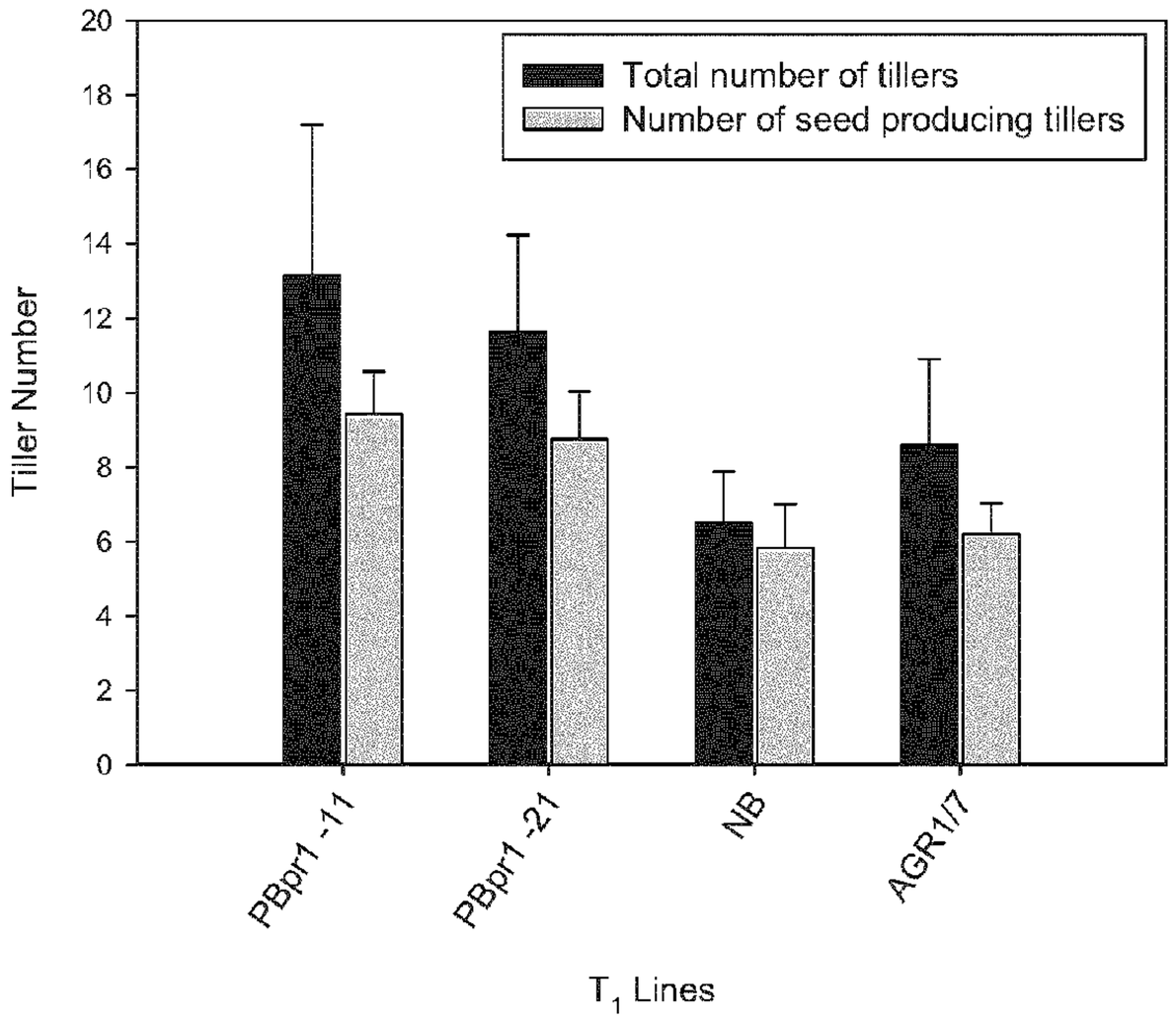

FIG. 3

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PLANTS HAVING ENHANCED NITROGEN EFFICIENCY

FIELD OF INVENTION

[0001] The present invention relates to monocot plants having enhanced yield and/or nitrogen utilization efficiency (NUE), to methods for enhancing yield and NUE in monocot plants, and to methods of increasing biomass and seed yield in monocot plants, for example, when grown under nitrogen limiting conditions.

BACKGROUND OF THE INVENTION

[0002] The productivity of plants is limited by the three primary nutrients: nitrogen, phosphorous and potassium, in most natural and agricultural ecosystems. Generally nitrogen is the most important of the three limiting nutrients and the major component in fertilizers. Since nitrogen is usually the rate-limiting element in plant growth, most field crops have a fundamental dependence on inorganic nitrogenous fertilizer. The nitrogen source in fertilizer is usually ammonium nitrate, potassium nitrate, or urea.

[0003] Increased nitrogen use efficiency by plants has a number of beneficial effects, for example, increased growth and yield when compared to conventional plants grown in nitrogen poor soils, and reduced requirement for the addition of nitrogenous fertilizers to crops. Fertilizers account for a significant percentage of the costs associated with crop production, therefore using less fertilizer would reduce the producers' costs. A reduction in fertilizer application would also lessen the environmental damage resulting from extensive nitrogenous fertilizer use. Excess fertilizer application causes increased eutrophication, acid rain, soil acidification and the greenhouse effect. These environmental disasters cause further problems such as fish kills, loss of biodiversity, increased algal blooms, loss of arable land and accelerated global climate change, affecting the world population on both social and economic scales.

[0004] Monocots represent a large percentage of the crops grown in the world with approximately 217 million hectares of wheat and 158 million hectares of both maize and rice planted in 2007. Approximately half of the global calorie and protein requirement is derived from wheat, rice and maize. Rice is routinely used as a model

crop for genetic and physiological studies in other monocot crops including maize, wheat, sugarcane, barley, sorghum, rye and grass. Rice has a small, diploid genome that is well conserved and syntenic across monocots.

[0005] Promoters are nucleic acid sequences that allow for regulation of transcription of a gene or nucleotide sequence. Promoters can allow for constitutive expression, such as the well known cauliflower mosaic virus promoter CaMV 35S, inducible expression, such as the stress inducible promoter *rd29A* (Pino et al., 2007), tissue specific expression, such as root specific *OsANT1* (U.S. Patent Application Publication No. 2009/0288224) and developmentally specific expression, such as the senescence induced *IPT* promoters (Ma et al., 2009). Promoters can also be weak or strong, suggesting that whenever or wherever they are induced, they will allow for expression of the attached gene or nucleotide sequence at varying levels.

[0006] When a promoter is fused to the 5' end of a gene or nucleotide sequence, it can regulate the expression of that gene or nucleotide sequence. However not all promoters will successfully express all genes or nucleotide sequences in all plant types. For example, a dicot promoter may not perform in the same manner when placed in a monocot system, and vice versa. Similarly, a monocot promoter may not function in the same manner when placed in a monocot of a different Genus, such as a rice promoter placed into wheat. For transgenic studies, there are different types of promoters that can be used, depending on the goal of the experiment. Promoters are often classified as constitutive, tissue specific and/or inducible. Many transgenic studies to date have used generic constitutive promoters such as the cauliflower mosaic virus (*CAMV35S*) and maize ubiquitin 1 promoter (*ubi-1*) to drive target gene over-expression in plants. This can be a disadvantage as it could be energetically unfavorable for plants to express the gene at all times and it could produce abnormal development, since expression levels of the transgene is not regulated (Shelton *et al.*, 2002). For example, constitutive over-expression of the cellulose synthase like gene *CslF6* by the oat globulin promoter *ProASGL* frequently resulted in reduced germination of seeds or seedlings with necrosis on the leaf tips leading to death in severe cases (Burton *et al.*, 2011).

[0007] Using inducible or tissue specific promoters may be a better option to drive transgene expression. Inducible promoters will only drive gene expression when a specific physical, environmental, biological or chemical stimulus is applied. The heat inducible promoter of the *Hvhsp17* gene from wheat can be used for high target gene expression when plants are exposed to 38 to 40°C for 1 to 2 hours (Freeman *et al.,* 2011). This allows for the short term gene or nucleotide sequence expression and control of developmental expression but is limited to tissues that are not severely affected by temperature changes. Over-expression of the *Triticum aestivum* NAC protein (*TaNAC69*), encoding a transcription factor involved in drought stress by the drought inducible promoter *HvDhn4s*, produced wheat plants with significantly higher shoot biomass at the early vegetative stage under mild salt stress and water limitation, compared to the wild-type. Conversely, the *HvDhn8s* constitutive promoter driving *TaNAC69* showed no significant difference from untransformed controls (Xue et. al., 2011). When the drought and cold stress inducible promoter *rdA29* was used to over-express the *DREB1A* gene, which encodes a transcription factor involved in stress tolerance in *Arabidopsis*, normal plants were generated, while constitutive expression driven by the *CAMV35S* promoter resulted in growth retardation under normal growing conditions (Kasuga *et al.,* 1999).

[0008] Tissue specific promoters are involved in organ specific and developmental stage specific expression of transgenes. For example, in potatoes, *StRCAp* is engineered into the leaves as a defense mechanism against predatory insects, but is not expressed in the eating parts of the plant that produces this toxin (Weber, 2003; Park and Jones, 2008). The use of the 35S promoter has also raised concerns of food safety where the toxin produced in non-target organs might cause the potatoes to be unsafe for consumption. In addition, it may be metabolically taxing for the plant to be constantly producing a secondary metabolite, regardless of the developmental stage or organ, therefore causing plants to be less healthy and potentially compromising yields.

[0009] In the case of NUE plant engineering, tissue specific expression of genes might increase the efficacy of N uptake, utilization or remobilization in the plant. In contrast, the use of a constitutive promoter might prove to be a waste of energy

because over-expression of non-rate limiting enzymes in certain organs may not produce any phenotype or may even decrease yield. Development of an NUE plant may also involve the transformation of multiple genes or gene stacking to achieve a satisfactory NUE phenotype, since N metabolism and transport are very complex processes.

[0010] In addition, the use of the *CAMV35S* promoter may not produce any phenotype because the gene expression or protein expression was not sufficient in a specific organ or developmental phase. Also, plants could turn off the expression of the transgene when it proves to be energetically unfavourable. When *HvAlaAT* was driven by the *CAMV35S* promoter, it did not exhibit any NUE phenotype. However, when the root specific *btg26* promoter was used, it produced plants that had higher NUE (Good *et al.,* 2007).

[0011] Similar to over-expression of target genes, promoters when used in a different species may not mimic the expression patterns of its native species. Seed specific promoters from barley (*B-hor* and *D-hor*) and wheat (*HMW-Glu*) did not direct seed specific expression in rice, instead the promoters drove high expression levels in leaf, shoot and maternal seed tissues of rice plants (Wu *et al.,* 1998; Qu and Takaiwa, 2004; Furtado *et al.,* 2008).

[0012] PBpr1 promoters are from genes encoding methyl-melonate semialdehyde dehydrogenase (MMSDH). The *PBpr1* promoter is upstream of the *OsALDH6* gene, which encodes a gene for methylmalonate semialdehyde dehydrogenase in rice (Accession number: gene: AK 121280.1 and mRNA: AF045770.1). AK121280.1 and AF045770.1 are splice variants of each other where AF045770 is shorter than the AK121280.1. AK121280.1 appears to be a hypothetical protein designated by GenBank, while AF045770.1 has been characterized (Oguchi *et al* (2004). *OsALDH6* is homologous to the A*LDH6B2* gene in *Arabidopsis,* which also encodes for a methlymalonate semialdehyde dehydrogenase. The *OsALDH6* gene is highly expressed in young roots and stems (Gao and Han, 2009).

[0013] Methylmalonate semialdehyde dehydrogenase (MMSDH) catalyzes the irreversible oxidative decarboxylation of malonate semialdehydes to acetyl-CoA and

methylmalonate-semialdehyde to propionyl-CoA in the distal portions of the valine and pyrimidine catabolic pathways. Since MMSDH generates acetyl-CoA, it is an important factor in the glyoxylate pathway, TCA cycle and fatty acid production.

[0014] MMSDH is down-regulated during oxidative stress due to the restriction in the TCA cycle and the production of ATP (Sweetlove *et al.,* 2002). In two week old rice plants, MMSDH mRNA was found at high levels in roots and leaf sheaths while protein accumulation was found highest in roots followed by leaf blades (Oguchi *et al..,* 2004). With the addition of auxin, MMSDH levels in roots increased along with an increase in rooting. Tanaka *et al.* (2005) has suggested that MMSDH is involved in root growth, tissue differentiation and thickening growth due to its expression in crown roots, lateral roots and root hairs. MMSDH was found to be localized in the mitochondrial matrix of Arabidopsis, rice, human, bovine and rats which suggests a similarity of function of MMSDH among all these organisms.

[0015] This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[0016] An object of the invention to provide plants having enhanced nitrogen efficiency. In accordance with one aspect of the present invention, there is provided a transgenic plant or plant part comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

[0017] In accordance with another aspect, there is provided a seed obtained from a transgenic plant of the invention.

[0018] In accordance with another aspect of the invention, there is provided a plant cell transformed with a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

[0019] In accordance with another aspect of the invention, there is provided a genetic construct comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

[0020] In accordance with another aspect, there is provided a vector comprising a genetic construct of the invention.

[0021] In accordance with another aspect, there is provided a host cell comprising a genetic construct or a vector according of the invention.

[0022] In accordance with another aspect of the invention, there is provided a method of generating a plant having increased nitrogen use efficiency comprising: transforming a plant cell with a genetic construct or vector of the invention, and growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter, thereby generating a plant having increased nitrogen use efficiency, wherein the increased nitrogen use efficiency is relative to the nitrogen use efficiency of a wild-type plant grown under identical conditions.

[0023] In accordance with another aspect of the invention, there is provided a method of generating a plant having increased biomass comprising:

transforming a plant cell with a genetic construct or vector of the invention, and growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter, thereby generating a plant having increased biomass, wherein the increased biomass is relative to the biomass of a wild-type plant grown under identical conditions.

[0024] In accordance with another aspect of the invention, there is provided a method of generating a plant having increased seed yield comprising: transforming a plant cell with a genetic construct or vector of the invention, and growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter, thereby generating a plant having increased seed yield, wherein the increased seed yield is relative to the seed yield of a wild-type plant grown under identical conditions.

[0025] In accordance with another aspect of the invention, there is provided a method for producing a plant having increased nitrogen use efficiency, increased biomass, increased seed yield, or a combination thereof, comprising: (a) providing a plant, a plant part or a seed comprising a genetic construct or vector of the invention, and (b) growing a plant from the plant, plant part or seed, thereby producing a plant having increased nitrogen uptake, increased biomass, increased seed yield, or a combination thereof, wherein the increased nitrogen uptake, increased biomass and increased seed yield is relative to the nitrogen uptake, biomass and seed yield of a wild-type plant grown under identical conditions.

[0026] In accordance with another aspect, there is provided a use of a transgenic plant of the invention for producing progeny.

[0027] In accordance with another aspect of the invention, there is provided a use of a genetic construct or vector of the invention to increase nitrogen use efficiency, biomass or seed yield in a plant.

[0028] In accordance with another aspect of the invention, there is provided a process for increasing nitrogen use efficiency, biomass, seed yield, or a combination thereof, in a plant comprising: providing a seed comprising a genetic construct or the vector of the invention, applying a herbicide, insecticide, fertilizer, or combination thereof, to the seed, and growing a plant from the seed, wherein the plant has increased nitrogen use efficiency, increased biomass, increased seed yield, or a combination thereof, and wherein the increased nitrogen use efficiency, increased biomass or increased seed yield is relative to the nitrogen use efficiency, biomass or seed yield of a wild-type plant grown under identical conditions.

[0029] In certain embodiments, the present invention provides a method of generating the plant with increased nitrogen use efficiency comprising, introducing a nucleic acid encoding the one or more nitrogen utilization protein operably linked to a root-epidermis-specific promoter, and producing the plant, the plant comprising elevated levels of one or more nitrogen utilization protein specifically localized to the root epidermis.

[0030] In certain embodiments, the present invention provides a method for directing tissue-specific expression of a target gene or nucleotide sequence in a plant, including producing a plant from a transformed plant cell such that tissue-specific expression of a target gene or nucleotide sequence occurs within a selected tissue of the plant, wherein the transformed plant cell contains a target gene or nucleotide sequence in operative linkage with a *PBpr1* promoter element. In one embodiment, the tissue-specific expression takes place in the root of the plant.

[0031] In certain embodiments, the present invention provides a method for directing tissue-specific and environmental or developmentally-regulated expression of a target gene or nucleotide sequence in a plant, including producing a plant from a transformed plant cell such that tissue-specific and environmentally or developmentally-regulated expression of a target gene or nucleotide sequence occurs within a selected tissue of the plant, wherein the transformed plant cell contains a target gene or nucleotide sequence in operative linkage with a *PBpr1* promoter element. In one embodiment, the tissue-specific expression takes place in the root or leaves of the plant and the environmentally or developmentally-regulated expression takes place under conditions of nutrient stress.

[0032] In certain embodiments, the present invention provides a method for increasing biomass of a plant growing under one or more environmentally adverse conditions comprising; transforming a plant with a target gene or nucleotide sequence in operative linkage with a *PBpr1* promoter element to produce a transformed plant, the target gene or nucleotide sequence encoding an enzyme involved in nitrogen assimilation or metabolism; and growing the transformed plant. In one embodiment, the enzyme is alanine aminotransferase (AlaAT).

[0033] In certain embodiments, the present invention provides a method for increasing biomass of a plant growing under conditions of low nitrogen comprising; transforming a plant with a target gene or nucleotide sequence in operative linkage with a *PBpr1* promoter element to produce a transformed plant, the target gene or nucleotide sequence encoding an enzyme involved in nitrogen assimilation; and growing the transformed plant. In one embodiment, the enzyme is AlaAT.

[0034] In certain embodiments, the present invention provides a method for increasing the biomass of a plant growing under one or more environmentally adverse conditions comprising; transforming a plant with a gene or nucleotide sequence encoding AlaAT in operative linkage with a promoter element to produce a transformed plant; and growing the transformed plant. In one embodiment, the promoter is *PBpr1*.

[0035] In certain embodiments, the present invention provides a method for increasing seed yield of a plant comprising; transforming the plant with a target gene or nucleotide sequence or nucleotide sequence in operative linkage with a *PBpr1* promoter element, to produce a transformed plant, the target gene or nucleotide sequence or nucleotide sequence encoding an enzyme involved in nitrogen assimilation or metabolism; and growing the transformed plant. In one embodiment, the enzyme is AlaAT.

[0036] In certain embodiments, the present invention provides a method for increasing seed yield of a plant growing under conditions of low nitrogen comprising; transforming a plant with a target gene or nucleotide sequence or nucleotide sequence in operative linkage with a *PBpr1* promoter element to produce a transformed plant, the target gene or nucleotide sequence or nucleotide sequence encoding an enzyme involved in nitrogen assimilation; and growing the transformed plant. Preferably, the enzyme is AlaAT.

[0037] In certain embodiments, the present invention provides a method for increasing seed yield of a plant growing under conditions of high nitrogen comprising; transforming a plant with a target gene or nucleotide sequence or nucleotide sequence in operative linkage with a *PBpr1* promoter element to produce a transformed plant, the target gene or nucleotide sequence or nucleotide sequence encoding an enzyme involved in nitrogen assimilation; and growing the transformed plant. In one embodiment, the enzyme is AlaAT.

[0038] This summary of the invention does not necessarily describe all features of the invention and is not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings.

[0040] FIGURE 1 shows a construct map of *PBpr1:: HvAlaAT* in pCAMBIA1300. The HvAlaAT is driven by *PBpr1* promoter and terminated by a nos terminator. The selection marker is kanamycin for plasmid selection and hygromycin.

[0041] FIGURE 2 shows a comparison of total above ground biomass (g) and seed yield (g) of T_1 PBpr1 lines, NB and AGR1/7 at maturation. Each measurement is from 5 to 7 replications and error bars are standard deviations. Black bars: Total above ground biomass (g); Grey bars: Total seed yield (g).

[0042] FIGURE 3 shows the number of tillers and productive tillers produced by T_1 PBpr1 plants compared to NB and AGR1/7 at maturation. Each measurement refers to an average of at least 4 replications and error bars show SD. Black bars: Total number of tillers; Grey bars: Number of seed producing tillers.

[0043] FIGURE 4 shows a comparison between T_2 PBpr1 plants, NB and AGR1/7 above ground biomass (g) and seed yield (g) at maturity. Values from each line are from 7 replications and error bars represents SD. Black bars: Total above ground biomass (g); Grey bars: Total seed yield (g).

[0044] FIGURE 5 shows (A) T_3 PBpr1 plant dry above ground biomass (g) at 52 DAG, each line was represented by 5 replication plants and error bars are SD, and (B) the fresh weight (g) of three *PBpr1:AlaAT* over-expressing rice lines versus WT (NB; Nipponbare) at maturity during soil growth conditions.

[0045] FIGURE 6 shows shoot specific AlaAT activity in µM NADH/min.mg of protein of T_1 PBpr1 lines grown in soil at 40 days after germination (DAG) compared to NB and AGR1/7. All lines labeled with N are null segregants. Each data point for the transgenic plants was taken from an average of 3 replications and error bars refer to SD of the 3 replicates.

[0046] FIGURE 7 shows alanine aminotransferase activity (μM NADH/min*mg of protein) of WT (NB), AGR 1/7 (*OsANT1:AlaAT*) and three independent PBpr1:AlaAT over-expressing T_3 rice lines (PBpr1-11, PBpr1-12, PBpr1-21) at five (5) different growth points during soil growth under high N conditions. Error bars refer to SD.

[0047] FIGURE 8 shows T_2 PBpr1 lines shoot AlaAT activity in μM NADH/min.mg of protein at 28 and 52 days old plant that were grown hydroponically. PBpr1-21-2 and PBpr1-21-3 are siblings of the same line. Each data point referred to 5 replications and error bars refer to their SD.

[0048] FIGURE 9 shows T_2 PBpr1 lines root AlaAT activity at 28 and 52 days old plant grown hydroponically. Each data point referred to at least 5 replications and error bars refer to their SD. PBpr1-21-2 and PBpr1-21-3 are siblings of the same line. Black bars: 28 day old plants; grey bars: 52 day old plants.

[0049] FIGURE 10 shows an immunoblot analysis of protein extracts of 52 Day old T_3 PBpr1 lines detected with *HvAlaAT*-2 specific 1^o antibody serum. Lane 1: AGR1/7, lane 2: wild-type Nipponbare, lane 3: PBpr1-10, lane 4: PBpr1-11, lane 5: PBpr1-11-2N, lane 6: PBpr1-12 and lane 7: PBpr1-21. Protein concentration was standardized across all lanes by using the Bradford protein quantifaction assay.

[0050] FIGURE 11 shows an immunoblot analysis of protein extract from T_2 hydroponically grown roots (A) and shoots (B) of 28 DAG rice lines with Hv AlaAT 1^o antibody detection to show amounts of *HvAlaAT* protein in each sample. Lane 1: PBpr1-11, lane 2: PBpr1-11-2N, lane 3: PBpr1-21-2, lane 4: PBpr1-21-3, lane 5: PBpr1-21-3 replicate, lane 6: PBpr1-21-N, lane 7: AGR1/7 and lane 8: wild-type Nipponbare. Protein concentration was standardized across all lanes by using the Bradford protein quantifaction assay.

[0051] FIGURE 12 shows a Log_2 of the relative quantification (ddCT) PBpr1 shoots, T_2 PBpr1 shoots and T_2 PBpr1 roots, AGR1/7 and null segregants of the PBpr1 lines relative to NB plants. 7 transcripts (*HvAlaAT :* Barley AlaAT, *GRP:* Glycine rich protein, *LRR:* Leucine rich repeat) were measured by qRT-PCR. Plants were raised in hydroponic conditions and harvested at 52 days after germination.

Tissue samples used were the same with those of the AlaAT activity assays in Figure 9. (2 fold change represents 1 ddCT. $Log_2 2=1$).

[0052] FIGURE 13 shows the nucleotide sequence of the PBpr1 promoter including the ATG start and initial nucleotides of the coding sequence (SEQ ID NO:6) and showing the *EcoRI* and *SacI* restriction enzyme sites used for cloning.

[0053] FIGURE 14 shows (A) the nucleotide sequence of the barley (*Hordeum vulgare*) AlaAT gene (SEQ ID NO:2) and (B) the amino acid sequence of the AlaAT protein (SEQ ID NO:3).

[0054] FIGURE 15 shows GUS staining of (A) 3 day old *PBpr1::GUSplus* (PBpr1G line) seedling, (B) 3 day old *OsANT1::GUSplus* (OsANT1::GUS lines) seedling as a positive control. (C) to (M) are GUS stained tissues from 7 to 10 day old seedlings of PBpr1G and OsANT1::GUS lines. (C) stem of PBpr1G, (D), (G), and (H) basal region of the shoot/shoot root interface containing remnant seed PBpr1G, (E) basal region of the shoot/shoot root interface containing remnant seed OsANT1GUS, (F) stem of OsANT1GUS, (I) leaf sheath and leaf blade interface OsANT1GUS, (J) leaf blades and veins PBpr1G, (K) leaf blades and veins OsANT1GUS, (L) roots and root hairs PBpr1G, and (M) roots and root hairs OsANT1GUS.

[0055] FIGURE 16 presents a table showing the foliar amino acid concentrations of wildtype (cv. Nipponbare) rice versus barley *AlaAT* over-expressing rice transgenic rice lines; AGR 1/7 (*OsANT1:HvAlaAT*) and PBpr1-11, PBpr1-12, PBpr1-21 (*OsPBpr1::HvAlaAT*). Amino acid concentration (nM/g FW) are averages of 4 replicates.

[0056] FIGURE 17 shows (A) PBpr2 promoter driving expression of AlaAT in rice, T1 generation, grown in soil growth chamber; comparison of plant height, total biomass and seed weight to wild-type rice cv. Nipponbare (NB), and (B) PBpr2 promoter driving expression of AlaAT in rice, T2 generation, grown in soil growth chamber; comparison of total biomass and seed weight to wild-type rice cv. Nipponbare (NB).

[0057] FIGURE 18 shows (A) the nucleotide sequence of the PBpr1 promoter (SEQ ID NO:1), and (B) the nucleotide sequence of the PBpr2 promoter (SEQ ID NO:4).

[0058] FIGURE 19 shows an alignment of the nucleotide sequences of the OsANT1 promoter (SEQ ID NO:5), the PBpr1 promoter (SEQ ID NO:1) and the PBpr2 promoter (SEQ ID NO:4).

DETAILED DESCRIPTION OF THE INVENTION

[0059] The present invention relates to monocot plants having enhanced yield and/or nitrogen utilization efficiency (NUE), to methods for enhancing yield and NUE in monocot plants, and to methods of increasing biomass and seed yield in monocot plants. This invention also relates to PBpr1 promoters, which are promoters from genes encoding methyl-melonate semialdehyde dehydrogenase (MMSDH).

[0060] Given the worldwide requirements for monocots and the diminishing fertility of existing fields, it is desirable to generate monocot plants that are able to grow under suboptimal nutrient conditions. One means for accomplishing this goal is to generate monocot plants that can use nitrogen more efficiently. Such monocot plants have the advantage of being able to grow in soils that are poorer in nitrogen, as a result of being able to more efficiently use the nitrogen that is available, with no loss in yield. Additionally, such monocot plants may also demonstrate enhanced yield in soils that have normal nitrogen levels as well. Nitrogen use efficiency in plants is a result of two main subcomponents; N uptake efficiency and N utilization efficiency. A plant exhibiting a nitrogen use efficiency phenotype may have improvements in its' ability to take up nitrogen from the soil, which is a desired trait in plants growing in lower nutrient-available soils. Or the NUE plant may have improvements in its' ability to utilize the N that it has taken up so that the available N (whether it be low or high) is efficiently incorporated into the subcellular components (such as nucleic acids, proteins, storage etc.), translocated to the necessary tissues and remobilized at the correct developmental stage into seed. Or, another possibility is that the NUE plant has improvements in both N uptake and utilization. Any one of these possibilities would allow for an increased yield from a NUE crop grown in normal nitrogen conditions because those plants would be able to take up a non-

limiting amount of nitrogen and be able to use the nitrogen to increase biomass and seed yield, either by increased number of seeds or an increase in seed weight, or both. Certain embodiments of the invention provide for transgenic plants having improved nitrogen utilization efficiency compared to wild-type plants.

[0061] Plants face a wide variety of non-optimal environmental conditions during growth and development. Such conditions may include water limitation, excess salinity, alkaline or acidic soil, infestation by pests, disease, or temperature stress, any of which individually may significantly hinder crop growth and/or yields. Certain embodiments of the present invention provide methods by which plants, and seeds thereof, may be engineered to grow and thrive under changing environmental conditions usually not conducive to the development of the plant. In some embodiments, methods are provided to develop plants that can maintain or increase their biomass and yield while being grown under conditions that are not optimal, in terms of nutrient applications are described.

[0062] In certain embodiments, the present invention advantageously allows the user to develop plants that have an environmental benefit in that they can maintain yield, while reducing the need for high levels of nutrient application. In certain embodiments, the present invention allows the user to develop plants that, under high nutrient levels, have improved nutrient up-take allowing the plants to extract more nutrients from their environment during times of nutrient abundance. Using the methods and compositions of certain embodiments of the invention, plants may be improved for growth and development under environmental conditions usually unsuitable for growth of the plant. In some embodiments, the methods and compositions of the invention permit the genetic engineering of a plant to alter one or more plant characteristics in only selected tissues of the plant.

[0063] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0064] As used herein, the terms “comprising,” “having,” “including” and “containing,” and grammatical variations thereof, are inclusive or open-ended and do

not exclude additional, unrecited elements and/or method steps. The term "consisting essentially of" when used herein in connection with a composition, use or method, denotes that additional elements and/or method steps may be present, but that these additions do not materially affect the manner in which the recited composition, method or use functions. The term "consisting of" when used herein in connection with a composition, use or method, excludes the presence of additional elements and/or method steps. A composition, use or method described herein as comprising certain elements and/or steps may also, in certain embodiments consist essentially of those elements and/or steps, and in other embodiments consist of those elements and/or steps, whether or not these embodiments are specifically referred to.

[0065] In addition, the use of the singular includes the plural, and "or" means "and/or" unless otherwise stated.

[0066] As used herein, the term "about" refers to an approximately +/-10% variation from a given value. It is to be understood that such a variation is always included in any given value provided herein, whether or not it is specifically referred to.

[0067] The term "substantially identical" as used herein in relation to a nucleotide or amino acid sequence indicates that, when optimally aligned, for example using the methods described below, the nucleotide or amino acid sequence shares at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity with a defined second nucleotide or amino acid sequence (or "reference sequence"). "Substantial identity" may be used to refer to various types and lengths of sequence, such as full-length sequence, functional domains, coding and/or regulatory sequences, promoters, and genomic sequences. Percent identity between two amino acid or nucleotide sequences can be determined in various ways that are within the skill of a worker in the art, for example, using publicly available computer software such as Smith Waterman Alignment (Smith, T. F. and M. S. Waterman (1981) *J Mol Biol* 147:195-7); "BestFit" (Smith and Waterman, *Advances in Applied Mathematics,* 482-489 10 (1981)) as incorporated into GeneMatcher Plus™, Schwarz and Dayhof (1979) *Atlas of Protein Sequence and Structure*, Dayhof, M. O., Ed pp 353-358; BLAST program (Basic

Local Alignment Search Tool (Altschul, S. F., W. Gish, *et al.* (1990) *J Mol Biol* 215: 403-10), and variations thereof including BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, and Megalign (DNASTAR) software. In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including algorithms needed to achieve maximal alignment over the length of the sequences being compared. In general, for amino acid sequences, the length of comparison sequences will be at least 10 amino acids. One skilled in the art will understand that the actual length will depend on the overall length of the sequences being compared and may be at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, or at least 200 amino acids, or it may be the full-length of the amino acid sequence. For nucleotide sequences, the length of comparison sequences will generally be at least 25 nucleotides, but may be at least 50, at least 100, at least 125, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, or at least 500, or it may be the full-length of the nucleotide sequence.

[0068] The term "tissue-specific expression" of a target nucleotide sequence is known in the art and includes the expression of a target nucleotide sequences predominantly in selected tissues. Tissue-specific expression may result in expression of the target nucleotide sequence only in the selected tissue, in which case the target nucleotide sequence may be present in multiple tissues, but is expressed in only a subset of those tissues. Alternatively, tissue-specific expression may result in higher levels of expression of the target nucleotide sequence in selected tissues, in which case expression may also be observed in other tissues, but at a lower level than in the target tissue(s), for example, 50% lower, 40% lower, 30% lower, 25% lower or 20% lower. Such selective expression may be due to the influence of one or more regulatory genetic elements, for example but not limited to promoter elements, repressor elements, enhancer elements, or other regulatory factors that may interact with DNA, RNA.

[0069] The terms "target gene" or "target nucleotide sequence" are art-recognized, and include nucleotide sequences which are desired to be expressed in one or more

selected plant tissues. Non-limiting examples of target genes or nucleotide sequences which may be utilized in conjunction with the methods of the invention include genes or nucleotide sequences involved in nitrogen assimilation and/or utilization, genes or nucleotide sequences involved stress resistance, genes or nucleotide sequences disease and pest resistance, and genes or nucleotide sequences involved in nutrient uptake and utilization. Such genes or nucleotide sequences are well known to one of skill in the art.

[0070] The terms "operative linkage," "operatively linked" and "operably linked" are used interchangeably herein to refer to the placement of a target nucleotide sequence relative to a nucleic acid regulatory sequence such that the expression of the target nucleotide sequence is controlled by the regulatory sequence. This regulatory sequence can have a positive effect (increase) on the expression of the target gene or nucleotide sequence (*e.g.*, the regulatory sequence is a promoter or an enhancer element), or the regulatory sequence can reduce the expression of the target gene or nucleotide sequence (*e.g.*, the regulatory sequence is a repressor element). The regulatory sequence may be physically located 5' or 3' of the target gene or nucleotide sequence, may be within the coding sequence of the target gene or nucleotide sequence, or may be contained on an intron within the target gene or nucleotide sequence.

[0071] The term "nulls" is art-recognized and includes a plant that has undergone tissue culturing but does not carry a transgene or selectable marker.

[0072] Production of plants which express one or more target genes or nucleotide sequences in a tissue-specific fashion are described herein. In certain embodiments, invention provides seeds containing one or more target genes or nucleotide sequences under the control of a promoter element which specifically directs the tissue-specific expression of the gene or nucleotide sequence. In certain embodiments, the methods of the invention allow production of plants having one or more desired traits or properties in selected tissues; *e.g.*, to alter specifically the genetic and/or physiological properties of the fruit or the roots of the plant. Certain embodiments of the invention further provide methods of producing plants having root and

leaf-specific expression of one or more desired nucleotide sequences using the *PBpr1* promoter element.

[0073] The methods of the invention for the production of plants having tissue-specific expression of one or more target genes or nucleotide sequences are accomplished through the use of a genetic regulatory element which directs the tissue-specific expression of the target gene(s). Regulatory elements may be either negative or positive in activity: a plant tissue-specific promoter or enhancer element permits the expression of the target gene or nucleotide sequence(s) in one or more specific tissues, whereas a plant tissue-specific repressor suppresses the expression of the target genes or nucleotide sequences in one or more specific tissues, while expression in the other tissue(s) continues unabated. For the purposes of the invention, it will be understood that promoter sequences constitute the preferred genetic regulatory elements of the invention.

[0074] The tissue-specific promoters useful in the present invention can be either homologous or heterologous to the plant to which they are to be used. Promoters which direct the expression of an operatively linked gene or nucleotide sequence in one or more plant tissues while excluding expression of the linked gene or nucleotide sequence in one or more other plant tissues may be used in the methods and constructs of the invention. Appropriate plant tissues include but are not limited to *e.g.*, root, leaf, petal, sepal, stamen, anther, stigma, ovary, style, pistil, epidermis, phloem, xylem, cortex, pith, cambium, stem or trunk, root hair, petiole, fruit and tuber.

[0075] Preferably, the promoter is a *PBpr1* promoter. In accordance with certain embodiments of the invention, the promoter is a *PBpr1* promoter having a nucleotide sequence substantially identical to the sequence as set forth in SEQ ID NO:1 (Figure 13).

[0076] It will be understood by one skilled in the art that modifications may be made to the promoters useful in the methods and constructs of the invention to improve or modulate the activity of the promoter. Multiple copies of a selected promoter may be operatively linked to a single target gene or nucleotide sequence to

thereby alter the expression level of the linked gene or nucleotide sequence, or a selected promoter may be operatively linked to one or more target genes or nucleotide sequences such that the expression of each target gene or nucleotide sequence is coordinately regulated. A promoter may be of any size appropriate to permit the tissue-specific functioning of the promoter. A promoter may be modified (*e.g.*, by mutagenesis, deletion, insertion, or truncation) to alter the degree to which the operatively linked gene or nucleotide sequence is expressed in the selected tissue, or to alter the specificity of tissue expression directed by the promoter. Further, the placement of the promoter relative to the operatively linked target gene or nucleotide sequence may be modulated (*e.g.*, moved further away or closer together) to attain a desired level of promoter-directed expression.

[0077] A promoter may direct the expression of an operatively linked gene or nucleotide sequence in one or more selected tissues of the plant, and also direct expression of the gene or nucleotide sequence in response to a specific environmental or physiological condition. For example, promoters may be activated under conditions of stress (*e.g.*, drought stress, saline stress, temperature stress, oxygen stress, pH stress, or heavy metal stress), under conditions of nutrient deprivation, under conditions of attack by disease or pests. In another example, promoters may be activated under specific developmental conditions (*e.g.*, upon sprouting, fruiting, or seed production) or in response to a change in environmental conditions (*e.g.*, from nutrient deprivation to provision or from hypoxia to normoxia).

[0078] In certain embodiments, the present invention provides for constructs comprising a target gene under control of a PBpr1 promoter that, when transformed into an appropriate plant host, result in tissue-specific expression of the gene product. For example, expression may preferentially be in the roots and/or shoots of the plant. In certain embodiments, the present invention provides for constructs comprising a target gene under control of a PBpr1 promoter that, when transformed into an appropriate plant host, result in developmentally regulated expression of the gene product.

[0079] A target gene or nucleotide sequence of the invention is a gene or nucleotide sequence which it is desired to be expressed in a plant. General classes of target genes

or nucleotide sequences which may be advantageously employed in the methods and constructs of the invention include genes or nucleotide sequences encoding plant structural proteins, genes or nucleotide sequences encoding proteins involved in the transport and/or uptake of nutrients, genes or nucleotide sequences encoding enzymes and proteins involved in nutrient utilization, genes or nucleotide sequences encoding proteins involved in plant resistance to herbicides, genes or nucleotide sequences encoding proteins involved in plant resistance to nematodes, viruses, insects, or bacteria, genes or nucleotide sequences encoding proteins involved in plant resistance to stress (*e.g.*, osmotic, temperature, pH, or oxygen stress), genes or nucleotide sequences encoding proteins involved in stimulation or continuation of plant growth, or genes or nucleotide sequences encoding proteins involved in phytoremediation. Further, the target gene or nucleotide sequence may be a nucleotide sequence which, when transcribed, is antisense to a native sequence, the transcription and translation of which is desired to be suppressed.

[0080] In certain embodiments of the invention, the target genes or nucleotide sequences are those encoding enzymes in the assimilation and/or metabolism of nitrogen. The genes or nucleotide sequences of interest may include genes or nucleotide sequences which encode proteins involved in assimilating ammonia into amino acids or use the formed amino acids in biosynthetic reactions, that is, “nitrogen utilization proteins”. Examples of nitrogen utilization proteins include, but are not limited to, nitrate ammonium and amino acid transporters, glutamine synthetase (GS), asparagine synthetase (AS), glutamate synthase (also known as glutamate 2:oxogluturate amino transferase and GOGAT), asparaginase (ANS), glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) and those genes or nucleotide sequences which may be involved in Nutrient Use Efficiency (NUE) as described by Beatty et al. (2009), and in U.S. Patent No. 7,589,257. Sequences of appropriate target genes and nucleotide sequences are readily available from public sources, for example GenBank, or can be determined by standard techniques well-known to those of skill in the art.

[0081] The target gene or nucleotide sequence may be a gene or nucleotide sequence naturally expressed in the selected plant, or it may be heterologous to the

selected plant. The gene or nucleotide sequence may originate from a variety of sources, including viral, bacterial, plant or animal sources.

[0082] Certain embodiment of the invention provide for genetic constructs comprising the target gene or nucleotide sequence operably linked to a *PBrp1* promoter. In certain embodiments, the gene or nucleotide sequence is heterologous to the *PBrp1* promoter to which it is linked.

[0083] The target gene or nucleotide sequence can be modified as required. For example, the gene or nucleotide sequence is modified to be transcribable and translatable in a plant system, for example by codon optimization. In certain embodiments, the gene or nucleotide sequence can be modified such that it contains all of the necessary poly-adenylation sequences, start sites and termination sites which allow the coding sequence to be transcribed into messenger ribonucleic acid (mRNA) and the mRNA to be translated into a functional protein in the selected plant system.

[0084] Certain embodiments of the invention thus provide for genetic constructs comprising the target gene or nucleotide sequence operably linked to a *PBrp1* promoter and further comprising one or more poly-adenylation sequences, transcription start sites, and/or transcription termination sequences. Such target gene or nucleotide sequence modifications and the methods by which they may be made are well known in the art. In certain embodiments, the target gene or nucleotide sequence encodes a nitrogen utilization protein., for example, a nitrate transporter, an ammonium transporter, an amino acid transporter, glutamine synthetase (GS), asparagine synthetase (AS), glutamate synthase (also known as glutamate 2:oxogluturate amino transferase and GOGAT), asparaginase (ANS), glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT), or alanine aminotransferase (AlaAT). Other examples include those genes or nucleotide sequences which may be involved in Nutrient Use Efficiency (NUE) as described by Beatty et al. (2009), and in U.S. Patent No. 7,589,257.

[0085] In certain embodiments, the present invention provides for vectors comprising the genetic constructs. One skilled in the art will appreciate that the precise vector used is not critical to the instant invention and suitable vectors can be

readily selected by the skilled person. Examples of expression vectors and cloning vehicles include, but are not limited to, viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, retrovirus vectors, viral DNA (for example, vaccinia, adenovirus, foul pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes, and other known vectors specific for specific host cells of interest.

[0086] Certain embodiments provide for host cells comprising the genetic constructs or vectors according to the invention. The genetic construct or vector can be introduced into a suitable host cell by one of a variety of standard methods. Such methods are well-known in the art and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors. One skilled in the art will understand that selection of the appropriate host cell will be dependent upon the vector chosen. Examples of host cells include, but are not limited to, bacterial, yeast, insect, plant and mammalian cells. In certain embodiments, the host cell is a plant cell.

[0087] The methods and genetic constructs disclosed herein may be used to produce a plant or a plant part of any species capable of utilizing the promoter such that the transgenic (non-natural) plant, has tissue-specific expression of one or more desired genes or nucleotide sequences. The invention is intended to be particularly applicable to, for example, crop plants (especially those of the genus *Oryza*), ornamental plants, and trees (particularly conifers and the genus *Populus*). Particularly suitable plants for the practice of the present invention include, but are not limited to, canola, barley, sugar cane, corn, canola, tobacco, soybean, cotton, alfalfa, tomato, wheat, potato, aspen, cottonwood, conifers and poplar, or parts of any of these plants, for example, roots, root tips, leaves, stems, flowers, apical buds, meristematic tissues, and the like. In certain embodiments, plants for use in the invention include canola, barley, maize, rice, tobacco, soybean, cotton, alfalfa, tomato, wheat, potato, and certain tree genera, including conifers and *Populus* species.

[0088] In certain embodiments, the methods, uses and constructs of the invention are applied to plants of the genus *Oryza* and other genera closely related to *Oryza,* such as *Porteresia* and *Leersia*. Examples of species in these genera include, but are

not limited to, *Oryza sativa, Oryza punctata, Oryza officinalis, Oryza malapuzhanensis, Oryza latifolia, Oryza australiensis, Oryza brachyantha, Oryza granulate, Oryza longiglumis, Oryza schlechteri, Porteresia coarctata, Leersia perrieri, Leersia hexandra* and *Leeria tisserantti.*

[0089] The transgenic (non-natural) plants, plant parts, and seeds produced according to the present invention may be further useful in breeding programs for the production of plant species having more than one desired trait. For example two transgenic plants of the invention each having expression of a desired transgene in differing plant tissues may be crossed to result in progeny transgenic plants having tissue-specific expression of both transgenes; or two transgenic plants of the invention each having expression of a different desired transgene in the same plant tissue may be crossed to result in progeny transgenic plants having tissue-specific expression of both transgenes. In this fashion it is possible to produce transgenic plants having a combination of desirable traits in selected tissue(s) of the plant.

[0090] Furthermore, it will be understood by one skilled in the art that different species of plants may be more or less amenable to genetic manipulation in general, and that, therefore, it may be advantageous to first transform a related species of the desired plant by the methods and with the constructs of the invention and to subsequently introduce the tissue-specific expression of the target gene or nucleotide sequence into the desired plant species by cross-breeding techniques. Such techniques and appropriately related plant species are well known to one skilled in the art.

[0091] Plant cells or protoplasts that have been transformed with the gene construct of the present invention can be regenerated into differentiated plants using standard nutrient media supplemented with shoot-inducing or root-inducing hormone, using methods known to those skilled in the art (see, for example, U.S. Patent No. 4,634,674 and references therein). Seeds may additionally be harvested from such transgenic plants using methods well known in the art and further used to re-grow the transgenic plants and hybrids of the invention.

Uses

[0092] The methods and constructs of the invention allow the production of plants and seeds having expression of one or more desired genes or nucleotide sequences, for example, in one or more selected tissues of the plant. Thus, in certain embodiments, the methods and constructs of the invention permit the production of plants having one or more desired traits limited to selected plant tissues, thereby enabling the targeting of a trait to the tissue to which it is best suited, or avoiding the expression of a desirable gene or nucleotide sequence in a tissue where its effects are unwanted. There are a wide variety of specific applications of the invention, including, but not limited to, the production of plants having increased yield, stress tolerance, having improved nutrient uptake and/or utilization, having improved nutrient content and/or yields of desired compounds, and having phytoremediative properties. Specific applications of the invention are further described below.

[0093] One application of the invention is in the production of plants better able to thrive on nutrient-poor soils. It is well known in the art that certain plant species, particularly crop plants, deplete the soil of nutrients necessary to sustain growth, such as nitrogen, phosphate, and potassium. In order to replenish the lacking nutrients, it is necessary either to fertilize the soil (an expensive and environmentally damaging procedure) or to cultivate plants known to deposit the depleted nutrient into the soil (*e.g.*, clover or soybean in the case of nitrogen depletion), which may be crops that are less profitable or nutritive and therefore less desirable to grow. Frequent fertilizing to maintain optimal nutrients in the soil is costly in terms of agriculture outputs such as labour and fuel therefore crop plants are usually in a state of either nutrient abundance or depletion. Another application of the invention is in the production of plants better able to capture and utilize nitrogen when it is present in either an adequate or abundant supply in the soil before nitrogen loss due to leaching, volatilization or microbial degradation occurs.

[0094] In certain embodiments, the methods of the invention permit the targeted expression of genes or nucleotide sequences involved in nutrient uptake (*e.g.*, transport molecules) to those tissues in which the uptake occurs (*e.g.*, the root or root hairs) to thereby improve the ability of the plant to absorb the nutrient from the

environment. In some embodiments, the invention may be used to produce plants which express heterologous nutrient utilization nucleotide sequences, or optimized (for example, optimized for plant expression) native nutrient utilization nucleotide sequences, that permit more efficient use of the nutrient, such that less of the nutrient is required for the normal growth and functioning of the plant. In certain embodiments, the expression of such sequences is targeted to selected tissues (*e.g.*, the root or leaves). In certain embodiments, the methods of the invention allow for expression of genes or nucleotide sequences involved in the use and uptake of nutrients not normally used by the plant in those plant tissues which are directly exposed to the different nutrient (*e.g.*, root and leaf). In this fashion, plants which are able to grow and thrive on different nutrient sources (*e.g.*, different nitrogen sources) may be produced. Particularly useful target genes or nucleotide sequences for the optimization of nitrogen efficiency of the plant include: nitrate transporters, ammonia transporters and amino acid transporters, glutamine synthetase (GS), asparagine synthetase (AS), glutamate synthase (also known as glutamate 2:oxogluturate amino transferase and GOGAT), asparaginase (ANS), glutamate dehydrogenase (GDH), alanine dehydrogenase, aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) and those genes or nucleotide sequences which may be involved in Nutrient Use Efficiency described by Beatty et al. (2009), and U.S. Patent No. 7,589,257.

[0095] In certain embodiments, plants produced by the methods of the invention can more efficiently utilize fertilizer input by rapidly taking up the nitrogen in the fertilizer and storing it at the time of application, to thereby reduce the amounts of nitrogenous fertilizer which are lost to leaching, etc. This may permit a reduction in the amount of nitrogenous fertilizer required to be applied to a crop, to obtain crop yields comparable to those obtained using normal cultivation techniques and plants which have not been modified according to the present invention. Additional agronomic advantages can include faster growth and crop yield, where nitrogenous fertilizer input is maintained at levels used in common crop cultivation techniques.

Development of Cereal Crops

[0096] As described below, transformed *Oryza sativa* plants ectopically expressing a nucleotide sequence encoding an enzyme involved in nitrogen assimilation or metabolism, for example but not limited to alanine dehydrogenase, glutamine synthetase, asparagine synthetase, glutamate synthase, asparaginase, glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and those nucleotide sequences which may be involved in Nutrient Use Efficiency described by Beatty et al. (2009), and U.S. Patent No. 7,589,257 were developed using a *PBpr1* promoter and grown under laboratory conditions to determine if the beneficial effects on plant growth and yield under controlled growth conditions could be obtained. For example, it was examined whether the amount of available nitrogen would have an effect on plant biomass and seed yield on plants expressing *PBpr1/AlaAT* grown under laboratory conditions when compared to similar plants that did not ectopically express the target nucleotide sequence.

[0097] Seed yield in control and transgenic plants grown under laboratory conditions was examined. Transgenic plants expressing a gene or nucleotide sequence encoding an enzyme involved in nitrogen assimilation or metabolism exhibited higher biomass and seed yields than control plants in the presence of an adequate supply of nitrogen. Collectively these results indicate that transgenic plants ectopically expressing an enzyme involved in nitrogen assimilation or metabolism are capable of optimising the utilization of available nitrogen under a range of environmental conditions thereby resulting in an increase in plant biomass, seed yield or a combination thereof.

[0098] Therefore, in one aspect, the present invention provides a method for increasing seed yield of a plant comprising; transforming the plant with a target gene or nucleotide sequence in operative linkage with a *PBpr1* promoter element, to produce a transformed plant, the target gene or nucleotide sequence encoding an enzyme involved in nitrogen assimilation or metabolism; and growing the transformed plant.

[0099] The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

[00100] To gain a better understanding of the invention described herein, the following examples are set forth. It will be understood that these examples are intended to describe illustrative embodiments of the invention and are not intended to limit the scope of the invention in any way.

EXAMPLES

EXAMPLE 1: PBpr1 Promoter Fused to the *alaAT* Gene

Construction of binary vectors and Agrobacterium mediated transformation

[00101] The *PBpr1* promoter was selected and designed to be cloned using GeneArt (Invitrogen, Life Technologies, Carlsbad, CA, USA). The *HvAlaAT* cDNA (SEQ ID NO: 2) was introduced into pCAMBIA1300 using a PstI/HindIII ligation while the *PBpr1* promoter was introduced upstream of *HvAlaAT* to drive expression using EcoRI/SacI sites (Figures 1,13,14). The *PBpr1* promoter was introduced into pCAMBIA1305.1 to drive *GUSplus* for promoter pattern analysis using the EcoRI/NcoI sites. The constructs were transformed separately into *Agrobacterium tumefaciens* strain EHA105 by the freeze thaw method (Weigel and Glazebrook, 2002). Rice callus (*Oryza sativa* c.v. Nipponbare (NB)) was transformed with both constructs, using an *Agrobacterium* transformation system developed in our laboratory (Shrawat and Good, 2011). Lines transformed with *PBpr1::HvAlaAT* are referred to herein as *PBpr1* lines while lines transformed with *PBpr1::GUSplus* are referred to herein as *PBpr1*G lines.

[00102] Lines transformed with *PBpr1::HvAlaAT* were previously named *Ap* lines. Correspondence between the previous nomenclature and the nomenclature used herein is shown in Table 1. Likewise, lines transformed with *PBpr1::GUSplus* were previously named *Ap*G lines.

[00103] Based on seed yield, total above soil biomass and tiller number at maturation, two T_0 lines containing *PBpr1::HvAlaAT* were selected to be continued through future generations. Leaf tissue was collected from two week old plants to determine if they were transgenic by means of a genomic DNA polymerase chain reaction (PCR) analysis using primers specific to the hygromycin resistant gene.

Transgenic plants and null segregants to be continued to further generations were selected based on uniform size and height. Primary transgenics are designated as T_0 lines.

Soil growth chamber experiments

[00104] T_0, T_1, and T_2 rice plant generations were planted in soilless-potting mixture Sunshine Mix #4 (Sun Gro Horticulture) in growth chambers at 28°C, 70% relative humidity, 14h/10h light/dark photoperiod, photon flux density 750 µE at bench height, as described in Shrawat et al., 2008).

[00105] In the T_3 generation, three seeds were germinated in a 7 inch round pot and allowed to grow for two weeks. Two of the three seedlings were culled based on uniformed height and size of plants remaining in each pot. A fertilization regime similar to that of the T_2 generation was started on the three week old plants. All statistical comparisons carried out for these growth chamber experiments were done using a Student's t-test.

Hydroponic growth chamber experiment

[00106] The hydroponic experiment was carried out in a growth chamber at 28°C, 70% humidity, 14h/10h light/dark photoperiod, photon flux density 750 µE at bench height, as described by Shrawat et al., 2008. Plant material was harvested on 28 and 52 day old plants for AlaAT assays and RNA experiments.

Selection of homozygous lines and maintenance of null lines

[00107] T_2 seed obtained from T_1 plants were germinated on MS and hygromycin and the ratio of resistance to the antibiotic was measured to determine homozygosity to heterozygosity as described in Shrawat et al., 2008. Null lines were identified by PCR at the T_1 plant stage using hygromycin specific primers. Subsequently, null lines were selected and maintained for a comparison between NB, AGR1/7 and *PBpr1* lines.

AlaAT Extraction and enzyme assays

[00108] Both shoot and root tissue was harvested for AlaAT enzyme assays in the hydroponic experiments, while only shoot tissue was harvested in potted experiments as per Muench and Good, 1994.

Protein SDS-PAGE electrophoresis and immunoblot analysis

[00109] In order to validate the results of the AlaAT enzyme assays, SDS-PAGE electrophoresis and immunoblot analyses was carried out using the protocol of Muench and Good (1994).

RNA extraction, cDNA synthesis and quantitative real time PCR

[00110] RNA extraction, cDNA synthesis and quantitative real time PCR was carried out using the protocol of Beatty et. al., 2009. Gene specific primer and probe pairs that were previously used by Beatty *et al.* (2009) were used to detect differences in gene expression due to over expression of *HvAlaAT*. NB plants were used as a negative control and 18srRNA was used as an endogenous control.

Bioinformatics analysis of promoter

[00111] The *PBpr1* nucleotide sequence was selected based on homology to the *OsANT1* promoter using NCBI's BLASTn program, and it was further investigated using the gene bioinformatic tools of NCBI Genbank and Refseq.

GUS histochemical staining

[00112] A total of 20 T_1 seeds of *PBpr1::GUSplus* transgenic lines, named PBpr1G lines, were sterilized and germinated in sterile liquid MS (4.4 g L^{-1}, pH 5.8) for staining. From each line, five seeds were selected at each sampling time for staining. Seedlings were sampled at 3 DAG and 7 to 10 DAG and stained for GUS expression.

RESULTS:

Heterozygous T_0 seed yield and biomass preliminary screens

[00113] At the T_0 stage, the independent PBpr1 plants had higher above ground biomass and seed yield compared to NB (Table 1). Two independent *PBpr1* lines

were selected based on high seed yield and biomass to continue on to the T_1 generation. When comparing the six lines selected with NB, they had significantly higher seed yield ($p<0.001$) and above ground biomass ($p<0.001$) compared to NB.

Table 1: Plant height, biomass and seed yield of T_0 PBpr1 plants and untransformed Nipponbare plants. SD refers to standard deviations.

Lines	Previous name of line	Plant height (cm)	Total above ground biomass (g)	% Change from NB total above ground biomass	Seed yield (g)	% change from NB seed yield	Total above ground biomass without seeds (g)
PBpr1 -3	Ap10	86.5	11.6	6	3.13	-20	8.47
PBpr1 -5	Ap12	69.2	12.5	15	3.34	-15	9.16
PBpr1 -7	Ap14	80.8	13.2	21	3.37	-14	9.83
PBpr1 -8	Ap15	79.2	14.2	30	4.27	9	9.93
PBpr1 -9	Ap16	74.0	12.4	14	4.04	3	8.36
PBpr1 -10	Ap17	73.8	17.9	64	7.33	87	10.57
PBpr1 -11	Ap18	84.3	17.7	62	10.01	156	7.69
PBpr1 -12	Ap19	87.2	20.3	86	8.31	113	11.99
PBpr1 -13	Ap20	85.0	17.8	63	7.01	79	10.79
PBpr1 -14	Ap21	78.5	12.2	12	4.62	18	7.58
PBpr1 -15	Ap22	78.0	12.1	11	3.49	-11	8.61
PBpr1 -16	Ap23	79.1	13.8	27	4.00	2	9.80
PBpr1 -17	Ap24	86.5	17.2	58	6.54	67	10.66
PBpr1 -18	Ap25	67.3	13.5	24	4.25	9	9.25
PBpr1 -19	Ap26	80.2	15.9	46	5.51	41	10.39
PBpr1 -20	Ap27	85.4	11.7	7	4.16	6	7.54
PBpr1 -21	Ap28	86.0	17.4	60	7.33	87	10.07
PBpr1 -22	Ap29	76.0	21.4	96	7.50	92	13.9
average		**78.0**	**14.1**	39	**5.45**	40	**9.70**
SD		6.8	3.63		2.05		1.62
Nipponbare		**75.2**	**10.9**		**3.91**		**7.03**
SD		6.3	2.17		1.63		1.25

Heterozygous T_1 PBpr1 seed yield and biomass preliminary screens

[00114] For further phenotypic analysis and to obtain homozygous seed, T_1 plants of *PBpr1* -11 and *PBpr1*-21 were grown to maturity and biomass and seed yield data collected. *PBpr1* -11 and *PBpr1*-21 had significantly higher above ground biomass

than NB ($p<0.05$) and AGR1/7 ($p<0.001$) (Figure 2). In terms of seed production, *PBpr1* -11and *PBpr1*-21 had significantly higher seed yield than NB ($p<0.05$).

Growth chamber biomass and seed yield analysis of homozygous lines

[00115] T_2 homozygous PBpr1 lines were grown in soil conditions to maturity and total above ground biomass and seed yield data was collected (Figure 4). Two of the five PBpr1 lines had higher above ground biomass than NB. Only one of these lines, PBpr1-21, was significantly larger than NB and AGR1/7 in terms of biomass ($p<0.001$). PBpr1-10 and PBpr1-11 were shown to have higher seed yield than NB and AGR 1/7, but these differences were not significant ($p>0.05$).

T_3 above ground biomass of homozygous PBpr1 lines

[00116] Homozygous PBpr1 T_3 plants were harvested at 52 DAG in order to assess the changes of biomass at a different developmental stage (Figure 5). PBpr1-11 and PBpr1-21, had higher dry above ground biomass than NB and the nulls at 52 DAG. PBpr1-21 had significantly higher above ground biomass than both NB and nulls ($p<0.03$). PBpr1-11-2N plants had similar above ground biomass compared to NB, which was consistent with previous experiments in the T_1 and T_2 generations. PBpr1-11, on the other hand, had higher biomass than NB and nulls.

PBpr1 biomass, seed yield and NUE

[00117] PBpr1-21, over all 3 generations, has significantly higher above ground biomass than NB. In the T_1 and T_2 generation, PBpr1-21 is higher in above ground biomass than AGR1/7 but not significantly (Figure 2 and 4). PBpr1-11 also exhibits a consistent trend of higher above ground biomass than NB in the T_1 and T_3 generation but not in the T_2 generation (Figures 2, 4 and 5). Across all generations, PBpr1-21 had higher seed yield than NB and AGR1/7 while PBpr1-11 had higher seed yield than NB in the T_1 but not in the T_2 generation.

T_1 PBpr1 lines tiller productivity

[00118] In addition to measuring biomass and seed yield at maturity, the number of productive and non-productive tillers was measured. Tiller productivity and plant

height was only recorded in soil grown plants. Tiller productivity of the PBpr1 lines, in comparison with AGR1/7 and NB was determined and all PBpr1 lines had more tillers and more productive tillers than NB (Figure 3). PBpr1-11 and PBpr1-21 had significantly ($p<0.01$) more tillers and more productive tillers than AGR1/7. PBpr1-11 had the highest total number of tillers and productive tillers among all PBpr1 lines, AGR1/7 and NB. This was consistent with other parameters assessing overall plant size as PBpr1-11 produced the largest plants among all other PBpr1 lines. In comparison to AGR1/7, PBpr1-11 and PBpr1-21 had significantly more productive tillers ($p<0.05$).

T_1 screens for AlaAT activity for HvAlaAT over-expression in soil grown plants

[00119] Shoots of T_1 soil grown PBpr1 plants were harvested between active tillering and maximum tillering stage (40 DAG) to determine their specific AlaAT activity. The different PBpr1 lines had 2.5 to 8 times higher shoot AlaAT activity compared to the null segregants and NB (Figure 6). AlaAT specific activity was highest in PBpr1-12 and PBpr1-21 at between 3.3 to 4.3 µmoles NADH min^{-1} mg of protein. AGR1/7 containing *OsANT1::HvAlaAT* had higher specific AlaAT activity than nulls and NB in agreement with Shrawat *et al.* (2008). Out of all the PBpr1 lines, only PBpr1-12 had higher average specific AlaAT activity than AGR1/7, but PBpr1-11 and PBpr1-21 had comparable levels of AlaAT activity as AGR1/7. The AGR1/7 used was a T_5 homozygous line while the T_1 PBpr1 lines tested were still segregating. Null segregants of the different PBpr1 lines (PBpr1-10-N, PBpr1-11-N, PBpr1-12-N and PBpr1-21-N) had similar AlaAT activity as NB. Once these lines were confirmed as true nulls, they were used as negative controls.

T_3 homozygous shoot AlaAT activity assay from soil grown plants

[00120] Independent plants of the T_3 generation from the lines PBpr1-10, PBpr1-11, PBpr1-12 and PBpr1-21 were identified as homozygous plants and were raised in the growth chamber until 52 DAG when tissue samples were harvested for AlaAT activity. Trends observed in the T_3 generation were consistent with those of the T_1 plants (Figure 7). PBpr1 lines had up to 5.3 times higher shoot AlaAT activity than NB and the nulls. In agreement with T_1 studies in soil, PBpr1-12 had the highest

AlaAT activity followed by PBpr1-11 and PBpr1-21 while PBpr1-10 had the lowest AlaAT activity.

T_2 homozygous AlaAT activity from hydroponic grown plants

[00121] To assess the AlaAT activity of roots, homozygous PBpr1 lines were selected to be grown hydroponically. PBpr1 roots and shoot tissue were harvested at the beginning of active tillering (28 DAG) and at maximum tillering (52 DAG).

Shoot AlaAT activity

[00122] The PBpr1 lines exhibit up to 8 times higher shoot AlaAT activity compared to nulls and NB (Figure 8). At both 28 DAG and 52 DAG, PBpr1-11 and PBpr1-21 had up to 1.2 and 1.7 times higher AlaAT activity than AGR1/7 respectively. Shoot AlaAT activity of PBpr1-11 and PBpr1-21 was higher at 52 DAG than 28 DAG, whereas native shoot AlaAT activity (the PBpr1 null lines and NB) was observed to drop slightly between 28 and 52 DAG.

[00123] Between 28 DAG and 52 DAG, AlaAT activities in some of the transgenic, nulls and NB shoots were observed to have decreased with the exception of PBpr1-21. For all PBpr1 lines, the changes in shoot AlaAT activity was only between 1.1 to 1.2 times between 28 and 52 DAG.

Root AlaAT activity

[00124] Although all PBpr1 lines had higher root AlaAT activity than NB, similar levels of root AlaAT activity were observed between PBpr1 lines and their corresponding null siblings (Figure 9). AGR1/7 was the only transgenic line that had clearly higher level of root AlaAT activity than all other lines. However, root AlaAT activity was observed to have dropped in all cases at 52 DAG compared to 28 DAG. The decrease was evident in null segregants and NB where levels of root AlaAT activity were observed to have dropped 2 to 3 times. A smaller decrease of 1.2 to 1.4 times in root AlaAT activity of 28 to 52 DAG was observed in the PBpr1 lines and AGR1/7. With the decrease of root AlaAT activity at 52 DAG, PBpr1 lines and AGR1/7 had up to 3 times higher AlaAT activity than NB and nulls. Shoot AlaAT

activities in the non-transgenics are always low at both 28 and 52 DAG (Figure 8). Root AlaAT activity, on the other hand, is high at 28 DAG and decreases drastically at 52 DAG. Overall root AlaAT activity in the non-transgenics was always higher than shoot AlaAT activity.

HvAlaAT immunoblotting to validate AlaAT assays

[00125] Fifty two day old soil grown T_3 PBpr1 lines were harvested to determine the stability of the *PBpr1* promoter in over-expressing *HvAlaAT,* by AlaAT activity assays and immunoblots. T_3 shoots of all the PBpr1 lines tested had high levels of AlaAT protein according to the immunoblot (Figure 10`). In addition, *HvAlaAT* protein levels in AGR1/7 were found to be comparable but slightly higher than that of the PBpr1 lines. This was consistent with the results obtained in the AlaAT assays (Figure 7). The 52kDa *HvAlaAT* band is absent in the nulls and NB indicating the absence of *HvAlaAT* protein.

[00126] Roots of hydroponically grown T_2 plants were harvested at 28 days (Figure 11). In lines that did not carry the *HvAlaAT* transgene insertion, the *HvAlaAT* antibody bound to native AlaAT. PBpr1 and AGR1/7 roots produced a thicker and more intense band that masked the two nonspecific thinner bands representing native AlaAT at the 52kDa region (Figure 11A). Therefore roots of 28 day old PBpr1 plants had higher levels of *HvAlaAT* protein than the nulls and NB.

[00127] Shoot *HvAlaAT* protein levels of hydroponically grown T_2 PBpr1 plants were also assessed at 28 (Figure 11B). *HvAlaAT* protein was observed to be high in the shoots of all PBpr1 lines. Again, AGR1/7 had the same level of banding intensity as all the PBpr1 lines indicating high levels of *HvAlaAT* protein compared to nulls and NB. At 28 days, protein levels of *HvAlaAT* appeared to be present and high in PBpr1 lines and absent in the non-transgenics.

qRT-PCR analysis to determine gene expression changes from over-expression of HvAlaAT

[00128] The mRNA expression levels of three target genes or nucleotide sequences (barley AlaAT (*HvAlaAT*), leucine rich repeat (*LRR*) and a glycine rich protein (GRP)

were selected from a list of genes or nucleotide sequences that had been found to be differentially expressed in a microarray experiment comparing AGR1/7 to NB (Beatty *et al.,* 2009).

[00129] *HvAlaAT* was approximately 400 to 1400 fold higher in mRNA expression in the T_3, soil grown, PBpr1 lines and AGR1/7 shoots compared to NB, except for the nulls which did not differentially express *HvAlaAT* compared to NB (Figure 12A). The qRT-PCR analysis, AlaAT assays and immunoblotting confirmed that the PBpr1 lines were over-expressing *HvAlaAT* compared to the null segregant. Among the PBpr1 lines tested, PBpr1-21 had the highest *HvAlaAT* expression at 1400 fold higher followed by PBpr1-12 with a 1000 fold higher expression relative to NB (Figure 2.18). *GRP* was up-regulated approximately 10 to 30 fold in all PBpr1 lines, null lines and AGR1/7. *LRR* was 9 to 20 fold up-regulated in the PBpr1 lines and AGR1/7 compared to NB and the nulls, which were 3.4 fold up-regulated compared to NB.

[00130] Hydronically grown T_2 PBpr1 shoots were analyzed using qRT-PCR to determine mRNA expression levels compared to NB. In agreement with previous AlaAT assays and immunoblots, *HvAlaAT* mRNA was highly expressed in all PBpr1 lines with 400 to 800 fold higher expression relative to NB with PBpr1-21 shoots having the highest up-regulation, while the null line did not differentially express *HvAlaAT* relative to NB (Figure 12B). All the PBpr1 lines, null line and AGR1/7 exhibited 20 to 40 fold up-regulation of *GRP* relative to NB. *LRR* was most highly up-regulated in AGR1/7 at a 29 fold change relative to NB, while the transgenic PBpr1 lines had a 7 to 11 fold up-regulation and the null line had 16 fold up-regulation.

[00131] Hydroponically grown T_2 PBpr1 roots RNA was analyzed for changes in transcript levels in comparison to AGR1/7 and NB. *HvAlaAT* mRNA was highly expressed in PBpr1 lines at an approximately 700 to 2000 fold increase compared to NB. AGR1/7 roots had 2500 fold higher expression of *HvAlaAT* relative to NB (Figure 12C). As expected, the null line did not show differential expression of *HvAlaAT* relative to NB. *GRP* was highly up-regulated at 200 to 500 fold in the PBpr1 lines, null line and AGR1/7 relative to NB.

[00132] *GRP* was more highly upregulated in roots than in shoots relative to NB in both the PBpr1 lines and AGR1/7. Similarly *HvAlaAT* mRNA was also more highly expressed in roots than in shoots of all the transgenic lines relative to NB (Figure 12B and C).

GUS histochemical staining of PBpr1G plants

[00133] Three and 7 to 10 day old PBpr1G plants were stained for GUS expression to determine the expression pattern of the *PBpr1* promoter at the seedling stage (Figure 15). GUS stains appeared at the tip of the newly emerged shoots in 3 day old plants. In 7 to 10 day old PBpr1G plants, staining was observed in the interface between the shoots and the roots, within the remnant seed coat. Faint GUS staining was also found in the veins of the leaf blades and tillers as well as exterior of the tiller of PBpr1G plants, which may be endogenous GUS stain. With the *OsANT1*::*GUSplus* lines used as a positive control, GUS staining appeared within one hour of incubation in GUS staining buffer and GUS stains appeared in the root hairs, root tips and veins of the leaves.

PBpr1 over-expresses *HvAlaAT*.

[00134] This conclusion is supported by the following observations: high levels of AlaAT activity were observed in all the PBpr1 lines consistently throughout all generations analyzed; and immunodetection showed that there is a high level of HvAlaAT protein in transgenic plants and finally, qPCR analysis demonstrated an increase in *HvAlaAT* mRNA in the PBpr1 lines.

Expression from PBpr1 promoter

[00135] At the transcript level, the *OsANT1* and *PBpr1* promoters both drive high level of *HvAlaAT* gene expression in roots and shoots compared to Nipponbare (NB) but *OsANT1* drives higher levels of *HvAlaAT* gene expression in the roots than *PBpr1* while *PBpr1* drives higher levels of *HvAlaAT* gene expression in shoots than *OsANT1* (Figure 12).

[00136] Pattern analysis of *PBpr1:: GUSplus* lines (PBpr1G lines) showed endogenous GUS staining in 3 day old and 7 to 10 day old seedlings while intense GUS staining was observed in the OsANT1::GUSplus line. PBpr1 may not show GUS expression but yet it drives high levels of HvAlaAT expression. This agrees with the fact that PBpr1 may be developmentally regulated in roots until after active tillering while OsANT1 drives high levels of expression at all times in both roots and shoots.

Transgenic HvAlaAT

[00137] At the protein level, *PBpr1::HvAlaAT* lines (PBpr1 lines) and *OsANT1::HvAlaAT* lines (AGR1/7) demonstrated higher levels of HvAlaAT protein in both roots and shoots from active to maximum tillering compared to NB (Figures 10 and 11).

[00138] At the enzymatic level, AlaAT activity of AGR1/7 was higher than NB at both active and maximum tillering in both roots and shoots. The PBpr1 lines exhibit similarly high levels of shoot AlaAT activity from active to maximum tillering compared to NB while root AlaAT activity of PBpr1 lines in roots was only observed to be higher than NB after active tillering and hence its activity seemed to be developmentally regulated in roots instead of constitutively regulated as seen in roots of AGR 1/7. In shoots, AlaAT activity appears to be high at all times using the *PBpr1* promoter.

HvAlaAT over-expressing plant lines

[00139] Morphologically, the *PBpr1* lines produced higher seed yield and biomass compared to NB and AGR1/7 in the T_1, T_2 and T_3 generations. In addition, the *PBpr1* lines exhibit increased tillering compared to NB and AGR1/7.

[00140] With the OsANT1 promoter, an increase in gene expression resulted in higher levels of HvAlaAT protein and consequently higher levels of AlaAT activity in both the roots and shoots of AGR1/7. In contrast, athough the PBpr1 promoter drove high levels of *HvAlaAT* expression and HvAlaAT protein levels, this did not result in an increase in AlaAT activity until after active tillering in roots. In shoots, an increase

in *HvAlaAT* gene expression driven by PBpr1 promoter resulted in both increased protein levels and AlaAT activity. This suggests that PBpr1 may be developmentally regulated in rice roots.

[00141] The nitrogen use efficiency (NUE) of three independent PBpr1 lines, AGR 1/7 and NB were calculated from the seed yield per gram of nitrogen applied per plant (Table 2). The three *PBpr1* lines were all higher in NUE than AGR 1/7 by 5-12 % and NB by 11-18%. The highest NUE line was *PBpr1*-21 and the lowest was NB.

[00142] Table 3 shows yield tests and agronomic traits from three *PBpr1:AlaAT* over-expressing rice lines (PBpr1-11, PBpr1-12, PBpr1-21 (*OsPBpr1::HvAlaAT*).) versus WT (NB; Nipponbare) at maturity during soil growth under high N conditions.

[00143] Figure 16 shows the foliar amino acid concentrations of wildtype (cv. Nipponbare) rice versus barley *AlaAT* over-expressing rice transgenic rice lines; AGR 1/7 (*OsANT1:HvAlaAT*) and PBpr1-11, PBpr1-12, PBpr1-21 (*OsPBpr1::HvAlaAT*). Measurements were taken at 28, 40, 52 and 95 days after germination (DAG) corresponding to active tillering, mid-tillering, maximum tillering and flag leaf emergence, respectively. Amino acid concentration (nM/g FW) are averages of 4 replicates.

Table 2: NUE of T_2 homozygous PBpr1 lines, AGR1/7 and Nipponbare (NB) plants (based on seed yield per gram of nitrogen applied per plant and the above ground biomass without seed. Each data point is taken from at least 4 replicates. (NUE= Grain yield/ N applied))

	Above ground biomass without seed (g)	Seed yield (g)	NUE (yield .unit applied^{-1})
PBpr1-10	18.26	8.55	43.63
PBpr1-11	18.03	8.29	42.27
PBpr1-21	24.20	8.83	45.07
NB	19.75	7.48	38.16
AGR1/7	19.94	7.89	40.23

Table 3. Yield tests and agronomic traits from three PBpr1:AlaAT over-expressing rice lines versus WT.

Trait	NB	PBpr1-11	PBpr1-12	PBpr1-21
Average days to flowering	92-105	88-99	93-100	94-110
Producing tillers	6.5	6.6	6.4	7.2
Nonproducing tillers	0.25	0.6	0.2	0.4
Seed weight	10.3±0.5	9.9±0.5	8.5±0.2	9.7±0.5
Fresh weight biomass per plant (g)	24.9±1.4	26.9±2.0	25.5±1.9	25.7±2.7
Dry weight per plant (g)	14.6±0.9	17.4±1.5	17.5±1.3	16.7±2.0
% dry weight of NB		108%	102%	103%

EXAMPLE 2: PBpr2 Promoter Fused to the *alaAT* Gene

[00144] The choice of promoter to use to drive expression of a gene, such as the alanine aminotransferase gene, in crop plants is important for optimal expression of the gene at the right time (developmental stage) and the right place in the plant (tissue specificity). The promoter chosen to drive the expression will affect the phenotypic outcome for the plant. There are examples in the literature where the promoter chosen to drive a gene of interest affects the phenotypic outcome. For example, Myashita *et al.,* (2007) used the CaMV35S promoter to drive expression of AlaAT in Arabidopsis and observed no phenotype. Furtado *et al.*, (2008) studied the ability of various promoters to drive expression of green fluorescent protein (GFP) in transgenic rice and showed that three out of five putative endosperm specific promoters from rice, barley and wheat did not allow GFP expression in glumes/lemma/palea of the flower tissues, while the other two did allow GFP expression in those plant parts. The choice of promoter to use therefore requires both careful design and experiments to determine its efficacy to improve NUE.

[00145] Prior to the studies outlined in Example 1, a promoter named "PBpr2" was selected because its native gene is homologous to rice aldehyde dehydrogenases, such as the OsANT1 gene. The sequence of the PBpr2 promter is provided in Figure 18 (SEQ ID NO: 4). Based on sequence similarity the PBpr2 gene is a putative aldehyde dehydrogenase, however, its function is unknown.

[00146] An alignment of the sequences of the PBpr1, PBpr2 and OsANT1 promoters (SEQ ID NOs: 1, 4 and 5, respectively) is shown in Figure 19. Although neither the Softberry nor the PlantpromoterDB software predicted a TATA box in PBpr1 at the same location as is present for OsANT1 or PBpr2, there is a conserved TATGAG sequence at that location. Also, PBpr1 ncludes a regulatory element about 30 nt downstream of the TATA boxes, and although the promoter prediction software does not predict a regulatory element at the corresponding position for the other two promoters, it is in a section of DNA with high conservation between the three sequences. The sequence identity between the PBpr1 and PBpr2 sequences, based on the alignment shown in Figure 19, is approximately 50% over the entire length (733 nucleotides) of the PBpr1 sequence.

[00147] The PBpr2 promoter was fused to the alaAT gene and a Nos terminator sequence and used to drive expression of AlaAT in rice following protocols similar to those outlined in Example 1. The results of these experiments are shown in Figure 17 and show that PBpr2 does not increase plant height, above ground biomass or seed yield compared to control wild-type (Nipponbare) rice, and so does not promote an NUE phenotype.

[00148] Collectively, the results from Examples 1 and 2 show that despite their similarities, including an approximately 50% sequence identity, the PBpr1 and PBpr2 promoters when fused to the AlaAT gene have different effects on phenotypic outcome.

[00149] The disclosures of all patents, patent applications, publications and database entries referenced in this specification are hereby specifically incorporated by reference in their entirety to the same extent as if each such individual patent, patent application, publication and database entry were specifically and individually indicated to be incorporated by reference.

[00150] Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention. All such modifications as

would be apparent to one skilled in the art are intended to be included within the scope of the following claims.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A transgenic plant or plant part comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

2. The transgenic plant or plant part according to claim 1, wherein the plant is selected from the group consisting of: corn, wheat, maize, rice, barley, canola, soybean, cotton, alfalfa, safflower, tomato and potato.

3. The transgenic plant or plant part according to claim 1, wherein the plant is rice, barley, wheat or maize.

4. The transgenic plant or plant part according to claim 1, wherein the plant is rice.

5. The transgenic plant or plant part according to any one of claims 1 to 4, wherein the nitrogen utilization protein is selected from the group consisting of: a nitrate transporter, an ammonia transporter, an amino acid transporter, nitrate reductase, glutamine synthetase, glutamate synthetase-1, glutamate dehydrogenase, an aminotransferase, alanine aminotransferase, aspartate aminotransferase, asparagine synthetase and asparaginase.

6. The transgenic plant or plant part according to any one of claims 1 to 4, wherein the nitrogen utilization protein is a nitrate transporter or an ammonium transporter.

7. The transgenic plant or plant part according to any one of claims 1 to 4, wherein the nitrogen utilization protein is alanine aminotransferase.

8. The transgenic plant or plant part according to any one of claims 1 to 7, wherein the PBpr1 promoter comprises a nucleotide sequence substantially identical to the sequence as set forth in SEQ ID NO: 1.

9. A seed obtained from the transgenic plant according to any one of claims 1 to 8.

10. A plant cell transformed with a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

11. The plant cell according to claim 10, wherein the plant cell is selected from the group consisting of: a corn plant cell, a wheat plant cell, a maize plant cell, a rice plant cell, a barley plant cell, a canola plant cell, a soybean plant cell, a cotton plant cell, an alfalfa plant cell, a safflower plant cell, a tomato plant cell and a potato plant cell.

12. The plant cell according to claim 10, wherein the plant cell is a rice plant cell, a barley plant cell, a wheat plant cell or a maize plant cell.

13. The plant cell according to claim 10, wherein the plant cell is a rice plant cell.

14. The plant cell according to any one of claims 10 to 13, wherein the nitrogen utilization protein is selected from the group consisting of: a nitrate transporter, an ammonia transporter, an amino acid transporter, nitrate reductase, glutamine synthetase, glutamate synthetase-1, glutamate dehydrogenase, an aminotransferase, alanine aminotransferase, aspartate aminotransferase, asparagine synthetase and asparaginase.

15. The plant cell according to any one of claims 10 to 13, wherein the nitrogen utilization protein is a nitrate transporter or an ammonium transporter.

16. The plant cell according to any one of claims 10 to 13, wherein the nitrogen utilization protein is alanine aminotransferase.

17. The plant cell according to any one of claims 10 to 16, wherein the PBpr1 promoter comprises a nucleotide sequence substantially identical to the sequence as set forth in SEQ ID NO: 1.

18. A genetic construct comprising a polynucleotide encoding a nitrogen utilization protein operably linked to a PBpr1 promoter.

19. The genetic construct according to claim 18, wherein the nitrogen utilization protein is selected from the group consisting of: a nitrate transporter, an ammonia transporter, an amino acid transporter, nitrate reductase, glutamine synthetase, glutamate synthetase-1, glutamate dehydrogenase, an aminotransferase, alanine aminotransferase, aspartate aminotransferase, asparagine synthetase and asparaginase.

20. The genetic construct according to claim 18, wherein the nitrogen utilization protein is a nitrate transporter or an ammonium transporter.

21. The genetic construct according to claim 18, wherein the nitrogen utilization protein is alanine aminotransferase.

22. The genetic construct according to any one of claims 18 to 21, wherein the construct further comprises a transcription termination sequence, a polyadenylation region, or both.

23. The genetic construct according to any one of claims 18 to 22, wherein the PBpr1 promoter comprises a nucleotide sequence substantially identical to the sequence as set forth in SEQ ID NO: 1.

24. A vector comprising the genetic construct according to any one of claims 18 to 23.

25. A host cell comprising the genetic construct according to any one of claims 18 to 23, or the vector according to claim 24.

26. A method of generating a plant having increased nitrogen use efficiency comprising:

transforming a plant cell with the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24, and

growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter,

thereby generating a plant having increased nitrogen use efficiency, wherein the increased nitrogen use efficiency is relative to the nitrogen use efficiency of a wild-type plant grown under identical conditions.

27. A method of generating a plant having increased biomass comprising:

transforming a plant cell with the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24, and

growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter,

thereby generating a plant having increased biomass, wherein the increased biomass is relative to the biomass of a wild-type plant grown under identical conditions.

28. A method of generating a plant having increased seed yield comprising:

transforming a plant cell with the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24, and

growing the transformed plant cell to produce a plant that expresses the nitrogen utilization protein from the PBpr1 promoter,

thereby generating a plant having increased seed yield, wherein the increased seed yield is relative to the seed yield of a wild-type plant grown under identical conditions.

29. A method for producing a plant having increased nitrogen use efficiency, increased biomass, increased seed yield, or a combination thereof, comprising:

(a) providing a plant, a plant part or a seed comprising the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24, and

(b) growing a plant from the plant, plant part or seed,

thereby producing a plant having increased nitrogen uptake, increased biomass, increased seed yield, or a combination thereof, wherein the increased nitrogen uptake, increased biomass and increased seed yield is relative to the nitrogen uptake, biomass and seed yield of a wild-type plant grown under identical conditions.

30. The method of claim 29, further comprising providing a fertilizer to the plant or the habitat of the plant in step (b).

31. The method of claim 30, wherein the fertilizer is a nitrogen containing fertilizer.

32. The method according to any one of claims 26 to 31, wherein the plant is selected from the group consisting of: corn, wheat, maize, rice, barley, canola, soybean, cotton, alfalfa, safflower, tomato and potato.

33. The method according to any one of claims 26 to 31, wherein the plant is rice, barley, wheat or maize.

34. The method according to any one of claims 26 to 31, wherein the plant is rice.

35. The method of any one of claims 26 to 34, wherein the increased nitrogen use efficiency, increased biomass or increased seed yield is observed in the mature plant.

36. Use of a transgenic plant according to any one of claims 1 to 7 for producing progeny.

37. Use of the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24 to increase nitrogen use efficiency, biomass or seed yield in a plant.

38. A process for increasing nitrogen use efficiency, biomass, seed yield, or a combination thereof, in a plant comprising:

providing a seed comprising the genetic construct according to any one of claims 18 to 23 or the vector according to claim 24,

applying a herbicide, insecticide, fertilizer, or combination thereof, to the seed, and

growing a plant from the seed, wherein the plant has increased nitrogen use efficiency, increased biomass, increased seed yield, or a combination thereof, and wherein the increased nitrogen use efficiency, increased biomass or increased seed yield is relative to the nitrogen use efficiency, biomass or seed yield of a wild-type plant grown under identical conditions.

Application number / Numéro de demande: 2,847,715

Figures:

Pages: 10, 11, 15,

Unscannable items received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés :
(Commander les documents originaux dans la section de la préparation des dossiers au 10ième étage)

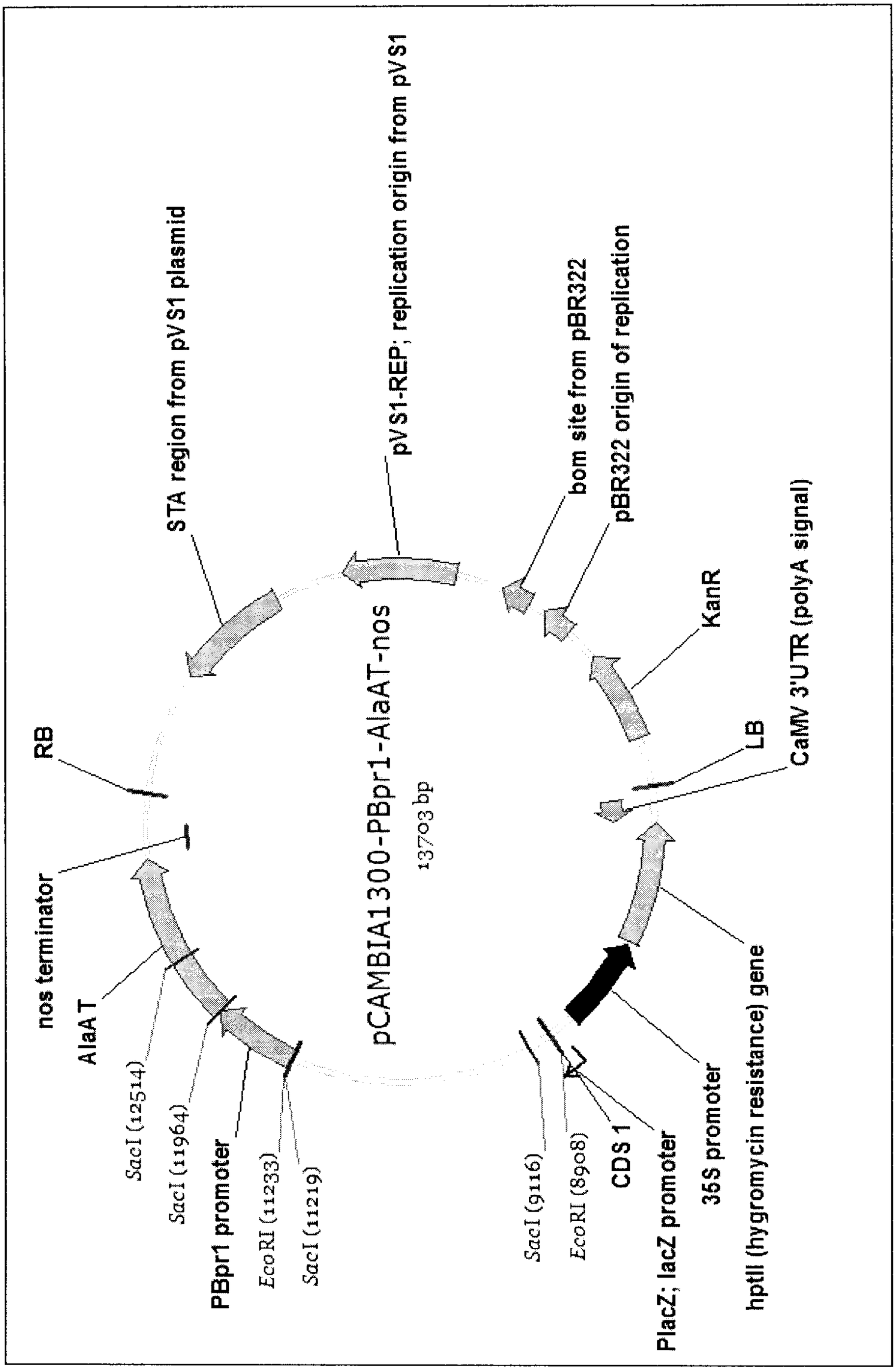

pCAMBIA1300-PBpr1-AlaAT
13703 bp
RB
nos terminator
AlaA T
SacI (12514)
SacI (11964)
PBpr1 promoter
EcoRI (11233)
SacI (11219)
SacI (9116)
EcoRI (8908)
CDS 1
PlacZ; lacZ promoter
35S promoter
hptII (hygromycin resistance) gene
LB
CaMV 3'UTR (polyA signal)
KanR
pBR322 origin of replication
bom site from pBR322
pVS1-REP; replication origin from pVS1
STA region from pVS1 plasmid

FIG. 1

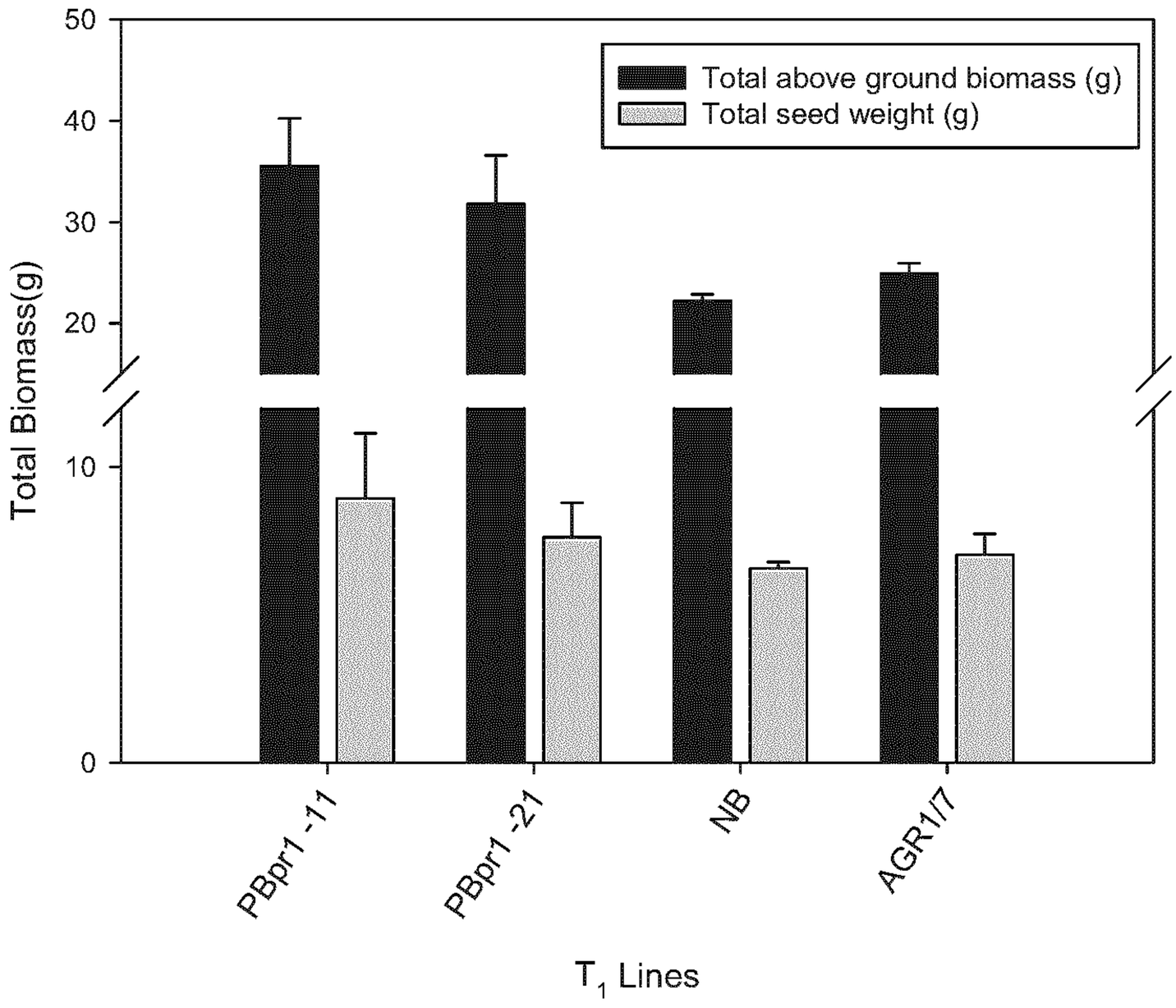

Total above ground biomass (g)
Total seed weight (g)
Total Biomass(g)
50
40
30
20
10
0
PBpr1 -11
PBpr1 -21
NB
AGR1/7
T1 Lines

FIG. 2

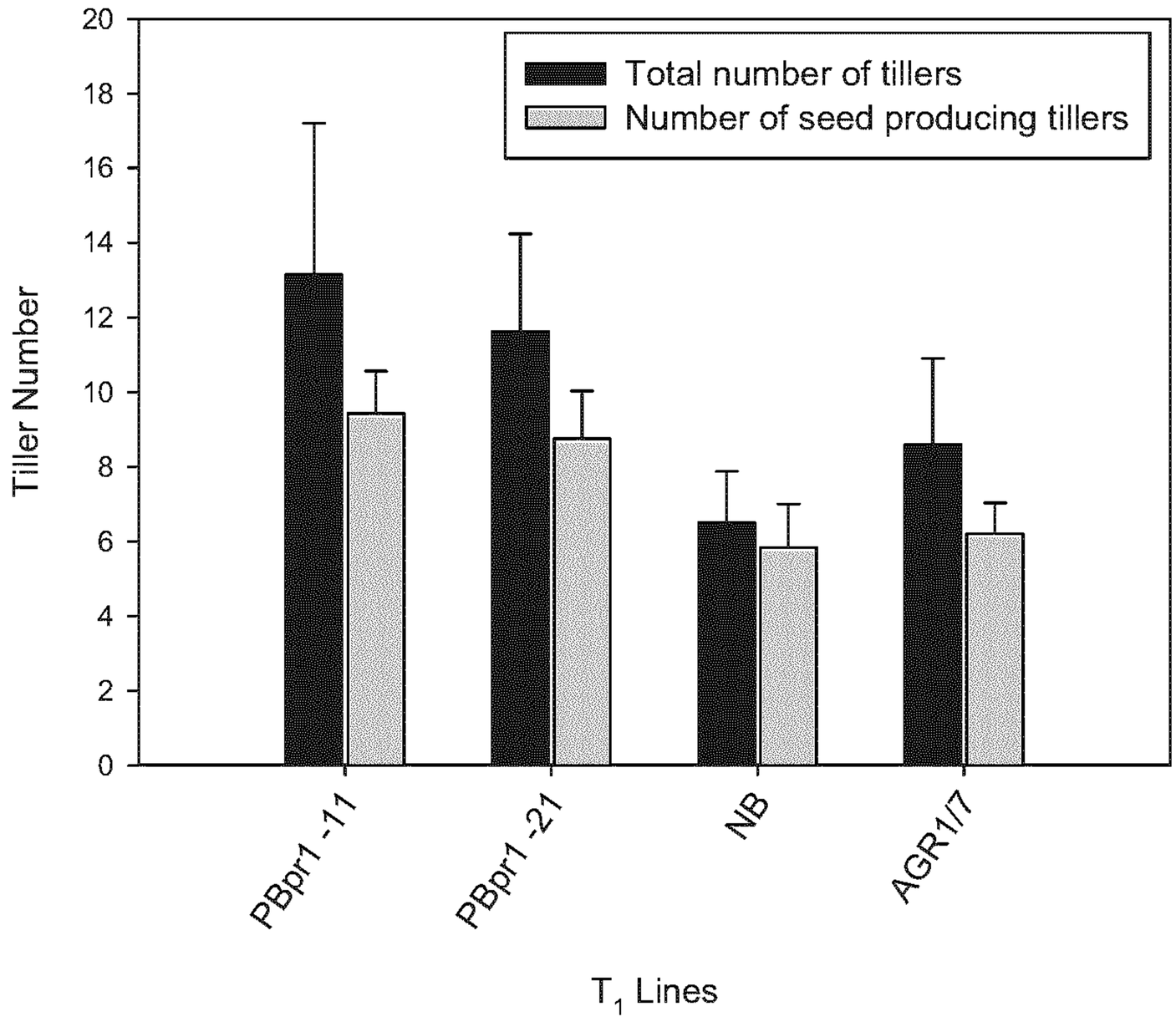

Total number of tillers
Number of seed producing tillers
Tiller Number
0
2
4
6
8
10
12
14
16
18
20
PBpr1 -11
PBpr1 -21
NB
AGR1/7
T_1 Lines

FIG. 3

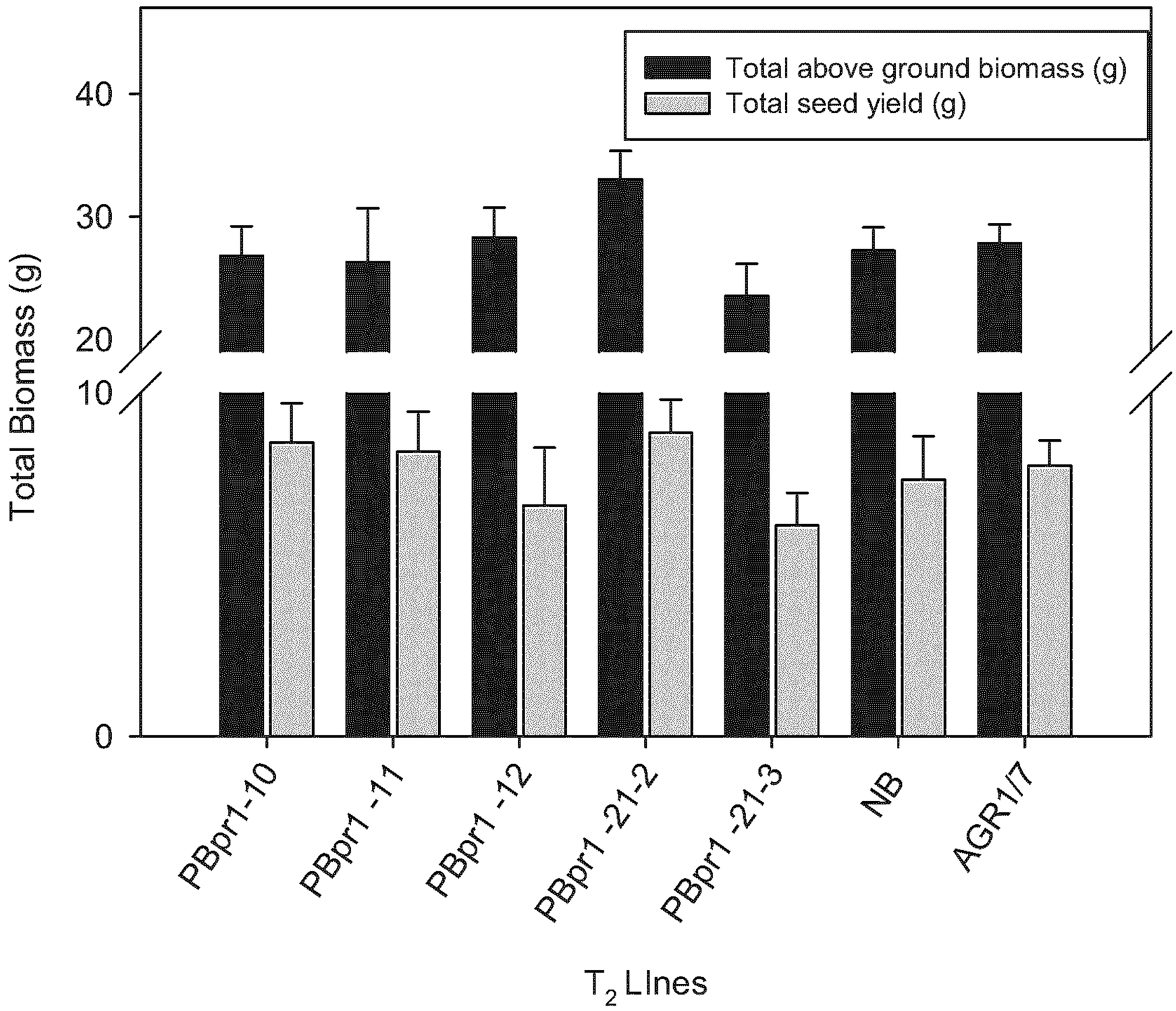
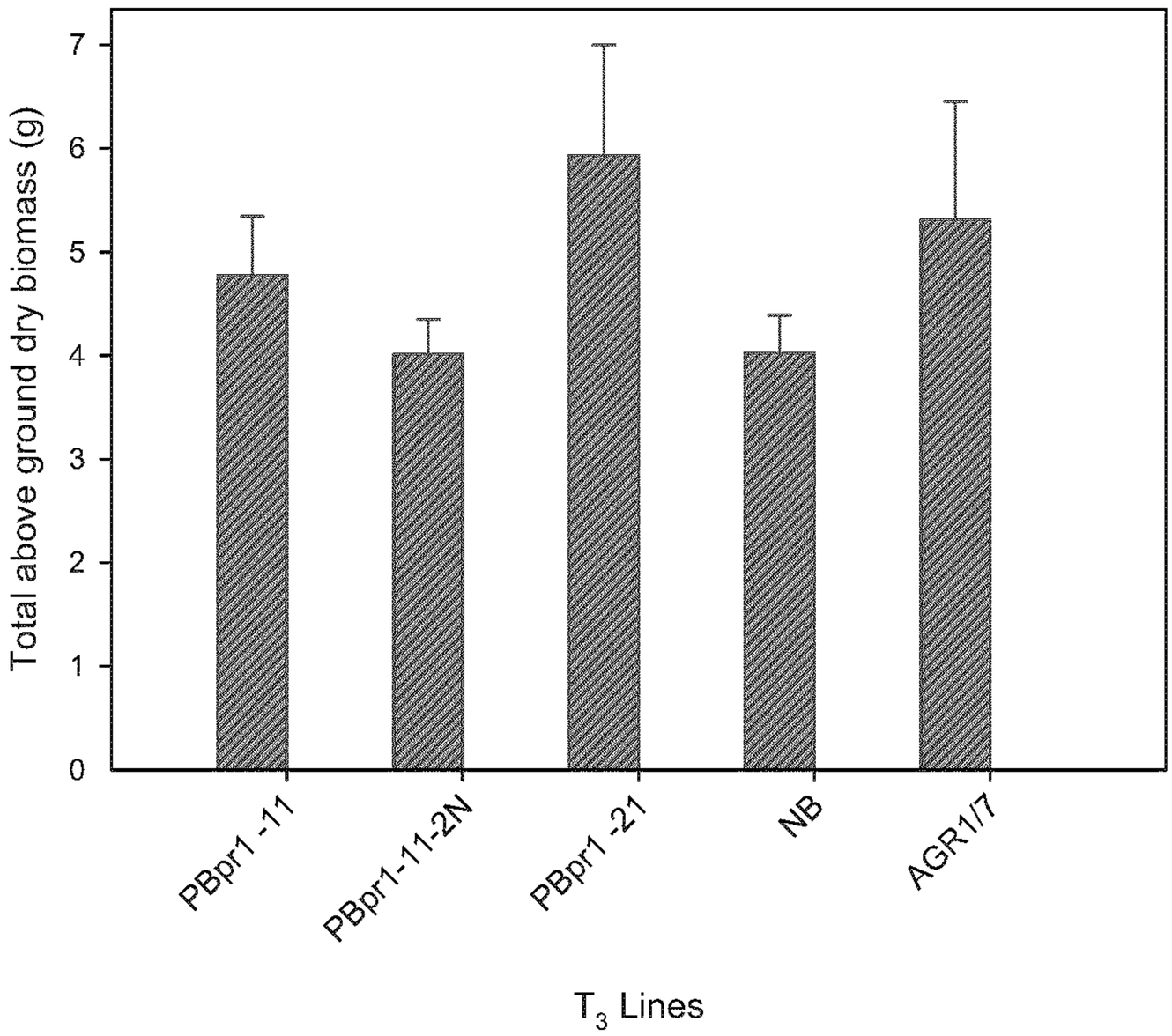

Total above ground biomass (g)
Total seed yield (g)
Total Biomass (g)
40
30
20
10
0
PBpr1-10
PBpr1 -11
PBpr1 -12
PBpr1 -21-2
PBpr1 -21-3
NB
AGR1/7
T2 LInes

FIG. 4

A.


Total above ground dry biomass (g)
0
1
2
3
4
5
6
7
PBpr1 -11
PBpr1-11-2N
PBpr1 -21
NB
AGR1/7
T3 Lines

B.

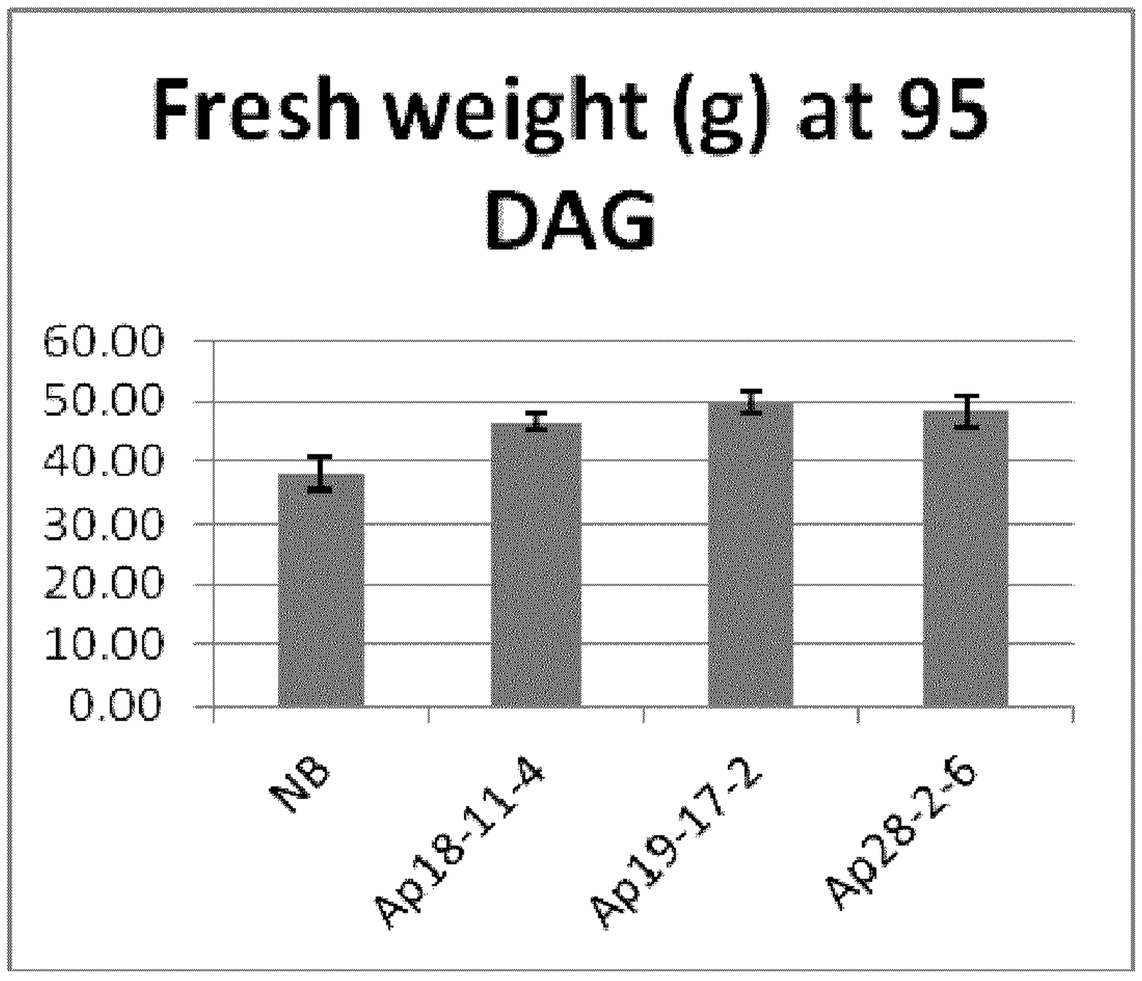

Fresh weight (g) at 95 DAG
60.00
50.00
40.00
30.00
20.00
10.00
0.00
NB
Ap18-11-4
Ap19-17-2
Ap28-2-6

FIG. 5

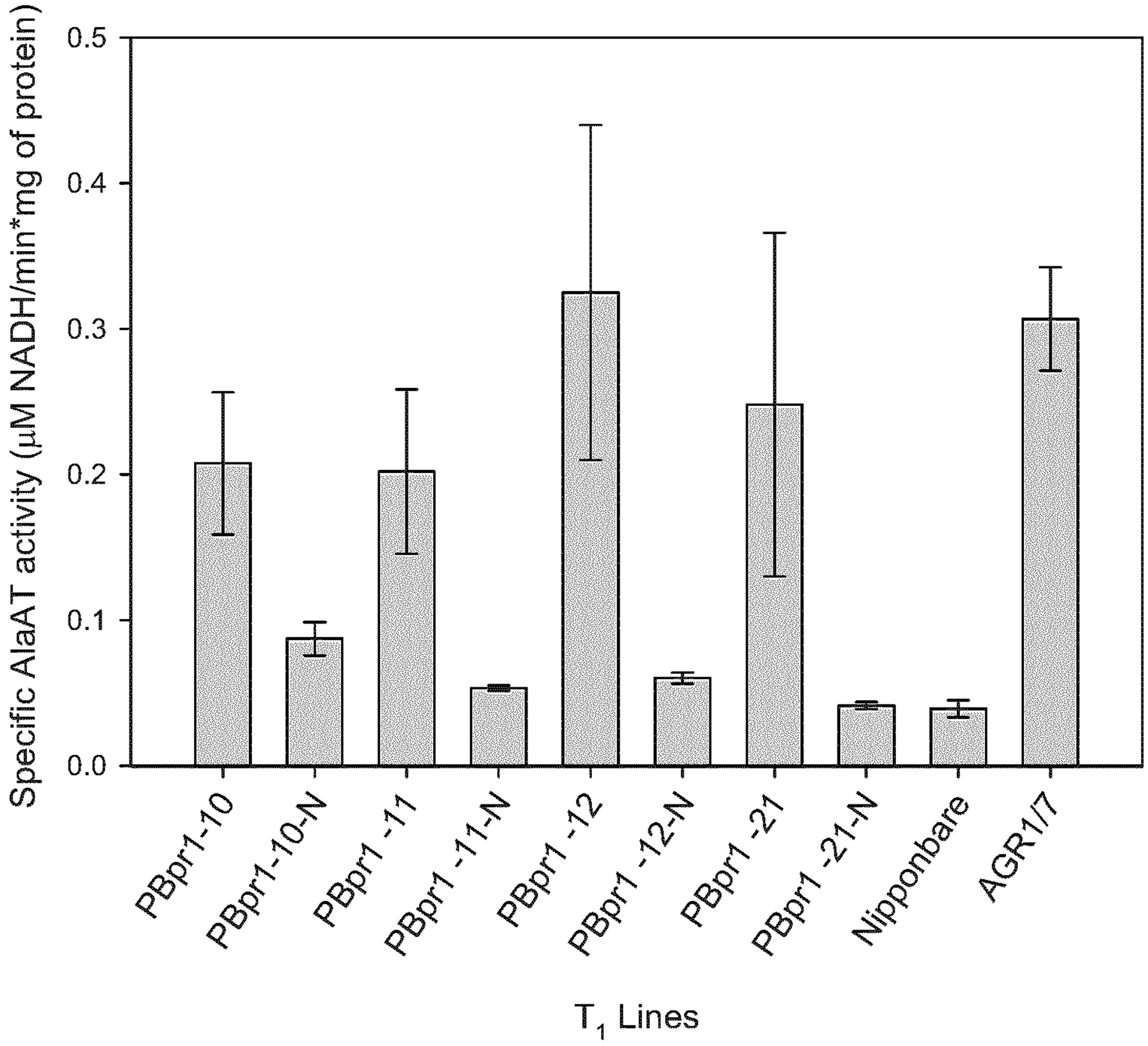

Specific AlaAT activity (μM NADH/min*mg of protein
0.0
0.1
0.2
0.3
0.4
0.5
PBpr1-10
PBpr1-10-N
PBpr1 -11
PBpr1 -11-N
PBpr1 -12
PBpr1 -12-N
PBpr1 -21
PBpr1 -21-N
Nipponbare
AGR1/7
T1 Lines

FIG. 6

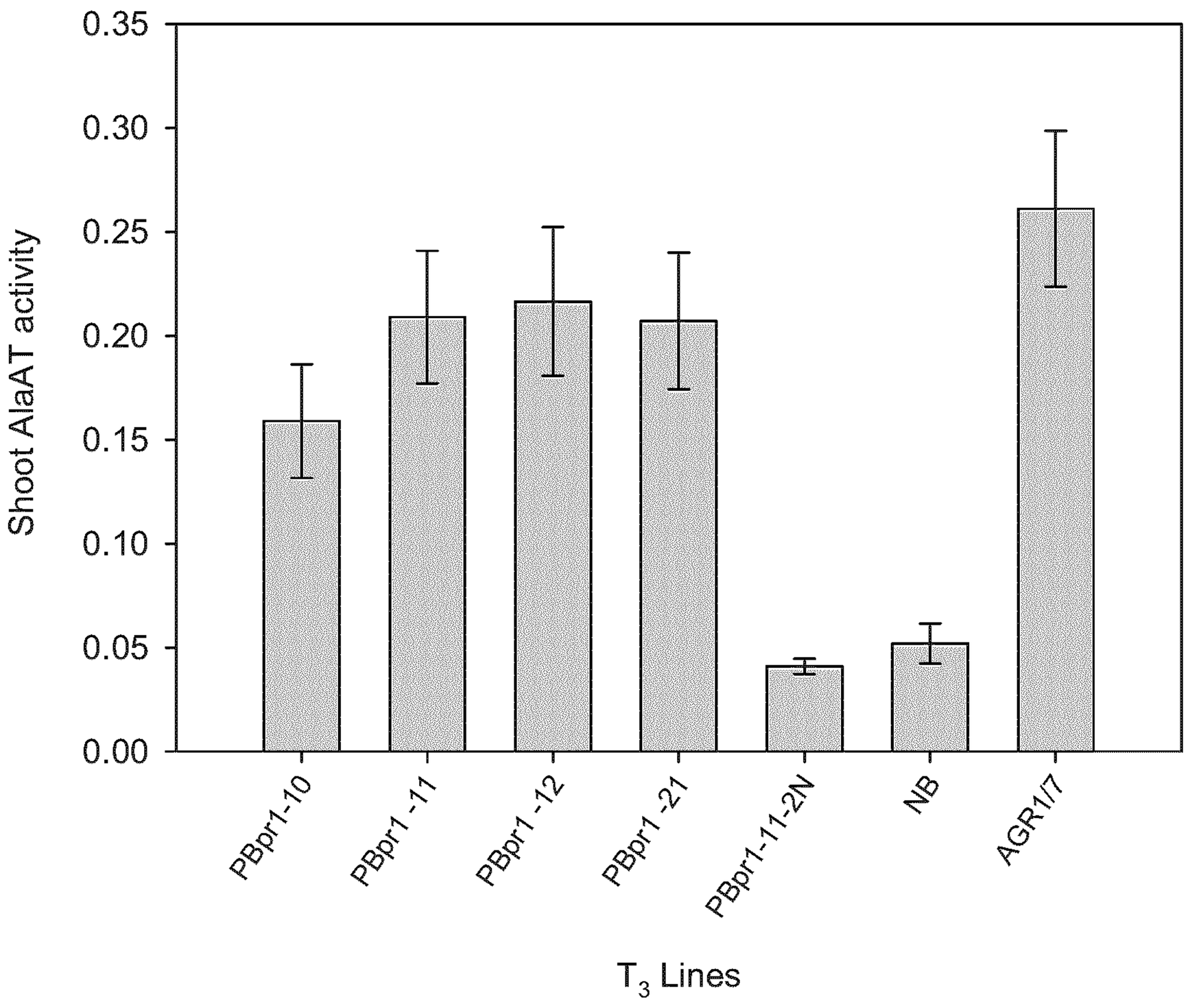

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Shoot AlaAT activity
PBpr1-10
PBpr1 -11
PBpr1 -12
PBpr1 -21
PBpr1-11-2N
NB
AGR1/7
T3 Lines

FIG. 7

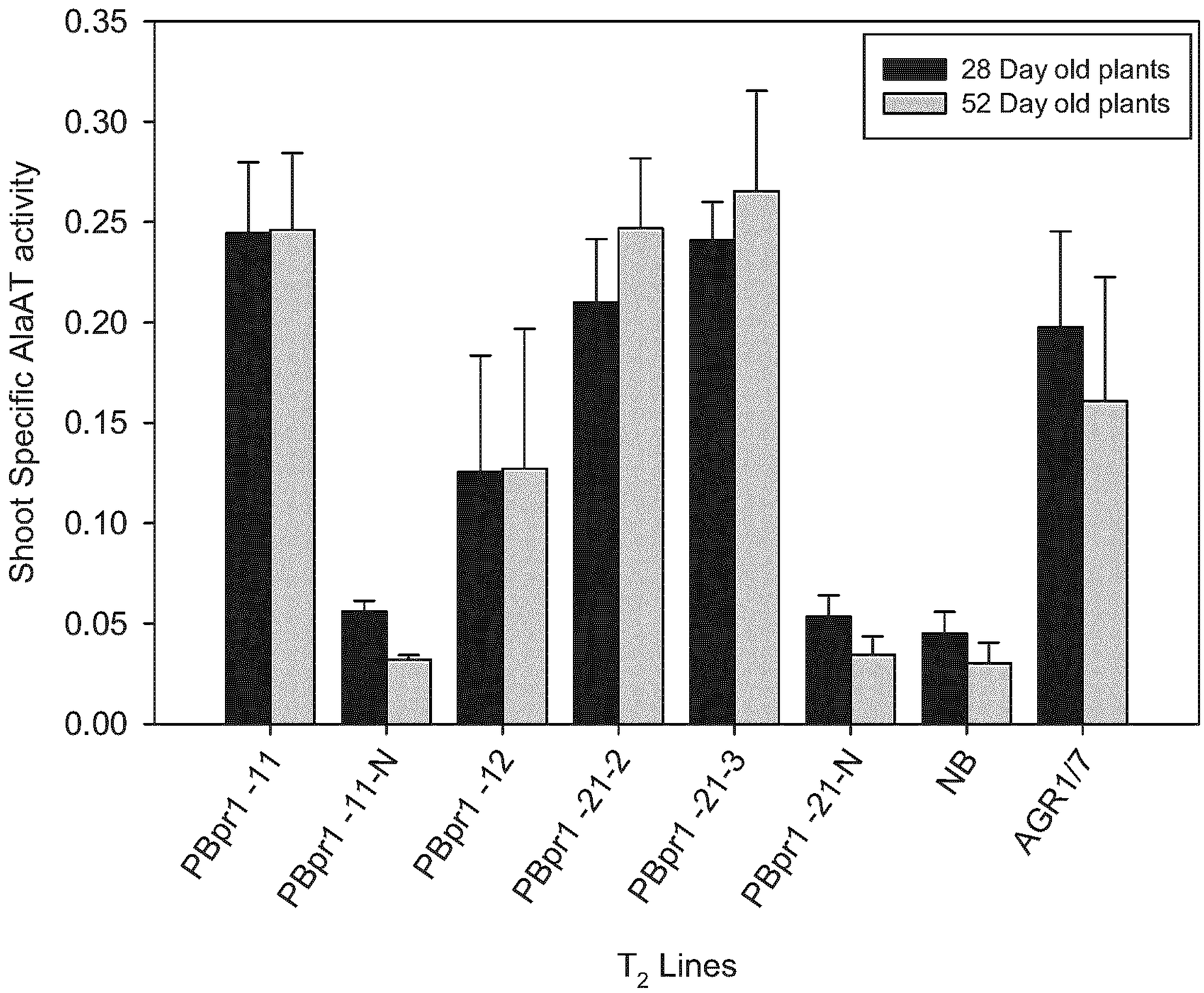

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Shoot Specific AlaAT activity
28 Day old plants
52 Day old plants
PBpr1 -11
PBpr1 -11-N
PBpr1 -12
PBpr1 -21-2
PBpr1 -21-3
PBpr1 -21-N
NB
AGR1/7
T2 Lines

FIG. 8

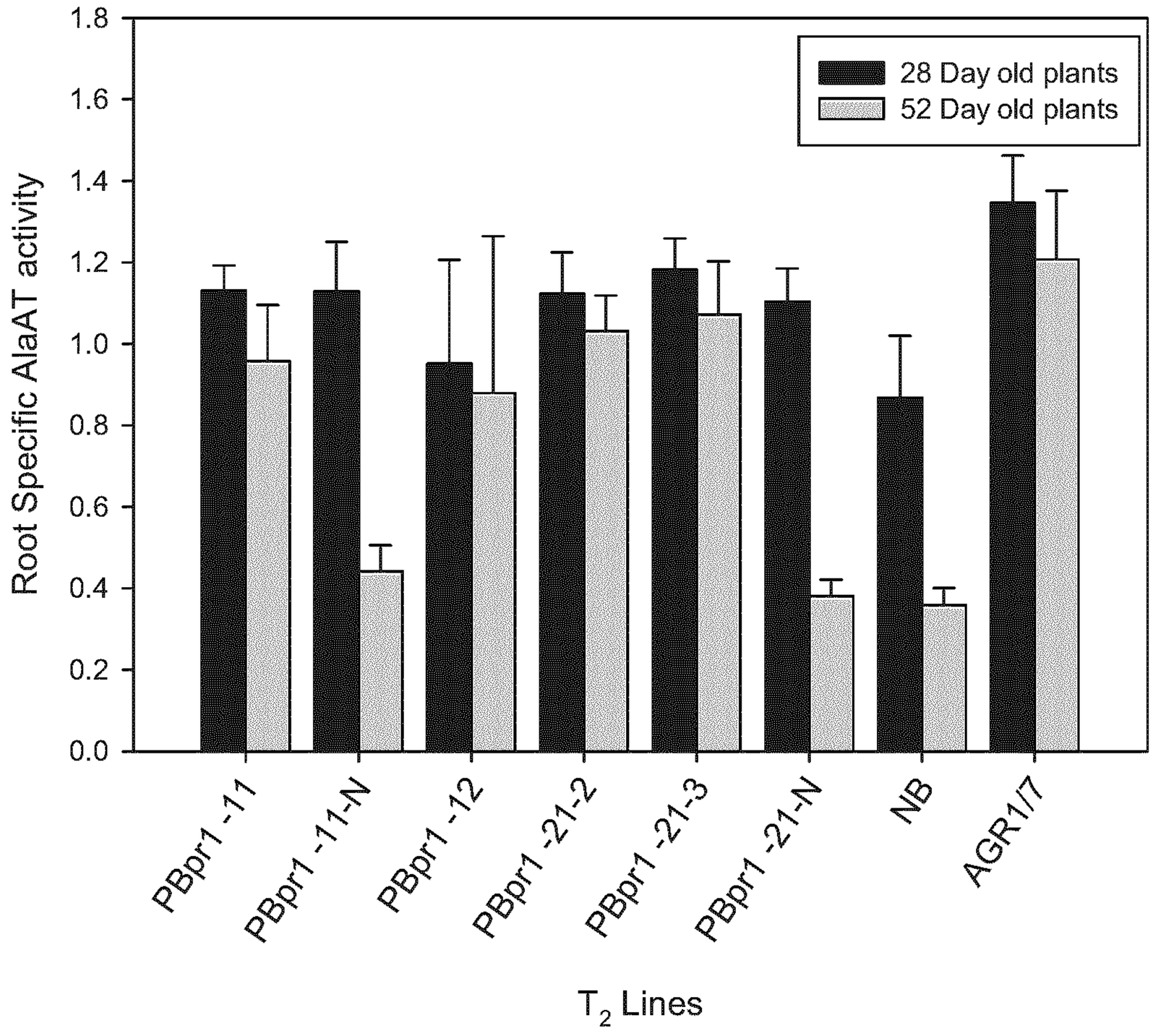

28 Day old plants
52 Day old plants
Root Specific AlaAT activity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PBpr1 -11
PBpr1 -11-N
PBpr1 -12
PBpr1 -21-2
PBpr1 -21-3
PBpr1 -21-N
NB
AGR1/7
T2 Lines

FIG. 9

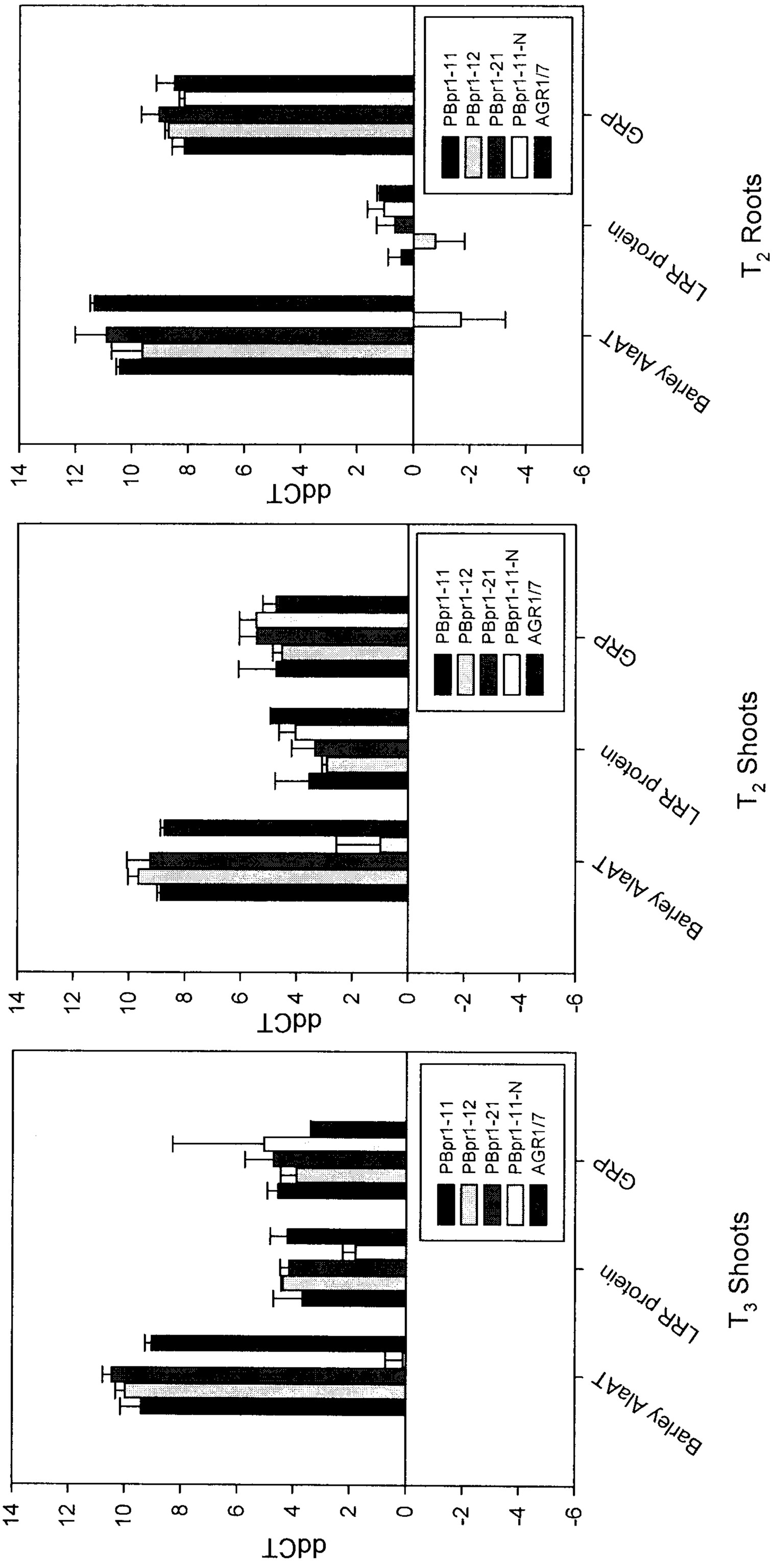

PBpr1-11
PBpr1-12
PBpr1-21
PBpr1-11-N
AGR1/7
GRP
LRR protein
Barley AlaAT
ddCT
14
12
10
8
6
4
2
0
-2
-4
-6
T2 Roots
T2 Shoots
T3 Shoots

FIG. 12

SUBSTITUTE SHEET (RULE 26)

EcoRI

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+
    1 GAATTCTGAA AGTTTCCGTC CAAATCGCAC CTTTTAACCG TTTGAAAAAC ATACAAACGA AAAATAATCT ATATCTTAAT CAGGAAGAAA GAGTACGAAA
-     CTTAAGACTT TCAAAGGCAG GTTTAGCGTG GAAAATTGGC AAACTTTTTG TATGTTTGCT TTTTATTAGA TATAGAATTA GTCCTTCTTT CTCATGCTTT

  101 TGGTGAACCG TCGAAACTAT TCATATACGT CGTCTGTCTC ATGAAAAAAA AAATCAATCC AGAAGGATAC GAGACACTTT TACTTCAACA AATATAGACA
      ACCACTTGGC AGCTTTGATA AGTATATGCA GCAGACAGAG TACTTTTTTT TTTAGTTAGG TCTTCCTATG CTCTGTGAAA ATGAAGTTGT TTATATCTGT

  201 TGAGCTTATT CTACTAGGTT TGGTTGTTTA ATAAGACGAA AGAAATACAT TGGTTAGTTT TTCATTAAAA AATAATCGTT TGACTGACAT AAACCTAGGA
      ACTCGAATAA GATGATCCAA ACCAACAAAT TATTCTGCTT TCTTTATGTA ACCAATCAAA AAGTAATTTT TTATTAGCAA ACTGACTGTA TTTGGATCCT

  301 AATACTGGAT TAAGATAGAT CAGTAGGATT AAGATCCACT GATGTAATTT CCCACTGATT TGGTGGCTGA CATGTGGACC TGAGAGTTGT GTGGGCTCAC
      TTATGACCTA ATTCTATCTA GTCATCCTAA TTCTAGGTGA CTACATTAAA GGGTGACTAA ACCACCGACT GTACACCTGG ACTCTCAACA CACCCGAGTG

  401 ATGTCAAATC ACGGTGAACA GTACGTCACG ATATGTTAGA GGTTCCTCTT CCGGAGATAC TTATACGAAT TTTGCGGAAA CCTGCAAACT TTGATGGACG
      TACAGTTTAG TGCCACTTGT CATGCAGTGC TATACAATCT CCAAGGAGAA GGCCTCTATG AATATGCTTA AAACGCCTTT GGACGTTTGA AACTACCTGC

  501 ATTGAGGCGA GTTTAGTTCT AAATTTTTTC TTCAAACTTC TAACTTTTTC ATCACATCGT TTCAATTTCA ATCAAACTTC CAATGTTGAC GTGAACTAAA
      TAACTCCGCT CAAATCAAGA TTTAAAAAAG AAGTTTGAAG ATTGAAAAAG TAGTGTAGCA AAGTTAAAGT TAGTTTGAAG GTTACAACTG CACTTGATTT

  601 CACACCTATG AGATATGAGA AGCGGGTTGA CACTTGACAA GTCCTGACAT GCTGTGTTGG CGTGGGCCCC ACCTGCCACG TCAGGTCCAG CTCCGGGTGG
      GTGTGGATAC TCTATACTCT TCGCCCAACT GTGAACTGTT CAGGACTGTA CGACACAACC GCACCCGGGG TGGACGGTGC AGTCCAGGTC GAGGCCCACC
```

SacI

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  701 TTGGGTTTGG TGCTTTCCGA TAGGCACGAG CTC ATGGCTG CCACC
      AACCCAAACC ACGAAAGGCT ATCCGTGCTC GAG TACCGAC GGTGG
```

FIG. 13

A.

atggctgccaccgtcgccgtggacaacctgaaccccaaggttttaaaatgtgag
tatgctgtgcgtggagagattgtcatccatgctcagcgcttgcaggaacagcta
aagactcaaccagggtctctacctttgatgagatcctctattgtaacattggg
aacccacaatctcttggtcagcaaccagttacattcttcagggaggttcttgcc
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gctgattctatttctcgagcaaagcagattcttgccatgatacctggaagagca
acaggagcatacagccatagccagggtattaaaggacttcgtgatgcaattgct
tctgggatcgcttcacgagatggattccctgctaatgctgatgacatttttctc
acagatggagcaagtcctggggtgcacctgatgatgcaattactgataaggaat
gagaaagatggcattcttgtcccgattcctcagtacccccttgtactcggcttcc
atagctcttcatggcggagctcttgtcccatactatctcaatgaatcgacgggc
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ggcatcaacgttagggctttggtggttatcaatccaggaaatccaactggacag
gtacttgctgaagaaaaccaatatgacatagtgaagttctgcaaaaatgagggt
cttgttcttctagctgatgaggtataccaagagaacatctatgttgacaacaag
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ctccctctagtatcatatcaatctgtttctaagggatattatggtgagtgtggt
aaaagaggtggttactttgagattactggcttcagtgctccagtaagagagcag
atctacaaaatagcatcagtgaacctatgctccaatatcactggccagatcctt
gctagtcttgtcatgaacccaccaaaggctagtgatgaatcatacgcttcatac
aaggcagaaaaagatggaatcctcgcatctttagctcgtcgtgcgaaggcattg
gagcatgcattcaataaacttgagggaattacttgcaacgaggctgaaggagca
atgtacgtgttccctcaaatctgtctgccacagaaggcaattgaggctgctaaa
gctgctaacaaagcacctgatgcattctatgctcttcgtctcctcgagtcgact
ggaatcgtcgttgtccctggatcaggatttggccaggttcctggcacatggcac
ttcaggtgcacgatccttccgcaggaggataagatcccggcagtcatctcccgc
ttcacggtgttccatgaggcgttcatgtcagagtatcgtgactaaactggt

B.

MAATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQEQLKTQPGSLPFDEILYCNIG
NPQSLGQQPVTFFREVLALCDHPDLLQREEIKTLFSADSISRAKQILAMIPGRA
TGAYSHSQGIKGLRDAIASGIASRDGFPANADDIFLTDGASPGVHLMMQLLIRN
EKDGILVPIPQYPLYSASIALHGGALVPYYLNESTGWGLETSDVKKQLEDARSR
GINVRALVVINPGNPTGQVLAEENQYDIVKFCKNEGLVLLADEVYQENIYVDNK
KFHSFKKIVRSLGYGEEDLPLVSYQSVSKGYYGECGKRGGYFEITGFSAPVREQ
IYKIASVNLCSNITGQILASLVMNPPKASDESYASYKAEKDGILASLARRAKAL
EHAFNKLEGITCNEAEGAMYVFPQICLPQKAIEAAKAANKAPDAFYALRLLEST
GIVVVPGSGFGQVPGTWH
FRCTILPQEDKIPAVISRFTVFHEAFMSEYRD

FIG. 14

28 DAG

Amino acids	NB	AGR 1/7	PBpr1-11	PBpr1-12	PBpr1-21
Glu	5313.8	4344.2	4321.1	4421.3	4426.4
Ala	3613.0	3143.7	3478.5	4549.0	3578.7
Asp	1578.7	1103.0	1258.7	1155.1	1034.8
Ser	1568.5	930.4	1566.1	1006.9	590.8
Thr	664.0	489.2	736.5	545.0	438.4
Asn	644.2	338.9	1129.6	620.8	389.2
Gln	597.5	1342.8	2805.3	1180.6	952.2
Gly	304.9	276.7	426.3	530.2	354.8
GABA	293.4	173.5	394.8	258.9	280.3
Val	204.0	246.5	414.6	279.6	239.7
His	167.6	189.3	202.2	164.8	133.1
Hyp	166.2	92.9	144.9	140.6	108.3
Pro	113.4	90.0	107.8	86.9	99.1
Lys	56.2	56.0	75.8	70.2	48.5
Leu	39.0	58.7	57.6	53.6	61.8
Tyr	32.3	50.4	86.7	34.4	49.2
Arg	nd	nd	294.2	81.1	nd
Cys	nd	nd	nd	nd	nd
Met	nd	nd	24.8	nd	nd
Trp	nd	16.4	58.0	16.7	nd
Phe	nd	nd	53.2	17.2	14.3
Ile	nd	27.1	124.9	38.9	35.5
TOTAL	15356.8	12969.6	17761.6	15251.8	12835.0

40 DAG

Amino acids	NB	AGR 1/7	PBpr1-11	PBpr1-12	PBpr1-21
Glu	4322.8	5299.0	5771.8	5042.7	5018.9
Ala	3442.4	4460.8	3801.1	4232.9	3692.7
Asp	1200.4	1264.9	1554.6	1168.4	1228.7
Ser	982.8	583.2	1392.0	635.4	492.0
Thr	453.7	535.2	686.9	482.7	457.2
Asn	79.5	80.1	157.3	56.3	58.4
Gln	199.6	429.1	629.7	253.1	309.1
Gly	197.9	276.2	330.6	315.2	254.7
GABA	229.3	201.2	196.9	163.2	144.7
Val	198.5	213.4	246.6	231.0	225.8
His	181.4	156.2	173.0	182.2	140.6
Hyp	152.4	134.3	152.9	126.1	107.3
Pro	83.0	65.8	114.0	86.4	74.7
Lys	52.5	42.6	63.1	50.3	45.4
Leu	37.6	49.0	53.1	47.5	53.3
Tyr	nd	nd	109.9	nd	nd
Arg	41.5	41.5	nd	44.1	52.3
Cys	nd	nd	nd	nd	nd
Met	nd	nd	nd	13.9	nd
Trp	nd	nd	24.3	nd	nd
Phe	nd	19.1	42.5	38.3	35.4
Ile	42.4	nd	55.3	33.1	41.2
TOTAL	11897.7	13851.7	15555.5	13202.8	12432.4

52 DAG

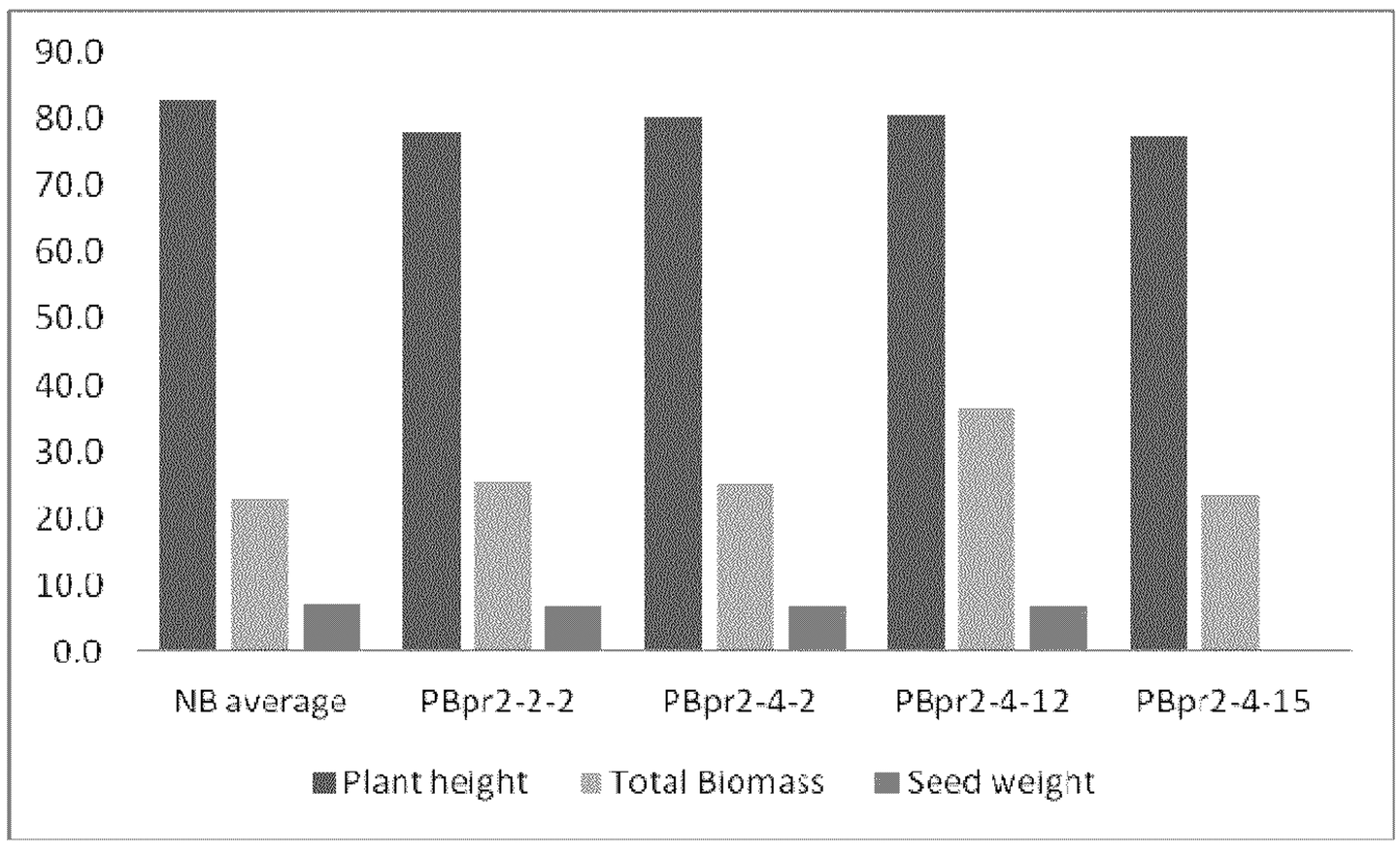
Amino acids	NB	AGR 1/7	PBpr1-11	PBpr1-12	PBpr1-21
Glu	5210.8	5186.8	4880.6	5340.4	5340.4
Ala	3080.7	3011.1	3536.2	2836.1	2836.1
Asp	1392.8	1423.2	1255.4	1673.0	1673.0
Ser	801.9	1199.7	1018.8	971.0	971.0
Thr	414.3	524.8	491.9	454.0	454.0
Asn	169.1	254.6	139.0	165.3	165.3
Gln	428.1	412.0	240.6	369.6	369.6
Gly	371.0	397.2	377.2	378.4	378.4
GABA	212.9	198.3	148.3	116.8	116.8
Val	289.0	247.6	272.1	224.4	224.4
His	333.7	307.3	304.7	291.4	291.4
Hyp	111.1	152.4	115.6	97.9	97.9
Pro	nd	45.9	57.2	45.8	45.8
Lys	nd	nd	nd	nd	nd
Leu	nd	nd	24.1	nd	nd
Tyr	nd	nd	nd	nd	nd
Arg	nd	nd	nd	nd	nd
Cys	nd	nd	nd	nd	nd
Met	nd	nd	nd	nd	nd
Trp	nd	nd	nd	nd	nd
Phe	nd	33.1	31.9	41.3	41.3
Ile	40.3	nd	32.1	31.7	31.7
TOTAL	12855.7	13393.9	12925.8	13037.2	13037.2

95 DAG

Amino acids	NB	AGR 1/7	PBpr1-11	PBpr1-12	PBpr1-21
Glu	4393.2	5500.2	4431.5	4512.8	3750.9
Ala	2388.6	1832.1	2142.7	2721.5	1801.4
Asp	1023.3	1667.9	1431.4	1266.8	1129.9
Ser	622.8	791.5	533.6	512.5	520.8
Thr	252.7	347.8	261.3	280.4	185.1
Asn	296.5	161.9	39.1	103.3	25.9
Gln	405.1	282.3	202.4	216.0	261.9
Gly	351.3	333.2	374.1	490.4	327.8
GABA	197.8	111.2	139.6	128.4	122.6
Val	396.4	367.8	253.2	226.0	226.7
His	430.3	451.4	377.8	386.2	425.3
Hyp	122.8	93.5	85.5	132.5	52.8
Pro	237.1	128.7	114.7	52.6	54.4
Lys	220.8	nd	nd	nd	nd
Leu	76.8	112.9	29.0	nd	nd
Tyr	nd	nd	nd	nd	nd
Arg	nd	nd	nd	nd	nd
Cys	nd	nd	nd	nd	nd
Met	nd	nd	nd	nd	nd
Trp	nd	nd	nd	nd	nd
Phe	103.4	120.7	80.8	61.5	58.0
Ile	85.7	137.2	27.1	nd	nd
TOTAL	11604.4	12440.2	10523.9	11091.0	8943.5

FIG. 16

A.


90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
NB average
PBpr2-2-2
PBpr2-4-2
PBpr2-4-12
PBpr2-4-15
Plant height
Total Biomass
Seed weight

B.

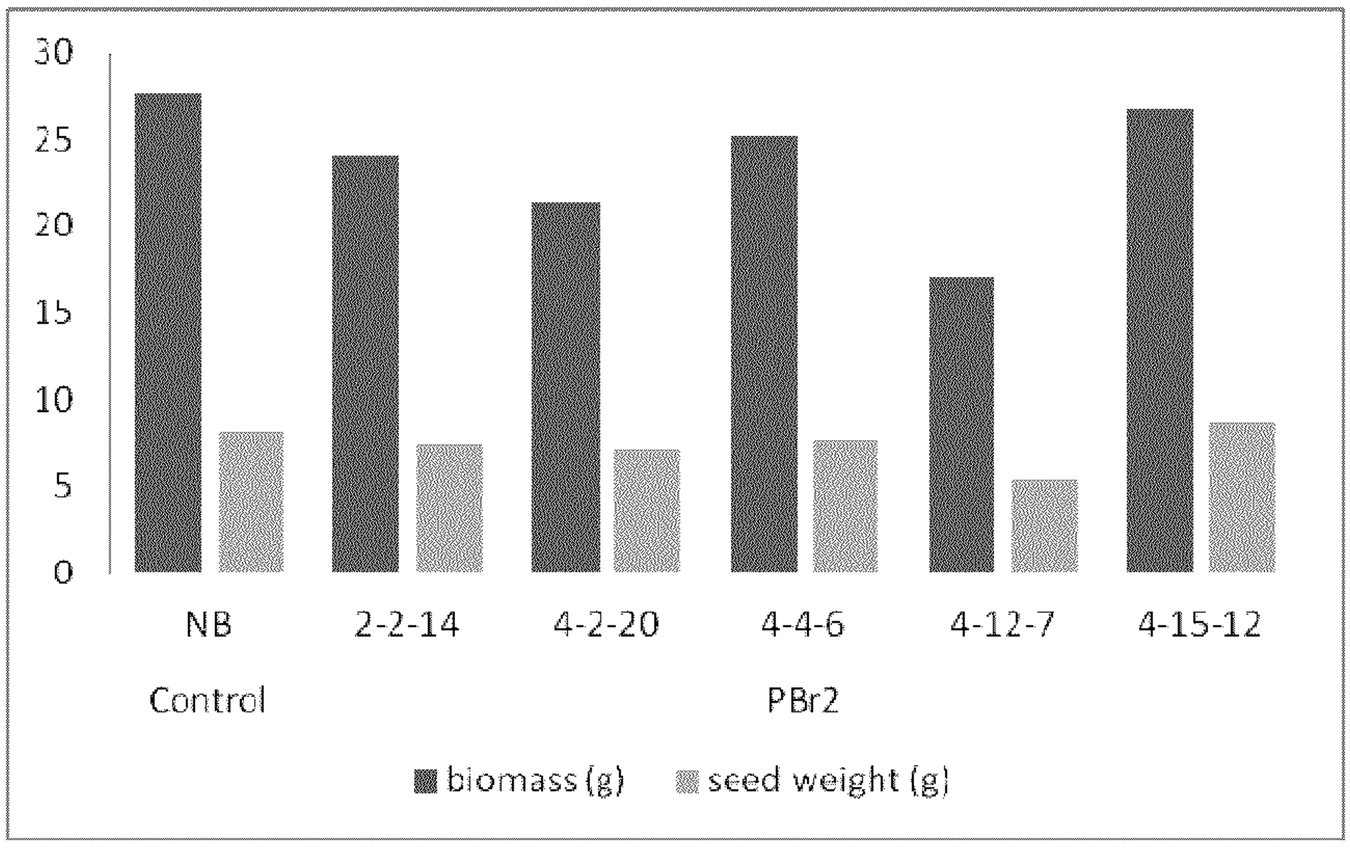

30
25
20
15
10
5
0
NB
2-2-14
4-2-20
4-4-6
4-12-7
4-15-12
Control
PBr2
biomass (g)
seed weight (g)

FIG. 17

A.

GAATTCTGAAAGTTTCCGTCCAAATCGCACCTTTTAACCGTTTGAAAA
ACATACAAACGAAAAATAATCTATATCTTAATCAGGAAGAAAGAGTA
CGAAATGGTGAACCGTCGAAACTATTCATATACGTCGTCTGTCTCATG
AAAAAAAAAATCAATCCAGAAGGATACGAGACACTTTTACTTCAACA
AATATAGACATGAGCTTATTCTACTAGGTTTGGTTGTTTAATAAGACG
AAAGAAATACATTGGTTAGTTTTTCATTAAAAAATAATCGTTTGACTG
ACATAAACCTAGGAAATACTGGATTAAGATAGATCAGTAGGATTAAG
ATCCACTGATGTAATTTCCCACTGATTTGGTGGCTGACATGTGGACCTG
AGAGTTGTGTGGGCTCACATGTCAAATCACGGTGAACAGTACGTCACG
ATATGTTAGAGGTTCCTCTTCCGGAGATACTTATACGAATTTTGCGGA
AACCTGCAAACTTTGATGGACGATTGAGGCGAGTTTAGTTCTAAATTT
TTTCTTCAAACTTCTAACTTTTTCATCACATCGTTTCAATTTCAATCAAA
CTTCCAATGTTGACGTGAACTAAACACACCTATGAGATATGAGAAGCG
GGTTGACACTTGACAAGTCCTGACATGCTGTGTTGGCGTGGGCCCCAC
CTGCCACGTCAGGTCCAGCTCCGGGTGGTTGGGTTTGGTGCTTTCCGAT
AGGCACGAGCTC

B.

GAATTCATATAGTATCTTTGTATGTTGAGCATTAGTATTCAGTGAGTTA
AAACTTTATAGTTGTTAGTGGATGAGTTTGAGTTTTAGGATTTAGTGGG
GTATTATAATTTCATGGTAAGATAGGATGAGTATATATAATTAGGGAT
TAAATTACAATATTTTAAGGATCATATCGATATTCAAAACAATATGTTT
TTTTAAGATGGATGGAATCAATTTTCTGAGATATGTCCCGCTGGGGTG
TCCACAATCTGCACGCCATATCTGACTACACATATACAACGCCCATCC
TCTGTCGATCTCCTTGTGTAAACACAACCGTCCGTGACACGTACGTAC
ATGGGGGATAGCTTGCCTCTCCGTTCAGTTGCGTGACAACTAATAAAA
TACTAACACAATGTCTTAATCCTCCAAAAAAAAACTCCTAAATAATTA
ATTAAAGATAAGGCAAGAAATTAATCCAAATAGTCCAAAACAATAGG
ATAAAGAGGAGAGCAAAACTCCAAATGGTCACACGTCTCTCGGTGTC
ATTTTCTCGTCGGCTTGCTTTGTTTGATTCCAGAGTTTCCCAAACTCTCT
AGAGAGCAAGTCAAACAGATCTCATCGATCGCCCAAGAAGAAGACAC
ATCCACAAATAATAATCTCATCTTATCGTACTAATTTATCTTAATACAG
TGTAATAAACTATATCAGTTTGAGATATTTTTTTCCTGTCTAAATTATC
TCCAAACCGTAGGAATTTTCGCACCCGACGATCTCTTTTCTTGATTTTT
CAAACCAAACGGACCAATCTCTCTCGCGTGTATATATAGCGAGGTGGA
GCTCGAGCTCCATGCCTGCTTCCTCCTGACTCCTCTCCTGCATTCTCTC
GCTTCTGACGGCGGCAACTAACCTTTTTGGGATTCGATTCGAGGAGGA
GGTGATGGGATCC

FIG. 18

```
                          1                                                  50
     O. sativa      (1)   AGGAAGTGATTTTTAGCGTAGCTGTGTTTGTAGCGTAATTGCGTAAAGTC
PBPr1 promoter      (1)   --------------------------------------------------
PBPr2 promoter      (1)   ------GAATTCATATAGTATCTTTGTATGTTGAGCATTAGTATTCAGTG
     Consensus      (1)         ATT  TA  GTA CT TGT TGT G G A T G  T  AGT
                         51                                                 100
     O. sativa     (51)   CTTTCAATTTTGCTATATCTCACTCGAA-AGATTTTTCTTATCTCTCAC
PBPr1 promoter      (1)   --------------------------------------------------
PBPr2 promoter     (45)   AGTTAAAACTTTATAGTTGTTAGTGGATGAGTTTGAGTTTTAGGATTTAG
     Consensus     (51)     TT AA  TT  TA  T T A T GA  AG TT   T TTA    T A
                        101                                                 150
     O. sativa    (100)   TCGATTTTCTCACTCAAATTTACAGTGTATTTTCTTGTA---AGTTACAG
PBPr1 promoter      (1)   --------------------------------------------------
PBPr2 promoter     (95)   TGGGGGTATTATAATTTCATGGTAAGATAGGATGAGTATATATAATTAGGG
     Consensus    (101)   T G  T T   A T   AT    AG      T   T TA   A TTA  G
                        151                                                 200
     O. sativa    (147)   TGTAATTTATGAAACTTACACTGTAACTTTTGTAAGTTACACTGTAATTT
PBPr1 promoter      (1)   ---GAATTCTGAAAGTTTCCGTC-CAAATCGCACCTTTTAACC----GTT
PBPr2 promoter     (145)   ATTAAATTACAATATTTTAAGGATCATATCGATATTCAAAACAATATGTT
     Consensus    (151)     TAAATTATGAAA TTTCAGT TCA ATCG TA TTTAAAC  TA GTT
                        201                                                 250
     O. sativa    (197)   TTGAATCTTCACATGTAAATTTTAAATTTTGTATTGGATTTGGTCTTTTT
PBPr1 promoter     (43)   TGAAAA----ACATACAAACGAAAAATAATCTAT--------ATCTTAAT
PBPr2 promoter    (195)   TTTTTA----AGATGGATGGAATCAATTTTCTGA--------GATATGTC
     Consensus    (201)   TT AAA    ACATG AAA  ATAAATTTTCTAT        GTCTT TT
                        251                                                 300
     O. sativa    (247)   CTTGAGGATATGGTAATTTAATGTTCATTATGGTGTTTCTTAAT-TGCTT
PBPr1 promoter     (81)   CAGGAAGAAAGAGTACGAAATGGTGAACCGTCGAAACTATTCATATACGT
PBPr2 promoter    (233)   CCGCTGGGGTGTCCACAATCTGCACGCCATATCTGACTACACATATACAA
     Consensus    (251)   C GGAGGA AG GTAC ATATGGT  AC  T GTGACTATTCATATAC T
                        301                                                 350
     O. sativa    (296)   TTTGCTTTTTATTATATCTATCGGATTTTAATACAAAGATTAAAAATCTG
PBPr1 promoter     (131)   CGTCTGTCTCATGAAAAAAAAAATCAATC--CAGAAGGATACGAGACACT
PBPr2 promoter     (283)   CGCCCATCCTCTGTCGATCTCCTTGTGTAAACACAACCGTCCGTGACACG
     Consensus    (301)   CGTCC TCTTATGA AA  A C T T T AACACAA GAT CGAGACACG
                        351                                                 400
     O. sativa    (346)   TGTGAT---ACGATTATAAAAATCTTTCGAAAGATGTATAGGTACTCCCA
PBPr1 promoter     (179)   TTTACTTCAACAAATATAGACATGAGCTTAT-TCTAC-TAGGTTTGGTTG
PBPr2 promoter     (333)   T--------ACGTACATGGGGGATAGCTTGCCTCTCCGTTCAGTTGCGTG
     Consensus    (351)   T T  T   ACGAATATAGA AT AGCTTA  TCT C TAGGTTTGC TG
                        401                                                 450
     O. sativa    (393)   AGCCCTTTTAAGAAAGTTTTTCAAGACAAAAGTTTTTGGATGAAAGGTAG
PBPr1 promoter     (227)   TTTAATAAGACGAAAGAAATACATTGG-TTAGTTTTTCATTAAAAAA---
PBPr2 promoter     (375)   ACAACTAATAAAATACTAACACAATGTCTTAATCCTCCAAAAAAAAACTC
     Consensus    (401)   A  ACTAATAAGAAAGTAATACAATG  TTAGTTTTTCAATAAAAAA
                        451                                                 500
     O. sativa    (443)   TTATAGGGAAAAAGGAATGTGCGTTTATGTTTATTTGCATTGCTTATTAG
PBPr1 promoter     (273)   -TAATCGTTTGACTGAC-ATAAACCTAGGAAATACTGGAT----TAAGAT
PBPr2 promoter     (425)   CTAAATAATTAATTAAAGATAAGGCAAGAAATTAATCCAAA---TAGTCC
     Consensus    (451)    TAAA G TTAA TGAA ATAAG CTAGGAATTA TGCAT    TA TA
                        501                                                 550
     O. sativa    (493)   CAACCAAAAACTAATCTATAAGTAAATCTTTTATATACGTGCGCTTAA-T
PBPr1 promoter     (317)   AGATCAGTAGG--AT-TAAGATC----CACTGATGTAATT--TCCCAC-T
PBPr2 promoter     (472)   AAAACAATAGG--AT-AAAGAGGAGAGCAAAACTCCAAATGGTCACACGT
     Consensus    (501)   AAA CAATAGG  AT TAAGAG A A CA T AT TAA TG TC CAC T
                        551                                                 600
     O. sativa    (542)   AATTCAAAAGCAAATTCATGTAAAATAAAATGCGATGAAG--AAACTTTA
```

FIG. 19

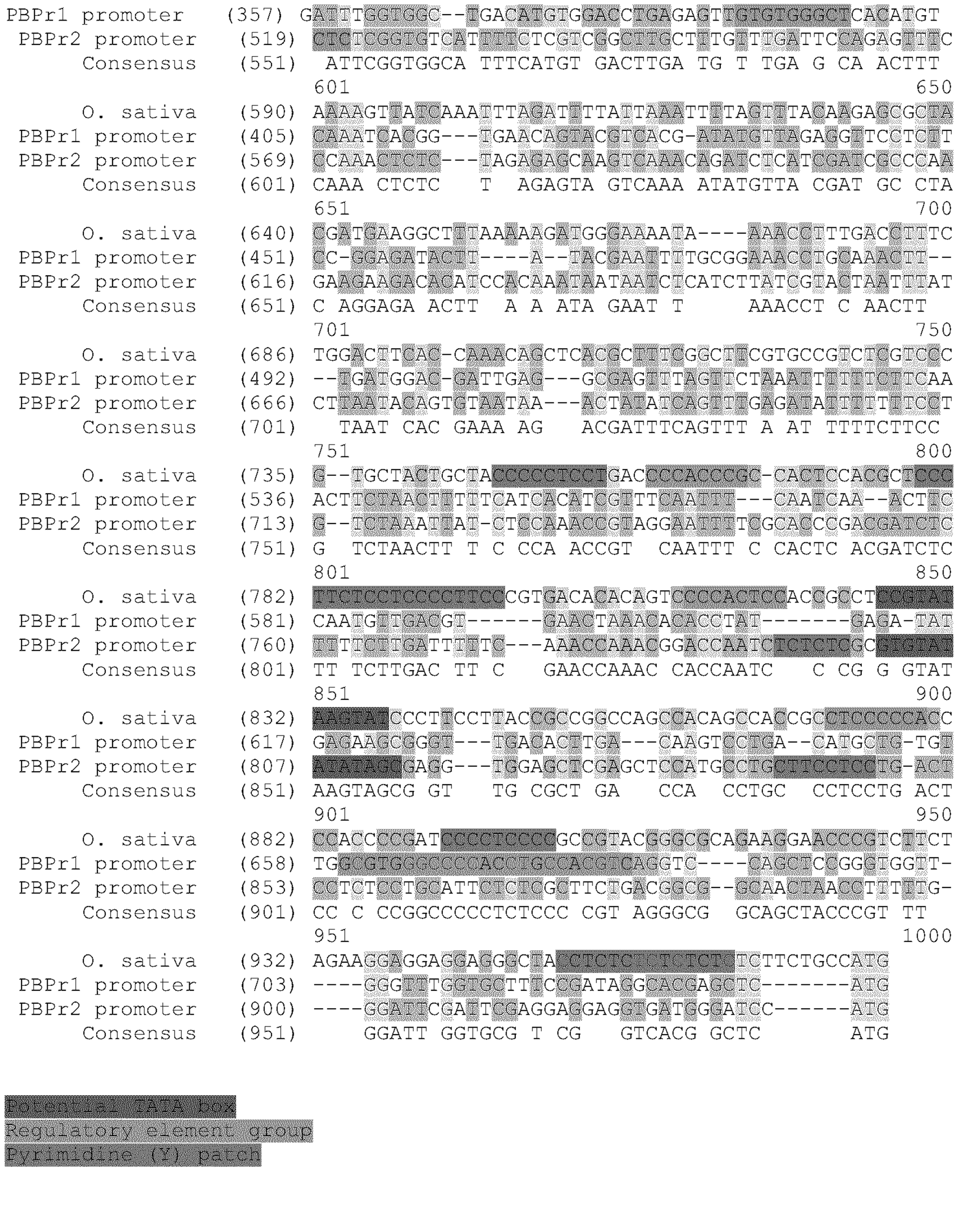

PBPr1 promoter (357) GATTTGGTGGC--TGACATGTGGACCTGAGAGTTGTGTGGGCTCACATGT
PBPr2 promoter (519) CTCTCGGTGTCATTTCTCGTCGGCTTGCTTTGTTTGATTCCAGAGTTTC
Consensus (551) ATTCGGTGGCA TTTCATGT GACTTGA TG T TGA G CA ACTTT
601 650
O. sativa (590) AAAAGTTATCAAATTTAGATTTTATTAAATTTTAGTTTACAAGAGCGCTA
PBPr1 promoter (405) CAAATCACGG---TGAACAGTACGTCACG-ATATGTTAGAGGTTCCTCTT
PBPr2 promoter (569) CCAAACTCTC---TAGAGAGCAAGTCAAACAGATCTCATCGATCGCCCAA
Consensus (601) CAAA CTCTC T AGAGTA GTCAAA ATATGTTA CGAT GC CTA
651 700
O. sativa (640) CGATGAAGGCTTTAAAAAGATGGAAAATA----AAACCTTTGACCTTTC
PBPr1 promoter (451) CC-GGAGATACTT----A--TACGAATTTTGCGGAAACCTGCAAACTT--
PBPr2 promoter (616) GAAGAAGACACATCCACAAATAATAATCTCATCTTATCGTACTAATTTAT
Consensus (651) C AGGAGA ACTT A A ATA GAAT T AAACCT C AACTT
701 750
O. sativa (686) TGGACTTCAC-CAAACAGCTCACGCTTTCGGCTTCGTGCCGTCTCGTCCC
PBPr1 promoter (492) --TGATGGAC-GATTGAG---GCGAGTTTAGTTCTAAATTTTTCTTCAA
PBPr2 promoter (666) CTTAATACAGTGTAATAA---ACTATATCAGTTTGAGATATTTTTTCCT
Consensus (701) TAAT CAC GAAA AG ACGATTTCAGTTT A AT TTTTCTTCC
751 800
O. sativa (735) G--TGCTACTGCTACCCCCTCCTGACCCCACCCGC-CACTCCACGCTCCC
PBPr1 promoter (536) ACTTCTAACTTTTTCATCACATCGTTTCAATTT---CAATCAA--ACTTC
PBPr2 promoter (713) G--TCTAAATTAT-CTCCAAACCGTAGGAATTTTCGCACCCGACGATCTC
Consensus (751) G TCTAACTT T C CCA ACCGT CAATTT C CACTC ACGATCTC
801 850
O. sativa (782) TTCTCCTCCCCTTCCCGTGACACACAGTCCCCACTCCACCGCCTCCGTAT
PBPr1 promoter (581) CAATGTTGACGT------GAACTAAACACACCTAT-------GAGA-TAT
PBPr2 promoter (760) TTTTCTTGATTTTTC---AAACCAAACGGACCAATCTCTCTCGCGTGTAT
Consensus (801) TT TCTTGAC TT C GAACCAAAC CACCAATC C CG G GTAT
851 900
O. sativa (832) AAGTATCCCTTCCTTACCGCCGGCCAGCCACAGCCACCGCCTCCCCCCACC
PBPr1 promoter (617) GAGAAGCGGGT---TGACACTTGA---CAAGTCCTGA--CATGCTG-TGT
PBPr2 promoter (807) ATATAGCGAGG---TGGAGCTCGAGCTCCATGCCTGCTTCCTCCTG-ACT
Consensus (851) AAGTAGCG GT TG CGCT GA CCA CCTGC CCTCCTG ACT
901 950
O. sativa (882) CCACCCCGATCCCCTCCCCGCCGTACGGGCGCAGAAGGAACCCGTCTTCT
PBPr1 promoter (658) TGGCGTGGGCCCCACCTGCCACGTCAGGTC----CAGCTCCGGGTGGTT-
PBPr2 promoter (853) CCTCTCCTGCATTCTCTCGCTTCTGACGGCG--GCAACTAACCTTTTTG-
Consensus (901) CC C CCGGCCCCCTCTCCC CGT AGGGCG GCAGCTACCCGT TT
951 1000
O. sativa (932) AGAAGGAGGAGGAGGCTACCTCTCTCTCTCTCTCTTCTGCCATG
PBPr1 promoter (703) ----GGGTTTGGTGCTTTCCGATAGGCACGAGCTC-------ATG
PBPr2 promoter (900) ----GGATTCGATTCGAGGAGGAGGTGATGGGATCC------ATG
Consensus (951) GGATT GGTGCG T CG GTCACG GCTC ATG
Potential TATA box
Regulatory element group
Pyrimidine (Y) patch

FIG. 19 (con.)

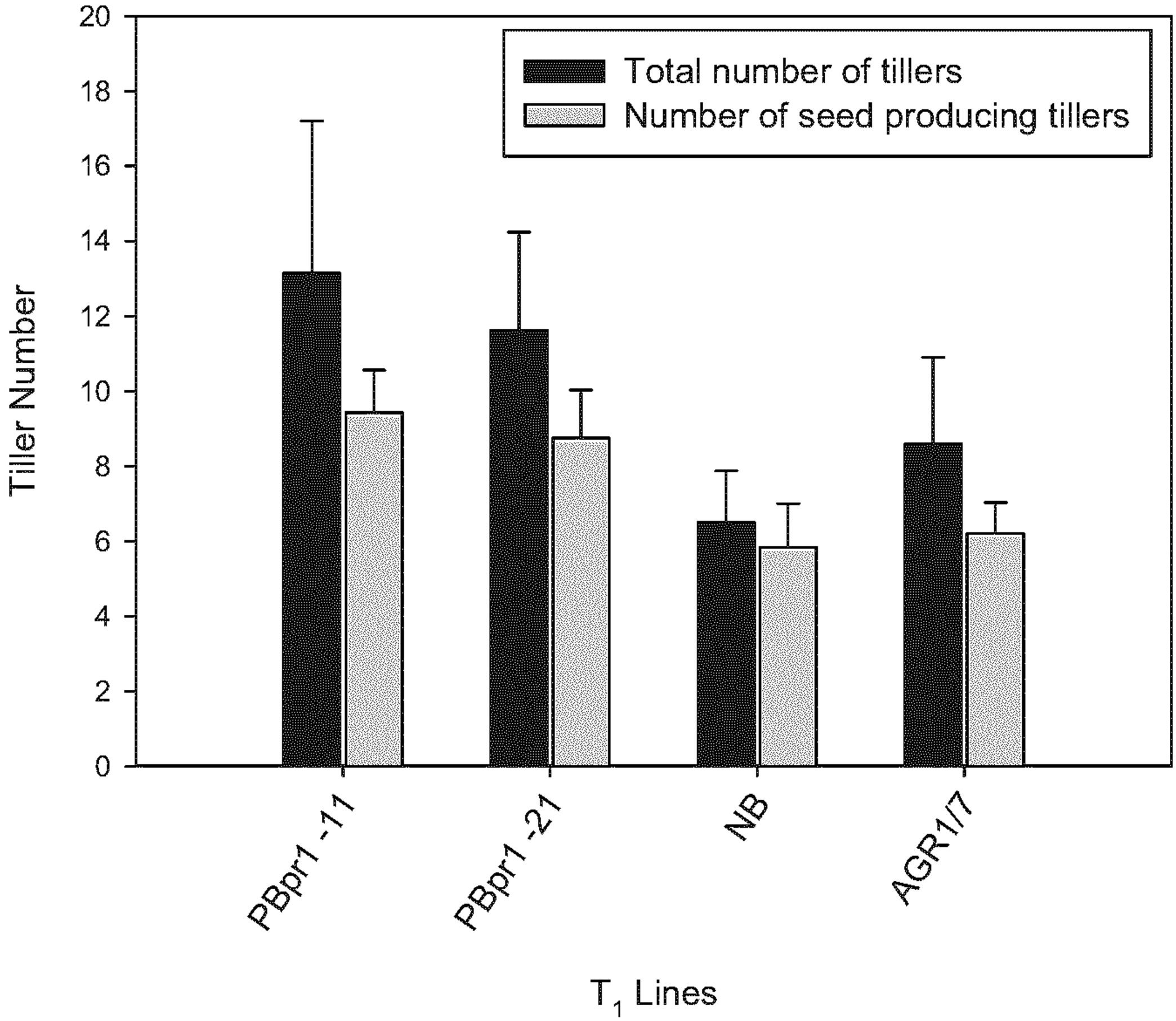

Total number of tillers
Number of seed producing tillers
Tiller Number
20
18
16
14
12
10
8
6
4
2
0
PBpr1 -11
PBpr1 -21
NB
AGR1/7
T1 Lines

FIG. 3