(12) STANDARD PATENT (11) Application No. **AU 2017248552 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Regulatory nucleic acid molecules for enhancing constitutive gene expression in plants

(51) International Patent Classification(s)
C12N 15/82 (2006.01) *A01H 5/00* (2006.01)

(21) Application No: **2017248552** (22) Date of Filing: **2017.10.20**

(43) Publication Date: **2017.11.09**
(43) Publication Journal Date: **2017.11.09**
(44) Accepted Journal Date: **2019.07.11**

(62) Divisional of:
2016202274

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(56) Related Art
WO 2007/107516 A2

2017248552 20 Oct 2017

Abstract

The present invention is in the field of plant molecular biology and provides methods for production of high expressing constitutive promoters and the production of plants with enhanced constitutive expression of nucleic acids wherein nucleic acid expression enhancing nucleic acids (NEENAs) are functionally linked to said promoters and/or introduced into plants.

2017248552 20 Oct 2017

Regulatory nucleic acid molecules for enhancing constitutive gene expression in plants

The entire disclosure in the complete specification of our Australian patent application no. 2016202274 is by this cross-reference incorporated into the present specification.

Field of the Invention

The present invention is in the field of plant molecular biology and provides methods for production of high expressing constitutive promoters and the production of plants with enhanced constitutive expression of nucleic acids wherein nucleic acid expression enhancing nucleic acids (NEENAs) are functionally linked to said promoters and/or introduced into plants.

Background

Expression of transgenes in plants is strongly affected by various external and internal factors resulting in a variable and unpredictable level of transgene expression. Often a high number of transformants have to be produced and analyzed in order to identify lines with desirable expression strength. As transformation and screening of lines with desirable expression strength is costly and labor intensive there is a need for high expression of one ore more transgenes in a plant. This problem is especially pronounced, when several genes have to be coordinately expressed in a transgenic plant in order to achieve a specific effect as a plant has to be identified in which each and every gene is strongly expressed.
For example, expression of a transgene can vary significantly, depending on construct design and positional effects of the T-DNA insertion locus in individual transformation events. Strong promoters can partially overcome these challenges. However, availability of suitable promoters showing strong expression with the desired specificity is often limited. In order to ensure availability of sufficient promoters with desired expression specificity, the identification and characterization of additional promoters can help to close this gap. However, natural availability of promoters of the respective specificity and strength and the time consuming characterization of promoter candidates impedes the identification of suitable new promoters.

In order to overcome these challenges, diverse genetic elements and/or motifs have been shown to positively affect gene expression. Among these, some introns have been recognized as genetic elements with a strong potential for improving gene expression. Although the mechanism is largely unknown, it has been shown that some introns positively affect the steady state amount of mature mRNA, possibly by enhanced transcriptional activity, improved mRNA maturation, enhanced nuclear mRNA export and/or improved translation initiation (e.g. Huang and Gorman, 1990; Le Hir et al., 2003; Nott et al., 2004). Since only selected introns were shown to increase expression, splicing as such is likely not accountable for the observed effects.

The increase of gene expression observed upon functionally linking introns to promoters is called intron mediated enhancement (IME) of gene expression and has been shown in various monocotyledonous (e.g. Callis et al., 1987; Vasil et al., 1989; Bruce et al., 1990; Lu et al., 2008) and dicotyledonous plants (e.g. Chung et al., 2006; Kim et al., 2006; Rose et al., 2008). In this respect, the position of intron in relation to the translational start site (ATG) was shown to be crucial for intron mediated enhancement of gene expression (Rose et al., 2004).

Next to their potential for enhancing gene expression, a few introns were shown to also affect the tissue specificity in their native nucleotide environment in plants. Reporter gene expression was found to be dependent on the presence of genomic regions containing up to two introns (Sieburth et al., 1997; Wang et al., 2004). 5' UTR introns have also been reported to be of importance for proper functionality of promoter elements, likely due to tissue specific gene control elements residing in the introns (Fu et al.,1995a; Fu et al., 1995b; Vitale et al., 2003; Kim et al., 2006). However, these studies also show that combination of introns with heterologous promoters can have strong negative impacts on strength and/or specificity of gene expression (Vitale et al., 2003; Kim et al., 2006, WO2006/003186, WO2007/098042). For example the strong constitutive Cauliflower Mosaic Virus CaMV35S promoter is negatively affected through combination with the sesame SeFAD2 5'UTR intron (Kim et al., 2006). In contrast to these observations, some documents show enhanced expression of a nucleic acid by IME without affecting the tissue specificity of the respective promoter (Schünmann et al., 2004).

It is to be understood that if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art in Australia or any other country.

Summary

A first aspect provides a method for producing a high expression constitutive plant promoter, the method comprising functionally linking to a promoter one or more nucleic acid expression enhancing nucleic acids (NEENA) heterologous to said promoter, the NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

2017248552 03 Jun 2019

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47.

A second aspect provides a method for producing a plant or part thereof with, compared to a respective control plant or part thereof, enhanced constitutive expression of one or more nucleic acid molecules comprising the steps of

a) introducing into the plant or part thereof one or more NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47,

and

b) functionally linking said one or more NEENA to a constitutive promoter and to a nucleic acid molecule being under the control of said constitutive promoter, wherein the NEENA is heterologous to said nucleic acid molecule and to said promoter.

A third aspect provides a recombinant expression construct comprising one or more NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid

03 Jun 2019 2017248552

molecule comprising the sequence of SEQ ID NO: 4, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47,

functionally linked to one or more constitutive promoter and one or more expressed nucleic acid molecule

wherein the one or more NEENA is heterologous to the promoter and the expressed nucleic acid molecule.

A fourth aspect provides a recombinant expression vector comprising one or more recombinant expression constructs of the third aspect.

A fifth aspect provides a transgenic plant or part thereof comprising one or more heterologous NEENA functionally linked to a heterologous promoter, the NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47.

A sixth aspect provides a transgenic cell or transgenic plant or part thereof comprising the recombinant expression construct of the third aspect or the recombinant expression vector of the fourth aspect.

A seventh aspect provides a transgenic cell cultue, transgenic seed, transgenic plant, part or propagation material derived from a transgenic cell or plant or part thereof of the sixth aspect comprising said heterologous NEENA, recombinant expression construct of the third aspect or recombinant expression vector of the fourth aspect

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An eighth aspect provides use of the recombinant expression construct of the third aspect or the recombinant expression vector of the fourth aspect for enhancing expression in a plant or part thereof.

A ninth aspect provides use of a transgenic cell culture, transgenic seed, transgenic plant, part or propagation material of the seventh aspect for producing foodstuffs, animal feeds, seeds, pharmaceuticals or fine chemicals.

Detailed description of the Invention

In the present application further nucleic acid molecules are described that enhance the expression of said promoters without affecting their specificity upon functionally linkage to constitutive promoters. These nucleic acid molecules are in the present application described as “nucleic acid expression enhancing nucleic acids” (NEENA). Introns have the intrinsic feature to be spliced out of the respective pre-mRNA. In contrast to that the nucleic acids presented in the application at hand, do not necessarily have to be included in the mRNA or, if present in the mRNA, have not necessarily to be spliced out of the mRNA in order to enhance the expression derived from the promoter the NEENAs are functionally linked to.

A first embodiment of the invention comprises a method for production of a high expression constitutive promoter comprising functionally linking to a promoter one or more nucleic acid expression enhancing nucleic acid (NEENA) molecule comprising

2017248552 20 Oct 2017

i) the nucleic acid molecule having a sequence as defined in any of SEQ ID NO: 1 to 19, preferably SEQ ID NO: 1 to 9, or

ii) a nucleic acid molecule having a sequence with an identity of 80% or more to any of the sequences as defined by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9, preferably, the identity is 85% or more, more preferably the identity is 90% or more, even more preferably, the identity is 95% or more, 96% or more, 97% or more, 98% or more or 99% or more, in the most preferred embodiment, the identity is 100% to any of the sequences as defined by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9 or

iii) a fragment of 100 or more consecutive bases, preferably 150 or more consecutive bases, more preferably 200 consecutive bases or more even more preferably 250 or more consecutive bases of a nucleic acid molecule of i) or ii) which has an expressing enhancing activity, for example 65% or more, preferably 70% or more, more preferably 75% or more, even more preferably 80% or more, 85% or more or 90% or more, in a most preferred embodiment it has 95% or more of the expression enhancing activity as the corresponding nucleic acid molecule having the sequence of any of the sequences as defined by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the previously mentioned nucleic acid molecules under i) to iii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 20 to 57, preferably SEQ ID NO: 20/21; 26/27; 30/31; 38/39; 42/43; 44/45; 46/47; 50;51 and 56/57 as shown in Table. 2 or

vi) a nucleic acid molecule of 100 nucleotides or more, 150 nucleotides or more, 200 nucleotides or more or 250 nucleotides or more, hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C or 65°C, preferably 65°C to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of a transcription enhancing nucleotide sequence described by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9 or the complement thereof. Preferably, said nucleic acid molecule is hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C or 65°C, preferably 65°C to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of a transcription enhancing nucleotide sequence described by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9 or the complement thereof, more preferably, said nucleic acid molecule is hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0,1 X SSC, 0.1% SDS at 50°C or 65°C, preferably 65°C to a nucleic acid molecule comprising at least 50, preferably at least 100, more preferably at least 150, even more preferably at least 200, most preferably at least 250 consecutive nucleotides of a transcription enhancing

20 Oct 2017 2017248552

nucleotide sequence described by any of the sequences as defined by SEQ ID NO:1 to 19, preferably SEQ ID NO: 1 to 9 or the complement thereof.

In one embodiment, the one or more NEENA is heterologous to the promoter to which it is functionally linked.

As described above under v) the nucleic acid molecule obtainable by PCR using oligonucleotides as defined by SEQ IDs 20 to 57, preferably SEQ ID NO: 20/21; 26/27; 30/31; 38/39; 42/43; 44/45; 46/47; 50/51 and 56/57 as shown in Table 2 is obtainable for example from genomic DNA from Arabidopsis plants such as A. thaliana using the conditions as described in Example 1 below.

The skilled person is aware of variations in the temperature profile, cycle number and/or buffer composition or concentration to obtain the respective NEENA molecule. The specific combination of oligonucleotides to be used in the respective PCR reaction for obtaining a respective NEENA molecule is described in Table 2.

A person skilled in the art is aware of methods for rendering a unidirectional to a bidirectional promoter and of methods to use the complement or reverse complement of a promoter sequence for creating a promoter having the same promoter specificity as the original sequence. Such methods are for example described for constitutive as well as inducible promoters by Xie et al. (2001) "Bidirectionalization of polar promoters in plants" nature biotechnology 19 pages 677 – 679. The authors describe that it is sufficient to add a minimal promoter to the 5´ prime end of any given promoter to receive a promoter controlling expression in both directions with same promoter specificity. Hence a high expression promoter functionally linked to a NEENA as described above is functional in complement or reverse complement and therefore the NEENA is functional in complement or reverse complement too.

A constitutive promoter as used herein means a promoter expressed in substantially all plant tissues throughout substantially the entire life span of a plant or part thereof. A promoter expressed in substantially all plant tissues may also encompass promoters that are expressed in at least two of the main plant tissues such as leaf, stem and/or root and may or may not be expressed in some or all minor tissues or cells such as epidermis, stomata, trichome, flower, seed or meristematic tissue. In a preferred embodiment a constitutive promoter as meant herein is expressed at least in green tissues such as leaf and stem. A promoter expressed throughout substantially the entire life span of a plant or part thereof may also encompass promoters that are expressed in young and developed tissue but may lack expression at specific time points in the lifespan of a plant or under specific conditions such as during germination and/or senescence or under biotic and/or abiotic stress conditions such as fungi or bacterial infection, drought, heat or cold. In a preferred

2017248552 20 Oct 2017

embodiment a constitutive promoter expressed in substantially the entire lifespan of a plant is expressed at least in fully expanded tissue until onset of senescence.

In principal the NEENA may be functionally linked to any promoter such as tissue specific, inducible, developmental specific or constitutive promoters. The respective NEENA will lead to an enhanced expression of the heterologous nucleic acid under the control of the respective promoter to which the at least one NEENA is functionally linked to. The enhancement of expression of promoters other than constitutive promoters, for example tissue specific promoters, will render the specificity of these promoters. Expression of the nucleic acid under control of the respective promoter will be detectable in additional tissues or developmental stages the transcript of said nucleic acid had not been detected without the NEENA. Hence, tissue- or developmental specific or any other promoter may be rendered to a constitutive promoter by functionally linking at least one of the NEENA molecules as described above to said promoter. It is therefore another embodiment of the invention to provide a method for rendering the specificity of any given promoter functional in plant to a constitutive promoter by linking the respective promoter to a NEENA molecule comprising a sequence as described above under i) to vi).

Preferably, the one or more NEENA is functionally linked to any constitutive promoter and will enhance expression of the nucleic acid molecule under control of said promoter. Constitutive promoters to be used in any method of the invention may be derived from plants, for example monocotyledonous or dicotyledonous plants, from bacteria and/or viruses or may be synthetic promoters. Constitutive promoters to be used are for example the PcUbi-Promoter from P. crispum (WO 2003102198), the ZmUbi-Promoter from Zea maize, AtNit-promoter from the A.thaliana gene At3g44310 encoding nitrilase 1, the 34S-promoter from figwort mosaiv virus, the 35S-promoter from tobacco mosaic virus, the nos and ocs-promoter derived from Agrobacteria, the ScBV-promoter (US 5 994 123), the SUPER-promoter (Lee et al. 2007, Plant. Phys.), the AtFNR-promoter from the A.thaliana gene At5g66190 encoding the ferredoxin NADH reductase, the ptxA promoter from Pisum sativum (WO2005085450), the AtTPT-promoter from the A.thaliana gene At5g46110 encoding the triose phosphate translocator, the bidirectional AtOASTL-promoter from the A.thaliana genes At4g14880 and At4g14890 , the PRO0194 promoter from the A.thaliana gene At1g13440 encoding the glyceraldehyde-3-phosphate dehydrogenase, the PRO0162 promoter from the A.thaliana gene At3g52930 encoding the fructose-bis-phosphate aldolase, the AHAS-promoter (WO2008124495) or the CaffeoylCoA-MT promoter and the OsCP12 from rice (WO2006084868).

The high expression constitutive promoters of the invention functionally linked to a NEENA may be employed in any plant comprising for example moss, fern, gymnosperm or angiosperm, for example monocotyledonous or dicotyledonous plant. In a preferred embodiment said promoter of the invention functionally linked to a NEENA may be employed

2017248552 20 Oct 2017

in monocotyledonous or dicotyledonous plants, preferably crop plant such as corn, soy, canola, cotton, potato, sugar beet, rice, wheat, sorghum, barley, musa, sugarcane, miscanthus and the like. In a preferred embodiment of the invention, said promoter which is functionally linked to a NEENA may be employed in monocotyledonous crop plants such as corn, rice, wheat, sorghum, musa, miscanthus, sugarcane or barley. In an especially preferred embodiment the promoter functionally linked to a NEENA may be employed in dicotyledonous crop plants such as soy, canola, cotton, sugar beet or potato.

A high expressing constitutive promoter as used in the application means for example a promoter which is functionally linked to a NEENA causing enhanced constitutive expression of the promoter in a plant or part thereof wherein the accumulation of RNA or rate of synthesis of RNA derived from the nucleic acid molecule under the control of the respective promoter functionally linked to a NEENA is higher, preferably significantly higher than the expression caused by the same promoter lacking a NEENA of the invention. Preferably the amount of RNA of the respective nucleic acid and/or the rate of RNA synthesis and/or the RNA stability in a plant is increased 50% or more, for example 100% or more, preferably 200% or more, more preferably 5 fold or more, even more preferably 10 fold or more, most preferably 20 fold or more for example 50 fold compared to a control plant of same age grown under the same conditions comprising the same constitutive promoter the latter not being functionally linked to a NEENA of the invention.

When used herein, significantly higher refers to statistical significance the skilled person is aware how to determine, for example by applying statistical tests such as the t-test to the respective data sets.

Methods for detecting expression conferred by a promoter are known in the art. For example, the promoter may be functionally linked to a marker gene such as GUS, GFP or luciferase and the activity of the respective protein encoded by the respective marker gene may be determined in the plant or part thereof. As a representative example, the method for detecting luciferase is described in detail below. Other methods are for example measuring the steady state level or synthesis rate of RNA of the nucleic acid molecule controlled by the promoter by methods known in the art, for example Northern blot analysis, qPCR, run-on assays or other methods described in the art.

A skilled person is aware of various methods for functionally linking two or more nucleic acid molecules. Such methods may encompass restriction/ligation, ligase independent cloning, recombineering, recombination or synthesis. Other methods may be employed to functionally link two or more nucleic acid molecules.

A further embodiment of the present invention is a method for producing a plant or part thereof with, compared to a respective control plant or part thereof, enhanced constitutive

20 Oct 2017 2017248552

expression of one or more nucleic acid molecule comprising the steps of introducing into the plant or part thereof one or more NEENA comprising a nucleic acid molecule as defined above under i) to vi) and functionally linking said one or more NEENA to a promoter, preferably a constitutive promoter and to a nucleic acid molecule being under the control of said promoter, preferably constitutive promoter, wherein the NEENA is heterologous to said nucleic acid molecule.

The NEENA may be heterologous to the nucleic acid molecule which is under the control of said promoter to which the NEENA is functionally linked or it may be heterologous to both the promoter and the nucleic acid molecule under the control of said promoter.

The term "heterologous" with respect to a nucleic acid molecule or DNA refers to a nucleic acid molecule which is operably linked to, or is manipulated to become operably linked to, a second nucleic acid molecule to which it is not operably linked in nature, or to which it is operably linked at a different location in nature. For example, a NEENA of the invention is in its natural environment functionally linked to its native promoter, whereas in the present invention it is linked to another promoter which might be derived from the same organism, a different organism or might be a synthetic promoter such as the SUPER-promoter. It may also mean that the NEENA of the present invention is linked to its native promoter but the nucleic acid molecule under control of said promoter is heterologous to the promoter comprising its native NEENA. It is in addition to be understood that the promoter and/or the nucleic acid molecule under the control of said promoter functionally linked to a NEENA of the invention are heterologous to said NEENA as their sequence has been manipulated by for example mutation such as insertions, deletions and the forth so that the natural sequence of the promoter and/or the nucleic acid molecule under control of said promoter is modified and therefore have become heterologous to a NEENA of the invention. It may also be understood that the NEENA is heterologous to the nucleic acid to which it is functionally linked when the NEENA is functionally linked to its native promoter wherein the position of the NEENA in relation to said promoter is changed so that the promoter shows higher expression after such manipulation.

A plant exhibiting enhanced constitutive expression of a nucleic acid molecule as meant herein means a plant having a higher, preferably statistically significant higher constitutive expression of a nucleic acid molecule compared to a control plant grown under the same conditions without the respective NEENA functionally linked to the respective nucleic acid molecule. Such control plant may be a wild-type plant or a transgenic plant comprising the same promoter controlling the same gene as in the plant of the invention wherein the promoter is not linked to a NEENA of the invention.

Producing a plant as used herein comprises methods for stable transformation such as introducing a recombinant DNA construct into a plant or part thereof by means of

2017248552 20 Oct 2017

Agrobacterium mediated transformation, protoplast transformation, particle bombardment or the like and optionally subsequent regeneration of a transgenic plant. It also comprises methods for transient transformation of a plant or part thereof such as viral infection or Agrobacterium infiltration. A skilled person is aware of further methods for stable and/or transient transformation of a plant or part thereof. Approaches such as breeding methods or protoplast fusion might also be employed for production of a plant of the invention and are covered herewith.

The method of the invention may be applied to any plant, for example gymnosperm or angiosperm, preferably angiosperm, for example dicotyledonous or monocotyledonous plants, preferably dicotyledonous plants. Preferred monocotyledonous plants are for example corn, wheat, rice, barley, sorghum, musa, sugarcane, miscanthus and brachypodium, especially preferred monocotyledonous plants are corn, wheat and rice. Preferred dicotyledonous plants are for example soy, rape seed, canola, linseed, cotton, potato, sugar beet, tagetes and *Arabidopsis*, especially preferred dicotyledonous plants are soy, rape seed, canola and potato

In one embodiment of the invention, the methods as defined above are comprising the steps of

a) introducing one or more NEENA comprising a nucleic acid molecule as defined above in i) to vi) into a plant or part thereof and

b) integrating said one or more NEENA into the genome of said plant or part thereof whereby said one or more NEENA is functionally linked to an endogenous preferably constitutively expressed nucleic acid heterologous to said one or more NEENA and optionally

c) regenerating a plant or part thereof comprising said one or more NEENA from said transformed cell.

The NEENA may be heterologous to the nucleic acid molecule which is under the control of said promoter to which the NEENA is functionally linked or it may be heterologous to both the promoter and the nucleic acid molecule under the control of said promoter.

The one or more NEENA molecule may be introduced into the plant or part thereof by means of particle bombardment, protoplast electroporation, virus infection, *Agrobacterium* mediated transformation or any other approach known in the art. The NEENA molecule may be introduced integrated for example into a plasmid or viral DNA or viral RNA. The NEENA molecule may also be comprised on a BAC, YAC or artificial chromosome prior to introduction into the plant or part of the plant. It may be also introduced as a linear nucleic acid molecule comprising the NEENA sequence wherein additional sequences may be present adjacent to the NEENA sequence on the nucleic acid molecule. These sequences neighboring the NEENA sequence may be from about 20 bp, for example 20 bp to several

2017248552 20 Oct 2017

hundred base pairs, for example 100 bp or more and may facilitate integration into the genome for example by homologous recombination. Any other method for genome integration may be employed, be it targeted integration approaches, such as homologous recombination or random integration approaches, such as illegitimate recombination.

The endogenous preferably constitutively expressed nucleic acid to which the NEENA molecule may be functionally linked may be any nucleic acid, preferably any constitutively expressed nucleic acid molecule. The nucleic acid molecule may be a protein coding nucleic acid molecule or a non coding molecule such as antisense RNA, rRNA, tRNA, miRNA, ta-siRNA, siRNA, dsRNA, snRNA, snoRNA or any other noncoding RNA known in the art.

The skilled person is aware of methods for identifying constitutively expressed nucleic acid molecules to which the method of the invention may preferably be applied for example by microarray chip hybridization, qPCR, Northern blot analysis, next generation sequencing etc.

A further way to perform the methods of the invention may be to
a) provide an expression construct comprising one or more NEENA comprising a nucleic acid molecule as defined above in i) to vi) functionally linked to a promoter, preferably a constitutive promoter as defined above and to one or more nucleic acid molecule the latter being heterologous to said one or more NEENA and which is under the control of said promoter, preferably constitutive promoter and
b) integrate said expression construct comprising said one or more NEENA into the genome of said plant or part thereof and optionally
c) regenerate a plant or part thereof comprising said one or more expression construct from said transformed plant or part thereof.

The NEENA may be heterologous to the nucleic acid molecule which is under the control of said promoter to which the NEENA is functionally linked or it may be heterologous to both the promoter and the nucleic acid molecule under the control of said promoter.

The expression construct may be integrated into the genome of the respective plant with any method known in the art. The integration may be random using methods such as particle bombardment or Agrobacterium mediated transformation. In a preferred embodiment, the integration is via targeted integration for example by homologous recombination. The latter method would allow integrating the expression construct comprising a high expression promoter functionally linked to a NEENA into a favorable genome region. Favorable genome regions are for example genome regions known to comprise genes that are highly expressed for example in seeds and hence may increase expression derived from said expression construct compared to a genome region which shows no transcriptional activity.

2017248552 20 Oct 2017

In another preferred embodiment said one or more NEENA is functionally linked to a promoter, preferably constitutive promoter close to the transcription start site of said heterologous nucleic acid molecule.

Close to the transcription start site as meant herein comprises functionally linking one or more NEENA to a promoter, preferably a constitutive promoter 2500 bp or less, preferentially 2000 bp or less, more preferred 1500 bp or less, even more preferred 1000 bp or less and most preferred 500 bp or less away from the transcription start site of said heterologous nucleic acid molecule. It is to be understood that the NEENA may be integrated upstream or downstream in the respective distance from the transcription start site of the respective promoter. Hence, the one or more NEENA must not necessarily be included in the transcript of the respective heterologous nucleic acid under control of the preferably constitutive promoter the one or more NEENA is functionally linked to. Preferentially the one or more NEENA is integrated downstream of the transcription start site of the respective promoter, preferably constitutive promoter. The integration site downstream of the transcription start site may be in the 5´ UTR, the 3´ UTR, an exon or intron or it may replace an intron or partially or completely the 5´ UTR or 3´ UTR of the heterologous nucleic acid under the control of the preferably constitutive promoter. Preferentially the one or more NEENA is integrated in the 5´ UTR or an intron or the NEENA is replacing an intron or a part or the complete 5´UTR, most preferentially it is integrated in the 5´UTR of the respective heterologous nucleic acid.

A further embodiment of the invention comprises a recombinant expression construct comprising one or more NEENA comprising a nucleic acid molecule as defined above in i) to vi).
The recombinant expression construct may further comprise one or more promoter, preferably constitutive promoter to which the one or more NEENA is functionally linked and optionally one or more expressed nucleic acid molecule the latter being heterologous to said one or more NEENA.

The NEENA may be heterologous to the nucleic acid molecule which is under the control of said promoter to which the NEENA is functionally linked or it may be heterologous to both the promoter and the nucleic acid molecule under the control of said promoter.

The expression construct may comprise one or more, for example two or more, for example 5 or more, such as 10 or more combinations of promoters, preferably constitutive promoters functionally linked to a NEENA and a nucleic acid molecule to be expressed heterologous to the respective NEENA. The expression construct may also comprise further promoters not comprising a NEENA functionally linked to nucleic acid molecules to be expressed homologous or heterologous to the respective promoter.

2017248552 20 Oct 2017

A recombinant expression vector comprising one or more recombinant expression construct as defined above is another embodiment of the invention. A multitude of expression vectors that may be used in the present invention are known to a skilled person. Methods for introducing such a vector comprising such an expression construct comprising for example a promoter functionally linked to a NEENA and optionally other elements such as a terminator into the genome of a plant and for recovering transgenic plants from a transformed cell are also well known in the art. Depending on the method used for the transformation of a plant or part thereof the entire vector might be integrated into the genome of said plant or part thereof or certain components of the vector might be integrated into the genome, such as, for example a T-DNA.

A transgenic plant or part thereof comprising one or more heterologous NEENA as defined above in i) to vi) is also enclosed in this invention. A NEENA is to be understood as being heterologous to the plant if it is synthetic, derived from another organism or the same organism but its natural genomic localization is rendered compared to a control plant, for example a wild type plant. It is to be understood, that a rendered genomic localization means the NEENA is located on another chromosome or on the same chromosome but 10 kb or more, for example 10 kb, preferably 5 kb or more, for example 5 kb, more preferably 1000 bp or more, for example 1000 bp, even more preferably 500 bp or more, for example 500 bp, especially preferably 100bp or more, for example 100 bp, most preferably 10 bp or more, for example 10 bp dislocated from its natural genomic localization, for example in a wild type plant.

A transgenic cell or transgenic plant or part thereof comprising a recombinant expression vector as defined above or a recombinant expression construct as defined above is a further embodiment of the invention. The transgenic cell, transgenic plant or part thereof may be selected from the group consisting of bacteria, fungi, yeasts or plant, insect or mammalian cells or plants. Preferably the transgenic cells are bacteria, fungi, yeasts or plant cells. Preferred bacteria are Enterobacteria such as *E. coli* and bacteria of the genus Agrobacteria, for example *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Preferred plants are monocotyledonous or dicotyledonous plants for example monocotyledonous or dicotyledonous crop plants such as corn, soy, canola, cotton, potato, sugar beet, rice, wheat, sorghum, barley, miscanthus, musa, sugarcane and the like. Preferred crop plants are corn, rice, wheat, soy, canola, cotton or potato. Especially preferred dicotyledonous crop plants are soy, canola, cotton or potato.

Especially preferred monocotyledonous crop plants are corn, wheat and rice.

A transgenic cell culture, transgenic seed, parts or propagation material derived from a transgenic cell or plant or part thereof as defined above comprising said heterologous

20 Oct 2017 2017248552

NEENA as defined above in i) to vi) or said recombinant expression construct or said recombinant vector as defined above are other embodiments of the invention. Transgenic parts or propagation material as meant herein comprise all tissues and organs, for example leaf, stem and fruit as well as material that is useful for propagation and/or regeneration of plants such as cuttings, scions, layers, branches or shoots comprising the respective NEENA, recombinant expression construct or recombinant vector.

A further embodiment of the invention is the use of the NEENA as defined above in i) to vi) or the recombinant construct or recombinant vector as defined above for enhancing expression in plants or parts thereof.

Hence the application at hand provides seed-specific and/or seed-preferential gene expression enhancing nucleic acid molecules comprising one or more promoter, preferably seed- specific and/or seed preferential promoter functionally linked to one ore more NEENA. Additionally use of such gene expression enhancing nucleic acid molecules and expression constructs, expression vectors, transgenic plants or parts thereof and transgenic cells comprising such gene expression enhancing nucleic acid molecules are provided.

A use of a transgenic cell culture, transgenic seed, parts or propagation material derived from a transgenic cell or plant or part thereof as defined above for the production of foodstuffs, animal feeds, seeds, pharmaceuticals or fine chemicals is also enclosed in this invention.

DEFINITIONS

Abbreviations: NEENA – nucleic acid expression enhancing nucleic acid, GFP – green fluorescence protein, GUS – beta-Glucuronidase, BAP – 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog medium; NAA - 1-naphtaleneacetic acid; MES, 2-(N-morpholino-ethanesulfonic acid, IAA indole acetic acid; Kan: Kanamycin sulfate; GA3 - Gibberellic acid; Timentin™: ticarcillin disodium / clavulanate potassium, microl: Microliter.

It is to be understood that this invention is not limited to the particular methodology or protocols. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a vector" is a reference to one or more vectors and includes equivalents thereof known to those skilled in the art, and so forth. The term "about" is used herein to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the

numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent, preferably 10 percent up or down (higher or lower). As used herein, the word "or" means any one member of a particular list and also includes any combination of members of that list. The words "comprise," "comprising," "include," "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of one or more stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof. For clarity, certain terms used in the specification are defined and used as follows:

Antiparallel: "Antiparallel" refers herein to two nucleotide sequences paired through hydrogen bonds between complementary base residues with phosphodiester bonds running in the 5'-3' direction in one nucleotide sequence and in the 3'-5' direction in the other nucleotide sequence.

Antisense: The term "antisense" refers to a nucleotide sequence that is inverted relative to its normal orientation for transcription or function and so expresses an RNA transcript that is complementary to a target gene mRNA molecule expressed within the host cell (e.g., it can hybridize to the target gene mRNA molecule or single stranded genomic DNA through Watson-Crick base pairing) or that is complementary to a target DNA molecule such as, for example genomic DNA present in the host cell.

Coding region: As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5'-side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3'-side by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA). In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5'- and 3'-end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5'-flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3'-flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

Complementary: "Complementary" or "complementarity" refers to two nucleotide sequences which comprise antiparallel nucleotide sequences capable of pairing with one another (by the base-pairing rules) upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences. For example, the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity can be "partial" or "total."

2017248552 20 Oct 2017

"Partial" complementarity is where one or more nucleic acid bases are not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acid molecules is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid molecule strands has significant effects on the efficiency and strength of hybridization between nucleic acid molecule strands. A "complement" of a nucleic acid sequence as used herein refers to a nucleotide sequence whose nucleic acid molecules show total complementarity to the nucleic acid molecules of the nucleic acid sequence.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprise” or variations such as “comprises” or “comprising” is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Double-stranded RNA: A "double-stranded RNA” molecule or “dsRNA" molecule comprises a sense RNA fragment of a nucleotide sequence and an antisense RNA fragment of the nucleotide sequence, which both comprise nucleotide sequences complementary to one another, thereby allowing the sense and antisense RNA fragments to pair and form a double-stranded RNA molecule.

Endogenous: An "endogenous" nucleotide sequence refers to a nucleotide sequence, which is present in the genome of the untransformed plant cell.

Enhanced expression: “enhance” or “increase” the expression of a nucleic acid molecule in a plant cell are used equivalently herein and mean that the level of expression of the nucleic acid molecule in a plant, part of a plant or plant cell after applying a method of the present invention is higher than its expression in the plant, part of the plant or plant cell before applying the method, or compared to a reference plant lacking a recombinant nucleic acid molecule of the invention. For example, the reference plant is comprising the same construct which is only lacking the respective NEENA. The term "enhanced” or “increased" as used herein are synonymous and means herein higher, preferably significantly higher expression of the nucleic acid molecule to be expressed. As used herein, an “enhancement” or “increase” of the level of an agent such as a protein, mRNA or RNA means that the level is increased relative to a substantially identical plant, part of a plant or plant cell grown under substantially identical conditions, lacking a recombinant nucleic acid molecule of the invention, for example lacking the NEENA molecule, the recombinant construct or recombinant vector of the invetion. As used herein, “enhancement” or “increase” of the level of an agent, such as for example a preRNA, mRNA, rRNA, tRNA, snoRNA, snRNA expressed by the target gene and/or of the protein product encoded by it, means that the level is increased 50% or more, for example 100% or more, preferably 200% or more, more

2017248552 20 Oct 2017

preferably 5 fold or more, even more preferably 10 fold or more, most preferably 20 fold or more for example 50 fold relative to a cell or organism lacking a recombinant nucleic acid molecule of the invention. The enhancement or increase can be determined by methods with which the skilled worker is familiar. Thus, the enhancement or increase of the nucleic acid or protein quantity can be determined for example by an immunological detection of the protein. Moreover, techniques such as protein assay, fluorescence, Northern hybridization, nuclease protection assay, reverse transcription (quantitative RT-PCR), ELISA (enzyme-linked immunosorbent assay), Western blotting, radioimmunoassay (RIA) or other immunoassays and fluorescence-activated cell analysis (FACS) can be employed to measure a specific protein or RNA in a plant or plant cell. Depending on the type of the induced protein product, its activity or the effect on the phenotype of the organism or the cell may also be determined. Methods for determining the protein quantity are known to the skilled worker. Examples, which may be mentioned, are: the micro-Biuret method (Goa J (1953) Scand J Clin Lab Invest 5:218-222), the Folin-Ciocalteau method (Lowry OH et al. (1951) J Biol Chem 193:265-275) or measuring the absorption of CBB G-250 (Bradford MM (1976) Analyt Biochem 72:248-254). As one example for quantifying the activity of a protein, the detection of luciferase activity is described in the Examples below.

Expression: "Expression" refers to the biosynthesis of a gene product, preferably to the transcription and/or translation of a nucleotide sequence, for example an endogenous gene or a heterologous gene, in a cell. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and - optionally - the subsequent translation of mRNA into one or more polypeptides. In other cases, expression may refer only to the transcription of the DNA harboring an RNA molecule.

Expression construct: "Expression construct" as used herein mean a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate part of a plant or plant cell, comprising a promoter functional in said part of a plant or plant cell into which it will be introduced, operatively linked to the nucleotide sequence of interest which is – optionally - operatively linked to termination signals. If translation is required, it also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region may code for a protein of interest but may also code for a functional RNA of interest, for example RNAa, siRNA, snoRNA, snRNA, microRNA, ta-siRNA or any other noncoding regulatory RNA, in the sense or antisense direction. The expression construct comprising the nucleotide sequence of interest may be chimeric, meaning that one or more of its components is heterologous with respect to one or more of its other components. The expression construct may also be one, which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression construct is heterologous with respect to the host, i.e., the particular DNA sequence of the expression construct does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The

2017248552 20 Oct 2017

expression of the nucleotide sequence in the expression construct may be under the control of a constitutive promoter or of an inducible promoter, which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a plant, the promoter can also be specific to a particular tissue or organ or stage of development.

Foreign: The term "foreign" refers to any nucleic acid molecule (e.g., gene sequence) which is introduced into the genome of a cell by experimental manipulations and may include sequences found in that cell so long as the introduced sequence contains some modification (e.g., a point mutation, the presence of a selectable marker gene, etc.) and is therefore distinct relative to the naturally-occurring sequence.

Functional linkage: The term "functional linkage" or "functionally linked" is to be understood as meaning, for example, the sequential arrangement of a regulatory element (e.g. a promoter) with a nucleic acid sequence to be expressed and, if appropriate, further regulatory elements (such as e.g., a terminator or a NEENA) in such a way that each of the regulatory elements can fulfill its intended function to allow, modify, facilitate or otherwise influence expression of said nucleic acid sequence. As a synonym the wording “operable linkage” or “operably linked” may be used. The expression may result depending on the arrangement of the nucleic acid sequences in relation to sense or antisense RNA. To this end, direct linkage in the chemical sense is not necessarily required. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are further away, or indeed from other DNA molecules. Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned behind the sequence acting as promoter, so that the two sequences are linked covalently to each other. The distance between the promoter sequence and the nucleic acid sequence to be expressed recombinantly is preferably less than 200 base pairs, especially preferably less than 100 base pairs, very especially preferably less than 50 base pairs. In a preferred embodiment, the nucleic acid sequence to be transcribed is located behind the promoter in such a way that the transcription start is identical with the desired beginning of the chimeric RNA of the invention. Functional linkage, and an expression construct, can be generated by means of customary recombination and cloning techniques as described (e.g., in Maniatis T, Fritsch EF and Sambrook J (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor (NY); Silhavy et al. (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY); Ausubel et al. (1987) Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience; Gelvin et al. (Eds) (1990) Plant Molecular Biology Manual; Kluwer Academic Publisher, Dordrecht, The Netherlands). However, further sequences, which, for example, act as a linker with specific cleavage sites for restriction enzymes, or as a signal peptide, may also be positioned between the two sequences. The insertion of sequences may also lead to the expression of fusion proteins. Preferably, the expression construct, consisting of a linkage of a regulatory

2017248552 20 Oct 2017

region for example a promoter and nucleic acid sequence to be expressed, can exist in a vector-integrated form and be inserted into a plant genome, for example by transformation.

Gene: The term "gene" refers to a region operably joined to appropriate regulatory sequences capable of regulating the expression of the gene product (e.g., a polypeptide or a functional RNA) in some manner. A gene includes untranslated regulatory regions of DNA (e.g., promoters, enhancers, repressors, etc.) preceding (up-stream) and following (downstream) the coding region (open reading frame, ORF) as well as, where applicable, intervening sequences (i.e., introns) between individual coding regions (i.e., exons). The term "structural gene" as used herein is intended to mean a DNA sequence that is transcribed into mRNA which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Genome and genomic DNA: The terms “genome” or “genomic DNA” is referring to the heritable genetic information of a host organism. Said genomic DNA comprises the DNA of the nucleus (also referred to as chromosomal DNA) but also the DNA of the plastids (e.g., chloroplasts) and other cellular organelles (e.g., mitochondria). Preferably the terms genome or genomic DNA is referring to the chromosomal DNA of the nucleus.

Heterologous: The term "heterologous” with respect to a nucleic acid molecule or DNA refers to a nucleic acid molecule which is operably linked to, or is manipulated to become operably linked to, a second nucleic acid molecule to which it is not operably linked in nature, or to which it is operably linked at a different location in nature. A heterologous expression construct comprising a nucleic acid molecule and one or more regulatory nucleic acid molecule (such as a promoter or a transcription termination signal) linked thereto for example is a constructs originating by experimental manipulations in which either a) said nucleic acid molecule, or b) said regulatory nucleic acid molecule or c) both (i.e. (a) and (b)) is not located in its natural (native) genetic environment or has been modified by experimental manipulations, an example of a modification being a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment refers to the natural chromosomal locus in the organism of origin, or to the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the sequence of the nucleic acid molecule is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least at one side and has a sequence of at least 50 bp, preferably at least 500 bp, especially preferably at least 1,000 bp, very especially preferably at least 5,000 bp, in length. A naturally occurring expression construct - for example the naturally occurring combination of a promoter with the corresponding gene - becomes a transgenic expression construct when it is modified by non-natural, synthetic “artificial” methods such as, for example, mutagenization. Such methods have been described (US 5,565,350; WO 00/15815). For example a protein encoding nucleic acid molecule operably linked to a promoter, which is not the native

2017248552 20 Oct 2017

promoter of this molecule, is considered to be heterologous with respect to the promoter. Preferably, heterologous DNA is not endogenous to or not naturally associated with the cell into which it is introduced, but has been obtained from another cell or has been synthesized. Heterologous DNA also includes an endogenous DNA sequence, which contains some modification, non-naturally occurring, multiple copies of an endogenous DNA sequence, or a DNA sequence which is not naturally associated with another DNA sequence physically linked thereto. Generally, although not necessarily, heterologous DNA encodes RNA or proteins that are not normally produced by the cell into which it is expressed.

High expression constitutive promoter: A “high expression constitutive promoter” as used herein means a promoter causing constitutive expression in a plant or part thereof wherein the accumulation or rate of synthesis of RNA or stability of RNA derived from the nucleic acid molecule under the control of the respective promoter is higher, preferably significantly higher than the expression caused by the promoter lacking the NEENA of the invention. Preferably the amount of RNA and/or the rate of RNA synthesis and/or stability of RNA is increased 50% or more, for example 100% or more, preferably 200% or more, more preferably 5 fold or more, even more preferably 10 fold or more, most preferably 20 fold or more for example 50 fold relative to a constitutive promoter lacking a NEENA of the invention.

Hybridization: The term "hybridization" as used herein includes "any process by which a strand of nucleic acid molecule joins with a complementary strand through base pairing." (J. Coombs (1994) Dictionary of Biotechnology, Stockton Press, New York). Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acid molecules) is impacted by such factors as the degree of complementarity between the nucleic acid molecules, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acid molecules. As used herein, the term "Tm" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the Tm of nucleic acid molecules is well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation: Tm=81.5+0.41(% G+C), when a nucleic acid molecule is in aqueous solution at 1 M NaCl [see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985)]. Other references include more sophisticated computations, which take structural as well as sequence characteristics into account for the calculation of Tm. Stringent conditions, are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

"Identity": "Identity" when used in respect to the comparison of two or more nucleic acid or amino acid molecules means that the sequences of said molecules share a certain degree of sequence similarity, the sequences being partially identical.

To determine the percentage identity (homology is herein used interchangeably) of two amino acid sequences or of two nucleic acid molecules, the sequences are written one underneath the other for an optimal comparison (for example gaps may be inserted into the sequence of a protein or of a nucleic acid in order to generate an optimal alignment with the other protein or the other nucleic acid).

The amino acid residues or nucleic acid molecules at the corresponding amino acid positions or nucleotide positions are then compared. If a position in one sequence is occupied by the same amino acid residue or the same nucleic acid molecule as the corresponding position in the other sequence, the molecules are homologous at this position (i.e. amino acid or nucleic acid "homology" as used in the present context corresponds to amino acid or nucleic acid "identity". The percentage identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e. % homology = number of identical positions/total number of positions x 100). The terms "homology" and "identity" are thus to be considered as synonyms.

For the determination of the percentage identity of two or more amino acids or of two or more nucleotide sequences several computer software programs have been developed. The identity of two or more sequences can be calculated with for example the software fasta, which presently has been used in the version fasta 3 (W. R. Pearson and D. J. Lipman, PNAS 85, 2444(1988); W. R. Pearson, Methods in Enzymology 183, 63 (1990); W. R. Pearson and D. J. Lipman, PNAS 85, 2444 (1988); W. R. Pearson, Enzymology 183, 63 (1990)). Another useful program for the calculation of identities of different sequences is the standard blast program, which is included in the Biomax pedant software (Biomax, Munich, Federal Republic of Germany). This leads unfortunately sometimes to suboptimal results since blast does not always include complete sequences of the subject and the query. Nevertheless as this program is very efficient it can be used for the comparison of a huge number of sequences. The following settings are typically used for such a comparisons of sequences:

-p Program Name [String]; -d Database [String]; default = nr; -i Query File [File In]; default = stdin; -e Expectation value (E) [Real]; default = 10.0; -m alignment view options: 0 = pairwise; 1 = query-anchored showing identities; 2 = query-anchored no identities; 3 = flat query-anchored, show identities; 4 = flat query-anchored, no identities; 5 = query-anchored no identities and blunt ends; 6 = flat query-anchored, no identities and blunt ends; 7 = XML Blast output; 8 = tabular; 9 tabular with comment lines [Integer]; default = 0; -o BLAST report Output File [File Out] Optional; default = stdout; -F Filter query sequence (DUST with blastn, SEG with others) [String]; default = T; -G Cost to open a gap (zero invokes default

behavior) [Integer]; default = 0; -E Cost to extend a gap (zero invokes default behavior) [Integer]; default = 0; -X X dropoff value for gapped alignment (in bits) (zero invokes default behavior); blastn 30, megablast 20, tblastx 0, all others 15 [Integer]; default = 0; -I Show GI's in deflines [T/F]; default = F; -q Penalty for a nucleotide mismatch (blastn only) [Integer]; default = -3; -r Reward for a nucleotide match (blastn only) [Integer]; default = 1; -v Number of database sequences to show one-line descriptions for (V) [Integer]; default = 500; -b Number of database sequence to show alignments for (B) [Integer]; default = 250; -f Threshold for extending hits, default if zero; blastp 11, blastn 0, blastx 12, tblastn 13; tblastx 13, megablast 0 [Integer]; default = 0; -g Perfom gapped alignment (not available with tblastx) [T/F]; default = T; -Q Query Genetic code to use [Integer]; default = 1; -D DB Genetic code (for tblast[nx] only) [Integer]; default = 1; -a Number of processors to use [Integer]; default = 1; -O SeqAlign file [File Out] Optional; -J Believe the query defline [T/F]; default = F; -M Matrix [String]; default = BLOSUM62; -W Word size, default if zero (blastn 11, megablast 28, all others 3) [Integer]; default = 0; -z Effective length of the database (use zero for the real size) [Real]; default = 0; -K Number of best hits from a region to keep (off by default, if used a value of 100 is recommended) [Integer]; default = 0; -P 0 for multiple hit, 1 for single hit [Integer]; default = 0; -Y Effective length of the search space (use zero for the real size) [Real]; default = 0; -S Query strands to search against database (for blast[nx], and tblastx); 3 is both, 1 is top, 2 is bottom [Integer]; default = 3; -T Produce HTML output [T/F]; default = F; -I Restrict search of database to list of GI's [String] Optional; -U Use lower case filtering of FASTA sequence [T/F] Optional; default = F; -y X dropoff value for ungapped extensions in bits (0.0 invokes default behavior); blastn 20, megablast 10, all others 7 [Real]; default = 0.0; -Z X dropoff value for final gapped alignment in bits (0.0 invokes default behavior); blastn/megablast 50, tblastx 0, all others 25 [Integer]; default = 0; -R PSI-TBLASTN checkpoint file [File In] Optional; -n MegaBlast search [T/F]; default = F; -L Location on query sequence [String] Optional; -A Multiple Hits window size, default if zero (blastn/megablast 0, all others 40 [Integer]; default = 0; -w Frame shift penalty (OOF algorithm for blastx) [Integer]; default = 0; -t Length of the largest intron allowed in tblastn for linking HSPs (0 disables linking) [Integer]; default = 0.

Results of high quality are reached by using the algorithm of Needleman and Wunsch or Smith and Waterman. Therefore programs based on said algorithms are preferred. Advantageously the comparisons of sequences can be done with the program PileUp (J. Mol. Evolution., 25, 351 (1987), Higgins et al., CABIOS 5, 151 (1989)) or preferably with the programs “Gap” and “Needle”, which are both based on the algorithms of Needleman and Wunsch (J. Mol. Biol. 48; 443 (1970)), and “BestFit”, which is based on the algorithm of Smith and Waterman (Adv. Appl. Math. 2; 482 (1981)). “Gap” and “BestFit” are part of the GCG software-package (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711 (1991); Altschul et al., (Nucleic Acids Res. 25, 3389 (1997)), “Needle” is part of the The European Molecular Biology Open Software Suite (EMBOSS) (Trends in Genetics 16 (6), 276 (2000)). Therefore preferably the calculations to determine

the percentages of sequence identity are done with the programs "Gap" or "Needle" over the whole range of the sequences. The following standard adjustments for the comparison of nucleic acid sequences were used for "Needle": matrix: EDNAFULL, Gap_penalty: 10.0, Extend_penalty: 0.5. The following standard adjustments for the comparison of nucleic acid sequences were used for "Gap": gap weight: 50, length weight: 3, average match: 10.000, average mismatch: 0.000.

For example a sequence, which is said to have 80% identity with sequence SEQ ID NO: 1 at the nucleic acid level is understood as meaning a sequence which, upon comparison with the sequence represented by SEQ ID NO: 1 by the above program "Needle" with the above parameter set, has a 80% identity. Preferably the identity is calculated on the complete length of the query sequence, for example SEQ ID NO:1.

Intron: refers to sections of DNA (intervening sequences) within a gene that do not encode part of the protein that the gene produces, and that is spliced out of the mRNA that is transcribed from the gene before it is exported from the cell nucleus. Intron sequence refers to the nucleic acid sequence of an intron. Thus, introns are those regions of DNA sequences that are transcribed along with the coding sequence (exons) but are removed during the formation of mature mRNA. Introns can be positioned within the actual coding region or in either the 5' or 3' untranslated leaders of the pre-mRNA (unspliced mRNA). Introns in the primary transcript are excised and the coding sequences are simultaneously and precisely ligated to form the mature mRNA. The junctions of introns and exons form the splice site. The sequence of an intron begins with GU and ends with AG. Furthermore, in plants, two examples of AU-AC introns have been described: the fourteenth intron of the RecA-like protein gene and the seventh intron of the G5 gene from Arabidopsis thaliana are AT-AC introns. Pre-mRNAs containing introns have three short sequences that are –beside other sequences- essential for the intron to be accurately spliced. These sequences are the 5' splice-site, the 3' splice-site, and the branchpoint. mRNA splicing is the removal of intervening sequences (introns) present in primary mRNA transcripts and joining or ligation of exon sequences. This is also known as cis-splicing which joins two exons on the same RNA with the removal of the intervening sequence (intron). The functional elements of an intron is comprising sequences that are recognized and bound by the specific protein components of the spliceosome (e.g. splicing consensus sequences at the ends of introns). The interaction of the functional elements with the spliceosome results in the removal of the intron sequence from the premature mRNA and the rejoining of the exon sequences. Introns have three short sequences that are essential -although not sufficient- for the intron to be accurately spliced. These sequences are the 5´ splice site, the 3´ splice site and the branch point. The branchpoint sequence is important in splicing and splice-site selection in plants. The branchpoint sequence is usually located 10-60 nucleotides upstream of the 3´ splice site.

Isogenic: organisms (e.g., plants), which are genetically identical, except that they may differ by the presence or absence of a heterologous DNA sequence.

Isolated: The term "isolated" as used herein means that a material has been removed by the hand of man and exists apart from its original, native environment and is therefore not a product of nature. An isolated material or molecule (such as a DNA molecule or enzyme) may exist in a purified form or may exist in a non-native environment such as, for example, in a transgenic host cell. For example, a naturally occurring polynucleotide or polypeptide present in a living plant is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides can be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and would be isolated in that such a vector or composition is not part of its original environment. Preferably, the term "isolated" when used in relation to a nucleic acid molecule, as in "an isolated nucleic acid sequence" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in its natural source. Isolated nucleic acid molecule is nucleic acid molecule present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acid molecules are nucleic acid molecules such as DNA and RNA, which are found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs, which encode a multitude of proteins. However, an isolated nucleic acid sequence comprising for example SEQ ID NO: 1 includes, by way of example, such nucleic acid sequences in cells which ordinarily contain SEQ ID NO:1 where the nucleic acid sequence is in a chromosomal or extrachromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid sequence may be present in single-stranded or double-stranded form. When an isolated nucleic acid sequence is to be utilized to express a protein, the nucleic acid sequence will contain at a minimum at least a portion of the sense or coding strand (i.e., the nucleic acid sequence may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (i.e., the nucleic acid sequence may be double-stranded).

Minimal Promoter: promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription.

NEENA: see “Nucleic acid expression enhancing nucleic acid”.

Non-coding: The term "non-coding" refers to sequences of nucleic acid molecules that do not encode part or all of an expressed protein. Non-coding sequences include but are not limited to introns, enhancers, promoter regions, 3' untranslated regions, and 5' untranslated regions.

Nucleic acid expression enhancing nucleic acid (NEENA): The term “nucleic acid expression enhancing nucleic acid” refers to a sequence and/or a nucleic acid molecule of a specific sequence having the intrinsic property to enhance expression of a nucleic acid under the control of a promoter to which the NEENA is functionally linked. Unlike promoter sequences, the NEENA as such is not able to drive expression. In order to fulfill the function of enhancing expression of a nucleic acid molecule functionally linked to the NEENA, the NEENA itself has to be functionally linked to a promoter. In distinction to enhancer sequences known in the art, the NEENA is acting in cis but not in trans and has to be located close to the transcription start site of the nucleic acid to be expressed.

Nucleic acids and nucleotides: The terms "Nucleic Acids" and "Nucleotides" refer to naturally occurring or synthetic or artificial nucleic acid or nucleotides. The terms “nucleic acids” and "nucleotides” comprise deoxyribonucleotides or ribonucleotides or any nucleotide analogue and polymers or hybrids thereof in either single- or double-stranded, sense or antisense form. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term "nucleic acid" is used inter-changeably herein with "gene", "cDNA, "mRNA", "oligonucleotide," and "polynucleotide". Nucleotide analogues include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil, and the like; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group selected from H, OR, R, halo, SH, SR, NH2, NHR, NR2, or CN. Short hairpin RNAs (shRNAs) also can comprise non-natural elements such as non-natural bases, e.g., ionosin and xanthine, non-natural sugars, e.g., 2'-methoxy ribose, or non-natural phosphodiester linkages, e.g., methylphosphonates, phosphorothioates and peptides.

Nucleic acid sequence: The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5'- to the 3'-end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. "Nucleic acid sequence" also refers to a consecutive list of abbreviations, letters, characters or words, which represent nucleotides. In one embodiment, a nucleic acid can be a "probe" which is a relatively short nucleic acid, usually less than 100 nucleotides in length. Often a nucleic acid probe is from

20 Oct 2017 2017248552

about 50 nucleotides in length to about 10 nucleotides in length. A "target region" of a nucleic acid is a portion of a nucleic acid that is identified to be of interest. A "coding region" of a nucleic acid is the portion of the nucleic acid, which is transcribed and translated in a sequence-specific manner to produce into a particular polypeptide or protein when placed under the control of appropriate regulatory sequences. The coding region is said to encode such a polypeptide or protein.

Oligonucleotide: The term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof, as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. An oligonucleotide preferably includes two or more nucleomonomers covalently coupled to each other by linkages (e.g., phosphodiesters) or substitute linkages.

Overhang: An "overhang" is a relatively short single-stranded nucleotide sequence on the 5'- or 3'-hydroxyl end of a double-stranded oligonucleotide molecule (also referred to as an "extension," "protruding end," or "sticky end").

Plant: is generally understood as meaning any eukaryotic single-or multi-celled organism or a cell, tissue, organ, part or propagation material (such as seeds or fruit) of same which is capable of photosynthesis. Included for the purpose of the invention are all genera and species of higher and lower plants of the Plant Kingdom. Annual, perennial, monocotyledonous and dicotyledonous plants are preferred. The term includes the mature plants, seed, shoots and seedlings and their derived parts, propagation material (such as seeds or microspores), plant organs, tissue, protoplasts, callus and other cultures, for example cell cultures, and any other type of plant cell grouping to give functional or structural units. Mature plants refer to plants at any desired developmental stage beyond that of the seedling. Seedling refers to a young immature plant at an early developmental stage. Annual, biennial, monocotyledonous and dicotyledonous plants are preferred host organisms for the generation of transgenic plants. The expression of genes is furthermore advantageous in all ornamental plants, useful or ornamental trees, flowers, cut flowers, shrubs or lawns. Plants which may be mentioned by way of example but not by limitation are angiosperms, bryophytes such as, for example, Hepaticae (liverworts) and Musci (mosses); Pteridophytes such as ferns, horsetail and club mosses; gymnosperms such as conifers, cycads, ginkgo and Gnetatae; algae such as Chlorophyceae, Phaeophpyceae, Rhodophyceae, Myxophyceae, Xanthophyceae, Bacillariophyceae (diatoms), and Euglenophyceae. Preferred are plants which are used for food or feed purpose such as the families of the Leguminosae such as pea, alfalfa and soya; Gramineae such as rice, maize, wheat, barley, sorghum, millet, rye, triticale, or oats; the family of the Umbelliferae,

2017248552 20 Oct 2017

especially the genus Daucus, very especially the species carota (carrot) and Apium, very especially the species Graveolens dulce (celery) and many others; the family of the Solanaceae, especially the genus Lycopersicon, very especially the species esculentum (tomato) and the genus Solanum, very especially the species tuberosum (potato) and melongena (egg plant), and many others (such as tobacco); and the genus Capsicum, very especially the species annuum (peppers) and many others; the family of the Leguminosae, especially the genus Glycine, very especially the species max (soybean), alfalfa, pea, lucerne, beans or peanut and many others; and the family of the Cruciferae (Brassicacae), especially the genus Brassica, very especially the species napus (oil seed rape), campestris (beet), oleracea cv Tastie (cabbage), oleracea cv Snowball Y (cauliflower) and oleracea cv Emperor (broccoli); and of the genus Arabidopsis, very especially the species thaliana and many others; the family of the Compositae, especially the genus Lactuca, very especially the species sativa (lettuce) and many others; the family of the Asteraceae such as sunflower, Tagetes, lettuce or Calendula and many other; the family of the Cucurbitaceae such as melon, pumpkin/squash or zucchini, and linseed. Further preferred are cotton, sugar cane, hemp, flax, chillies, and the various tree, nut and wine species.

Polypeptide: The terms "polypeptide", "peptide", "oligopeptide", "polypeptide", "gene product", "expression product" and "protein" are used interchangeably herein to refer to a polymer or oligomer of consecutive amino acid residues.

Pre-protein: Protein, which is normally targeted to a cellular organelle, such as a chloroplast, and still comprising its transit peptide.

Primary transcript: The term “primary transcript” as used herein refers to a premature RNA transcript of a gene. A “primary transcript” for example still comprises introns and/or is not yet comprising a polyA tail or a cap structure and/or is missing other modifications necessary for its correct function as transcript such as for example trimming or editing.

Promoter: The terms "promoter", or "promoter sequence" are equivalents and as used herein, refer to a DNA sequence which when ligated to a nucleotide sequence of interest is capable of controlling the transcription of the nucleotide sequence of interest into RNA. Such promoters can for example be found in the following public databases http://www.grassius.org/grasspromdb.html, http://mendel.cs.rhul.ac.uk/mendel.php?topic=plantprom, http://ppdb.gene.nagoya-u.ac.jp/cgi-bin/index.cgi. Promoters listed there may be addressed with the methods of the invention and are herewith included by reference. A promoter is located 5' (i.e., upstream), proximal to the transcriptional start site of a nucleotide sequence of interest whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. Said promoter comprises for example the at least 10 kb, for example 5 kb or 2 kb proximal to the

transcription start site. It may also comprise the at least 1500 bp proximal to the transcriptional start site, preferably the at least 1000 bp, more preferably the at least 500 bp, even more preferably the at least 400 bp, the at least 300 bp, the at least 200 bp or the at least 100 bp. In a further preferred embodiment, the promoter comprises the at least 50 bp proximal to the transcription start site, for example, at least 25 bp. The promoter does not comprise exon and/or intron regions or 5´ untranslated regions. The promoter may for example be heterologous or homologous to the respective plant. A polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (e.g. a genetically engineered coding sequence or an allele from a different ecotype or variety). Suitable promoters can be derived from genes of the host cells where expression should occur or from pathogens for this host cells (e.g., plants or plant pathogens like plant viruses). A plant specific promoter is a promoter suitable for regulating expression in a plant. It may be derived from a plant but also from plant pathogens or it might be a synthetic promoter designed by man. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. Also, the promoter may be regulated in a tissue-specific or tissue preferred manner such that it is only or predominantly active in transcribing the associated coding region in a specific tissue type(s) such as leaves, roots or meristem. The term "tissue specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue (e.g., petals) in the relative absence of expression of the same nucleotide sequence of interest in a different type of tissue (e.g., roots). Tissue specificity of a promoter may be evaluated by, for example, operably linking a reporter gene to the promoter sequence to generate a reporter construct, introducing the reporter construct into the genome of a plant such that the reporter construct is integrated into every tissue of the resulting transgenic plant, and detecting the expression of the reporter gene (e.g., detecting mRNA, protein, or the activity of a protein encoded by the reporter gene) in different tissues of the transgenic plant. The detection of a greater level of expression of the reporter gene in one or more tissues relative to the level of expression of the reporter gene in other tissues shows that the promoter is specific for the tissues in which greater levels of expression are detected. The term "cell type specific" as applied to a promoter refers to a promoter, which is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue. The term "cell type specific" when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide sequence of interest in a region within a single tissue. Cell type specificity of a promoter may be assessed using methods well known in the art, e.g., GUS activity staining, GFP protein or immunohistochemical staining. The term "constitutive" when made in reference to a

20 Oct 2017 2017248552

promoter or the expression derived from a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid molecule in the absence of a stimulus (e.g., heat shock, chemicals, light, etc.) in the majority of plant tissues and cells throughout substantially the entire lifespan of a plant or part of a plant. Typically, constitutive promoters are capable of directing expression of a transgene in substantially any cell and any tissue.

Promoter specificity: The term “specificity” when referring to a promoter means the pattern of expression conferred by the respective promoter. The specificity describes the tissues and/or developmental status of a plant or part thereof, in which the promoter is conferring expression of the nucleic acid molecule under the control of the respective promoter. Specificity of a promoter may also comprise the environmental conditions, under which the promoter may be activated or down-regulated such as induction or repression by biological or environmental stresses such as cold, drought, wounding or infection.

Purified: As used herein, the term "purified" refers to molecules, either nucleic or amino acid sequences that are removed from their natural environment, isolated or separated. "Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated. A purified nucleic acid sequence may be an isolated nucleic acid sequence.

Recombinant: The term "recombinant" with respect to nucleic acid molecules refers to nucleic acid molecules produced by recombinant DNA techniques. Recombinant nucleic acid molecules may also comprise molecules, which as such does not exist in nature but are modified, changed, mutated or otherwise manipulated by man. Preferably, a "recombinant nucleic acid molecule" is a non-naturally occurring nucleic acid molecule that differs in sequence from a naturally occurring nucleic acid molecule by at least one nucleic acid. A “recombinant nucleic acid molecule” may also comprise a “recombinant construct” which comprises, preferably operably linked, a sequence of nucleic acid molecules not naturally occurring in that order. Preferred methods for producing said recombinant nucleic acid molecule may comprise cloning techniques, directed or non-directed mutagenesis, synthesis or recombination techniques.

Sense: The term "sense" is understood to mean a nucleic acid molecule having a sequence which is complementary or identical to a target sequence, for example a sequence which binds to a protein transcription factor and which is involved in the expression of a given gene. According to a preferred embodiment, the nucleic acid molecule comprises a gene of interest and elements allowing the expression of the said gene of interest.

Significant increase or decrease: An increase or decrease, for example in enzymatic activity or in gene expression, that is larger than the margin of error inherent in the measurement

2017248552 20 Oct 2017

technique, preferably an increase or decrease by about 2-fold or greater of the activity of the control enzyme or expression in the control cell, more preferably an increase or decrease by about 5-fold or greater, and most preferably an increase or decrease by about 10-fold or greater.

Small nucleic acid molecules: "small nucleic acid molecules" are understood as molecules consisting of nucleic acids or derivatives thereof such as RNA or DNA. They may be double-stranded or single-stranded and are between about 15 and about 30 bp, for example between 15 and 30 bp, more preferred between about 19 and about 26 bp, for example between 19 and 26 bp, even more preferred between about 20 and about 25 bp for example between 20 and 25 bp. In a especially preferred embodiment the oligonucleotides are between about 21 and about 24 bp, for example between 21 and 24 bp. In a most preferred embodiment, the small nucleic acid molecules are about 21 bp and about 24 bp, for example 21 bp and 24 bp.

Substantially complementary: In its broadest sense, the term "substantially complementary", when used herein with respect to a nucleotide sequence in relation to a reference or target nucleotide sequence, means a nucleotide sequence having a percentage of identity between the substantially complementary nucleotide sequence and the exact complementary sequence of said reference or target nucleotide sequence of at least 60%, more desirably at least 70%, more desirably at least 80% or 85%, preferably at least 90%, more preferably at least 93%, still more preferably at least 95% or 96%, yet still more preferably at least 97% or 98%, yet still more preferably at least 99% or most preferably 100% (the later being equivalent to the term "identical" in this context). Preferably identity is assessed over a length of at least 19 nucleotides, preferably at least 50 nucleotides, more preferably the entire length of the nucleic acid sequence to said reference sequence (if not specified otherwise below). Sequence comparisons are carried out using default GAP analysis with the University of Wisconsin GCG, SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-453; as defined above). A nucleotide sequence "substantially complementary " to a reference nucleotide sequence hybridizes to the reference nucleotide sequence under low stringency conditions, preferably medium stringency conditions, most preferably high stringency conditions (as defined above).

Transgene: The term "transgene" as used herein refers to any nucleic acid sequence, which is introduced into the genome of a cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (i.e., "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence, which is naturally found in the cell into which it is introduced so long as it does not contain some modification (e.g., a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence.

2017248552 20 Oct 2017

Transgenic: The term transgenic when referring to an organism means transformed, preferably stably transformed, with a recombinant DNA molecule that preferably comprises a suitable promoter operatively linked to a DNA sequence of interest.

Vector: As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid molecule to which it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromosomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid molecule capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context. Expression vectors designed to produce RNAs as described herein in vitro or in vivo may contain sequences recognized by any RNA polymerase, including mitochondrial RNA polymerase, RNA pol I, RNA pol II, and RNA pol III. These vectors can be used to transcribe the desired RNA molecule in the cell according to this invention. A plant transformation vector is to be understood as a vector suitable in the process of plant transformation.

Wild-type: The term "wild-type", "natural" or "natural origin" means with respect to an organism, polypeptide, or nucleic acid sequence, that said organism is naturally occurring or available in at least one naturally occurring organism which is not changed, mutated, or otherwise manipulated by man.

EXAMPLES

Chemicals and common methods

Unless indicated otherwise, cloning procedures carried out for the purposes of the present invention including restriction digest, agarose gel electrophoresis, purification of nucleic acids, Ligation of nucleic acids, transformation, selection and cultivation of bacterial cells were performed as described (Sambrook et al., 1989). Sequence analyses of recombinant DNA were performed with a laser fluorescence DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the Sanger technology (Sanger et al., 1977). Unless described otherwise, chemicals and reagents were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, USA), from Promega (Madison, WI, USA), Duchefa (Haarlem, The Netherlands) or Invitrogen (Carlsbad, CA, USA). Restriction endonucleases were from New England Biolabs (Ipswich, MA, USA) or Roche Diagnostics GmbH (Penzberg, Germany). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Example 1: Identification of Nucleic Acid Expression Enhancing Nucleic Acids (NEENA) from genes with constitutive expression

1.1 Identification of NEENA molecules from *A. thaliana* genes

Using publicly available genomic DNA sequences (e.g. http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html) and transcript expression data (e.g. http://www.weigelworld.org/resources/microarray/AtGenExpress/), a set of 18 potential NEENA candidates deriving from *Arabidopsis thaliana* transcripts from highly expressing constitutive genes were selected for detailed analyses. In addition, a putative NEENA molecule deriving from parsley was also included in the analysis. The candidates were named as follows:

Table 1: constitutive NEENA candidates (NEENAc).

NEENA name	Locus	Annotation	SEQ ID NO
NEENAc24		*Petroselinum crispum* gene Pcubi4-2 for polyubiquitin	1
NEENAc17	At2g47170	ADP-ribosylation factor 1 (ARF1)	2
NEENAc5	At1g56070	elongation factor 2, putative / EF-2, putative	3
NEENAc18	At5g54760	eukaryotic translation initiation factor SUI1, putative	4
NEENAc7	At4g02890	polyubiquitin (UBQ14)	5
NEENAc13	At3g03780	AtMS2 (Arabidopsis thaliana methionine synthase 2)	6
NEENAc1	At5g60390	elongation factor 1-alpha / EF-1-alpha	7
NEENAc21	At1g14400	ubiquitin-conjugating enzyme 1 (UBC1)	8
NEENAc16	At4g14880	cysteine synthase / O-acetylserine (thiol)-lyase / O-acetylserine sulfhydrylase (OAS1)	9
NEENAc2	At4g27960	ubiquitin-conjugating enzyme E2-17 kDa 9 (UBC9)	10
NEENAc14	At1g64230	ubiquitin-conjugating enzyme, putative	11
NEENAc4	At2g37270	40S ribosomal protein S5 (RPS5A)	12
NEENAc6	At4g05050	polyubiquitin (UBQ11)	13
NEENAc8	At1g43170	60S ribosomal protein L3 (RPL3A)	14

NEENAc11	At1g01100	60S acidic ribosomal protein P1 (RPP1A)	15
NEENAc12	At5g04800	40S ribosomal protein S17 (RPS17D)	16
NEENAc19	At4g34110	polyadenylate-binding protein 2 (PABP2)	17
NEENAc22	At2g34770	fatty acid hydroxylase (FAH1) (anticipated IME effect)	18
NEENAc23	At5g17920	cobalamin-independent methionine synthase (CIMS)	19

1.2 Isolation of the NEENA candidates

Genomic DNA was extracted from *A. thaliana* green tissue using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the putative NEENA molecule with the SEQ ID NO1, DNA of the vector construct 1bxPcUbi4-2GUS (WO 2003102198) was used. Genomic DNA fragments containing putative NEENA molecules were isolated by conventional polymerase chain reaction (PCR). The polymerase chain reaction comprised 19 sets of primers (Table 2). Primers were designed on the basis of the *A. thaliana* genome sequence with a multitude of NEENA candidates. The nucleotide sequence of the vector construct 1bxPcUbi4-2GUS (WO 2003102198) was used for the design of primers (SEQ ID NO56 and 57) for amplification of the NEENA candidate with SEQ ID NO1 (Table 2). The polymerase chain reaction followed the protocol outlined by Phusion High Fidelity DNA Polymerase (Cat No F-540L, New England Biolabs, Ipswich, MA, USA). The isolated DNA was used as template DNA in a PCR amplification using the following primers:

Table 2: Primer sequences

Primer name	Sequence	SEQ ID NO	PCR yielding SEQ ID NO
NEENAc1_for	tttatggtaccagccgcaagactcctttcagattct	20	7
NEENAc1_rev	aaattccatggtagctgtcaaaacaaaaacaaaaatcga	21	
NEENAc2_for	aaaaaggtacctcgaagaaccaaaaccaaaaacgtga	22	10
NEENAc2_rev	tttttccatggttatttatccaaaatcccacgatccaaattcca	23	
NEENAc4_for	tttttggtaccgatccctacttctctcgacact	24	12
NEENAc4_rev	ttttaccatggtgactggaggatcaatagaagat	25	
NEENAc5_for	tttttggtacctttctctcgttctcatctttctctct	26	3
NEENAc5_rev	taatagatatctttgtcaaacttttgattgtcacct	27	
NEENAc6_for	tataaggtaccaaatcaatctctcaaatctctca	28	13
NEENAc6_rev	tttatccatggtctgttaatcagaaaaaccgagat	29	
NEENAc7_for	tatatggtaccaaatcgttctttcaaatctctca	30	5
NEENAc7_rev	ttataccatggtctgtaattcacaaaaaactgaga	31	
NEENAc8_for	tttttggtacctcatcgttggagcttagaagc	32	14

NEENAc8_rev	tttttccatggtcttcttcttcttctacatca	33	
NEENAc11_for	tatatggtaccaaagcattttcgatcttactcttaggt	34	15
NEENAc11_rev	tttttccatggtttttatcctgaaacgattca	35	
NEENAc12_for	tttttggtacctttgacgccgccgcttcttcttct	36	16
NEENAc12-rev	tttttccatggtctttcagttacctgtgtgacttacct	37	
NEENAc13_for	tttaaggtacccatctctcatctccactcttct	38	6
NEENAc13_rev	tttttgatatcttttgtttgttttttgttttttact	39	
NEENAc14_for	ttataggtaccaagtgaatcgtcaaaaccgagtt	40	11
NEENAc14_rev	tttttccatggttctcaaccaaaaaaaaactcct	41	
NEENAc16_for	tttttggtaccacgattcgggtcaaggttattga	42	9
NEENAc16_rev	tttttccatggtgattcaagcttcactgcttaaattcaca	43	
NEENAc17_for	tttttggtaccttagatctcgtgccgtcgtgcga	44	2
NEENAc17_rev	tttttccatggtttgatcaagcctgttcaca	45	
NEENAc18_for	aaaaaggtacctcatcagatcttcaaaaccccaa	46	4
NEENAc18_rev	aaaaaccatggtgatttgagggtagtactaaccgggaa	47	
NEENAc19_for	ttttaggtaccatacgttaacttcaccaatccccaa	48	17
NEENAc19_rev	tttttccatggttaattaatgcagtgctttgtggtcgatgga	49	
NEENAc21_for	tttttcccgggatctttacctcaacaacgagat	50	8
NEENAc21_rev	tttttccatggttatcctcctttctttctaataaacaaaaccca	51	
NEENAc22_for	tttttggtacctctcttccgtctcgagtcgctgaga	52	18
NEENAc22_rev	tttttccatggtttgcagacctttactgat	53	
NEENAc23_for	tttttggtaccttccttcctcctctccgattcttcct	54	19
NEENAc23_rev	tttttccatggttattgattttctttactgcat	55	
NEENAc24_for	ttttttggtaccttaagaaatcctctcttctcct	56	1
NEENAc24_rev	ttttttccatggtctgcacatacataacatatca	57	

Amplification during the PCR was carried out with the following composition (50 microl):

3,00 microl *A. thaliana* genomic DNA (50 ng/microl genomic DNA, 5 ng/microl vector construct)
10,00 microl 5x Phusion HF Buffer
4,00 microl dNTP (2,5 mM)
2,50 microl for Primer (10 microM)
2,50 microl rev Primer (10 microM)
0,50 microl Phusion HF DNA Polymerase (2U/microl)

A touch-down approach was employed for the PCR with the following parameters: 98,0°C for 30 sec (1 cycle), 98,0°C for 30 sec, 56,0°C for 30 sec and 72,0°C for 60 sec (4 cycles), 4 additional cycles each for 54,0°C, 51,0°C and 49,0°C annealing temperature, followed by 20 cycles with 98,0°C for 30 sec, 46,0°C for 30 sec and 72,0°C for 60 sec (4 cycles) and 72,0°C for 5 min. The amplification products was loaded on a 2 % (w/v) agarose gel and separated

at 80V. The PCR products were excised from the gel and purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Following a DNA restriction digest with *KpnI* (10 U/microl) and *NcoI* (10 U/microl) or *EcoRV* (10U/microl) restriction endonuclease, the digested products were again purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

1.3 Vector construction

1.3.1 Generation of vector constructs with potential NEENA molecules

Using the Multisite Gateway System (Invitrogen, Carlsbad, CA, USA), the promoter::NEENA::reporter-gene cassettes were assembled into binary constructs for plant transformation. The *A. thaliana* p-AtNit1 (At3g44310, GenBank X86454; WO03008596, with the prefix p- denoting promoter) promoter was used in the reporter gene construct, and firefly luciferase (Promega, Madison, WI, USA) was utilized as reporter protein for quantitatively determining the expression enhancing effects of the putative NEENA molecules to be analyzed.

The pENTR/A vector holding the p-AtNit1 promoter was cloned via site specific recombination (BP-reaction) between the pDONR/A vector and p-AtNit1 amplification products with primers p-AtNit1-for and p-AtNit1-rev (Table 3) on genomic DNA (see above) with site specific recombination sites at either end according to the manufacturers manual (Invitrogen, Carlsbad, CA, USA). Positive pENTR/A clones underwent sequence analysis to ensure correctness of the p-AtNit1 promoter.

Table 3: Primer sequences (p-AtNit1)

Primer name	Sequence	SEQ ID NO.
p-AtNit1-for	ggggacaactttgtatagaaaagttgtcgagaccagatgttttacacttga	58
p-AtNit1-rev	ggggactgctttttgtacaaacttggacactcagagacttgagagaagca	59

An ENTR/B vector containing the firefly luciferase coding sequence (Promega, Madison, WI, USA) followed by the t-nos nopalin synthase transcriptional terminator (Genbank V00087) was generated. NEENA candidate PCR fragments (see above) were cloned separately upstream of the firefly luciferase coding sequence using *KpnI* and *NcoI* or *EcoRV* restriction enzymes. The resulting pENTR/B vectors are summarized in table 4, with promoter molecules having the prefix p-, coding sequences having the prefix c-, and terminator molecules having the prefix t-.

Table 4: all pENTR/B vectors plus and minus NEENA candidates

pENTR/B vector	Composition of the partial expression cassette SEQ ID NO::reporter gene::terminator
LJK1	MCS::c-LUC::t-nos
LJK4	SEQ ID NO1::c-LUC::t-nos
LJK40	SEQ ID NO7::c-LUC::t-nos
LJK41	SEQ ID NO10::c-LUC::t-nos
LJK43	SEQ ID NO12::c-LUC::t-nos
LJK44	SEQ ID NO3::c-LUC::t-nos
LJK46	SEQ ID NO13::c-LUC::t-nos
LJK47	SEQ ID NO5::c-LUC::t-nos
LJK48	SEQ ID NO14::c-LUC::t-nos
LJK51	SEQ ID NO15::c-LUC::t-nos
LJK52	SEQ ID NO16::c-LUC::t-nos
LJK53	SEQ ID NO6::c-LUC::t-nos
LJK54	SEQ ID NO11::c-LUC::t-nos
LJK56	SEQ ID NO9::c-LUC::t-nos
LJK57	SEQ ID NO2::c-LUC::t-nos
LJK58	SEQ ID NO4::c-LUC::t-nos
LJK59	SEQ ID NO17::c-LUC::t-nos
LJK61	SEQ ID NO8::c-LUC::t-nos
LJK62	SEQ ID NO18::c-LUC::t-nos
LJK63	SEQ ID NO19::c-LUC::t-nos

The pENTR/C vector was constructed by introduction of a multiple cloning site (SEQ ID NO60) via *KpnI* and *HindIII* restriction sites. By performing a site specific recombination (LR-reaction), the created pENTR/A, pENTR/B and pENTR/C were combined with the pSUN destination vector (pSUN derivative) according to the manufacturers (Invitrogen, Carlsbad, CA, USA) Multisite Gateway manual. The reactions yielded 1 binary vector with p-AtNit1 promoter, the firefly luciferase coding sequence c-LUC and the t-nos terminator and 19 vectors harboring SEQ ID NO1, NO2, NO3, NO4, NO5, NO6, NO7, NO8, NO9, NO10, NO11, NO12, NO13, NO14, NO15, NO16, NO17, NO18 and NO19 immediately upstream of the firefly luciferase coding sequence (Table 5), for which the combination with SEQ ID NO1 is given exemplary (SEQ ID NO61). Except for varying SEQ ID NO2 to NO19, the nucleotide sequence is identical in all vectors (Table 5). The resulting plant transformation vectors are summarized in table 5:

Table 5: Plant expression vectors for *A. thaliana* transformation

plant expression vector	Composition of the expression cassette Promoter::SEQ ID NO::reporter gene::terminator	SEQ ID NO
LJK132	p-AtNit1::-::c-LUC::t-nos	
LJK133	p-AtNit1::SEQ ID NO1::c-LUC::t-nos	61
LJK91	p-AtNit1::SEQ ID NO7::c-LUC::t-nos	
LJK92	p-AtNit1::SEQ ID NO10::c-LUC::t-nos	
LJK94	p-AtNit1::SEQ ID NO12::c-LUC::t-nos	
LJK95	p-AtNit1::SEQ ID NO3::c-LUC::t-nos	
LJK97	p-AtNit1::SEQ ID NO13::c-LUC::t-nos	
LJK98	p-AtNit1::SEQ ID NO5::c-LUC::t-nos	
LJK99	p-AtNit1::SEQ ID NO14::c-LUC::t-nos	
LJK102	p-AtNit1::SEQ ID NO15::c-LUC::t-nos	
LJK103	p-AtNit1::SEQ ID NO16::c-LUC::t-nos	
LJK104	p-AtNit1::SEQ ID NO6::c-LUC::t-nos	
LJK105	p-AtNit1::SEQ ID NO11::c-LUC::t-nos	
LJK107	p-AtNit1::SEQ ID NO9::c-LUC::t-nos	
LJK108	p-AtNit1::SEQ ID NO2::c-LUC::t-nos	
LJK109	p-AtNit1::SEQ ID NO4::c-LUC::t-nos	
LJK110	p-AtNit1::SEQ ID NO17::c-LUC::t-nos	
LJK112	p-AtNit1::SEQ ID NO8::c-LUC::t-nos	
LJK113	p-AtNit1::SEQ ID NO18::c-LUC::t-nos	
LJK114	p-AtNit1::SEQ ID NO19::c-LUC::t-nos	

The resulting vectors were subsequently used to transform *A. thaliana* leaf protoplasts transiently.

1.3.2 Renilla luciferase control construct

Renilla luciferase cDNA was amplified using 10ng of the plasmid pRL-null from Promega (Madison, WI, USA) as DNA template and primers R-LUC_for and R-LUC_rev (Table 6) with PCR parameters as described above.

Table 6: Primer sequences (c-RLUC)

Primer name	Sequence	SEQ ID NO
RLUC_for	aaaaaggtaccatgacttcgaaagtttatgatc	62
RLUC_rev	aaattgagctcttattgttcatttttgagaactc	63

Following a DNA restriction digest with *KpnI* (10 U/microl) and *SacI* (10 U/microl) restriction endonuclease, the digested products were again purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

The fragment was cloned into a ENTR/B vector containing the nopaline synthase constitutive promoter p-nos (Genbank V00087) followed by the t-nos nopalin synthase transcriptional terminator (Genbank V00087) via *KpnI* and *SacI* restriction sites, yielding a pENTR/B clone, which underwent sequence analysis to ensure correctness of the Renilla luciferase containing expression cassette.

Example 2: Screening for NEENA candidate molecules enhancing gene expression in *A. thaliana* transiently transformed leaf protoplasts

This example illustrates that only selected NEENA molecules are capable of enhancing gene expression.

2.1 Isolation and transient transformation of *A. thaliana* leaf protoplasts

Isolation and transient transformation of *A. thaliana* leaf protoplasts was amended according to established protocols (Damm and Willmitzer, 1988; Damm et al., 1989) Leaves of 4 week old *A. thaliana* plants were cut in small pieces using a razor blade and transferred to a solution with 1,5 % Cellulase R10 (Duchefa, Haarlem, The Netherlands), 0,3 % Mazerozyme R10 (Duchefa, Haarlem, The Netherlands), 400 mM Mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$, pH5,7 and incubated over night at room temperature. Due to a variability of transient *A. thaliana* leaf protoplast transformation, Renilla luciferase (Dual-Luciferase® Reporter Assay System, Promega, Madison, WI, USA) was used to normalize the firefly luciferase expression capabilities of the constructs above. The transient transformation of the NEENA-less (LJK132) and each NEENA-containing vector construct (LJK66 – LJK114) was performed in triplicate with 6 microg plasmid DNA, which was mixed with 25 microg of Renilla luciferase containing construct prior to transformation, using PEG (poly ethylene glycol) and 1 x 10^4 protoplasts.

2.2 Dual luciferase reporter gene assay

Transfected *A. thaliana* protoplasts were collected by centrifugation at 100 g and frozen in liquid nitrogen after removal of supernatant. The assay for detection of firefly and Renilla luciferase activity in the transfected cells was performed according to the manufacturers (Promega, Madison, WI, USA) Dual-Luciferase Reporter Assay System manual. Luminescence measurements were conducted in a MicroLumat Plus LB96V (Berthold Technologies, Bad Wildbad, Germany) recorded after addition of the luciferase substrates. Instrument readings of both luciferase recordings were normalized by generating a ratio between firefly luciferase and Renilla luciferase. The data from three experiments were averaged for each construct and based on these average expression values, fold change values were calculated to assess the impact of presence of a putative NEENA over reporter gene constructs lacking the respective putative NEENA. In comparison to p-AtNit1 promoter-

20 Oct 2017 2017248552

only NEENA-less reporter gene constructs, the 19 tested NEENA candidates containing constructs showed negative as well as positive effects, ranging from 0,1-fold to 18,1-fold induction in firefly Luciferase activity (Fig. 1). In total, 9 putative NEENA molecules comprising sequences with SEQ ID NO1, NO2, NO3, NO4, NO5, NO6, NO7, NO8 and NO9 conferred a greater than 2-fold increase in gene expression based on luciferase reporter gene activity compared to the NEENA-less promoter-only reporter gene construct (Fig. 1) and hence are functional NEENA molecules. Since a number of the tested NEENA candidate molecules have marginal or even negative effects on the enhancement of gene expression, not all putative NEENA molecules are mediating a common stimulatory effect, but rather that the selected NEENA sequences convey significant enhancement of gene expression (SEQ ID NO. 1 to 9).

Example 3: Test of NEENA molecules for enhancement of gene expression in oilseed rape plants

This example illustrates that NEENA molecules can be used across species to enhance gene expression in all tissues tested compared to a NEENA-less promoter-only approach. NEENA molecules mediating the strongest enhancement in gene expression in the pre-screening (cp. Example 2, SEQ ID NO1, NO2, NO3, NO4 and NO5) were selected for determining the enhancement on gene expression levels in transgenic oilseed rape plants.

3.1 Vector construction for *B. napus* plant transformation

For transformation of oilseed rape plants, reporter gene expression cassettes without and with gene expression control molecules (SEQ IDs NO1 – NO5) were combined with a gene expression cassette carrying a selectable marker gene for detecting transgenic plant lines within a pENTR/C vector. By performing a site specific recombination (LR-reaction), as previously described (see above, 1.4), the pENTR/A, pENTR/B and the pENTR/C carrying the selectable marker cassette were combined with the pSUN destination vector according to the manufacturers (Invitrogen, Carlsbad, CA, USA) Multisite Gateway manual. The reactions yielded one binary vector with p-AtNit1 promoter, the firefly luciferase coding sequence c-LUC, the t-nos terminator and the selectable marker cassette as well as 5 vectors harboring SEQ ID NO1, NO2, NO3, NO4, and NO5 immediately upstream of the firefly luciferase coding sequence (Table 7), for which the combination with SEQ ID NO1 is given exemplary (SEQ ID NO64). Except for varying SEQ ID NO2 to NO5, the nucleotide sequence is identical in all vectors (Table 7). The resulting plant transformation vectors are summarized in table 7:

Table 7: Plant expression vectors for *B. napus* transformation

plant expression vector	Composition of the expression cassette Promoter::SEQ ID NO::reporter gene::terminator	SEQ ID NO
LJK138	p-AtNit1::-::c-LUC::t-nos	
LJK139	p-AtNit1::SEQ ID NO1::c-LUC::t-nos	64
LJK141	p-AtNit1::SEQ ID NO3::c-LUC::t-nos	
LJK142	p-AtNit1::SEQ ID NO5::c-LUC::t-nos	
LJK143	p-AtNit1::SEQ ID NO2::c-LUC::t-nos	
LJK144	p-AtNit1::SEQ ID NO4::c-LUC::t-nos	

3.2 Generation of transgenic rapeseed plants (amended protocol according to Moloney et al., 1992, Plant Cell Reports, 8: 238-242).

In preparation for the generation of transgenic rapeseed plants, the binary vectors were transformed into *Agrobacterium tumefaciens* C58C1:pGV2260 (Deblaere et al., 1985, Nucl. Acids. Res. 13: 4777-4788). A 1:50 dilution of an overnight culture of Agrobacteria harboring the respective binary construct was grown in Murashige-Skoog Medium (Murashige and Skoog, 1962, Physiol. Plant 15, 473) supplemented with 3 % saccharose (3MS-Medium). For the transformation of rapeseed plants, petioles or hypocotyledons of sterile plants were incubated with a 1:50 *Agrobacterium* solution for 5 – 10 minutes followed by a three-day co-incubation in darkness at 25°C on 3 MS. Medium supplemented with 0,8 % bacto-agar. After three days, the explants were transferred to MS-medium containing 500 mg/l Claforan (Cefotaxime-Sodium), 100 nM Imazetapyr, 20 microM Benzylaminopurin (BAP) and 1,6 g/l Glucose in a 16 h light / 8 h darkness light regime, which was repeated in weekly periods. Growing shoots were transferred to MS-Medium containing 2 % saccharose, 250 mg/l Claforan and 0,8 % Bacto-agar. After 3 weeks, the growth hormone 2-Indolbutyl acid was added to the medium to promote root formation. Shoots were transferred to soil following root development, grown for two weeks in a growth chamber and grown to maturity in greenhouse conditions.

3.3 Plant analysis

Tissue samples were collected from the generated transgenic plants from leaves, flowers and siliques, stored in a freezer at -80°C subjected to a Luciferase reporter gene assay (amended protocol after Ow et al., 1986). After grinding the frozen tissue samples were resuspended in 800 microl of buffer I (0,1 M Phosphate buffer pH7,8, 1 mM DTT (Sigma Aldrich, St. Louis, MO, USA), 0,05 % Tween 20 (Sigma Aldrich, St. Louis, MO, USA)) followed by centrifugation at 10 000 g for 10 min. 75 microl of the aqueous supernatant were transferred to 96-well plates. After addition of 25 microl of buffer II (80 mM gycine-glycyl (Carl Roth, Karlsruhe, Germany), 40 mM MgSO4 (Duchefa, Haarlem, The Netherlands), 60 mM ATP (Sigma Aldrich, St. Louis, MO, USA), pH 7,8) and D-Luciferin to a final concentration of 0,5 mM (Cat No: L-8220, BioSynth, Staad, Switzerland), luminescence was

recorded in a MicroLumat Plus LB96V (Berthold Technologies, Bad Wildbad, Germany) yielding the unit relative light unit RLU per minute (RLU/min).

In order to normalize the luciferase activity between samples, the protein concentration was determined in the aqueous supernatant in parallel to the luciferase activity (adapted from Bradford, 1976, Anal. Biochem. 72, 248). 5 microl of the aqueous cell extract in buffer I were mixed with 250 microl of Bradford reagent (Sigma Aldrich, St. Louis, MO, USA), incubated for 10 min at room temperature. Absorption was determined at 595 nm in a plate reader (Thermo Electron Corporation, Multiskan Ascent 354). The total protein amounts in the samples were calculated with a previously generated standard concentration curve. Values resulting from a ratio of RLU/min and mg protein/ml sample were averaged for transgenic plants harboring identical constructs and fold change values were calculated to assess the impact of NEENA molecule presence over NEENA-less reporter gene constructs.

3.4 NEENA sequences mediate strong enhancement of gene expression in oilseed rape plants

For assessing the potential of enhancing gene expression of selected NEENA molecules (SEQ ID NO:1, 2, 3, 4 and 5) in oilseed rape plants, leafs, flowers and siliques harboring seeds of plants having identical developmental stages and which were grown under equal growth conditions were collected. The samples were taken from individual transgenic oilseed rape plant lines harboring either a promoter-only reporter gene construct or Luciferase reporter gene constructs containing a NEENA (SEQ ID NO1, 2, 3, 4 and 5). 10 seeds were collected from each transgenic event, processed and analyzed for Luciferase activity as described above (Example 3.3).

In comparison to the constitutive p-AtNit1 promoter-only NEENA-less reporter gene construct, the five tested NEENA molecules all mediated strong enhancements in gene expression in leaf tissues (Fig. 2, a). Comparable enhancement of gene expression mediated by NEENAs (SEQ ID NO1, 2, 3, 4 and 5) was detected in oilseed rape flowers and siliques including seeds (Fig. 2, b and c).

Example 4: Analysis of constitutive enhancement of gene expression in soybean plants

This example illustrates that NEENA molecules can be used in a wide array of plant species and across species borders from different plant families to enhance gene expression in all tissues compared to a NEENA-less promoter-only approach.

NEENA sequence molecules mediating the strongest enhancement in gene expression in the pre-screening (cp. Example 2, SEQ ID NO1, 2, 3, 4 and 5) were selected for determining the enhancement on gene expression levels in transgenic soybean plants. Plant expression vectors LJK138, LJK139, LJK141, LJK142, LJK143 and LJK144 (cp. example 3.1) were used for stable soybean transformation.

4.1 Generation of transgenic soybean plants (amended protocol according to WO2005/121345; Olhoft et al., 2007).

Soybean seed germination, propagation, *A. rhizogenes* and axillary meristem explant preparation, and inoculations were done as previously described (WO2005/121345; Olhoft et al., 2007) with the exception that the constructs LJK138, LJK139, LJK141, LJK142, LJK143 and LJK144 (cp. example 3.1) each contained a mutated AHAS gene driven by the parsley ubiquitin promoter PcUbi4-2, mediating tolerance to imidazolinone herbicides for selection.

4.2 NEENA sequences mediate strong enhancement of gene expression in soybean plants

Tissue samples were collected from the generated transgenic plants from leaves, flowers and seeds. The tissue samples were processed and analyzed as described above (cp. example 3.3)

In comparison to the constitutive p-AtNit1 promoter-only NEENA-less reporter gene construct, the five tested NEENA molecules all mediated strong enhancements in gene expression in leaves (Fig. 3a). Comparable enhancement of gene expression mediated by NEENAs (SEQ ID NO1 to NO5) was detected in soybean flowers and siliques (Fig. 3, b and c).

Example 5: Analysis of NEENA activity in monocotyledonous plants

This example describes the analysis of NEENA sequences with SEQ ID NO 1, 2, 3, 4 and 5 in monocotyledonous plants.

5.1 Vector Construction

For analyzing NEENA sequences with SEQ ID NO 1, 2, 3, 4 and 5 in monocotyledonous plants, a pUC-based expression vector harboring an expression cassette composed of the NEENA-less, constitutive monocotyledonous promoter p-Ubi from *Z. mais* is combined with a coding sequence of the beta-Glucuronidase (GUS) gene followed by the nopaline synthase (NOS) transcriptional terminator. Genomic DNA is extracted from *A. thaliana* green tissue using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA fragments containing NEENA molecules are isolated by conventional polymerase chain reaction (PCR). Primers are designed on the basis of the *A. thaliana* genome sequence with a multitude of NEENA candidates. The reaction comprises 5 sets of primers (Table 8) and follows the protocol outlined by Phusion High Fidelity DNA Polymerase (Cat No F-540L, New England Biolabs, Ipswich, MA, USA) using the following primers:

Table 8: Primer sequences

Primer name	Sequence	SEQ ID NO	PCR yielding SEQ ID NO
NEENAc5_forII	tttttggcgcgcctttctctcgttctcatctttctctct	65	3
NEENAc5_revII	taataggcgcgcctttgtcaaacttttgattgtcacct	66	
NEENAc7_forII	tatatggcgcgccaaatcgttctttcaaatctctca	67	5
NEENAc7_revII	ttataggcgcgcctctgtaattcacaaaaaactgaga	68	
NEENAc17_forII	tttttggcgcgccttagatctcgtgccgtcgtgcga	69	2
NEENAc17_revII	tttttggcgcgcctttgatcaagcctgttcaca	70	
NEENAc18_forII	aaaaaggcgcgcctcatcagatcttcaaaaccccaa	71	4
NEENAc18_revII	aaaaaggcgcgcctgatttgagggtagtactaaccgggaa	72	
NEENAc24_forII	ttttttggcgcgccttaagaaatcctctcttctcct	73	1
NEENAc24_revII	ttttttggcgcgccctgcacatacataacatatca	74	

Amplification during the PCR and purification of the amplification products is carried out as detailed above (example 1.2). Following a DNA restriction digest with *AscI* (10 U/microl) restriction endonuclease, the digested products are purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

NEENA PCR fragments (see above) are cloned separately upstream of the beta-Glucuronidase coding sequence using *AscI* restriction sites. The reaction yields one binary vector with the p-Ubi promoter, the beta-Glucuronidase coding sequence c-GUS and the t-nos terminator and five vectors harboring SEQ ID NO1, NO2, NO3, NO4 and NO5, immediately upstream of the beta-Glucuronidase coding sequence (Table 9), for which the combination with SEQ ID NO1 is given exemplary (SEQ ID NO75). Except for varying SEQ ID NO2 to NO5, the nucleotide sequence is identical in all vectors (Table 9). The resulting vectors are summarized in table 9, with promoter molecules having the prefix p-, coding sequences having the prefix c-, and terminator molecules having the prefix t-.

Table 9: Plant expression vectors

plant expression vector	Composition of the expression cassette Promoter::SEQ ID NO::reporter gene::terminator	SEQ ID NO
RTP2940	p-Ubi::-::c-GUS::t-nos	
LJK361	p-Ubi::SEQ ID NO1::c-GUS::t-nos	75
LJK362	p-Ubi::SEQ ID NO2::c-GUS::t-nos	
LJK363	p-Ubi::SEQ ID NO3::c-GUS::t-nos	
LJK364	p-Ubi::SEQ ID NO4::c-GUS::t-nos	
LJK365	p-Ubi::SEQ ID NO5::c-GUS::t-nos	

The resulting vectors are used to analyze NEENA molecules in experiments outlined below (Example 5.2).

5.2 Analysis of NEENA molecules enhancing gene expression in monocotyledonous plant tissues

These experiments are performed by bombardment of monocotyledonous plant tissues or culture cells (Example 6.2.1), by PEG-mediated (or similar methodology) introduction of DNA to plant protoplasts (Example 6.2.2), or by *Agrobacterium*-mediated transformation (Example 6.3.3). The target tissue for these experiments can be plant tissues (*e.g.* leaf tissue), cultured plant cells (*e.g.* maize Black Mexican Sweetcorn (BMS), or plant embryos for *Agrobacterium* protocols.

5.2.1 Transient assay using microprojectile bombardment

The plasmid constructs are isolated using Qiagen plasmid kit (cat# 12143). DNA is precipitated onto 0.6 microM gold particles (Bio-Rad cat# 165 -2262) according to the protocol described by Sanford *et al.* (1993) (Optimizing the biolistic process for different biological applications. Methods in Enzymology, 217: 483-509) and accelerated onto target tissues (*e.g.* two week old maize leaves, BMS cultured cells, *etc.*) using a PDS-1000/He system device (Bio-Rad). All DNA precipitation and bombardment steps are performed under sterile conditions at room temperature. Black Mexican Sweet corn (BMS) suspension cultured cells are propagated in BMS cell culture liquid medium [Murashige and Skoog (MS) salts (4.3 g/L), 3% (w/v) sucrose, myo-inositol (100 mg/L), 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), casein hydrolysate (1 g/L), thiamine (10 mg/L) and L-proline (1.15 g/L), pH 5.8]. Every week 10 mL of a culture of stationary cells are transferred to 40 mL of fresh medium and cultured on a rotary shaker operated at 110 rpm at 27°C in a 250 mL flask.

60 mg of gold particles in a siliconized Eppendorf tube are resuspended in 100% ethanol followed by centrifugation for 30 seconds. The pellet is rinsed once in 100% ethanol and twice in sterile water with centrifugation after each wash. The pellet is finally resuspended in 1 mL sterile 50% glycerol. The gold suspension is then divided into 50 microL aliquots and stored at 4°C. The following reagents are added to one aliquot: 5 microL of 1 microg/microL total DNA, 50 microL 2.5 M $CaCl_2$, 20 microL 0.1 M spermidine, free base. The DNA solution is vortexed for 1 minute and placed at -80°C for 3 min followed by centrifugation for 10 seconds. The supernatant is removed. The pellet is carefully resuspended in 1 mL 100% ethanol by flicking the tube followed by centrifugation for 10 seconds. The supernatant is removed and the pellet is carefully resuspended in 50 microL of 100% ethanol and placed at -80°C until used (30 min to 4 hr prior to bombardment). If gold aggregates are visible in the solution the tubes are sonicated for one second in a waterbath sonicator just prior to use.

For bombardment, two -week-old maize leaves are cut into pieces approximately 1 cm in length and placed ad-axial side up on osmotic induction medium M-N6-702 [N6 salts (3.96

g/L), 3% (w/v) sucrose, 1.5 mg/L 2,4 -dichlorophenoxyacetic acid (2,4-D), casein hydrolysate (100 mg/L), and L-proline (2.9 g/L), MS vitamin stock solution (1 mL/L), 0.2 M mannitol, 0.2 M sorbitol, pH 5.8]. The pieces are incubated for 1-2 hours.

In the case of BMS cultured cells, one-week-old suspension cells are pelleted at 1000 g in a Beckman/Coulter Avanti J25 centrifuge and the supernatant is discarded. Cells are placed onto round ash-free No 42 Whatman filters as a 1/16 inch thick layer using a spatula. The filter papers holding the plant materials are placed on osmotic induction media at 27°C in darkness for 1-2 hours prior to bombardment. Just before bombardment the filters are removed from the medium and placed onto on a stack of sterile filter paper to allow the calli surface to partially dry.

Each plate is shot with 6 microL of gold -DNA solution twice, at 1,800 psi for the leaf materials and at 1,100 psi for the BMS cultured cells. To keep the position of plant materials, a sterilized wire mesh screen is laid on top of the sample. Following bombardment, the filters holding the samples are transferred onto M-N6-702 medium lacking mannitol and sorbitol and incubated for 2 days in darkness at 27°C prior to transient assays.

The transient transformation via microprojectile bombardment of other monocotyledonous plants are carried out using, for example, a technique described in Wang et al., 1988 (Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. Plant Molecular Biology, 11 (4), 433-439), Christou, 1997 (Rice transformation: bombardment. Plant Mol Biol. 35 (1-2)).

Expression levels of the expressed genes in the constructs described above (example 5.1) are determined by GUS staining, quantification of luminescence /fluorescence, RT-PCR and protein abundance (detection by specific antibodies) using the protocols in the art. GUS staining is done by incubating the plant materials in GUS solution [100 mM NaHPO4, 10 mM EDTA, 0.05% Triton X100, 0.025% X-Gluc solution (5-bromo-4-chloro -3-indolyl-beta-D-glucuronic acid dissolved in DMSO), 10% methanol, pH 7.0] at 37 °C for 16-24 hours. Plant tissues are vacuum-infiltrated 2 times for 15 minutes to aid even staining. Analyses of luciferase activities are performed as described above (example 2 and 3.3).

In comparison to the constitutive p-Ubi promoter-only NEENA-less reporter gene construct, the NEENA molecules all mediate strong enhancement in gene expression in these assays.

5.2.2 Transient assay using protoplasts

Isolation of protoplasts is conducted by following the protocol developed by Sheen (1990) (Metabolic Repression of Transcription in Higher Plants. The Plant Cell 2 (10), 1027-1038). Maize seedlings are kept in the dark at 25°C for 10 days and illuminated for 20 hours before protoplast preparation. The middle part of the leaves are cut to 0.5 mm strips (about 6 cm in length) and incubated in an enzyme solution containing 1% (w/v) cellulose RS, 0.1% (w/v) macerozyme R10 (both from Yakult Honsha, Nishinomiya, Japan), 0.6 M mannitol, 10 mM Mes (pH 5.7), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM beta-mercaptoethanol, and 0.1% BSA (w/v) for 3 hr at 23°C followed by gentle shaking at 80 rpm for 10 min to release protoplasts.

Protoplasts are collected by centrifugation at 100 x g for 2 min, washed once in cold 0.6 M mannitol solution, centrifuged, and resuspended in cold 0.6 M mannitol (2 x 10^6/mL).

A total of 50 microg plasmid DNA in a total volume of 100 microL sterile water is added into 0.5 mL of a suspension of maize protoplasts (1 x 10^6 cells/mL) and mixed gently. 0.5 mL PEG solution (40 % PEG 4,000, 100 mM $CaNO_3$, 0.5 mannitol) is added and pre-warmed at 70°C with gentle shaking followed by addition of 4.5 mL MM solution (0.6 M mannitol, 15 mM $MgCl_2$, and 0.1 % MES). This mixture is incubated for 15 minutes at room temperature. The protoplasts are washed twice by pelleting at 600 rpm for 5 min and resuspending in 1.0 mL of MMB solution [0.6 M mannitol, 4 mM Mes (pH 5.7), and brome mosaic virus (BMV) salts (optional)] and incubated in the dark at 25°C for 48 hr. After the final wash step, the protoplasts are collected in 3 mL MMB medium, and incubated in the dark at 25°C for 48 hr.
The transient transformation of protoplasts of other monocotyledonous plants are carried out using, for example, a technique described in Hodges et al., 1991 (Transformation and regeneration of rice protoplasts. Biotechnology in agriculture No. 6, Rice Biotechnology. International Rice Research Institute, ISBN: 0-85198-712-5) or Lee et al., 1990 (Transient gene expression in wheat (*Triticum aestivum*) protoplasts. Biotechnology in agriculture and forestry 13 – Wheat. Springer Verlag, ISBN-10: 3540518096).
Expression levels of the expressed genes in the constructs described above (Example 5.1) are determined by GUS staining, quantification of luminescence /fluorescence, RT-PCR or protein abundance (detection by specific antibodies) using the protocols in the art. GUS staining is done by incubating the plant materials in GUS solution [100 mM NaHPO4, 10 mM EDTA, 0.05% Triton X100, 0.025% X-Gluc solution (5-bromo-4-chloro -3-indolyl-beta-D-glucuronic acid dissolved in DMSO), 10% methanol, pH 7.0] at 37 °C for 16-24 hours. Analyses of luciferase activities are performed as described above (Example 2 and 3.3).
In comparison to the constitutive p-Ubi promoter-only NEENA-less reporter gene construct, the NEENA molecules mediate strong enhancement in gene expression in these assays.

5.2.3 Transformation and regeneration of monocotyledonous crop plants

The *Agrobacterium*-mediated plant transformation using standard transformation and regeneration techniques may also be carried out for the purposes of transforming crop plants (Gelvin and Schilperoort, 1995, Plant Molecular Biology Manual, 2nd Edition, Dordrecht: Kluwer Academic Publ. ISBN 0-7923-2731-4; Glick and Thompson (1993) Methods in Plant Molecular Biology and Biotechnology, Boca Raton: CRC Press, ISBN 0-8493-5164-2). The transformation of maize or other monocotyledonous plants can be carried out using, for example, a technique described in US 5,591,616. The transformation of plants using particle bombardment, polyethylene glycol-mediated DNA uptake or via the silicon carbonate fiber technique is described, for example, by Freeling & Walbot (1993) "The maize handbook" ISBN 3-540-97826-7, Springer Verlag New York).
Expression levels of the expressed genes in the constructs described above (Example 5.1) are determined by GUS staining, quantification of luminescence or fluorescence, RT-PCR,

protein abundance (detection by specific antibodies) using the protocols in the art. GUS staining is done by incubating the plant materials in GUS solution [100 mM NaHPO4, 10 mM EDTA, 0.05% Triton X100, 0.025% X-Gluc solution (5-bromo-4-chloro -3-indolyl-beta-D-glucuronic acid dissolved in DMSO), 10% methanol, pH 7.0] at 37 °C for 16-24 hours. Plant tissues are vacuum-infiltrated 2 times for 15 minutes to aid even staining. Analyses of luciferase activities are performed as described above (Examples 2 and 3.3).
In comparison to the constitutive p-Ubi promoter-only NEENA-less reporter gene constructs, the NEENA molecules mediate strong enhancement in gene expression in plants.

Example 6: Quantitative analysis of NEENA activity in corn plants

This example describes the analysis of NEENA sequences with SEQ ID NO 1 and 2 in corn plants.

6.1 Vector Construction
For analyzing NEENA sequences with SEQ ID NO 1 and 2 in monocotyledonous plants quantitatively, a pUC-based expression vector harboring an expression cassette composed of the NEENA-less, constitutive monocotyledonous promoter p-Ubi from *Z. mais* was combined with a coding sequence of the firefly luciferase (LUC) gene (Promega, Madison, WI, USA) followed by the nopaline synthase (NOS) transcriptional terminator. Genomic DNA was extracted from *A. thaliana* green tissue using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA fragments containing NEENA molecules were isolated by conventional polymerase chain reaction (PCR). Primers were designed on the basis of the *A. thaliana* genome sequence with a multitude of NEENA candidates. The reaction comprised 2 sets of primers (Table 10) and followed the protocol outlined by Phusion High Fidelity DNA Polymerase (Cat No F-540L, New England Biolabs, Ipswich, MA, USA) using the following primers:

Table 10: Primer sequences

Primer name	Sequence	SEQ ID NO	PCR yielding SEQ ID NO
NEENAc17_forIII	atatacgcgtttagatctcgtgccgtcg	76	2
NEENAc17_revIII	atatggcgcgcctttgatcaagcctgttcaca	77	
NEENAc24_forIII	atatacgcgtttaagaaatcctctcttctcctc	78	1
NEENAc24_revIII	atatggcgcgccctgcacatacataacatatcaagatc	79	

Amplification during the PCR and purification of the amplification products was carried out as detailed above (example 1.2). Following a DNA restriction digest with *MluI* (10 U/microl) and

AscI (10 U/microl) restriction endonucleases, the digested products were purified with the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

NEENA PCR fragments (see above) were cloned separately upstream of the firefly luciferase coding sequence using *AscI* restriction sites. The reaction yielded one binary vector with the p-Ubi promoter, the firefly luciferase coding sequence c-LUC and the t-nos terminator and two vectors harboring SEQ ID NO1 and NO2, immediately upstream of the firefly luciferase coding sequence (Table 11), for which the combination with SEQ ID NO1 is given exemplary (SEQ ID NO80). Except for varying SEQ ID NO2, the nucleotide sequence is identical in the vectors (Table 11). The resulting vectors are summarized in table 11, with promoter molecules having the prefix p-, coding sequences having the prefix c-, and terminator molecules having the prefix t-.

Table 11: Plant expression vectors

plant expression vector	Composition of the expression cassette Promoter::SEQ ID NO::reporter gene::terminator	SEQ ID NO
LJK309	p-Ubi::-::c-LUC::t-nos	
LJK327	p-Ubi::SEQ ID NO1::c-LUC::t-nos	80
LJK326	p-Ubi::SEQ ID NO2::c-LUC::t-nos	

The resulting vectors were used to analyze NEENA molecules in experiments outlined below (Example 6.2).

6.2 Generation of transgenic maize plants

Maize germination, propagation, *A. tumefaciens* preparation and inoculations were done as previously described (WO2006136596, US20090249514) with the exception that the constructs LJK309, LJK326 and LJK327 (cp. example 6.1) each contained a mutated AHAS gene driven by the corn ubiquitin promoter p-Ubi, mediating tolerance to imidazolinone herbicides for selection.

6.3 NEENA sequences mediate strong enhancement of gene expression in corn plants

Tissue samples were collected from the generated transgenic plants from leaves and kernels. The tissue samples were processed and analyzed as described above (cp. example 3.3)

In comparison to the constitutive p-Ubi promoter-only NEENA-less reporter gene construct, the two tested NEENA molecules mediated strong enhancements in gene expression in leaves (Fig. 4a). Comparable enhancement of gene expression mediated by NEENAs (SEQ ID NO1 to NO2) was detected in maize kernels (Fig. 4b).

Example 7: Quantitative analysis of NEENA activity in rice plants

This example describes the analysis of NEENA sequences with SEQ ID NO 1 in rice plants.

7.1 Vector Construction

For analyzing NEENA sequences with SEQ ID NO 1 in rice plants quantitatively, pENTR/B vectors LJK1 and LJK4 (compare example 1.3) were combined with a destination vector harboring the constitutive PRO0239 upstream of the recombination site using site specific recombination (LR-reaction) according to the manufacturers (Invitrogen, Carlsbad, CA, USA) Gateway manual. The reactions yielded one binary vector with PRO0239 promoter, the firefly luciferase coding sequence c-LUC and the t-nos terminator as well as 1 vector harboring SEQ ID NO1 immediately upstream of the firefly luciferase coding sequence (Table 12),. The resulting vectors are summarized in table 12, with promoter molecules having the prefix p-, coding sequences having the prefix c-, and terminator molecules having the prefix t-.

Table 12: Plant expression vectors

plant expression vector	Composition of the expression cassette Promoter::SEQ ID NO::reporter gene::terminator	SEQ ID NO
CD30963	p-PRO0239 ::-::c-LUC::t-nos	
CD30964	p-PRO0239 ::SEQ ID NO1::c-LUC::t-nos	-

The resulting vectors were used to analyze NEENA molecules in experiments outlined below (Example 7.2).

7.2 Generation of transgenic rice plants

The *Agrobacterium* containing the respective expression vector was used to transform *Oryza sativa* plants. Mature dry seeds of the rice japonica cultivar Nipponbare were dehusked. Sterilization was carried out by incubating for one minute in 70% ethanol, followed by 30 minutes in 0.2% $HgCl_2$, followed by a 6 times 15 minutes wash with sterile distilled water. The sterile seeds were then germinated on a medium containing 2,4-D (callus induction medium). After incubation in the dark for four weeks, embryogenic, scutellum-derived calli were excised and propagated on the same medium. After two weeks, the calli were multiplied or propagated by subculture on the same medium for another 2 weeks. Embryogenic callus pieces were sub-cultured on fresh medium 3 days before co-cultivation (to boost cell division activity).

Agrobacterium strain LBA4404 containing the respective expression vector was used for co-cultivation. *Agrobacterium* was inoculated on AB medium with the appropriate antibiotics and cultured for 3 days at 28°C. The bacteria were then collected and suspended in liquid co-cultivation medium to a density (OD_{600}) of about 1. The suspension was then transferred to a Petri dish and the calli immersed in the suspension for 15 minutes. The callus tissues were then blotted dry on a filter paper and transferred to solidified, co-cultivation medium and incubated for 3 days in the dark at 25°C. Co-cultivated calli were grown on 2,4-D-containing medium for 4 weeks in the dark at 28°C in the presence of a selection agent. During this period, rapidly growing resistant callus islands developed. After transfer of this material to a regeneration medium and incubation in the light, the embryogenic potential was released and shoots developed in the next four to five weeks. Shoots were excised from the calli and incubated for 2 to 3 weeks on an auxin-containing medium from which they were transferred to soil. Hardened shoots were grown under high humidity and short days in a greenhouse.

Approximately 35 independent T0 rice transformants were generated for one construct. The primary transformants were transferred from a tissue culture chamber to a greenhouse. After a quantitative PCR analysis to verify copy number of the T-DNA insert, only single copy transgenic plants that exhibit tolerance to the selection agent were kept for harvest of T1 seed. Seeds were then harvested three to five months after transplanting. The method yielded single locus transformants at a rate of over 50 % (Aldemita and Hodges1996, Chan et al. 1993, Hiei et al. 1994).

7.3 NEENA sequences mediate strong enhancement of gene expression in rice plants

Tissue samples were collected from the generated transgenic plants from leaves and kernels. The tissue samples were processed and analyzed as described above (cp. example 3.3)

In comparison to the constitutive p-PRO239 promoter-only NEENA-less reporter gene construct, the tested NEENA molecule (SEQ ID NO 1) mediated strong enhancements in gene expression in leaves (Fig. 5a). Strong enhancement of gene expression mediated by the NEENA (SEQ ID NO1) was detected in rice seeds (Fig. 5b).

Figure legends:

Fig. 1: Luciferase reporter gene expression analysis in transiently transformed *A. thaliana* leaf protoplasts of NEENA-less (LJK132) and NEENA-containing constructs (LJK91 – LJK133) representing putative NEENA molecules deriving from constitutively expressed genes under the control of the p-AtNit1 promoter. Normalization was performed by cotransformation and analysis of the Renilla luciferase and expression values are shown in relation to the NEENA-less control construct (LJK132 = 1). Expression values are shown in relation to the NEENA-less control construct (LJK134 = 1).

2017248552 20 Oct 2017

Fig. 2: Bar graph of the luciferase reporter gene activity shown as relative light units (RLU) of independent transgenic oilseed rape plant lines harboring NEENA-less (LJK138) or NEENA-containing reporter gene constructs representing NEENA molecules from constitutively expressed genes (LJK139 – LJK144) under the control of the p-AtNit1 promoter and after normalization against the protein content of each sample (averaged values, tissues of 20 independent transgenic plants analyzed). A) leaf tissue, B) flowers, C) siliques

Fig. 3: Bar graph of the luciferase reporter gene activity shown as relative light units (RLU) of independent transgenic soybean plant lines harboring NEENA-less (LJK138) or NEENA-containing reporter gene constructs representing NEENA molecules from constitutively expressed genes (LJK139 – LJK144) under the control of the p-AtNit1 promoter and after normalization against the protein content of each sample (averaged values, tissues of 10 independent transgenic plants analyzed). A) leaf tissue, B) flowers, C) seeds

Fig. 4: Bar graph of the luciferase reporter gene activity shown as relative light units (RLU) (log scale) of independent transgenic maize plant lines harboring NEENA-less (LJK309) or NEENA-containing reporter gene constructs representing NEENA molecules from constitutively expressed genes (LJK326 – LJK327) under the control of the p-ZmUbi promoter and after normalization against the protein content of each sample (averaged values, tissues of 15 independent transgenic plants analyzed). A) leaf tissue, B) kernels

Fig. 5: Bar graph of the luciferase reporter gene activity shown as relative light units (RLU) of independent transgenic rice plant lines harboring NEENA-less (CD30963) or the NEENA-containing reporter gene construct representing a NEENA molecule from constitutively expressed genes (CD30964) under the control of the constitutive p-PRO239 promoter and after normalization against the protein content of each sample (averaged values, tissues of 15 independent transgenic plants analyzed). A) leaf tissue, B) seeds

2017248552 03 Jun 2019

What is claimed is:

1. A method for producing a high expression constitutive plant promoter, the method comprising functionally linking to a promoter one or more nucleic acid expression enhancing nucleic acids (NEENA) heterologous to said promoter, the NEENA comprising

   i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

   ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

   iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

   iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

   v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47.

2. A method for producing a plant or part thereof with, compared to a respective control plant or part thereof, enhanced constitutive expression of one or more nucleic acid molecules comprising the steps of

   a) introducing into the plant or part thereof one or more NEENA comprising

      i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

      ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

      iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

      iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

      v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47,

2017248552 03 Jun 2019

and

b) functionally linking said one or more NEENA to a constitutive promoter and to a nucleic acid molecule being under the control of said constitutive promoter, wherein the NEENA is heterologous to said nucleic acid molecule and to said promoter.

3. The method of claim 1 or claim 2 comprising the steps of

a) introducing the one or more NEENA into a plant or part thereof, and

b) integrating said one or more NEENA into the genome of said plant or part thereof wherein said one or more NEENA is functionally linked to an endogenous constitutively expressed nucleic acid heterologous to said one or more NEENA, and optionally

c) regenerating a plant or part thereof comprising said one or more NEENA from said plant or part thereof.

4. The method of any one of claims 1 to 3 comprising the steps of

a) integrating an expression construct comprising the one or more NEENA functionally linked to a constitutive promoter and to one or more nucleic acid molecules the latter being heterologous to said one or more NEENA and which is under the control of said constitutive promoter into the genome of said plant or part thereof, and optionally

b) regenerating a plant or part thereof comprising said one or more expression construct from said plant or part thereof.

5. The method of any one of claims 1 to 4 wherein the plant is a monocot or dicot plant.

6. The method of claim 5 wherein the plant is a dicot plant.

7. The method of claim 5 wherein the plant is a monocot plant.

8. The method of any one of claims 1 to 7 wherein said one or more NEENA is functionally linked to a constitutive promoter close to the transcription start site of said heterologous nucleic acid molecule.

9. The method of claim 8 wherein said one or more NEENA is functionally linked to a constitutive promoter 500 bp or less away from the transcription start site of said heterologous nucleic acid molecule.

2017248552 03 Jun 2019

10. The method of any one of claims 1 to 9 wherein said one or more NEENA is functionally linked to a constitutive promoter upstream of the translational start site of the nucleic acid molecule the expression of which is under the control of said constitutive promoter.

11. The method of any one of claims 1 to 10 wherein said one or more NEENA is functionally linked to a constitutive promoter within the 5´UTR of the nucleic acid molecule the expression of which is under the control of said constitutive promoter.

12. A recombinant expression construct comprising one or more NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47,

functionally linked to one or more constitutive promoter and one or more expressed nucleic acid molecule

wherein the one or more NEENA is heterologous to the promoter and the expressed nucleic acid molecule.

13. A recombinant expression vector comprising one or more recombinant expression constructs of claim 12.

14. A transgenic plant or part thereof comprising one or more heterologous NEENA functionally linked to a heterologous promoter, the NEENA comprising

i) a nucleic acid molecule comprising a sequence as defined in SEQ ID NO: 4, or

ii) a nucleic acid molecule comprising a sequence with an identity of at least 80% to SEQ ID NO: 4, or

2017248552 03 Jun 2019

iii) a fragment of 100 or more consecutive bases of a nucleic acid molecule of i) or ii) which has expression enhancing activity as the corresponding nucleic acid molecule comprising the sequence of SEQ ID NO: 4, or

iv) a nucleic acid molecule which is the complement or reverse complement of any of the nucleic acid molecule of i) or ii), or

v) a nucleic acid molecule which is obtainable by PCR using oligonucleotide primers described by SEQ ID NO: 46/47.

15. A transgenic cell or transgenic plant or part thereof comprising the recombinant expression construct of claim 12 or the recombinant expression vector of claim 13.

16. The transgenic cell or transgenic plant or part thereof of claim 15, selected or derived from the group consisting of bacteria, fungi, yeasts or plants.

17. The transgenic plant or part thereof of claim 16, wherein said plant or part thereof is a dicotyledonous plant.

18. The transgenic plant or part thereof of claim 16, wherein said plant or part thereof is a monocotyledonous plant.

19. A transgenic cell culture, transgenic seed, transgenic plant, part or propagation material derived from a transgenic cell or plant or part thereof of any one of claims 14 to 18 comprising said heterologous NEENA, recombinant expression construct of claim 12 or recombinant expression vector of claim 13.

20. Use of the recombinant expression construct of claim 12 or the recombinant expression vector of claim 13 for enhancing expression in a plant or part thereof.

21. Use of a transgenic cell culture, transgenic seed, transgenic plant, part or propagation material of claim 19 for producing foodstuffs, animal feeds, seeds, pharmaceuticals or fine chemicals.

2017248552 20 Oct 2017

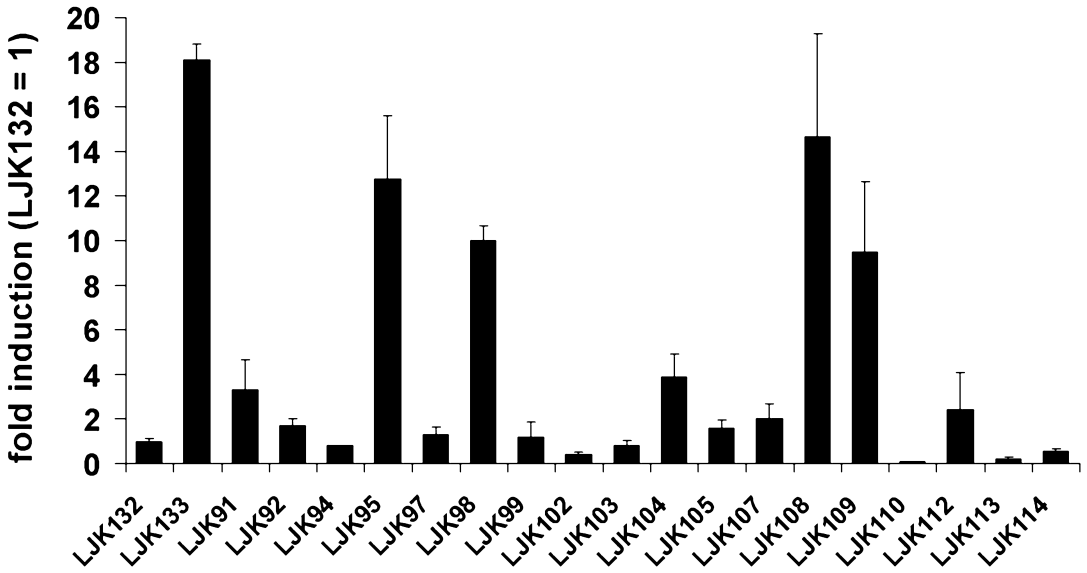

FIGURE 1

2017248552 20 Oct 2017

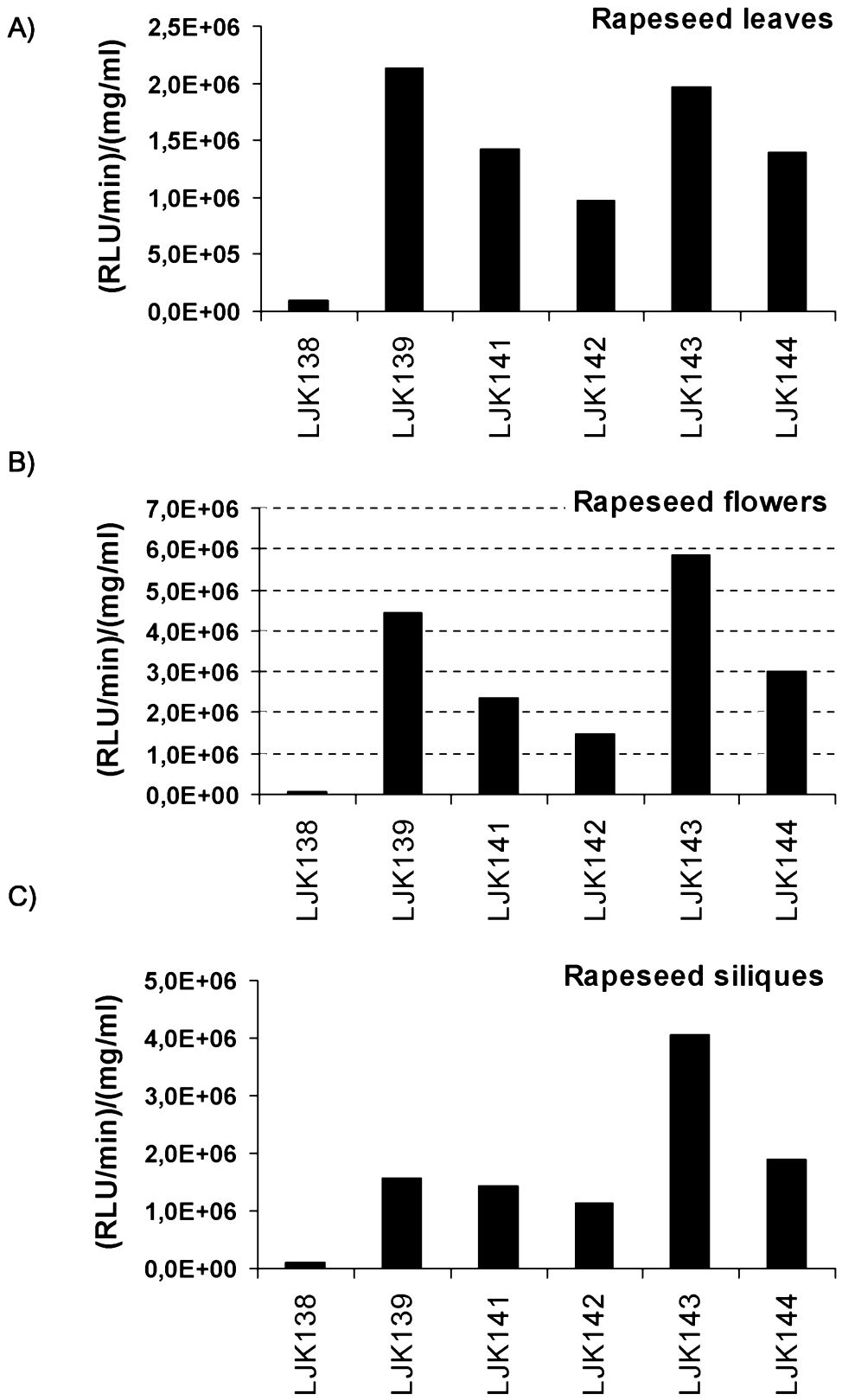

FIGURE 2

2017248552 20 Oct 2017

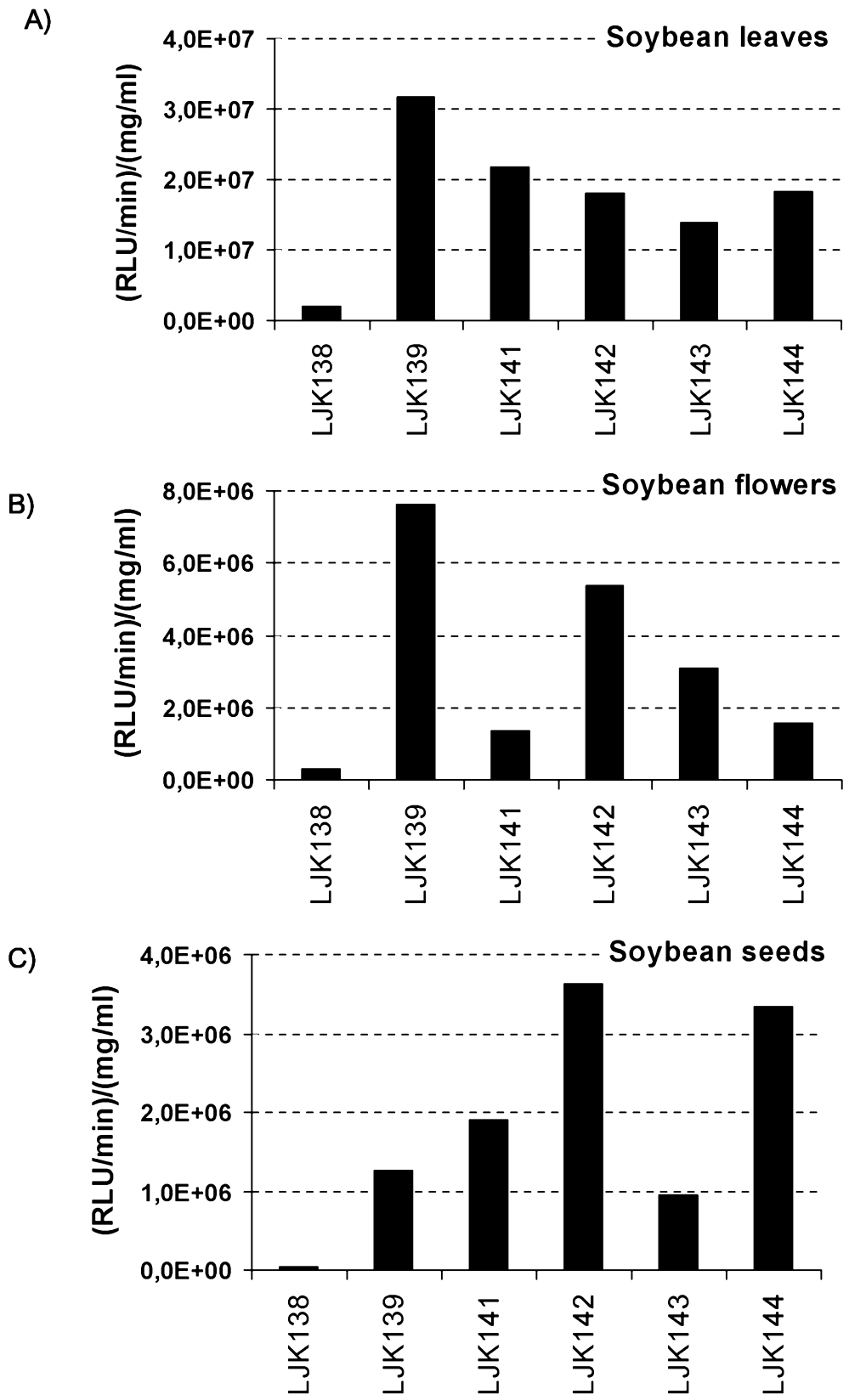

FIGURE 3

2017248552 20 Oct 2017

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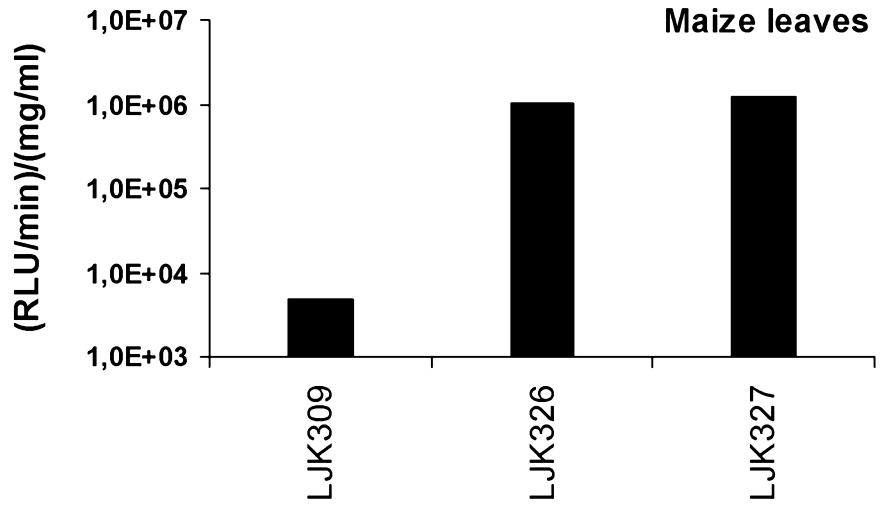

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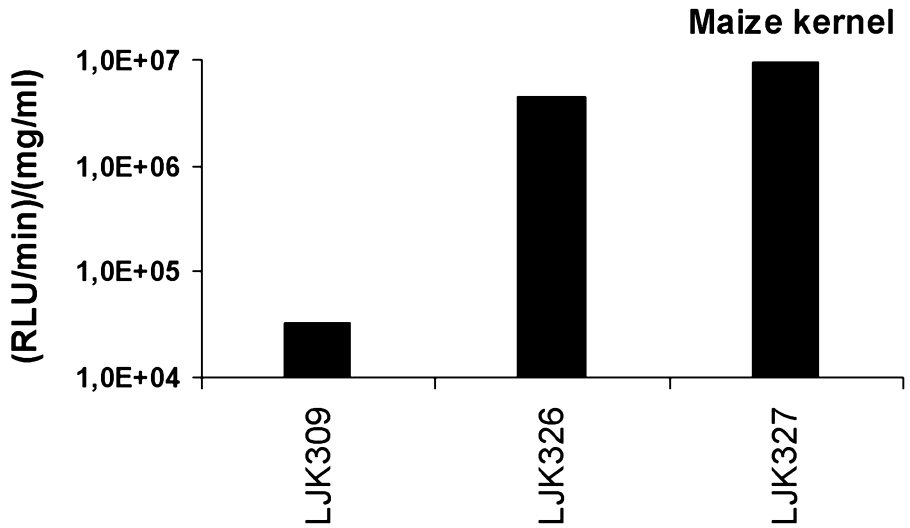

FIGURE 4

2017248552 20 Oct 2017

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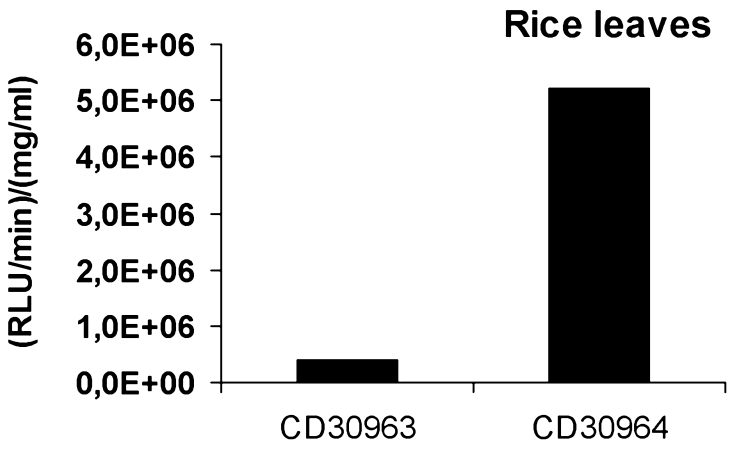

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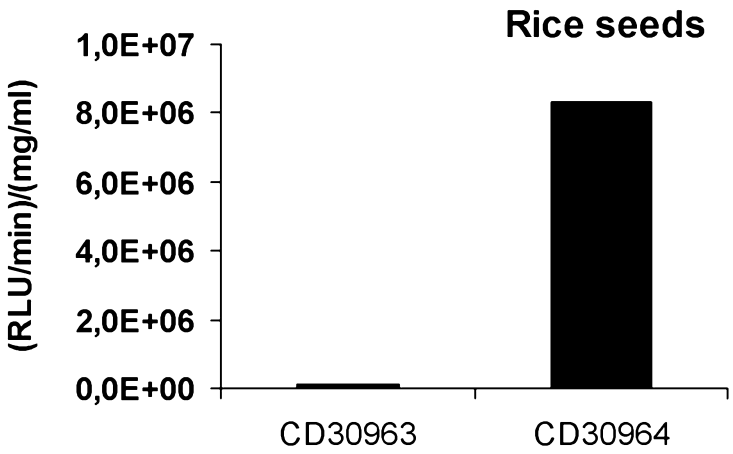

FIGURE 5

SEQUENCE LISTING

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tgagacgacc agttatgtaa catgttgtta actatcttca tgtttgaatc gattttgatt 660

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taatctttga tcggagataa atatctgagt ttcatcgcta gatctcatct atattctgaa 180

ctagggttcg tagatttcac agatttgtat atttaggcgt cgattctttg aatttgatcc 240

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aaattttcaa cctttgcttt tcctttgttt gttgcgttgc tgaatctatc tagggtttgg 120

ttttttggat ctaggagttt ttttttggtt gagaa 155

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<212> DNA
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tgtctctgta gcttttgaat cttaggtttg gttagattcg ttaataagat aatggaaaag 180

ttatgaaatg ggttgatttt tattaccgga tactgccgct gcatcttcat tcttttagtt 240

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caggtaactg aaag 674

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aaaatgctca attgttgcta gaaaggaagg tgtctcgaaa acatgtgaat ttatttttg 300

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gatttataga tctggtttct ggatctgttt acgatctatc gtcatctttc ctttgaaaat 300

gattggtgtt tctgtgttcg tattcgttta gatctaaagt ttttgatcga tgaatgtcgc 360

atgtgttttt atctgaaagt tttcgattac agtatcaagt ggtggtagta gtagtagtag 420

actcaaaaag ctgcacaaac tttttataca cgtgaattgt gattgcttta cggttttctt 480

ggagtttgtt aattaaatca tttaatatta agaagtttat gaattaagag aacgttattt 540

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