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(54) Title: METHOD OF ENHANCING STRESS TOLERANCE OF MONOCOTYLEDONOUS PLANTS

(57) Abstract: A method is provided for producing a transformed plant of the family Poaceae. One or more genes selected from the group consisting of *OTS1*, *OTS2* and *ICE1* are introduced into the genome of the plant, resulting in transformed plants having enhanced tolerance to an abiotic stress compared to untransformed plants. The gene can be under the control of a drought inducible promoter, such as the *Rab17* promoter. Examples of the abiotic stress are drought, heat, cold and salinity. The plant can be a cereal or grass, such as crops or forage grasses including wheat, barley, sorghum, maize, sugarcane, oats, rye, triticale and commercial fodder grass species. The invention also extends to a vector for transforming the plants and to transformed plants and plant parts.

METHOD OF ENHANCING STRESS TOLERANCE OF MONOCOTYLEDONOUS PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from South African provisional patent application number 2015/04699 filed on 30 June 2015, which is incorporated by reference herein.

5

FIELD OF THE INVENTION

The invention provides methods for enhancing the tolerance of monocotyledonous crops to
10 abiotic stresses such as salt (high salinity), drought, heat and cold.

BACKGROUND OF THE INVENTION

15 Abiotic stresses, defined as the negative impact of non-living factors on the living organisms in a specific environment, are the primary causes of crop loss for crop species such as wheat and other crops in the grass family Poaceae (e.g. maize, rice, sorghum and sugarcane). Abiotic stresses include high and low temperatures, salinity, drought, flooding, heavy metal stress and many other environmental factors.

20

Wheat (*Triticum aestivum* L.) is one of the main cereal crops grown worldwide providing essential proteins and carbohydrates to the human diet (Feuillet *et al.,* 2007). Global wheat production in the 2012/13 growth season was 660 million tons with major producers being China, India and the USA (FAOStat, 2013). In sub-Saharan Africa, South Africa is the second largest wheat producing
25 country after Ethiopia with production standing at 1.8 million tons in the 2014 season according to the Mundi index (www.indexmundi.com/agriculture/country). However, the wheat industry has been declining in South Africa over the past two decades due to a number of environmental factors such as inconsistent precipitation levels as well as biotic factors such as bollworms, aphids and mite infestation (van der Vyver, 2013).

30

Traditional approaches to breeding crop plants with improved abiotic stress tolerances have so far met with limited success. This is due to a number of contributing factors, including: (1) the

focus has been on yield rather than on specific traits; (2) the difficulties in breeding for tolerance traits; and (3) desired traits can only be introduced from closely related species.

Most cereals and grasses are moderately sensitive to a wide range of abiotic stresses, and variability in the gene pool generally appears to be relatively small and may provide few opportunities for major step changes in tolerance. Of potentially larger impact on abiotic stress tolerance is the use of genetic manipulation technologies to generate such step changes.

Arabidopsis (*Arabidopsis thaliana*) is often used as a model plant for plant transformation. However, knowledge gained from working on Arabidopsis is not particularly suitable for transfer to the grasses (including both cereals and forage species). In addition to the obvious fundamental differences in development and anatomy between monocotyledons and dicotyledons, many of the mechanisms of tolerance to abiotic stresses can have fundamentally different characteristics between these two major plant groups. For example, when grown in saline soils, many dicotyledonous halophytes accumulate much higher concentrations of $Na+$ in their shoots than monocotyledonous halophytes, a feature that may be related to the observation that succulence is observed more commonly in dicotyledons than monocotyledons, particularly the grasses.

The possibilities for increasing tolerance to abiotic stresses are enormous, although it is notable that the actual production of transgenic plants with demonstrably improved abiotic stress tolerance has been slow. There have been many published reviews outlining the possibilities for inducing stress tolerance, but, to date, only a handful of papers have been published presenting results from transgenic plants. Unfortunately, some of the transgenic alterations appear to come with a growth and/or yield penalty in conditions of reduced stress (Tester and Bacic, 2005).

Thus, there is still a need to provide cereal and grass crops with increased abiotic stress tolerance.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided a method for producing a transformed plant of the family Poaceae, the method comprising the step of introducing one or more nucleic acids encoding genes selected from the group consisting of *OTS1*, *OTS2* and *ICE1* into the genome of the plant, wherein the transformed plant has enhanced tolerance to an abiotic stress compared to an untransformed plant.

The plant may be a cereal or grass, such as crops or forage grasses including wheat, barley, sorghum, maize, sugarcane, oats, rye, triticale and commercial fodder grass species. More preferably, the plant is wheat or sugarcane.

5 The abiotic stress may be drought, heat, cold or salinity.

The gene may be under the control of a drought inducible promoter, such as the *Rab17* promoter.

The nucleic acid may have a nucleotide sequence which is at least 80% identical to SEQ ID NO.
10 3, at least 80% identical to SEQ ID NO. 9 or at least 80% identical to SEQ ID NO. 11.

According to a second embodiment of the invention, there is provided vector for transforming a plant as described above, the vector comprising one or more genes selected from the group consisting of *OTS1*, *OTS2* and *ICE1* under the control of a *Rab17* promoter.

15

According to a third embodiment of the invention, there is provided a transformed plant or plant part produced according to the method described above. The transformed part plant may be a seed, plantlet, leaf or the like.

20

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Donor plant cultivar Gamtoos R (*Dn7+)* and Gamtoos S (*Dn7-)* in a glasshouse at 24ºC with natural light. **A)** Plant at 2 months, **B)** Plant begins anthesis, **C)** Caryopses collected
25 12-16 days post-anthesis.

Figure 2: Vector map of the pGFP 510b (6167 bp) vector containing the green fluorescent protein gene (*gfp*) under the control of a maize ubiquitin promoter and cauliflower mosaic virus terminator (CaMV-t), used to assess bombardment parameters.

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Figure 3: Sequence of ICE with *Rab17* promotor (SEQ ID NO. 1), where underlined region = *Rab17*-promoter region (647 bp; SEQ ID NO. 2) and non-underlined region = *ICE* gene CDS (1485 bp, SEQ ID NO. 3).

35 **Figure 4**: Explants from experiments 1 - 6 expressing *gfp* (see Table 1 for quantitative data which correlate with this figure).

Figure 5: Amplification of *OTS1* (1.7 kb), *OTS2* (1.7 kb) and *ICE* (1.5 kb) using Phusion High-Fidelity DNA Polymerase. Ladder used Thermo Scientific O'GeneRuler 1 kb DNA Ladder.

Figure 6: Vector maps of plant expression vector, pUbi510 and selection vector, pEmuKN.

Figure 7: Nest PCR to amplify *Rab17* (650 bp) drought promoter from maize callus. The promoter was confirmed by sequencing.

Figure 8: Vector map of the initial pAHC20 vector prior to the removal of its endogenous promoter (*UBI*) and reporter gene (*Bar*).

Figure 9: Vector map of pAHC20 contain *Rab17* promoter in-frame with *ICE1* gene.

Figure 10: Particle bombardment of immature embryos with WP (5 µg/µl) with an osmoticum treatment for 16 hours, and helium pressure at 80 kPa. **A)** Immature embryos (1 mm), **B)** 2-6 day old callus (3 mm), **C)** Individual cells expressing GFP, D) Immature embryos expressing GFP visualization with overlaying filters.

Figure 11: Transgene confirmation of *OTS1*. **A)** Confirming stable expression of *OTS1* in wheat, where *(P+)*: Positive control (pUbi510:OTS1 plasmid); *N(-)*: negative control; *(NT)*: non-transformed plant; *(P1-P3)*: transformed plant. Only P3 tested positive for transgene integration, **B)** The plant which tested positive (P3).

Figure 12: Full length cDNA sequence of OTS1 (AT1G60220) (SEQ ID NO. 4). Bold and red indicates the coding sequence (SEQ ID NO. 9), underlined letters are areas on which primers were designed. Italicised letters are start and stop codons.

Figure 13: Full length cDNA sequence of ICE1 (AT3G26744.4) (SEQ ID NO. 5). Bold and red indicate the coding sequence (SEQ ID NO. 10), underlined letters are areas on which primers were designed. Italicised letters are start and stop codons.

Figure 14: Full length cDNA sequence of OTS2 (AT1G10570) (SEQ ID NO. 6). Bold and red indicate the coding sequence (SEQ ID NO. 11), underlined letters are areas on which primers were designed. Italicised letters are start and stop codons.

Figure 15: Sugarcane tissue ready for hardening off.

Figure 16: **(A)** Four *OTS1* transgenics (1-4); **(B)** two *OTS2* transgenics and; **(C)** one *ICE1* transgenic. *P (+)* refers to plasmid DNA used for bombardment; *W* refers to non-transformed
5 wheat, *N* negative water control.

Figure 17: Phenotypes of spikelet and wheat plants**.** **(A)** Spikelet of (pUBI-*ICE1*); **(B)** Control spikelet; and **(C)** transgenic plants. Note a single plant was chosen as a representative for this photo. However there is only one transgenic plant for pUBI-*ICE1*

10

Figure 18: **(A-C)** *OTS1* transgenic plants; **(D-E)** *OTS2* transgenic plants

Figure 19: Levels of **p**eroxidase activity measured in the transgenic and control (untransformed) plants, where plants 1 to 6 represent transgenic lines and plant 7 the
15 untransformed control wheat plant.

Figure 20: Levels of Glutathion-S-transferase activit; measured in the transgenic and control (untransformed) plants, where plants 1 to 6 represent transgenic lines and plant 7 the untransformed control wheat plant.

20

Figure 21: Levels of ß-1,3-Glucanase activity measured in the transgenic and control (untransformed) plants, where plants 1 to 6 represent transgenic lines and plant 7 the untransformed control wheat plant.

25

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for producing a transformed plant of the family Poaceae. One or more nucleic acids encoding genes selected from the group consisting of *OTS1*, *OTS2* and
30 *ICE1* transformed into the genome of the plant, and expression of these genes in the mature plant confers upon the plant enhanced tolerance to an abiotic stress such as heat, cold, drought and/or salinity. These genes were originally isolated from *Arabidopsis thaliana*.

The plant is a grass or cereal, such as crops or forage grasses including wheat, barley, sorghum,
35 maize, sugarcane, oats, rye, triticale, commercial fodder grass species and the like. In one

embodiment, the plant is wheat, a hexaploid monocotyledonous food crop. In another embodiment, the plant is sugarcane.

The gene is typically under the control of an inducible promoter, and in particular a drought inducible promoter, such as the *Rab17* promoter.

In an embodiment of the invention, the plant is transformed with only one of the genes, such as any one of *OTS1*, *OTS2* or *ICE1*. The nucleic acid encoding the *OTS1* gene can have a nucleotide sequence which is at least 80% identical to SEQ ID NO. 9, at least 80% identical to SEQ ID NO. 9, at least 90% identical to SEQ ID NO. 9, at least 95% identical to SEQ ID NO. 9 or may even be identical to SEQ ID NO. 9. The nucleic acid encoding the *OTS2* gene can have a nucleotide sequence which is at least 80% identical to SEQ ID NO. 11, at least 80% identical to SEQ ID NO. 11, at least 90% identical to SEQ ID NO. 11, at least 95% identical to SEQ ID NO. 11 or may even be identical to SEQ ID NO. 11. The nucleic acid encoding the *ICE1* gene can have a nucleotide sequence which is at least 80% identical to SEQ ID NO. 3, at least 80% identical to SEQ ID NO. 3, at least 90% identical to SEQ ID NO. 3, at least 95% identical to SEQ ID NO. 3 or may even be identical to SEQ ID NO. 3.

or at least 80% identical to SEQ ID NO. 11.

at least 80% identical to SEQ ID NO. 9

Alternatively, the plant can be transformed with two of the genes, such as *OTS1* and *ICE1, OTS2* and *ICE1* or *OTS1* and *OTS2*. More preferably, the plant is transformed with *OTS1* and *ICE1* or *OTS2* and *ICE1*.

The transformation is typically performed by way of particle bombardment. Preferred conditions under which the plants are transformed include:

- A circular plasmid is used;
- The whole plasmid is used rather than a minimal cassette;
- Transgenic DNA is delivered to the plant under a pressure of less than 90 kPa and more preferably at about 80 kPa; and
- After bombardment, the plants are subjected to osmoticum treatment on non-metabolisable osmotica including sorbitol and mannitol for a maximum period of 16 hours.

The invention will now be described in more detail by way of the following non-limiting examples.

Example 1: Transformation of wheat to enhance stress tolerance

Gene identification, vector construction and delivery

5

The most commonly used methods for wheat transformation include polyethylene glycerol (PEG)-mediated pollen tube pathway *Agrobacterium tumefaciens*-mediated or biolistics techniques (Yi *et al.*, 2015). However, the latter remains the most frequently used method globally. Biolistics, also referred to as particle bombardment (PB), has achieved stable expression of various 10 transgenes in wheat through the application of this technique (Southgate *et al.*, 1995; Rasco-Gaunt *et al.*, 1999; Folling, 2001; Liu *et al.*, 2011; Jackson *et al.*, 2013). It involves the use of a helium driven system which propels DNA-coated tungsten or gold particles into target tissue, leading to the establishment of mature transgenic plants. PB is relatively genotype-dependant, and for this reason various parameters need to be optimized (Lacock, 1990). These parameters 15 include explants pre- and post-treatment, DNA format and quantity, propellant force, target distance and helium pressure leading to effective DNA delivery. However, this technique is not without its pitfalls (Lacock, 1990; Southgate *et al.,* 1995), one of which is tissue damage and also the integration of complex trans-gene patterns, which ultimately lead to difficulties in subsequent analysis. Thus, prior to engineering wheat, regardless of the method of choice to introduce a 20 foreign gene, it is of great importance to fully establish the platform, which entails extensive optimization (Ziemienowicz, 2013). This section therefore addresses the optimization in the context of particle bombardment for transient expression. These results are then mimicked to obtain stable expression in wheat.

25 <u>Materials and methods</u>

Donor plant and explant isolation and initiation

Due to superior *in vitro* regeneration capabilities, the cultivars Gamtoos R (*Dn7*+) and Gamtoos 30 *S (Dn7-)* were chosen for the establishment of the engineering platform. The cultivars were grown in soil mixed with vermiculite and sand (ratio of 1:1:1) until maturity (3 months) in a glasshouse at 24ºC with natural light (Figure 1a-b). Caryopses were collected 12-16 days post-anthesis (Figure 1c) and surface sterilized for 15 minutes in 20% (v/v) commercial bleach (sodium hypochlorite) and 2 minutes in 70% (v/v) ethanol, containing 0.01% (v/v) Tween-20, and then rinsed with 35 sterilised distilled water. Immature embryos were extracted under sterile conditions with the aid of a binocular microscope. The scutella was removed from the immature embryo axis, and placed on callus-embryogenic tissue induction media (INDUC A). Explants were maintained for 4-5 days

at 25ºC in the dark on INDUC A media. A total of ±433 immature embryos were used and divided onto ten plates with a minimum of ±30 immature embryos per plate.

Tissue pre-treatment

5

Prior to bombardment, explants underwent turgidity treatments (osmoticum) by exposure to sorbitol (0.2 M) and mannitol (0.2 M), within basal MS medium (pH 5.6) (Southgate *et al.*, 1995; Rivera *et al.*, 2012). Subsequently, the most efficient turgidity treatment was optimised with respect to the duration of exposure (3, 4 or 16 hours, respectively).

10

Plasmid construct and purification

Transient expression and PB optimization

A pGFP 510b (6167 bp) vector containing the green fluorescent protein gene (*gfp*) under the
15 control of a maize ubiquitin promoter and cauliflower mosaic virus terminator (CaMV-t) was used to assess the bombardment parameters (Figure 2). The plant expression vector was transformed into *Escherichia coli* (DH5α), followed by large-scale purification of plasmid DNA according to the manufacturer's instructions of the GenEluteTM Plasmid Midiprep Kit (Sigma-Aldrich, SA). The circular plasmid DNA concentrations were adjusted to 1 µg/µl and used in all downstream
20 applications. Assessment of DNA format (e.g. minimal cassette and whole plasmid), for efficient transient expression was evaluated. The minimal cassette was prepared with two restriction enzymes, i.e. *HindIII* and *KpnI,* to remove the vector backbone, resulting in a fragment containing only the ubiquitin promoter, reporter gene (*gfp*) and nos terminator. The digest was separated on a 1% agarose gel, and the appropriate fragment (3015 bp) was isolated and purified with the
25 GeneJET Gel Extraction Kit according to manufacture protocol (Thermo Scientific, Inqaba, South Africa).

Particle bombardment

Bombardment was done as described by Indra *et al.* (2006) with modifications. Briefly, all DNA
30 deliveries were done via a locally engineered particle inflow gun. Tungsten particles (M10 Bio-Rad, South Africa) were sterilized with absolute ethanol and washed 3 times and re-suspended in sterile water, followed by the simultaneously addition of 2.4 M $CaCl_2$ and 0.1 M spermidine, vortexed and placed on ice. The aliquots of tungsten suspension were vortexed with 1 µg/ul concentrations of DNA. Incubation occurred at 4ºC, followed by the removal of 100 µl of the
35 aqueous layer prior to bombardment. For the bombardment, explants were clustered together (still exposed on turgidity treatment (osmoticum)), and placed 13 cm below the particle expelling tip, covering a 2 cm diameter circle and enclosed with a metal grid. The particle tip containing 1

mm^2 metal grid was loaded with 5 µl of the DNA suspensions. Air within the chamber was relinquished, until 80 kPa and 90 kPa, and the suspensions (DNA) were expelled when helium (1000 kPa) was released by a timer relay (0.05 s) 16 hours after bombardment, and the current media (turgidity treatment) was replaced with INDUC A media and maintained at 24ºC in the dark
5 for 3-4 weeks.

Regeneration and selection

At 5 weeks, embryogenic material was transferred to regeneration media for shoot formation, under a photoperiod of 16 h/8 h (day/night) at 24ºC. This was also the selection phase, as the
10 regeneration media (RDZA) also contained the antibiotic geneticin (45 mg/l) (note that selection started in either the first or second round of regeneration and continued in subsequent rounds). The selection phased lasted for 4-5 weeks. Plantlets that did not have adventitious roots were transferred to magentas containing either half or full strength MS media (Murashige and Skoog, 1962) until roots formed.
15

Ex vitro acclimatization

In vitro generated plantlets were hardened off in closed glass or plastic containers in a greenhouse at 28ºC. Over time the containers were partially opened to allow acclimatization into the bigger, drier greenhouse environment. Plantlets were then regularly irrigated to ensure moist
20 soil conditions.

Transient expression using GFP

Three to four days after bombardment, explants were assessed for transient GFP expression using a florescence microscope with special GFP filters, to eliminate the auto florescence. The
25 fluorescence microscope was connected to an imaging system (Leica DC 200) to capture the image. The number of total cells per explant per plate, permitting the expression, was counted.

Results

30 Several experiments were conducted to assess four parameters that could lead to efficient transient *gfp* expression of immature embryos after bombardment. These parameters included the DNA format, concentration and pressure settings used during bombardment, and the osmoticum treatments of tissue pre and post-bombardment. A single wheat cultivar, Gamtoos *S*, was chosen due to its superior *in vitro* regeneration capabilities for all bombardment experiments.
35 Whole plasmid (WP) and minimal cassette (MC) were assessed in combination with other parameters as listed in Table 1. The highest *gfp* expression was observed when pGFP was kept as a circular plasmid. The weakest transient expression was seen when pGFP was linearized.

MC (0.5-1 µg/µl) in combination with WP (0.5 µg/µl -3 µg/µl) was not as effective as WP (5 µg/µl) on its own. WP (5 µg/µl) resulted in a transformation efficiency of between 43.3% and 46.6%, with an osmoticum treatment lasting maximally for 16 hours, and helium pressure at 80 kPa (Table 1; Figures 3 and 4). Although only transient GFP expression was assessed, these results served as the foundation in subsequent experiments for stable gene transfer and expression.

Table 1: Factors affecting the bombardment of 2-6 day old immature embryos, for transient GFP expression

Induction A											
Experiment	**pGFP** (µg/ul)		**pEmuKN** (µg/ul)	**Pressure (kPa)**	**Embryos amount**		**Osmoticum duration (hours)**	**Total embryos with GFP**	**GFP foci****		**TTE**
	Linear	Circular	Circular		1mm	3mm			1mm	3mm	
1	1		3	90	30	30	3	--	--	--	0%
2	---	1	1	80	73	--	4	1	11		1.37%
3	---	5	5	80	30	30	16	14	229	--	46.6%
4	0.5	---	0. 5	90	30	30	16	1	1	--	3.3%
5	1	---	3	80	30	30	3	7*	10	--	23.3%
6	---	5	5	90	30	30	16	13	149	--	43.3%
7	0.5	---	0.5	80	30	30	16	4*	20	0	13.3%

**There is the possibility for human error during counting the foci, thus transient-transformation efficiency (TTE) is an approximation

Discussion

Particle bombardment is the most widely used method for transformation of cereal crops (Southgate *et al.*, 1995; Rasco-Gaunt *et al.*, 1999; Harwood *et al.*, 2000; Rivera *et al.*, 2012). However, optimization of parameters is crucial to improve on the widely published 1% transformation efficiency rate of wheat (Li *et al.*, 2012). Transient GFP expression in Gamtoos allowed for the effective optimization of bombardment variables in this particular study. Since there are many parameters that can influence the transformation efficiency of wheat, those that are believed to be the most influential were evaluated when optimizing bombardment parameters.

Distance of micro-carrier to explant and embryo size made no significant difference in the transient GFP expression profile. However, pressure exerted on explants when delivering DNA had a significant difference, e.g. with the higher pressure (90 kPa) showing less GFP expression (149 foci). This might be due to tissue/cell damage (Folling, 2001). Using a pressure of 80 kPa
5 resulted in much higher GFP expression levels (229 foci). This finding is supported by reports from Rasco-Gaunt *et al.* (1999). Low pressure is associated with uniform distribution of GFP expression, with substantial reduction in tissue shock and damage (Finer and McMullen, 1991).

Furthermore, DNA format, i.e. the use of minimal cassettes (MC), is often a requirement for
10 commercialized GM products (Vianna *et al.*, 2011). Multiple studies support the use of minimal cassettes to obtain transgenic monocot plants such as rice (Loc *et al.*, 2002). However, with the investigation of DNA format, the results show that the use of a MC resulted in less transient GFP expression (0%-23.3%) in contrast to WP (1.3%-46.6%). DNA format is also closely linked to the DNA concentration used for bombardment (Jackson *et al.*, 2013). 5 µg/µl of circular DNA resulted
15 in the highest transient *gfp* expression (Table 1; Figures 3 and 4).

The final parameter assessed was the osmoticum treatment after bombardment. This is crucial since it minimises cytoplasm leakage from target cells, preventing the loss of DNA that has penetrated the bombarded cells. The results show that post-conditioning of explants for a
20 maximum of 16 hours on non-metabolisable osmotica such as mannitol and sorbitol leads to effective plasmolysis, which is also supported by Indra *et al.* (2006). Although only transient *gfp* expression was evaluated, the purpose of this particular study was not to obtain stable transgenic plants.

25 ***Transformation using SUMO targets***

Gene identification and vector construction

Arabidopsis thaliana seeds were growth in a growth chamber under controlled humidity and light. At 4 weeks leaf material was harvested and total RNA isolated and quantified (ng/µl). RNA was
30 then used as template for cDNA synthesis using First-strand cDNA Synthesis Kit, and the integrity of the cNDA was confirmed by amplifying a house-keeping gene known as actin. Bioinformatics tools were applied to receive information regarding the gene ontology (OTS1:AT1G60220 (SEQ ID NO. 4, Figure 12), OTS2:AT1G10570 (SEQ ID NO. 6, Figure 14) and ICE1: AT3G26744.4 (SEQ ID NO. 5, Figure 13), which was used during primer design. All primers were diluted
35 according to manufacturer's recommendation (Inqaba Biotec). With extensive optimization all three genes were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Gel electrophoresis confirmed the correct size fragments (Figure 5), which were then excised using a

GenElute Gel Extraction Kit and quantified (ng/µl). Fragments were independently cloned into a storing vector known as *pJET1.2/blunt* and heat-shock transformed into *E.coli* DH5α, followed by a colony PCR using the gene specific primers to confirm gene presence. GenElute Plasmid Miniprep Kit was done on positive clones followed by sequencing at a local sequencing unit (CAF)
5 which confirmed 100% identity to the corresponding genes on "*The Arabidopsis Information Resource* (*TAIR*)" database.

Since nBLAST results confirmed that *OTS1, OTS2* and *ICE1* had indeed been amplified and isolated, these genes were excised (double enzymatic digest) from storage vectors (pJect::ots1,
10 pjet::ots2 and pjet::ICE), blunted and phosphorylated for ligation into a prepared plant expression vector, known as pUbi510, under the control of a well characterized constitutive ubiquitin promoter and CamV-t terminator (Figure 6). The plant expression vector required further preparation, which entailed a single blunt-end digest (SmaI) to linearize the vector followed by dephosphorylation. After successfully ligating the genes independently into pUbi510, each construct was heat-shock
15 transformed into *E.coli* DH5α, followed by a colony PCR to confirm the 5'-3' orientation of each gene. Again, sequencing confirmed the aforementioned. This resulted in three constitutive constructs known as a) Pubi510::OTS1, b) Pubi510::OTS2, and c) Pubi510::ICE1.

According to literature regarding *ICE1*, constitutive expression of this gene could potentially result
20 in a metabolically skewed plant with severely retarded growth. This is of particular concern in the context of the present study as this could affect the yield of wheat and other cereals or grasses. However, this has not been confirmed in a hexaploid species such as wheat. It was hypothesised that placing *ICE1* in a vector under the control of a drought inducible promoter could not only avoid yield penalty, but increase the plant abiotic fitness (drought, heat, cold, etc). The promoter
25 *Rab17* (Accession: x15994.1) from maize was selected, as data supports that this drought inducible promoter contains all necessary *cis*-elements and is not leaky in hexaploids.

Bioinformatics tools were again applied, where the entire gene (1989 bp) was examined, gDNA was isolated from four week old maize callus, followed by High-Fidelity nested amplification of
30 the *Rab17* promoter region (45…-654bp)(Figure 7), and again this was cloned into a storage vector for glycerol stock and sequencing purposes. The identity (100%) of *Rab17* was confirmed on NCBI. The vector pAHC20 (Figure 8) was then modified by removing its endogenous promoter (ubiquitin) by single restriction digest (XbaI), blunting and dephosphorylating, and replacing it with the phosphorylated *Rab17*. The construct was transformed into *E.coli* followed by colony PCR,
35 which confirmed pAHC20::Rab17 and its 5'-3' orientation. This plasmid was further manipulated by removing its reporter gene (*Bar*) through double digestion (BamH I) and dephosphorylation. Finally the *ICE1* gene was phosphorylated and ligation set up overnight (pAHC20::Rab17::ICE).

After successful ligation, the construct was heat-shock transformed into *E.coli* DH5α, followed by a colony PCR to confirm RAB17:ICE 5’-3’ orientation in pAHC20 (Figures 9 and 10). Sequencing confirmed the aforementioned orientation.

5 Six vectors (Pubi510:OTS1, Pubi510:OTS2, Pubi510:ICE1, pAHC20:RAB17:ICE1, Pubi510 (empty-control) and pEmuKN) were transformed into *Escherichia coli* (DH5α) followed by large-scale purification according to the manufacturer’s instructions of the GenEluteTM Plasmid Midiprep Kit (Sigma-Aldrich, SA). Note that pEmuKN is a selection vector which houses the selection gene known as *npt*II. All plasmid DNA concentrations were adjusted to 1 µg/µl (the 10 aforementioned concentration was determined as describer earlier herein).

Characterization of the transgene and modified plant

Verifying stable expression in transformants and progeny

15 Plants were firstly assessed through amplification of the sequences of the vector boundaries of the inserted constructs using PCR. If PCR amplicons were produced (and thus sequences are present in the genomic DNA of the samples), then an insertion event was confirmed. Secondly, the expression of the gene inserted into the tissue was quantified using RT-qPCR. Again, primer sequences specific to the gene of interest were applied. However, in this instance, RNA was 20 extracted and cDNA (complementary-DNA) was synthesized, which were then used as template for the amplification of products.

Transgene confirmation

T_0 generation plants were analyzed for the presence of transgene using specific primers. 25 Genomic DNA was isolated from fresh 4 leaf stage transformed and non-transformed (NT) plants using Fermentas Gene JET Plant Genomic DNA Purification Mini Kit (#k0791) according to the manufacturer’s instructions. Each PCR reaction had approximately 150 ng of the genomic DNA template, 10 mM of dNTP, 10 µl of 5xGreen GoTaq buffer reaction buffer® (containing 1.5 mM $MgCl_2$), GoTaq® DNA Polymerase (5 µ/ul). One primer was promoter-specific (Ubi-exp: 5′-30 ATACGCTATTTATTTGCTTGG-′3) (SEQ ID NO. 7) and the second primer gene-specific 5’-CTGGTTTACTCTGTCTGGTCACT-3’ (*OTS1-REV*) (SEQ ID NO. 8). PCR was initiated by denaturation at 95 ℃, for 3 min followed by 35 cycles of 1 min at 95 ℃ (denaturation), 30s at 60 ℃ (annealing) and 72 ℃ for 2 min (extension), ending off with 1 cycles of 72 ℃ for 10 min (final extension). Sample was loaded with appropriate controls and was subjected to electrophoresis 35 on 1% Agrose gel at 90V/aM for 40 min.

Enzymatic activity: Assaying reactive oxygen species (ROS) and pathogenesis related (PR) protein activity

To verify the potential biotic and abiotic stress tolerance, selected marker protein activities (i.e., peroxidise, GST and β-1,3-glucanase) were assayed in some of the transgenic plants. The T_1 generation plants were assayed for ROS and PR protein activities using the method as previously described by Botha *et al.* (2014).

Results

DNA was extracted from T_1 generation plants and genomic DNA used as template to confirm transgene insertion in the putative transgenic plants. Five plants tested positive for pUBI:*OTS1* with fragment size of 1700 bp, two plants for pUBI:*OTS2* with fragment size of 1700 bp, and one for pUBI:*ICE1* with fragment size of 1500 bp (one pUBI:*OTS1* plant died) (Figures 16 to 18). The transgenic plants pUBI:*OTS1* and pUBI:*OTS2* plants displayed normal phenotypes. However, the transgenic pUBI:*ICE1* plant displayed stunted growth (Figures 17 and 18).

Functional characterisation of these plants revealed enhanced increased expression of reactive oxygen species (i.e., peroxidase and GTS activity) and pathogenesis related (PR, i.e., β-1,3-glucanase) protein activity (Figures 19-21) in transgenic plants when compared to untransformed control wheat plants, suggesting increased abiotic and biotic stress tolerance in these transgenic plants.

Example 2: Transformation of sugarcane to enhance stress tolerance

Sugarcane *in vitro* regeneration

Materials and methods

Plasmids

Gene inserts (as described above) were cloned into the multiple cloning site of plant expression vector, pUbi510 (Figure 6). This vector contained an ubiquitin promoter and a 35S cauliflower mosaic virus terminator sequence. A second 5.6 kB size plasmid, pEmuKN, contained the *npt*II coding sequence driven by the strong monocot Emu promoter sequence, and nos terminator (Figure 6), allowing efficient selection on geneticin (Last *et al.*, 1990).

Explant material and culture conditions

In order to produce embryonic callus, the basal part of sugarcane (*Saccharum* spp. hybrids) cultivar NCo310 leaf roll, just above the apical meristem, was excised, cut into 2 mm thick transverse sections and placed on MS medium (Murashige and Skoog, 1962; Highveld Biological,
5 South Africa [SA]) containing 2% (*w/v*) sucrose, 3 mg/l 2,4-D, 0.5 g casein, and 0.22% (*w/v*) gelrite (Sigma-Aldrich, SA), pH 6.0 (MS3). Cultures were incubated for 6 to 8 weeks at 24℃ in the dark and placed on fresh medium every two weeks.

Transformation via particle bombardment

10

Materials and methods

Plant transformation

A particle inflow gun, constructed locally, was used for all DNA deliveries. Bombardment was
15 done as described by Franks and Birch (1991) with modifications. Tungsten particles (5 mg; M10; Biorad, CA) were sterilized with 100% ethanol, vortex and rinsed three times with sterile water and re-suspended in 50 µl sterile water. The tungsten suspension was mixed with 10 µl plasmid DNA (5 µg of each plasmid), 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. The mixture was incubated on ice and 100 µl of the aqueous layer removed prior to plant explant bombardment.
20 For bombardment, 5 µl of the particle suspension was placed in the centre of a 1 mm^2 metal grid above the explants. Target tissue was placed 16.5 cm below the particle source and covered with a metal grid. The chamber was set under a vacuum of 80 kPa and the particles were discharged when helium, 1000 kPa, was released by a timer relay, 0.05 sec.

25 Harvested embryogenic sugarcane calli clumps (on average 3-4 mm diameter a piece) were stacked closely together in a circle, with a size of 3 cm diameter, on osmoticum medium consisting of the basal MS3 medium with 0.2 M each of sorbitol and mannitol 4 h prior to and after bombardment (Vain and McMullen, 1993). After bombardment, the plant tissue was maintained on MS3 medium for 3 days before selection started on 45 mg/l geneticin (Sigma, UK) for 8-10
30 weeks in the dark as outlined by Bower and Birch (1992). On average ten plates of embryogenic callus circle portions were bombarded for each transgene. After selection, newly regenerated calli, surviving selection, were placed on MS0 medium, lacking 2.4-D for 8 weeks until roots has formed. Regeneration was done in a growth room at 26℃ in a 16/8 light/dark regime. Plantlets, 5 cm high with roots (Figure 15), were hardened off in a mix of potting soil, sand and vermiculite
35 at a ratio of 2:1:1 in pots in the glasshouse.

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CLAIMS:

1. A method for producing a transformed plant of the family Poaceae, the method comprising the step of introducing at least one nucleic acid encoding a gene selected from the group consisting of *OTS1*, *OTS2* and *ICE1* into the genome of the plant, wherein the nucleic acid has a sequence which is at least 80% identical to SEQ ID NO. 3, SEQ ID NO. 9 or SEQ ID NO. 11, respectively, and wherein transformed plant has enhanced tolerance to an abiotic stress compared to an untransformed plant.

2. A method according to claim 1, wherein the gene is under the control of a drought inducible promoter.

3. A method according to claim 2, wherein the promoter is the *Rab17* promoter (SEQ ID NO. 2).

4. A method according to any one of claims 1 to 3, wherein the plant is a cereal or grass.

5. A method according to claim 4, wherein cereal or grass is selected from the group consisting of wheat, barley, sorghum, maize, sugarcane, oats, rye, triticale and fodder grass species.

6. A method according to claim 5, wherein the plant is wheat.

7. A method according to claim 5, wherein the plant is sugarcane.

8. A method according to any one of claims 1 to 7, wherein the abiotic stress is drought.

9. A method according to any one of claims 1 to 7, wherein the abiotic stress is heat.

10. A method according to any one of claims 1 to 7, wherein the abiotic stress is cold.

11. A method according to any one of claims 1 to 7, wherein the abiotic stress is salinity.

12. A method according to any one of claims 1 to 11, wherein the gene is *OTS1* and the sequence of the introduced nucleic acid is at least 80% identical to SEQ ID NO. 9.

13. A method according to claim 12, wherein the sequence of the introduced nucleic acid is identical to SEQ ID NO. 9.

14. A method according to any one of claims 1 to 11, wherein the gene is *OTS2* and the sequence of the introduced nucleic acid is at least 80% identical to SEQ ID NO. 11.

15. A method according to claim 14, wherein the sequence of the introduced nucleic acid is identical to SEQ ID NO. 11.

16. A method according to any one of claims 1 to 11, wherein the gene is *ICE1* and the sequence of the introduced nucleic acid is at least 80% identical to SEQ ID NO. 3.

17. A method according to claim 16, wherein the sequence of the introduced nucleic acid is identical to SEQ ID NO. 3.

18. A vector for transforming a plant so as to provide the transformed plant with enhanced tolerance to an abiotic stress compared to an untransformed plant, the vector comprising at least one nucleic acid encoding a gene selected from the group consisting of *OTS1*, *OTS2* and *ICE1* under the control of a drought inducible promoter, wherein the nucleic acid has a sequence which is at least 80% identical to SEQ ID NO. 3, SEQ ID. NO 9 or SEQ ID NO. 11, respectively.

19. A vector according to claim 18, wherein the drought inducible is the *Rab17* promoter (SEQ ID NO. 2).

20. A transformed plant or plant part produced according to the method of any one of claims 1 to 17.

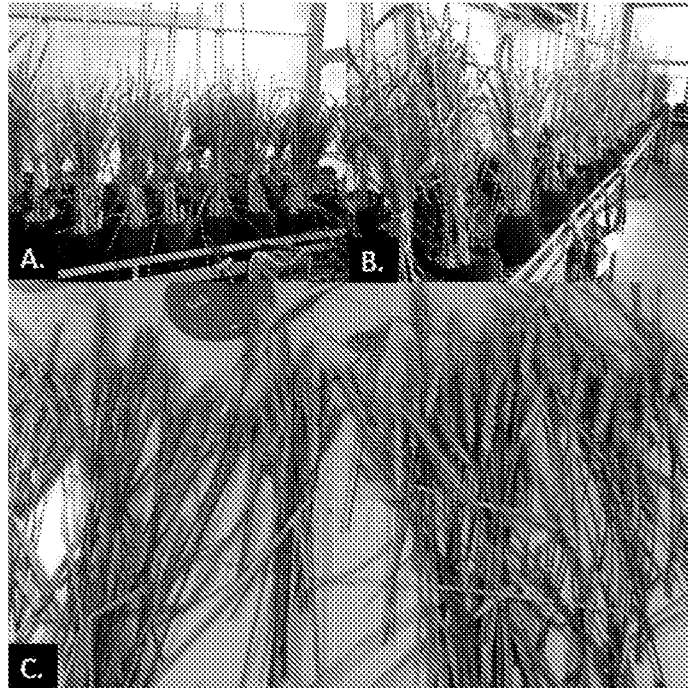

Figure 1

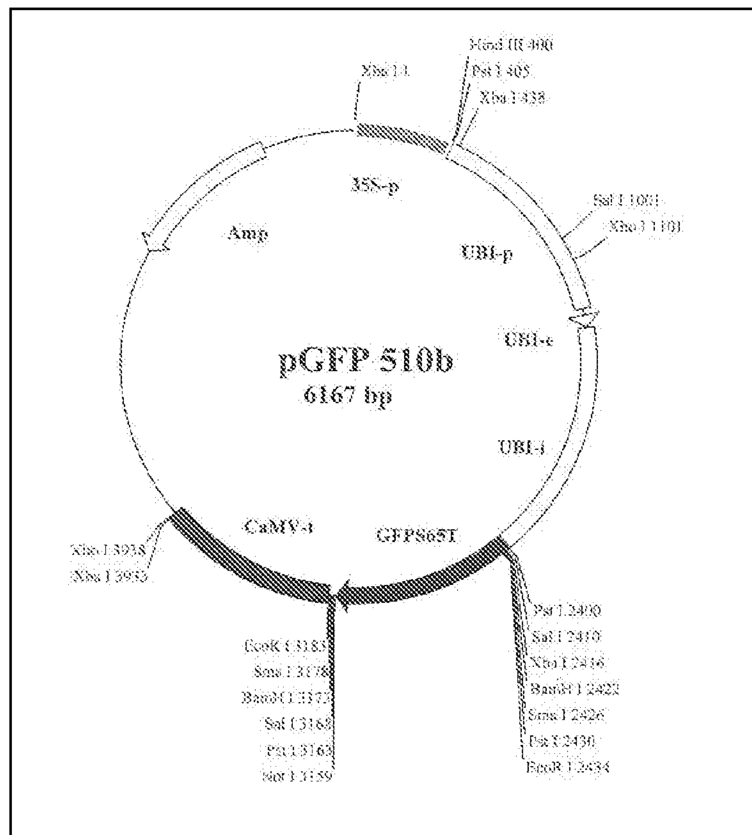

Figure 2

GTTGCCGTACTGGTCGACATGGCCCGTGGCGCCGTGGCCGCGCTGCCCCTGCTGACCGTACTCCATGA
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CAGAACGGCGAAAGGATTAGGATCTGGGCGCGCCACGGCAGGGGTGTACGTGGGCATGCATGAGTGGA
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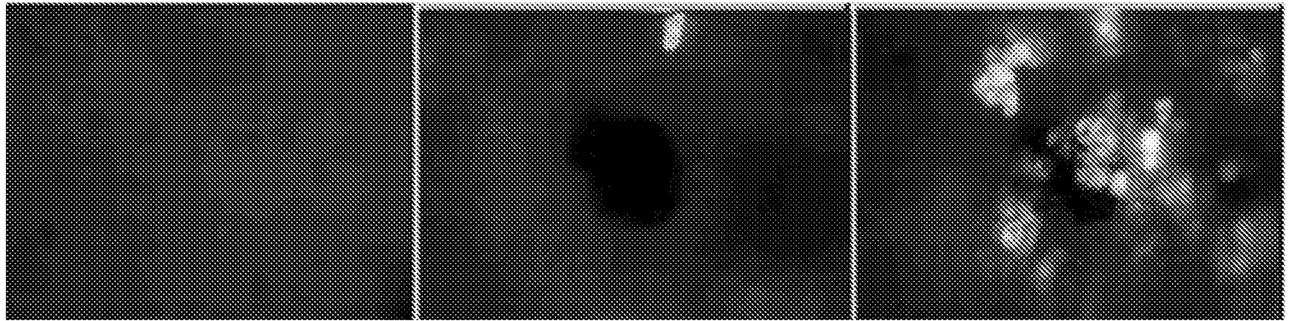
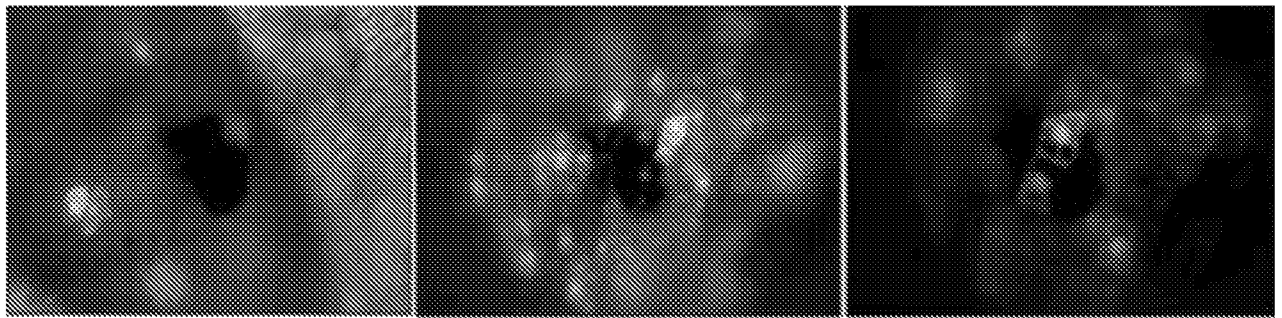
Figure 3

Experiment 1 **Experiment 2** **Experiment 3**



Experiment 4 **Experiment 5** **Experiment 6**



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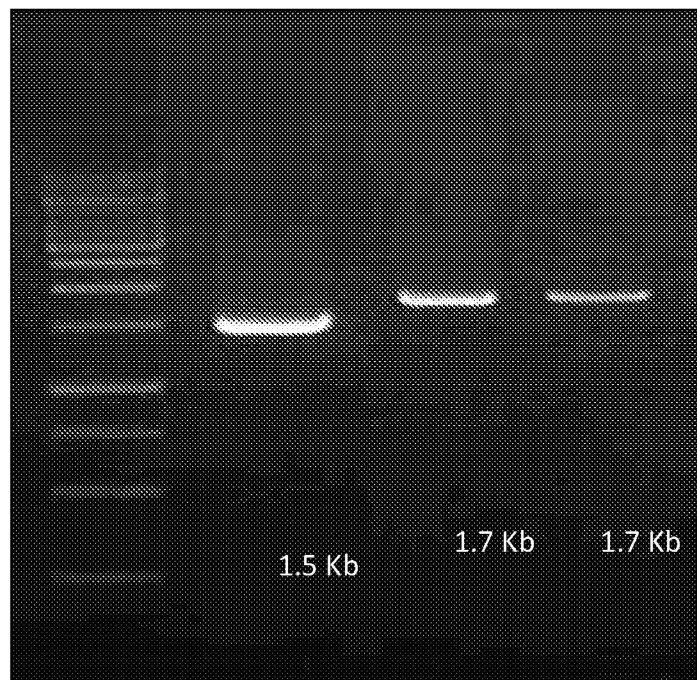

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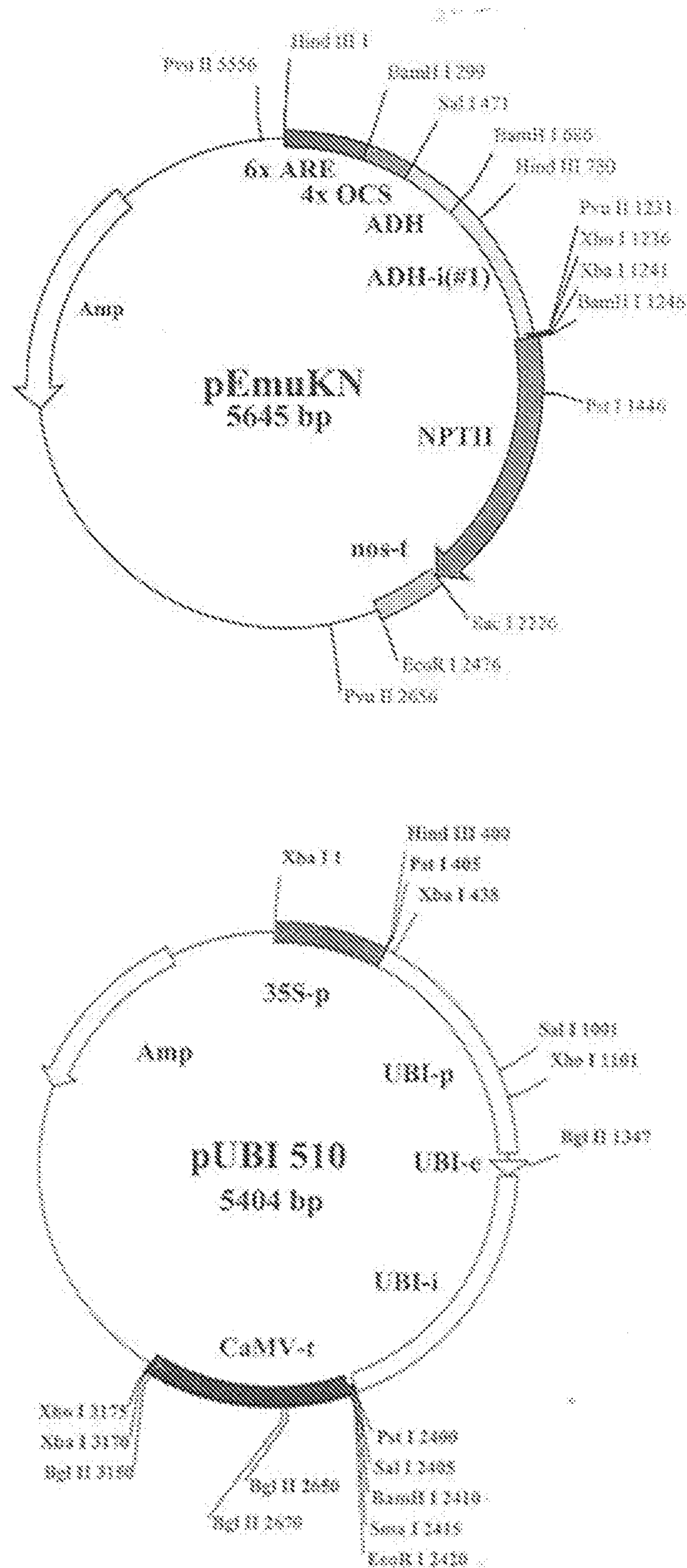

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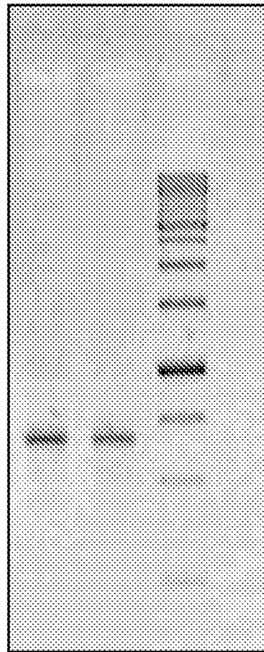

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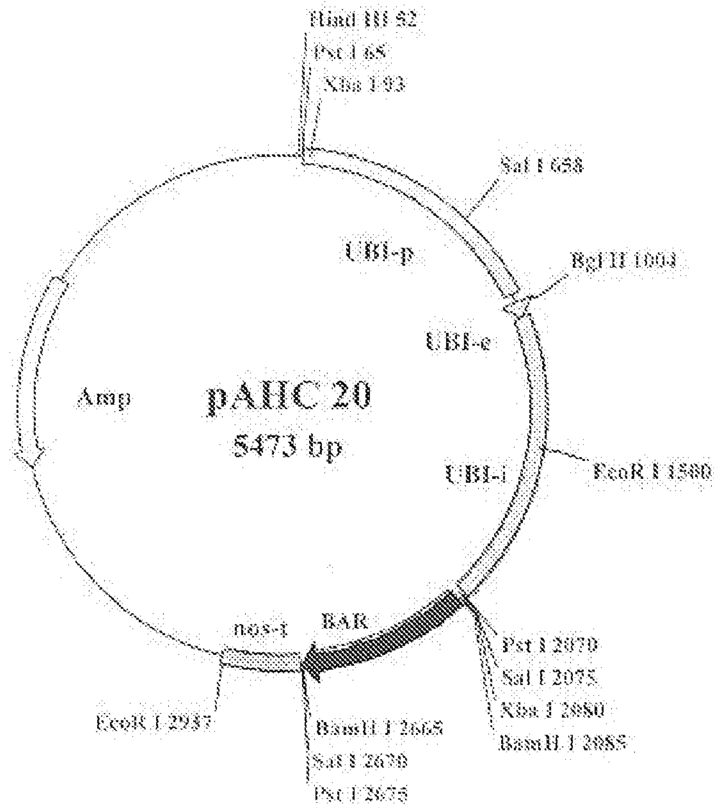

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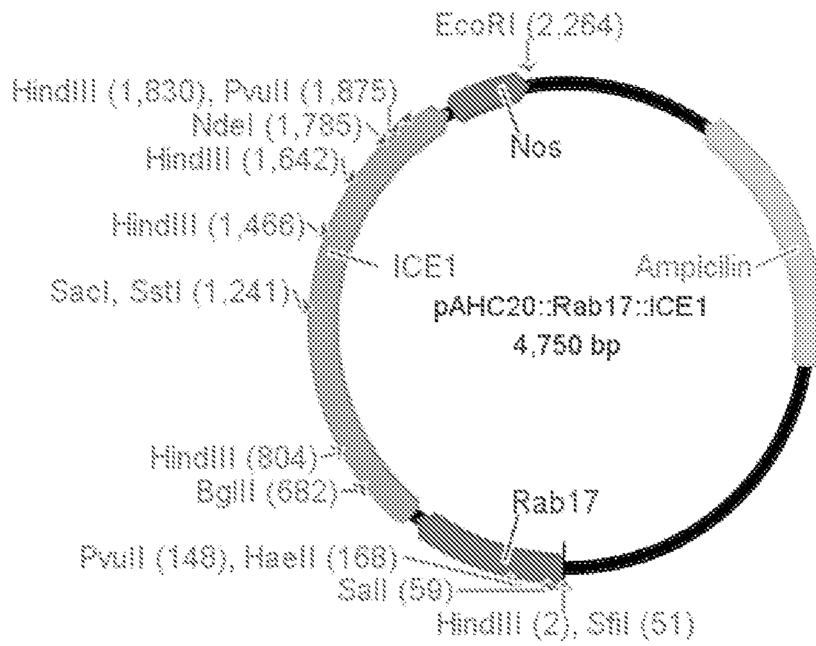

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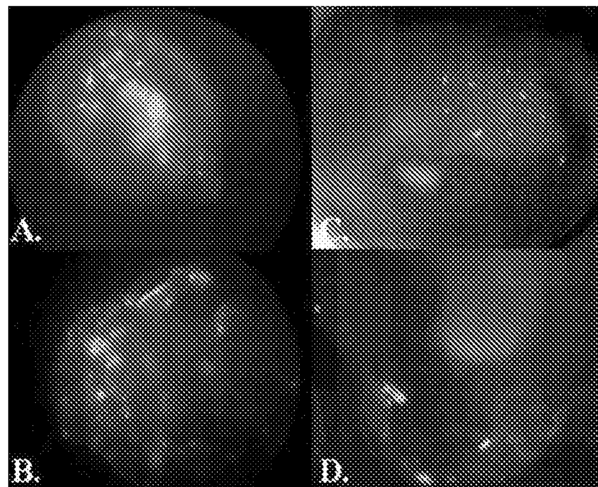

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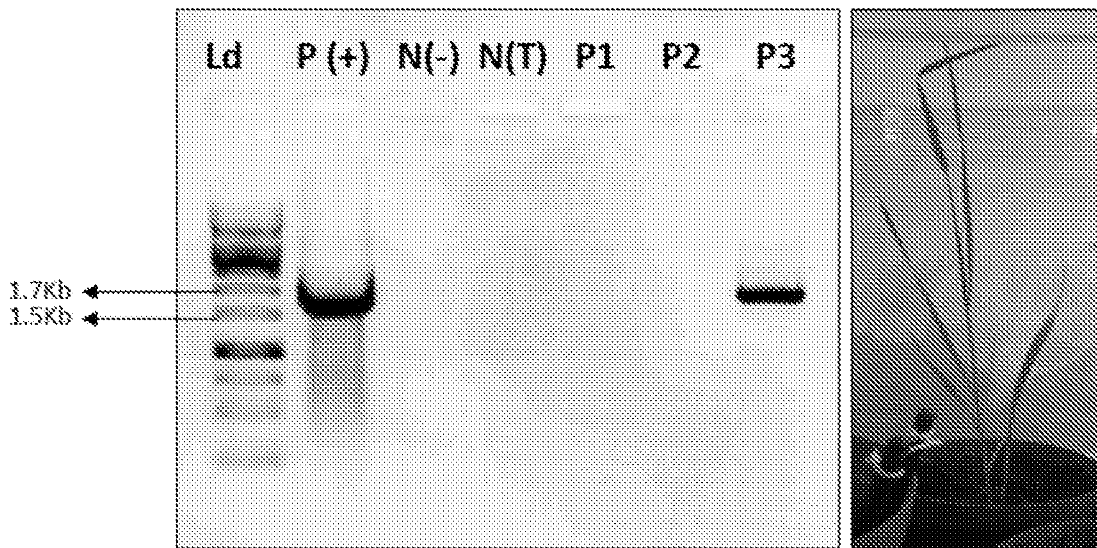

Figure 11

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Figure 12

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Figure 13

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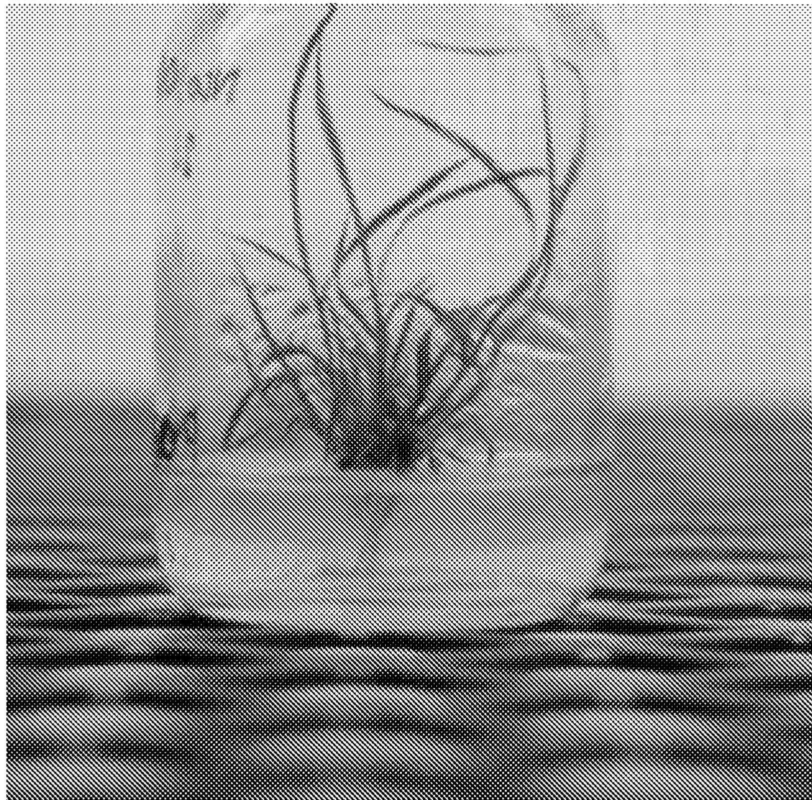

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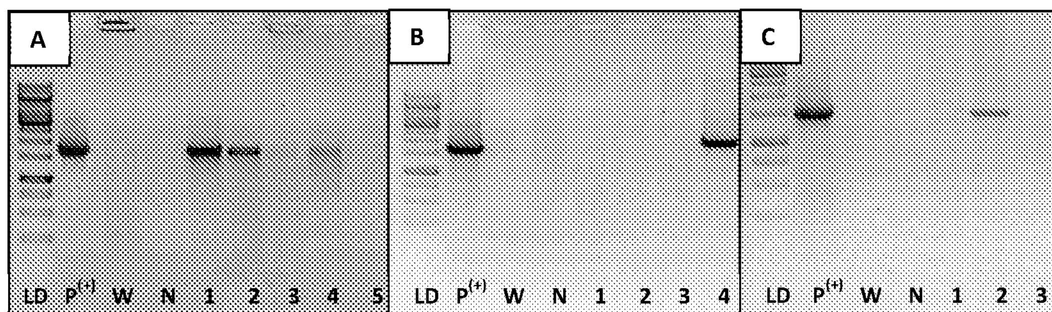

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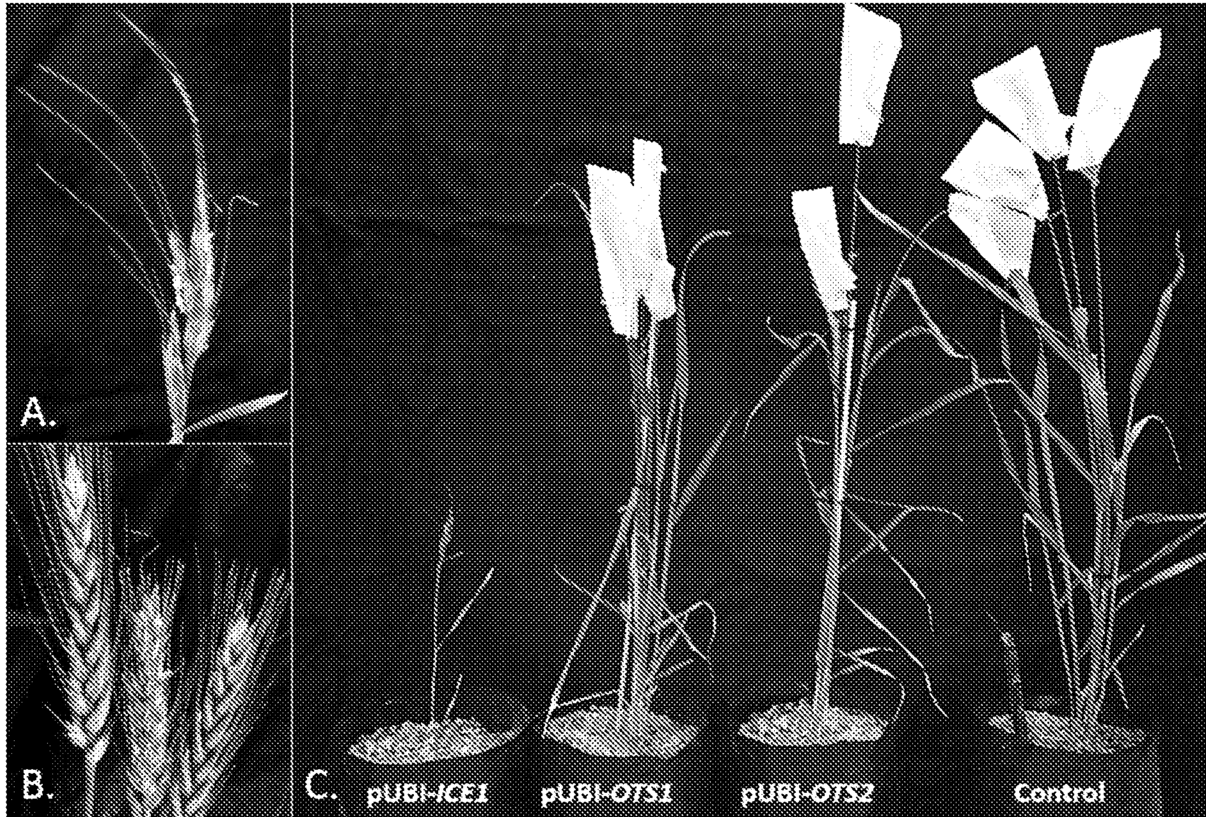

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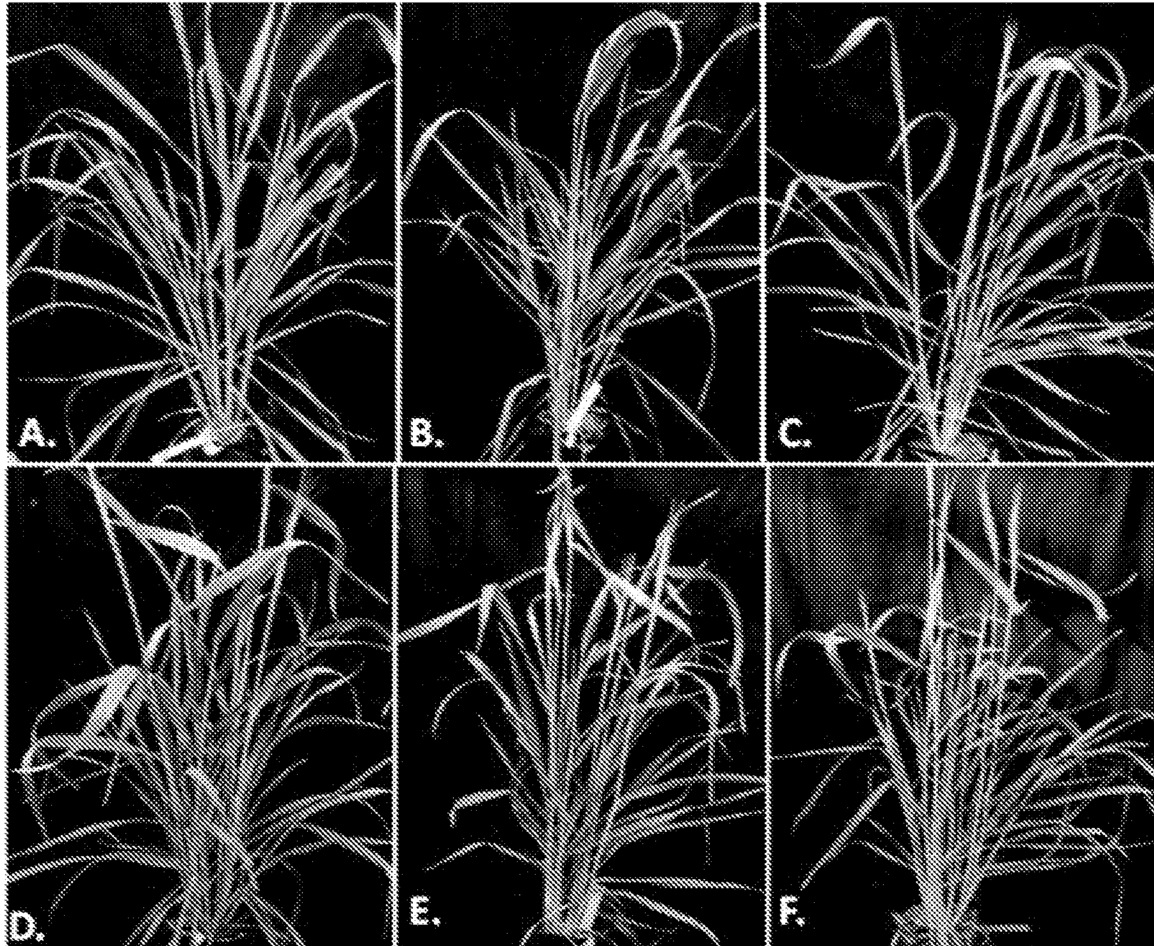

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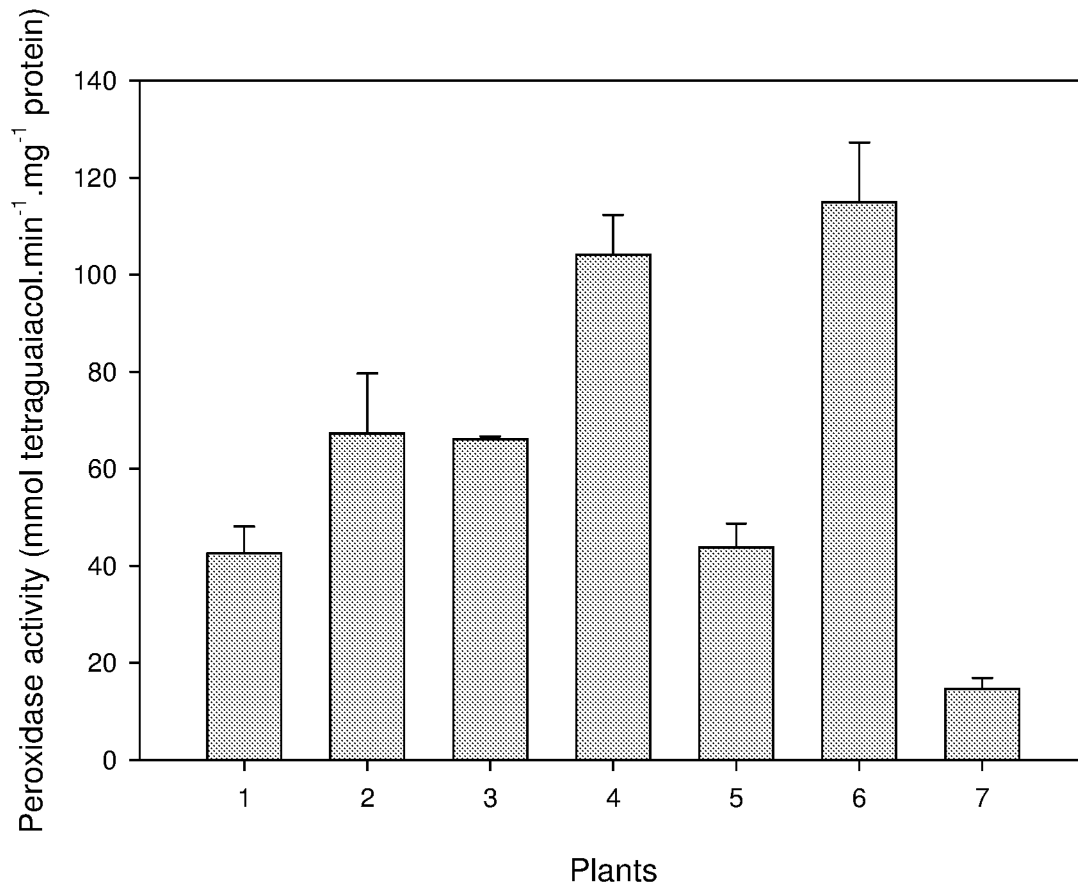

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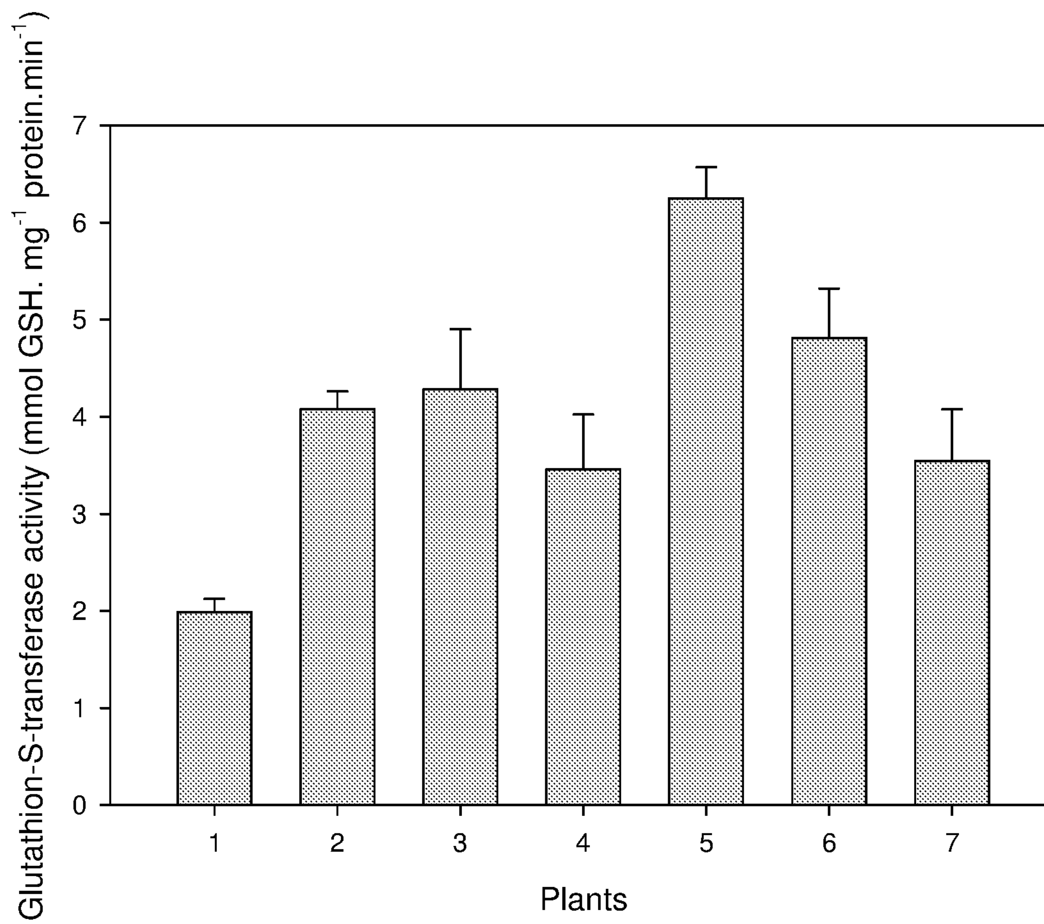

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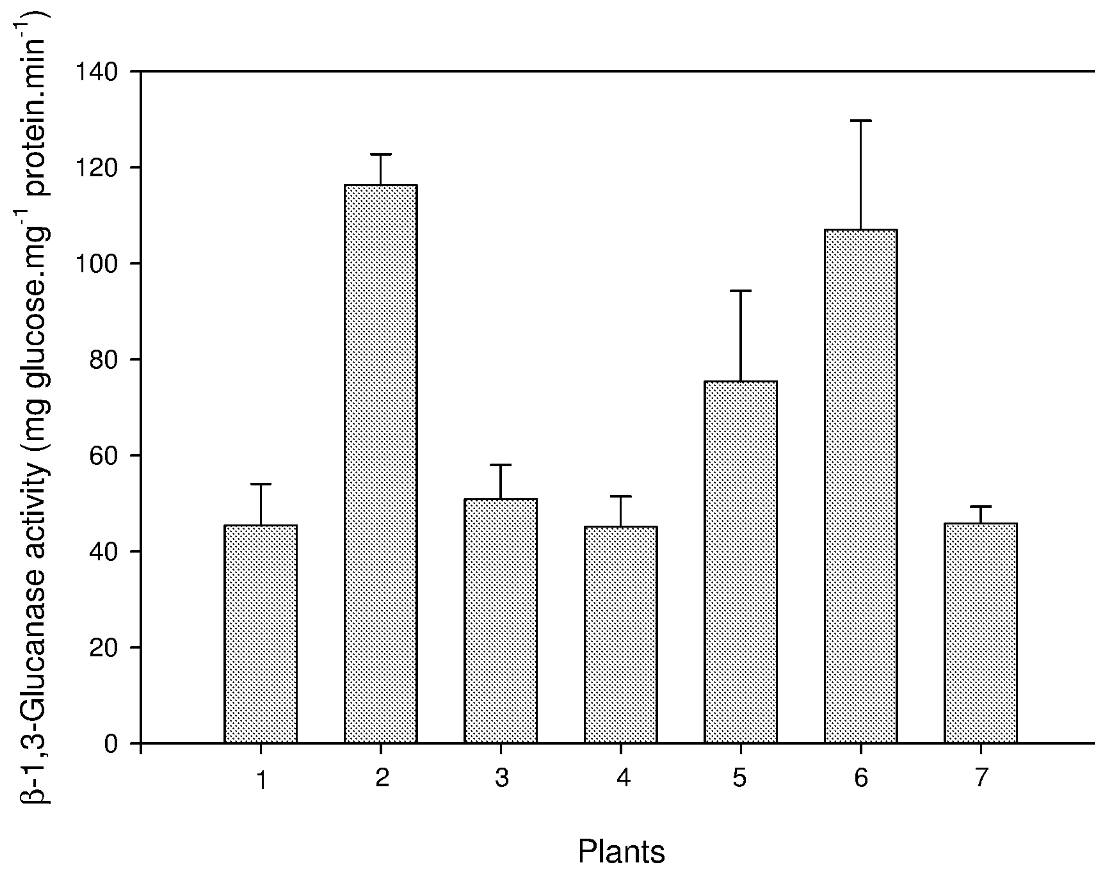

Figure 21