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(71) Applicant *(for all designated States except US)*: **SYNGENTA PARTICIPATIONS AG** [CH/CH]; Schwarzwaldallee 215, Basel, 4058 (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LEE, Mikyong** [KR/US]; 3054 E. Cornwallis Road, Durham, NC 27709 (US). **NUCCIO, Michael** [US/US]; 3054 E. Cornwallis Road, Durham, NC 27709 (US). **CLARKE, Joseph** [US/US]; 3054 E. Cornwallis Road, Durham, NC 27709 (US).

(74) Common Representative: **SYNGENTA PARTICIPATIONS AG**; 3054 E. Cornwallis Road, Durham, NC 27709 (US).

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(54) Title: PLANT REGULATORY SEQUENCES

(57) Abstract: The present invention relates to regulatory sequences. In particular, the invention relates to a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of an operably associated protein encoding polynucleotide of interest to non-pollen tissue, but not or substantially not to pollen. The invention further relates to chimeric genes and expression cassettes comprising said regulatory sequence and to transgenic plants comprising said chimeric genes and expression cassettes.

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Plant Regulatory Sequence

The present invention is in the field of plant biotechnology and relates to regulatory sequences. In particular, the invention relates to a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of an operably associated protein encoding polynucleotide of interest to basically all plant tissues, but essentially excluding expression in the tissues of the reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent. The invention further relates to chimeric genes and expression cassettes comprising said regulatory sequence in association with an expressible protein encoding polynucleotide of interest and to transgenic plants comprising said chimeric genes and expression cassettes, respectively, expressing the protein encoding polynucleotide of interest in basically all plant tissues, but essentially excluding expression in the tissues of the reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

BACKGROUND OF THE INVENTION

In many agricultural crops such as corn, devastating pests tend to feed on vegetative tissues such as the leaf, stalk and root and also reproductive tissues such as the ear. One technique used to protect plants from pests is the application of chemical compounds. An alternative technique involves genetic recombination, wherein a gene or genes are introduced into the plant to express protein products that are directly or indirectly involved in the control of the pest organisms. Current protein products produced by genetic recombination are expressed constitutively, i.e., throughout the plant at all times and in most tissues and organs. Such protein products are also expressed specifically, either in response to particular stimuli or confined to specific cells or tissues. In contrast, the present invention includes

expression of the protein or polynucleotide of interest in basically all plant tissues, but essentially excludes expression in the tissues of the reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

Several insect control trait genes target the larval stage of development. Under certain circumstances, these proteins also affect unintended insects, which are not corn pests, but do occasionally feed on corn pollen. These insects may be harmed by insecticidal proteins expressed in pollen tissue. This was seen as a problem in early BT-corn events which had high insecticidal protein expression in pollen. This issue was addressed in later BT-corn events through the development of alternative transgene expression systems. These newer events remained effective against target pests and accumulated less insecticidal protein in pollen, but are still viewed as potentially harmful to non-target pests due to the presence of insecticidal protein in pollen.

In some instances, useful insect control trait genes may also compromise the development of reproductive structures of the plant such as, for example, the tassel.

It is, therefore, desirable to provide plants, particularly corn plants that exclude expression of the transgene in the tissues of the reproductive structures of the plant such as the tissues of the pollen and/or the tassel. This could be achieved within the scope of the present invention by providing a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of an operably associated protein encoding a polynucleotide of interest to basically all plant tissues, but essentially excluding expression in the tissues of the male reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent. This regulatory nucleotide sequence can then be used to develop expression systems that enable effective accumulation of the polypeptide or protein of interest such as, for example, an insecticidal protein, in tissues that target pests normally feed on, and eliminate or reduce accumulation of the insecticidal protein in non-target tissues or organs and/or in those tissues that may be compromised by the polypeptide or protein of interest.

SUMMARY OF THE INVENTION

In one embodiment, the invention relates to a transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct, particularly a chimeric construct, comprising a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, associated with and/or under control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of said protein encoding polynucleotide of interest to basically all tissues of said plant, particularly the tissues target insects normally feed on, but essentially excluding the tissues of the reproductive plant structures, particularly the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, is not transcribed to any significant extent in the tissues of the reproductive plant structures, particularly in pollen and/or tassel tissue of the transgenic plant according to the invention. Therefore, essentially no expression of the polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, occurs in the tissues of the male reproductive plant structures, particularly in the tissues of the pollen and/or the tassel, and only residual amounts of the expression product, if any, can be detected in said tissues, which is not sufficient for the expression product to fulfil its envisaged biological function in said tissues, particularly in the tissues of the pollen and/or the tassel, and therefore also does not exhibit any toxic effects on insects feeding on said tissues or on the plant reproductive structures.

In one embodiment of the invention, a transgenic plant is provided as described herein, wherein a chimeric polynucleotide construct, particularly a chimeric DNA construct, comprises a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function and is obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which polypeptide or protein is highly expressed in

most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent.

In one embodiment, said actin depolymerizing factor 3 (ABP3) gene is obtainable from maize.

In one embodiment of the invention, a transgenic plant is provided as described herein, wherein a chimeric polynucleotide construct, particularly a chimeric DNA construct, comprises a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function and is obtainable from a gene represented by a DNA probe, particularly a DNA probe exhibiting a DNA sequence as depicted in SEQ ID NOs: 47 to 56, which DNA probe shows a signal pattern in tissue samples, which is indicative of expression of said gene in all tissues and of no or substantially no expression in pollen.

In one embodiment, a transgenic plant according to the invention and as described herein is provided comprising a regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, at least part of which has a transcription initiation function and mediates expression of an operably associated protein encoding polynucleotide of interest in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, which regulatory sequence can be obtained in a PCR reaction from a genomic *Zea mays* DNA template using

i) a first primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 1, particularly a first primer of SEQ ID NO: 1; or

ii) second primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 2, particularly a second primer of; SEQ ID NO: 2; or

iii) a first primer as a forward primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 1 and a second primer as a reverse primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 2, particularly the forward primer of SEQ ID NO: 1 and the reverse primer of SEQ ID NO: 2.

In one embodiment, the invention relates to a transgenic plant as described herein, wherein the nucleotide sequence providing the transcription initiation function has at least between 80% and 85% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13, or a fragment thereof, and wherein said regulatory nucleotide sequence or fragment thereof mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide of interest such that said polynucleotide of interest is transcribed in most of the plant tissues but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a transgenic plant as described herein, wherein the complementary strand of the nucleotide sequence providing the transcription initiation function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 13, particularly under moderate hybridization conditions, more particularly under stringent hybridization conditions, and wherein said regulatory nucleotide sequence mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide of interest such that said polynucleotide of interest is transcribed in

most of the plant tissues but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a transgenic plant as described herein,, wherein the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 13 or a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription termination function obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which regulatory sequence mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide molecule of interest such that said polynucleotide of interest is transcribed in most of the plant tissues excluding the tissues of the pollen but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly from a maize actin depolymerizing factor 3 (ABP3) gene, wherein

i) said regulatory nucleotide sequence comprises a transcription termination sequence which has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 14; or a fragment thereof, which still exhibits the functionality of a termination sequence; or

ii) the complementary strand of said regulatory nucleotide sequence hybridizes to a nucleotide sequence depicted in SEQ ID NO: 14, particularly under moderate hybridization conditions, more particularly

under moderate-stringent hybridization conditions, particularly under stringent hybridization conditions and mediates termination of transcription of an operably associated protein encoding polynucleotide of interest; or.

iii) said regulatory nucleotide sequence has a sequence as depicted in SEQ ID NO: 14, including complements thereof,.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which is expressed in most plant tissues but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly from a maize actin depolymerizing factor 3 (ABP3) gene, and which regulatory nucleotide sequence comprises a transcription initiation sequence and a transcription termination sequence, respectively, which have at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13 and SEQ ID NO:14, respectively, or a fragment thereof which still exhibits the full functionality as a transcription initiation and a termination sequence, respectively.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which is

expressed in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly from a maize actin depolymerizing factor 3 (ABP3) gene, and which regulatory nucleotide sequence comprises a transcription initiation sequence as depicted in SEQ ID NO: 13 and a transcription termination sequence as depicted in SEQ ID NO:14.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a gene represented by a DNA probe, particularly a DNA probe exhibiting a DNA sequence as depicted in SEQ ID NOs: 47 to 56, which DNA probe shows a signal pattern in tissue samples, which is indicative of expression of said gene in all tissues and of no or substantially no expression in pollen.

In one embodiment of the invention, a transgenic plant is provided as described herein, wherein the chimeric polynucleotide construct, particularly the chimeric DNA construct, comprises a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function and is obtainable from plant genomic DNA, particularly from maize genomic DNA, which polypeptide or protein is expressed in most tissues of the plant but essentially excluding tissues of the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment of the invention, a transgenic plant is provided as described herein, wherein the chimeric polynucleotide construct, particularly the chimeric DNA construct, comprises a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function and is obtainable from a gene represented by a DNA

probe, particularly a DNA probe exhibiting a DNA sequence as depicted in SEQ ID NOs: 57 to 79, which DNA probe shows a signal pattern in tissue samples, which is indicative of expression of said gene in all tissues and of no or substantially no expression in the tissues of the tassel.

In one embodiment, a transgenic plant according to the invention and as described herein is provided comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which regulatory sequence can be obtained in a PCR reaction from a genomic *Zea mays* DNA template using

i) a first primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 19, particularly the primer of SEQ ID NO: 19; or

ii) a second primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 20, particularly the reverse primer of SEQ ID NO: 20; or

iii) a first primer as a forward primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 19 and a second primer as a reverse primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 20, particularly the forward primer of SEQ ID NO: 19 and the reverse primer of SEQ ID NO: 20.

In one embodiment, the invention relates to a transgenic plant as described herein, wherein the nucleotide sequence providing the transcription initiation function has at least between 80% and 85% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity with a

nucleotide sequence depicted in SEQ ID NO: 35, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35, or a fragment thereof, and wherein said regulatory nucleotide sequence or fragment thereof mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide of interest such that said polynucleotide of interest is transcribed in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent,.

In one embodiment, the invention relates to a transgenic plant as described herein, wherein the complementary strand ofthe nucleotide sequence providing the transcription initiation function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 35, particularly under moderate hybridization conditions, more particularly under stringent hybridization conditions and wherein said regulatory nucleotide sequence mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide of interest such that said polynucleotide of interest is transcribed in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a transgenic plant as described herein, wherein the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 35 or a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription termination function obtainable from a plant genomic DNA, particularly a maize genomic DNA and mediates transcription of an operably associated polynucleotide molecule,

particularly of an operably associated protein encoding polynucleotide of interest such that said polynucleotide of interest is transcribed in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent, wherein

i) said regulatory nucleotide sequence comprises a transcription termination sequence which has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 36; or a fragment thereof which still exhibits the full functionality as a transcription initiation sequence; or

ii) the complementary strand of said regulatory nucleotide sequence hybridizes to a nucleotide sequence depicted in SEQ ID NO: 36, particularly under moderate hybridization conditions, particularly under moderate-stringent hybridization conditions, particularly under stringent hybridization conditions and mediates termination of transcription of an operably associated protein encoding polynucleotide of interest; or

iii) said regulatory sequence has a sequence as depicted in SEQ ID NO: 36, or a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence including complements thereof.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a genomic plant DNA, particularly from a genomic maize DNA and is expressed in most tissues of the plant but essentially excluding tissues of the tassel so that no expression product is present in said tissues to any significant

extent, which regulatory nucleotide sequence comprises a transcription initiation sequence and a transcription termination sequence, respectively, which sequences have at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35 and SEQ ID NO:36, respectively, including a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence and a termination sequence, respectively.

In one embodiment the invention relates to a transgenic plant according to the invention and as described herein comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a genomic plant DNA, particularly a genomic maize DNA and is expressed in most tissues of the plant but essentially excluding tissues of the tassel so that no expression product is present in said tissues to any significant extent, which regulatory nucleotide sequence comprises a transcription initiation sequence as depicted in SEQ ID NO: 35 and a transcription termination sequence as depicted in and SEQ ID NO:36 respectively, including a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence and a termination sequence, respectively.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes a polypeptide product exhibiting an insecticidal activity, particularly an endotoxin of *Bacillus thuringiensis*.

In one embodiment, the concentration of the polypeptide product expressed from the protein encoding polynucleotide of interest in the tissues of the plant reproductive structures, particularly in the tissues of the pollen and/or the tassel is

such that no insecticidal activity can be detected in a standard insect feeding assay. In particular, the concentration of the expression product in the tassel is below a basic level of not more than 10 ng/mg soluble protein, particularly of not more than 5 ng/mg soluble protein, more particularly of not more than 3 ng/mg soluble protein, but especially of not more than 2 ng/mg soluble protein or less.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes an endotoxin of *Bacillus thuringiensis* which has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO:15.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes an endotoxin of *Bacillus thuringiensis* which has the nucleotide sequence as depicted in SEQ ID NO: 15.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes a polypeptide product contributing to the enhancement of drought tolerance, particularly a deregulated form of a H^+-pyrophosphatase, wherein said polypeptide or protein is under control of a regulatory sequence according to the invention at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues but essentially excluding expression in the tissues of the pollen and/or the tissues of the tassel so that no expression product is present in said tissues to any significant extent,.

In one embodiment, the transgenic plant according to the invention and as described herein is a *Zea mays* plant.

In one embodiment, the invention relates to a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a

polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence, at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding a polynucleotide of interest in most plant tissues but essentially excluding expression in the tissues of the male reproductive structures, particularly the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment of the invention, the regulatory nucleotide sequence is obtainable from a gene represented by a DNA probe, particularly a DNA probe exhibiting a DNA sequence as depicted in SEQ ID NOs: 47 to 56, which DNA probe shows a signal pattern in tissue samples which is indicative of expression of said gene in all tissues and of no or substantially no expression in pollen.

In one embodiment of the invention, the regulatory nucleotide sequence is obtainable from a gene encoding an actin depolymerizing factor 3, which is expressed in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly an actin depolymerizing factor 3 gene from maize.

In one embodiment of the invention, the regulatory nucleotide sequence is obtainable from a gene represented by a DNA probe, particularly a DNA probe exhibiting a DNA sequence as depicted in SEQ ID NOs: 57 to 79, which DNA probe shows a signal pattern in tissue samples, which is indicative of expression of said gene in all tissues and of no or substantially no expression in the tissues of the tassel...

In one embodiment, the invention provides a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which sequence is obtainable from a genomic *Zea mays* DNA template using

i) a first primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity

with a nucleotide sequence as depicted in SEQ ID NO: 1, particularly a first primer of SEQ ID NO: 1; or

ii) second primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 2, particularly a second primer of; SEQ ID NO: 2; or

iii) a first primer as a forward primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 1 and a second primer as a reverse primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 2, particularly the forward primer of SEQ ID NO: 1 and the reverse primer of SEQ ID NO: 2.

In one embodiment, the regulatory nucleotide sequence according to the invention and as described herein is modified using one or more oligonucleotides selected from the group of oligonucleotides depicted in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

In one embodiment the invention relates to a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which regulatory nucleotide sequence provides a transcription initiation function, wherein the nucleotide sequence providing said function has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13 and wherein said regulatory nucleotide sequence mediates transcription of an operably associated protein encoding polynucleotide of interest in most tissues of the plant but essentially excluding the

tissues of the pollen so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which regulatory nucleotide sequence provides a transcription initiation function, wherein the complementary strand of the nucleotide sequence providing said function hybridizes to a nucleotide sequence depicted in SEQ ID NO: 13, particularly under moderate hybridization conditions, more particularly under moderately stringent hybridization conditions and wherein said regulatory nucleotide sequence mediates transcription of an operably associated protein encoding polynucleotide of interest in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent. In particular, said hybridization occurs under stringent hybridization conditions.

In one embodiment of the invention, the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 13, or a fragment thereof which still exhibits full functionality as a transcription initiation sequence, and complements thereof.

In one embodiment, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence according to the invention and as described herein is provided comprising approximately 1 kb of the nucleotide sequence upstream of the ZmABP3 transcription start site of a ZmABP3 gene, particularly upstream of the ZmABP3 transcription start site of the ZmABP3 gene as depicted in SEQ ID NO: 17.

In one embodiment of the invention, said regulatory nucleotide sequence comprises in addition the ZmABP3 5'-untranslated sequence, the ZmABP3 first exon, the ZmABP3 first intron and a portion of the ZmABP3 second exon, particularly a portion of the ZmABP3 second exon terminating at the translation initiation codon, particularly a portion of the ZmABP3 second exon comprising between about 10 to

about 20 nucleotides, particularly between about 12 and about 16 nucleotides, particularly about 14 nucleotides, of the second exon.

In one embodiment, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence is provided at least part of which has a transcription termination function, which sequence is obtainable in a PCR amplification reaction from a gDNA template, particularly a maize gDNA template, using a forward primer (P3 (5'-tatatagagctcgcatcatgatcatgcatcatggact-3') which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 9 and a reverse primer (P4 (5'-atatatactagtggcgcgccacactttctgtcgcatgtgatttgca-3') which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 10. In particular, said regulatory nucleotide sequence comprises a transcriptional terminator and poly-adenylation signal. In particular, a forward primer (P3 (5'-tatatagagctcgcatcatgatcatgcatcatggact-3')) which has a nucleotide sequence as depicted in SEQ ID NO: 9 and a reverse primer (P4 (5'-atatatactagtggcgcgccacactttctgtcgcatgtgatttgca-3') which has a nucleotide sequence as depicted in SEQ ID NO: 10 are used.

In one embodiment of the invention, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence is provided which comprises a transcription termination sequence obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which regulatory sequence mediates transcription of an operably associated polynucleotide molecule, particularly of an operably associated protein encoding polynucleotide molecule of interest such that said polynucleotide of interest is transcribed in most of the plant tissues but not or substantially not in the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly from a maize actin depolymerizing factor 3 (ABP3) gene, wherein

i) said regulatory nucleotide sequence comprises a transcription termination sequence which regulatory sequence has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 14; or

ii) the complementary strand of said regulatory nucleotide sequence hybridizes to a nucleotide sequence depicted in SEQ ID NO: 14, particularly under moderate hybridization conditions, more particularly under moderate-stringent hybridization conditions, particularly under stringent hybridization conditions and mediates termination of transcription of an operably associated protein encoding polynucleotide of interest; or

iii) said regulatory nucleotide sequence has a sequence as depicted in SEQ ID NO: 14, or a fragment thereof which still exhibits full functionality as a termination sequence, including complements thereof.

In one embodiment of the invention, a regulatory nucleotide sequence is provided or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which is expressed in most tissues of the plant but not or substantially not in the tissues of the pollen so that no expression product is present in said tissues to any significant extent, particularly from a maize actin depolymerizing factor 3 (ABP3) gene, and which regulatory nucleotide sequence comprises a transcription initiation sequence as depicted in SEQ ID NO: 13 and a transcription termination sequence as depicted in SEQ ID NO:14.

In one embodiment of the invention, the regulatory nucleotide sequence is obtainable from maize genomic DNA, particularly from a putative gene on the maize genome, which is highly expressed in most tissues of the plant but not or substantially not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence according to the invention and as described herein is provided comprising approximately 2.6 kb of the 5'-sequence including approximately 2 kb of 5'-non-transcribed sequence, a 5'-UTR, and exon 1 and part of exon 2 and intron 1, particularly approximately 0.6 kb representing exon 1, intron 1 and about 16 bp of exon 2.

In one embodiment, the invention provides a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence at least part of which has a transcription initiation function as described herein, which regulatory sequence is obtainable from a genomic *Zea mays* DNA template using

i) a first primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 19, particularly the primer of SEQ ID NO: 19; or

ii) a second primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 20, particularly the reverse primer of SEQ ID NO: 20; or

iii) a first primer as a forward primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 19 and a second primer as a reverse primer which has at least 90%,

particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, .97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 20, particularly the forward primer of SEQ ID NO: 19 and the reverse primer of SEQ ID NO: 20.

In one embodiment, the regulatory nucleotide sequence according to the invention and as described herein is modified using one or more oligonucleotides selected from the group of oligonucleotides depicted in SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26.

In one embodiment, the invention relates to a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which regulatory nucleotide sequence provides a transcription initiation function, wherein the nucleotide sequence providing said function has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35 and wherein said regulatory nucleotide sequence mediates transcription of an operably associated protein encoding polynucleotide of interest in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence as described herein, which regulatory nucleotide sequence provides a transcription initiation function, wherein the complementary strand of the nucleotide sequence providing said function hybridizes to a nucleotide sequence depicted in SEQ ID NO: 35, particularly under moderate hybridization conditions, more particularly under moderately stringent hybridization conditions and

wherein said regulatory nucleotide sequence mediates transcription of an operably associated protein encoding polynucleotide of interest in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent. In particular, said hybridization occurs under stringent hybridization conditions.

In one embodiment of the invention, the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 35, or a fragment thereof, which still exhibits the full functionality as a transcription initiation sequence and complements thereof.

In one embodiment, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence is provided at least part of which has a transcription termination function which sequence is obtainable in a PCR amplification reaction from a gDNA template, particularly a maize gDNA template, using a forward primer which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 29 and a reverse primer, which has at least 90%, particularly at least 91%, particularly at least 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO: 30. In particular, said regulatory nucleotide sequence comprises a transcriptional terminator and poly-adenylation signal. In particular, a forward primer, which has a nucleotide sequence as depicted in SEQ ID NO: 29 and a reverse primer, which has a nucleotide sequence as depicted in SEQ ID NO: 30 are used.

In one embodiment of the invention, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence is provided wherein

i) said regulatory nucleotide sequence comprises a transcription termination sequence which has at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith,

particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 36; or

ii) the complementary strand of said regulatory nucleotide sequence hybridizes to a nucleotide sequence depicted in SEQ ID NO: 36, particularly under moderate hybridization conditions, particularly under moderate-stringent hybridization conditions, particularly under stringent hybridization conditions and mediates termination of transcription of an operably associated protein encoding polynucleotide of interest; or

iii) said regulatory sequence has a sequence as depicted in SEQ ID NO: 36, or a fragment thereof, which still exhibits the full functionality as a termination sequence, including complements thereof.

In one embodiment of the invention, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence, is provided at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a maize genomic DNA, which is expressed in most tissues of the plant but not or substantially not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent, and which regulatory nucleotide sequence comprises a transcription initiation sequence and a transcription termination sequence, respectively, which have at least between 80% and 85% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least between 85% and 90% sequence identity, with all integers falling within this range also being comprised herewith, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35 and SEQ ID NO:36, respectively.

In one embodiment of the invention, a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence, is provided at least part of which has a transcription initiation function and a termination function, respectively, which regulatory nucleotide sequence is obtainable from a maize genomic DNA, which is expressed in most tissues of the plant but not or substantially not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent, and which regulatory nucleotide sequence comprises a transcription initiation sequence as depicted in SEQ ID NO: 35 and a transcription termination sequence as depicted in SEQ ID NO: 36.

It is apparent to the skilled artisan that, based on the nucleotide sequences shown in SEQ ID NO: 13, SEQ ID NO 14, SEQ ID NO: 35 and SEQ ID NO: 36, fragments of various length can be obtained from said sequences, for example by using any primer combinations of interest to generate fragments that still exhibit the specific regulatory function according to the invention that is driving expression of an operably associated polynucleotide of interest in most plant tissues but tissues of the pollen and the tassel, respectively. The invention thus includes fragments derived from a full-length transcript promoter and a full-length terminator of the invention and as described herein, respectively that function according to the invention, i.e. are capable of conferring expression and termination of an operably associated nucleotide sequence in most plant tissues but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent and/or the tassel.

The function of the promoter and terminator fragments, once obtained, can be easily tested by fusing them to a selectable or screenable marker gene and assaying the fusion constructs for retention of the specific promoter activity. Such assays are within the ordinary skill of the person skilled in the art.

In one embodiment, the invention relates to nucleotide fragments, particularly to nucleotide fragments obtainable from the regulatory sequences of an action depolymerizing factor 3 (ABP3) gene, which nucleotide fragments are of at least about 50 bases, preferably of between about 400 bases and about 650 bases, more

preferably of between about 200 bases and about 400 bases and most preferably of about 350 bases in length and still exhibit the specific regulatory function according to the invention that is driving expression of an operably associated polynucleotide of interest in most plant tissues but essentially excluding the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to nucleotide fragment comprising a nucleotide sequence comprising a consecutive stretch of at least 50 nt, particularly of between about 400 nt and about 650 nt, particularly of between about 200 nt and about 400 nt, particularly of about 350 nt in length of the nucleotide sequence depicted in SEQ ID NO:13 and SEQ ID NO: 35, respectively, wherein said nucleotide sequences still exhibit the specific regulatory function according to the invention that is driving expression of an operably associated polynucleotide of interest in most plant tissues but essentially excluding the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

It is also clear to the skilled artisan that variant sequences may be obtained without affecting the specific properties of the regulatory sequences according to the invention by introducing mutations, i.e. insertions, deletions and/or substitutions of one or more nucleotides, into the DNA sequences of SEQ ID NO: 13, SEQ ID NO 14, SEQ ID NO: 35 and SEQ ID NO: 36, respectively, using methods known in the art. In addition, an unmodified or modified nucleotide sequence of the present invention may be further varied by shuffling the sequence of the invention. To test for a function of variant DNA sequences according to the invention, the sequence of interest is operably linked to a selectable or screenable marker gene and expression of the marker gene is tested in transient expression assays with protoplasts or in whole plant tissues or in stably transformed plants. It is known to the skilled artisan that DNA sequences capable of driving expression of an operably associated nucleotide sequence are build in a modular way. Accordingly, expression levels from shorter DNA fragments may be different than the one from the longest fragment and may be different from each other. For example, deletion of a down-regulating upstream element will lead to an increase in the expression levels of the associated

nucleotide sequence while deletion of an up-regulating element will decrease the expression levels of the associated nucleotide sequence.

In one embodiment, the invention relates to an expression cassette comprising a regulatory nucleotide sequence or an expression cassette comprising said regulatory nucleotide sequence or a polynucleotide construct, particularly a chimeric polynucleotide construct, comprising said regulatory sequence according to the invention and as described herein.

In one embodiment, the expression cassette according to the invention comprises about 2.3 kb of the 5'-sequence of ZmABP3 which consists of about 1.1 kb of 5'-non-transcribed sequence, about 0.25 kb of 5'-UTR and about 0.98 kb representing ZmABP3-intron 1, about 1.013 kb of the 3'-sequence starting just past the ABP3 translation stop codon including about 0.3 kb of 3'-UTR and about 0.7 kb of non-transcribed sequence, which functions as the transcriptional terminator and poly-adenylation signal.

In one embodiment, an expression cassette according to the invention is provided wherein the natural translation start codon is silenced and moved to the second exon, particularly moved within 15 nucleotides of the 5'-end of ZmABP3 exon 2.

In one embodiment, an expression cassette according to the invention is provided wherein the start codon is preceded by the Kozak sequence 5'-...CCACC...-3'.

In one embodiment, the expression cassette according to the invention comprises a regulatory nucleotide sequence comprising approximately 2.6 kb of the 5'-sequence, which consists of approximately 2 kb of 5'-non-transcribed sequence, and about 12 bp of 5'-UTR, approximately 0.6 kb representing exon 1, intron 1 and about 16 bp of exon 2; and approximately 1 kb of the 3'-sequence that begins just past the translation stop codon and includes approximately 0.6 kb of 3'-UTR and about 0.4 kb of non-transcribed sequence, and functions as the transcriptional terminator and poly-adenylation signal.

In one embodiment, an expression cassette according to the invention is provided wherein the natural translation start codon is silenced and moved to the second exon.

In one embodiment, a polypeptide or protein encoding nucleotide sequence is provided encoding an endotoxin of *Bacillus thuringiensis* which has at least 80% sequence identity, particularly at least 85% sequence identity, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO:15.

In one embodiment, a polypeptide or protein encoding nucleotide sequence is provided encoding an endotoxin of *Bacillus thuringiensis* which has the nucleotide sequence as depicted in SEQ ID NO: 15.

In one embodiment, the invention relates to a transgenic plant comprising an expression cassette according to the invention and as described herein.

In one embodiment, the invention provides a transgenic plant, particularly a transgenic maize plant comprising a regulatory sequence according to the invention and as described herein.

In one embodiment, the invention provides a transgenic plant, particularly a transgenic maize plant comprising a regulatory sequence according to the invention and as described herein in association with a polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest.

In one embodiment, the invention provides a transgenic plant, particularly a transgenic maize plant comprising an expression cassette according to the invention and as described herein.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding nucleotide sequence encodes an endotoxin of *Bacillus thuringiensis* which has at least 80% sequence identity, particularly at least 85% sequence identity, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO:15 and is under the control of a regulatory sequences operable in said plant.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding nucleotide sequence encodes an endotoxin of *Bacillus thuringiensis* which has the nucleotide sequence

as depicted in SEQ ID NO: 15 and is under the control of a regulatory sequences operable in said plant.

The invention also provides methods for preparing expression cassettes comprising the regulatory sequence according to the invention comprising linking an expressible polynucleotide encoding a polypeptide or a protein of interest with the regulatory sequence according to the invention and as described herein to obtain an expression construct, wherein the polynucleotide of interest is operably linked or associated with the regulatory sequence such that expression of the polypeptide or a protein of interest is mediated by the regulatory sequence according to the invention and results in the expression of said polypeptide or a protein of interest in essentially all plant tissues, but essentially excludes expression in the tissues of the reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a method of producing a transgenic plant expressing a DNA sequence of interest in non-pollen tissue but not or substantially not in the tissues of the pollen and/or the tassel, comprising

a. transforming an expression cassette according to the invention and as described herein into a plant cell which comprises a regulatory nucleotide sequence, at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues but essentially excluding the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent; and

b. regenerating the plant cell transformed in step a) into a plant.

In one embodiment, the invention relates to a method of controlling insect target-pests feeding on vegetative plant tissues such as the leaf, stalk and root and/or on reproductive tissues such as the ear, but protecting non-target pests feeding on pollen comprising

a. growing a plant according to the invention and as described herein in an area that is infested with the target pest;

b. expressing a polypeptide or protein that is capable of controlling said target pest under the control of a regulatory sequence according to the invention and as described herein.

In one embodiment, the invention relates to a method of protecting the reproductive tissues of a plant, particularly the tissues of the pollen and/or the tassel against damage caused by expression in said tissues of a polypeptide or protein of interest comprising

a. growing a plant according to the invention and as described herein;

b. expressing in said plant a polypeptide or protein of interest under the control of a regulatory sequence according to the invention and as described herein.

In one embodiment the present invention relates to the use of a regulatory sequence according to the present invention and as disclosed herein for protecting the reproductive tissues of a plant, particularly the tissues of the pollen and/or the tassel against damage caused by expression in said tissues of a polypeptide or protein of interest comprising expressing in said plant said polypeptide or protein of interest under the control of a regulatory sequence according to the invention and as described herein.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 depicts the nucleotide sequence of forward primer P1
SEQ ID NO: 2 depicts the nucleotide sequence of reverse primer P2
SEQ ID NO: 3 depicts the nucleotide sequence of oligonucleotide Patg
SEQ ID NO: 4 depicts the nucleotide sequence of oligonucleotide Pnco
SEQ ID NO: 5 depicts the nucleotide sequence of oligonucleotide ADPc-1
SEQ ID NO: 6 depicts the nucleotide sequence of oligonucleotide ADPc-2
SEQ ID NO: 7 depicts the nucleotide sequence of oligonucleotide ADPc-4

SEQ ID NO: 8 depicts the nucleotide sequence of oligonucleotide adp3-a
SEQ ID NO: 9 depicts the nucleotide sequence of forward primer P3
SEQ ID NO: 10 depicts the nucleotide sequence of reverse primer P4
SEQ ID NO: 11 depicts the nucleotide sequence of forward primer Tnco
SEQ ID NO: 12 depicts the nucleotide sequence of forward primer T2
SEQ ID NO: 13 depicts the nucleotide sequence of modified *Zm*ABP3 regulatory sequence including the transcription initiation sequence
SEQ ID NO: 14 depicts the nucleotide sequence of *Zm*ABP3 terminal sequence
SEQ ID NO: 15 depicts the nucleotide sequence of Cry1AbG6
SEQ ID NO: 16 depicts the nucleotide sequence of maize-optimized AtAVP1D coding sequence
SEQ ID NO: 17 depicts the nucleotide sequence of the ZmABP3 gene
SEQ ID NO: 18 depicts the nucleotide sequence of the pNOV1321 plasmid
SEQ ID NO: 19 depicts the nucleotide sequence of forward primer ABT P1 forw
SEQ ID NO: 20 depicts the nucleotide sequence of reverse primer ABT P2 rev
SEQ ID NO: 21 depicts the nucleotide sequence of oligonucleotide pABT mut1
SEQ ID NO: 22 depicts the nucleotide sequence of oligonucleotide pABT mut2
SEQ ID NO: 23 depicts the nucleotide sequence of oligonucleotide pABT mut3
SEQ ID NO: 24 depicts the nucleotide sequence of oligonucleotide pABT mut4
SEQ ID NO: 25 depicts the nucleotide sequence of oligonucleotide pABT mut5
SEQ ID NO: 26 depicts the nucleotide sequence of oligonucleotide pABT mut6
SEQ ID NO: 27 depicts the nucleotide sequence of forward primer pABT amp1
SEQ ID NO: 28 depicts the nucleotide sequence of reverse primer pABT amp2
SEQ ID NO: 29 depicts the nucleotide sequence of forward primer ABT P4
SEQ ID NO: 30 depicts the nucleotide sequence of reverse primer ABT P5
SEQ ID NO: 31 depicts the nucleotide sequence of oligonucleotide ABTt m1
SEQ ID NO: 32 depicts the nucleotide sequence of oligonucleotide ABTt m2
SEQ ID NO: 33 depicts the nucleotide sequence of ZmABT1 cDNA
SEQ ID NO: 34 depicts the nucleotide sequence of ZmABT2 cDNA
SEQ ID NO: 35 depicts the nucleotide sequence of the ZmABT promoter
SEQ ID NO: 36 depicts the nucleotide sequence of the ZmABT terminal sequence.

SEQ ID NO: 37 depicts the nucleotide sequence of the ZmABP3-Cry1AbG6 Assembly construct.

SEQ ID NO: 38 depicts the nucleotide sequence of the ZmABP3-Cry1AbG6 binary construct.

SEQ ID NO: 39 depicts the nucleotide sequence of the enhanced ZmABP3-Cry1AbG6 binary construct.

SEQ ID NO: 40 depicts the nucleotide sequence of the ZmABP3-AmCyan assembly construct.

SEQ ID NO: 41 depicts the nucleotide sequence of the ZmABP3-AmCyan binary construct.

SEQ ID NO: 42 depicts the nucleotide sequence of the ZmABP3-AtAVP1D assembly construct.

SEQ ID NO: 43 depicts the nucleotide sequence of the ZmABP3-AtAVP1D binary construct.

SEQ ID NO: 44 depicts the nucleotide sequence of plasmid 15772 (ZmABT Assembly)

SEQ ID NO: 45 depicts the nucleotide sequence of plasmid 15773

SEQ ID NO: 46 depicts the nucleotide sequence of ZmABT gDNA

SEQ ID NO: 47 depicts the nucleotide sequence of Ctrl_ZMU45855-3_at

SEQ ID NO: 48 depicts the nucleotide sequence of AF032370_at

SEQ ID NO: 49 depicts the nucleotide sequence of Zm001747_s_at

SEQ ID NO: 50 depicts the nucleotide sequence of Zm005803_s_at

SEQ ID NO: 51 depicts the nucleotide sequence of Zm007728_s_at

SEQ ID NO: 52 depicts the nucleotide sequence of Zm009722_s_at

SEQ ID NO: 53 depicts the nucleotide sequence of Zm015335_s_at

SEQ ID NO: 54 depicts the nucleotide sequence of Zm021004_s_at

SEQ ID NO: 55 depicts the nucleotide sequence of Zm058948_s_at

SEQ ID NO: 56 depicts the nucleotide sequence of Zm061393_s_at

SEQ ID NO: 57 depicts the nucleotide sequence of Zm016864_s_at

SEQ ID NO: 58 depicts the nucleotide sequence of Zm018791_at

SEQ ID NO: 59 depicts the nucleotide sequence of ZMMETALL_x_at

SEQ ID NO: 60 depicts the nucleotide sequence of Zm000019_at

SEQ ID NO: 61 depicts the nucleotide sequence of Zm002987_at
SEQ ID NO: 62 depicts the nucleotide sequence of Zm002990_s_at
SEQ ID NO: 63 depicts the nucleotide sequence of Zm002990_x_at
SEQ ID NO: 64 depicts the nucleotide sequence of Zm004433_at
SEQ ID NO: 65 depicts the nucleotide sequence of Zm005761_at
SEQ ID NO: 66 depicts the nucleotide sequence of Zm006285_at
SEQ ID NO: 67 depicts the nucleotide sequence of Zm006481_s_at
SEQ ID NO: 68 depicts the nucleotide sequence of Zm010323_s_at
SEQ ID NO: 69 depicts the nucleotide sequence of Zm011554_at
SEQ ID NO: 70 depicts the nucleotide sequence of Zm011554_x_at
SEQ ID NO: 71 depicts the nucleotide sequence of Zm021403_at
SEQ ID NO: 72 depicts the nucleotide sequence of Zm028405_s_at
SEQ ID NO: 73 depicts the nucleotide sequence of Zm032921_s_at
SEQ ID NO: 74 depicts the nucleotide sequence of Zm033444_s_at
SEQ ID NO: 75 depicts the nucleotide sequence of Zm035082_s_at
SEQ ID NO: 76 depicts the nucleotide sequence of Zm040564_x_at
SEQ ID NO: 77 depicts the nucleotide sequence of Zm054116_s_at
SEQ ID NO: 78 depicts the nucleotide sequence of Zm066342_at
SEQ ID NO: 79 depicts the nucleotide sequence of Zm051284_at
SEQ ID NO: 80 depicts the nucleotide sequence of Vector 15289
SEQ ID NO: 81 depicts the nucleotide sequence of ZmABP-948-binary
SEQ ID NO: 82 depicts the nucleotide sequence of ZmABT-990-binary
SEQ ID NO: 83 depicts the nucleotide sequence of 5' Bfr1 primer
SEQ ID NO: 84 depicts the nucleotide sequence of 3' Xba1 primer
SEQ ID NO: 85 depicts the nucleotide sequence of 5'Gfix primer
SEQ ID NO: 86 depicts the nucleotide sequence of 3'Gfix primer
SEQ ID NO: 87 depicts the nucleotide sequence of 5'1Ab5Xbal primer
SEQ ID NO: 88 depicts the nucleotide sequence of 3'1Ab3d6 primer
SEQ ID NO: 89 depicts the nucleotide sequence of cy2'
SEQ ID NO: 90 depicts the nucleotide sequence of cy1
SEQ ID NO: 91 depicts the nucleotide sequence of cy2

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The technical terms and expressions used within the scope of this application are generally to be given the meaning commonly applied to them in the pertinent art of plant molecular biology if not otherwise indicated herein below.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes one or more plants, and reference to "a cell" includes mixtures of cells, tissues, and the like.

As used in this specification and the appended claims, the plural form "tissues", includes also the singular form unless the context clearly dictates otherwise. Thus, for example, reference to "tissues of the tassel" includes one or more tissues present in the tassel.

As used in this specification and the appended claims, the phrase “most tissues of the plant” or “essentially all tissues of the plant” is used interchangeably and refers to the majority to the tissues present in the plant with the exception of the tissues of the reproductive structures, particularly the tissues of the pollen and the tassel. In particular, “most tissues” refer to those tissues of the plant where target insects mainly feed on, with the exception of the tissues of the male reproductive structures, such as the tissues of the stalk, the roots, the leaves, the ear, the ear sheath, the silks and the developing kernels.

The term "polynucleotide” is understood herein to refer to polymeric molecule of high molecular weight which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and a base which is either a purine or pyrimidine. A "polynucleotide fragment" is a fraction of a given polynucleotide molecule. In higher plants, deoxyribonucleic acid (DNA) is the

genetic material while ribonucleic acid (RNA) is involved in the transfer of information contained within DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism, including the genomes of the mitochondria and the plastids. The term "polynucleotide" thus refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers. Unless otherwise indicated, a particular nucleic acid sequence of this invention also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer, et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka, et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini, et al., Mol. Cell. Probes 8:91-98 (1994)). The term polynucleotide is used interchangeably with nucleic acid, nucleotide sequence and may include genes, cDNAs, and mRNAs encoded by a gene, etc.

A "regulatory nucleotide sequence at least part of which has a transcription initiation function" is understood herein to refer to a nucleotide sequence, which controls the expression of an operably associated coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription and is located usually upstream (5') to its coding sequence. "Regulatory nucleotide sequences" include 5' regulatory sequences located proximal and more distal elements upstream of the associated coding region, which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. "Regulatory nucleotide sequences" may further include 3' sequences, including 3' non-translated and/or 3' non-transcribed sequences, located downstream of the associated coding region, and can include a transcription termination site. "Regulatory nucleotide sequences" may include enhancers, promoters, untranslated leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences that may be a combination of synthetic and natural sequences. An "enhancer" is a

DNA sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. The meaning of the term "regulatory nucleotide sequences" includes "transcription initiation" or "promoter" sequences and "promoter regulatory sequences." These terms are used interchangeably herein after.

For purposes of this invention, the definition of the term "3'-nontranscribed sequence" includes modifications to the nucleotide sequence of a 3'-nontranscribed sequence derived from a target gene, provided the modified 3'-nontranscribed sequence does not significantly reduce the activity of its associated 3' regulatory sequence. The 3'-nontranscribed sequence extends approximately 0.5 to 1.5 kb downstream of the transcription termination site.

The polynucleotide of the invention is understood to be provided in isolated form. The term "isolated" means that the polynucleotide disclosed and claimed herein is not a polynucleotide as it occurs in its natural context, if it indeed has a naturally occurring counterpart. Accordingly, the other compounds of the invention described further below are understood to be isolated. If claimed in the context of a plant genome, the polynucleotide of the invention is distinguished over naturally occurring counterparts by i.e. modifications introduced into the naturally occurring counterpart sequence and/or the insertion side in the genome and the flanking sequences at the insertion side.

"Operably associated" and "operably-linked" are used interchangeably and refer to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is associated or operably-linked with a coding sequence or functional RNA when it is capable of affecting the expression of that coding sequence or functional RNA (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences in sense or antisense orientation can be operably-linked to regulatory sequences.

The term "present to any significant extent" as used within the context of the present invention refers to the fact that only negligible expression occurs in pollen resulting in only minor amounts of the expression product in pollen tissue at concentrations that may be detectable by high-resolution detection methods such as HPLC, ELISA-based assays, Western analysis, insect feeding assays, enzyme activity assays etc., but stay below a certain threshold level that would be needed to effect the envisaged biological function of the expression product. For example, in case of the Cry1AbG6 endotoxin of *Bacillus thuringiensis* the threshold level is in the range of between 5ng/mg soluble protein and 60 ng/mg soluble protein, particularly in the range of between 20 ng/mg soluble protein and 50 ng/mg soluble protein.

The term "chimeric gene" refers to any gene that contains 1) DNA sequences, including regulatory and coding sequences that are not found together in nature in this specific combination or 2) sequences encoding parts of proteins not naturally adjoined, or 3) parts of promoters that are not naturally adjoined. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or comprise regulatory sequences and coding sequences derived from the same source, but arranged in a manner different from that found in nature.

The terms "heterologous DNA sequence," "exogenous DNA segment" or "heterologous nucleic acid," as used herein, each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling or mutation. These terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell genome in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides. A "homologous" DNA sequence is a DNA sequence that is naturally associated with a host cell into which it is introduced.

A "transgene" refers to a gene that has been introduced into the genome by

transformation and is stably maintained. Transgenes may include, for example, genes that are either heterologous or homologous to the genes of a particular plant to be transformed. Additionally, transgenes may comprise native genes inserted into a non-native organism, or chimeric genes. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the protein encoding polynucleotide of interest which is operably linked to a terminator. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the protein encoding polynucleotide of interest may be chimeric.

"Intron" refers to an intervening section of DNA which occurs almost exclusively within a eukaryotic gene, but which is not translated to amino acid sequences in the gene product. The introns are removed from the pre-mature mRNA through a process called splicing, which leaves the exons untouched, to form an mRNA. For purposes of the present invention, the definition of the term "intron" includes modifications to the nucleotide sequence of an intron derived from a target gene, provided the modified intron does not significantly reduce the activity of its associated 5' regulatory sequence.

"Exon" refers to a section of DNA which carries the coding sequence for a protein or part of it. Exons are separated by intervening, non- coding sequences (introns). For purposes of the present invention, the definition of the term "exon" includes modifications to the nucleotide sequence of an exon derived from a target gene, provided the modified exon does not significantly reduce the activity of its associated 5' regulatory sequence.

The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

A “probe” as used herein refers to a defined nucleic acid (DNA or RNA) fragment of variable length which may be used to detect in a DNA or RNA containing sample nucleotide sequences that are complementary to the sequence represented by the probe molecule.

The probe molecules may be used in a microarray set up, where they are covalently attached to a chemical matrix on an inert surface, such as coated glass slides or silicon based gene chips. Hybridization of the probe molecules to a target nucleic acid in the sample usually occurs under high stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative transcript abundance of nucleic acid sequences in the target. DNA microarrays may be used in expression profiling experiments to quantify transcript abundance for a target molecule in tissue samples such as the tissues of the pollen and/or the tassel, calculated based on the strength of the signal detected in the respective samples.

The term "hybridize" as used herein refers to conventional hybridization conditions, preferably to hybridization conditions at which 5xSSPE, 1% SDS, 1xDenhardts solution is used as a solution and/or hybridization temperatures are between 35°C and 70°C, preferably 65°C. After hybridization, washing is preferably carried out first with 2xSSC, 1% SDS and subsequently with 0.2xSSC at temperatures between 35°C and 75°C, particularly between 45°C and 65°C, but especially at 59°C (regarding the definition of SSPE, SSC and Denhardts solution see Sambrook et al. loc. cit.). High stringency hybridization conditions as for instance described in Sambrook et al, supra, are particularly preferred. Particularly preferred stringent hybridization conditions are for instance present if hybridization and washing occur at 65°C as indicated above. Non-stringent hybridization conditions for instance with hybridization and washing carried out at 45°C are less preferred and at 35°C even less.

“Sequence Homology or Sequence Identity” is used herein interchangeably. The terms "identical" or percent "identity" in the context of two or more nucleic acid or protein sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are

the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. If two sequences which are to be compared with each other differ in length, sequence identity preferably relates to the percentage of the nucleotide residues of the shorter sequence which are identical with the nucleotide residues of the longer sequence. Sequence identity can be determined conventionally with the use of computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive Madison, WI 53711). Bestfit utilizes the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2 (1981), 482-489, in order to find the segment having the highest sequence identity between two sequences. When using Bestfit or another sequence alignment program to determine whether a particular sequence has for instance 95% identity with a reference sequence of the present invention, the parameters are preferably so adjusted that the percentage of identity is calculated over the entire length of the reference sequence and that homology gaps of up to 5% of the total number of the nucleotides in the reference sequence are permitted. When using Bestfit, the so-called optional parameters are preferably left at their preset ("default") values. The deviations appearing in the comparison between a given sequence and the above-described sequences of the invention may be caused for instance by addition, deletion, substitution, insertion or recombination. Such a sequence comparison can preferably also be carried out with the program "fasta20u66" (version 2.0u66, September 1998 by William R. Pearson and the University of Virginia; see also W.R. Pearson (1990), Methods in Enzymology 183, 63-98, appended examples and http://workbench.sdsc.edu/). For this purpose, the "default" parameter settings may be used.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase: "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe

nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize to its target subsequence, but to no other sequences.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook, infra, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 0.1X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0 M Na^+ ion, typically about 0.01 to 1.0 M Na^+ ion concentration (or other salts) at pH 7.0 to 8.3,

and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

A "plant" is any plant at any stage of development, particularly a seed plant.

A "plant cell" is a structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, plant tissue, a plant organ, or a whole plant.

"Plant cell culture" means cultures of plant units such as, for example, protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, ovules, embryo sacs, zygotes and embryos at various stages of development.

"Plant material" refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

A "plant organ" is a distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

"Plant tissue" as used herein means a group of plant cells organized into a structural and functional unit. Any tissue of a plant in planta or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. "Plant tissue" includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, leaves, pollen, seeds, tumor tissue and various forms of cells and culture such as single cells, protoplasts, embryos, and callus tissue. The plant tissue may be in plants or in organ, tissue or cell culture.

The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

The terms "maize", "corn" and "*Zea mays*" are used herein interchangeably and refer to plants belonging to the genus Zea including, for example, different strains, races or varieties, commercial and non-commercial, of the species *Zea mays*.

The present invention relates to a transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct, particularly a chimeric DNA construct, comprising a protein encoding polynucleotide of interest, particularly a polypeptide or protein encoding polynucleotide of interest, under control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of said protein encoding polynucleotide of interest to essentially all tissues of the plant with the exception of the tissues of the male reproductive structures, particularly the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

A regulatory nucleotide sequence according to the present invention at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues but the tissues of the male reproductive structures, particularly the tissues of the pollen and/or the tassel, may be obtained in an expression profiling experiment to screen for probes that give strong signals in all samples, but only a weak or no signal in the pollen and/or the tassel sample, which is indicative of expression of the respective polynucleotides represented by said probes in most plant tissues and of no or substantially no expression in the tissues of the pollen and/or the tassel. In particular, maize plant tissues and tissues of the reproductive structures, particularly tissues of the pollen and/or the tassel may be screened to identify and obtain a regulatory sequence according to the present invention.

In particular, samples of all plant tissues, particularly samples of the green tissues and the root of a maize plant, may be directly compared to tissue samples from the male reproductive structures, particularly tissue samples of the pollen and/or the tassel. Probes representing polynucleotides that do not meet the target expression

profile are eliminated. Only those probes with the strongest signal across all non-pollen/non-tassel tissues and weak of no signal in pollen and/or the tassel are selected for further analysis that is probes representing polynucleotides that are highly expressed in all tissue samples, but show substantially no expression in pollen and/or the tassel. Said probes may then be aligned with plant cDNA assembly datasets to detect *bona fide* plant genes, particularly maize genes or putative maize genes.

The DNA sequence representing probes on the maize chip identified as representing genes that are highly expressed in all tissue samples but essentially not expressed in pollen, particularly probes represented by the DNA sequence as given in SEQ ID NOs: 47 to 56 and those representing genes that are highly expressed in all tissue samples and have essentially no or reduced expression in tassel samples, particularly probes represented by the DNA sequence a given in SEQ ID NOs: 57-79, can easily be extended to designed expression cassettes following the steps outlined in the Examples.

Probe candidate sequences from the expression profiling analysis for each expression category may be selected and progressed to a finished binary vector with the designed expression cassette linked to a gene of interest such as, for example, a reported gene, i.e., the GUS reporter gene.

In a first step, each expression cassette is flanked with one or more suitable restriction sites such as, for example, SanDI/RsrII sites and cloned into the vector molecule. The regulatory region including the transcription initiation function typically resides within a fragment of about 1000-1500 bp upstream of the transcription start site and extends into the second exon, or to the natural translation start codon if it is not on the first exon. It typically terminates with the maize optimized Kozak sequence 'gtaaaccatgg'. The engineered translation start codon is then embedded in a suitable restriction site such as the NcoI restriction endonuclease site 'ccatgg'. All translation start codons in the theoretical transcript that are upstream of the engineered restriction site are eliminated. At least one stop codon should be present in each reading frame upstream of the engineered restriction site. The regulatory region including the transcription initiation function is designed to be flanked by

suitable restriction sites such as, for example, XhoI/SanDI sites at the 5'-end and a NcoI site at the 3'-end.

The Gene Of Interest (GOI) such as the GUS reporter gene is provided as a suitable restriction fragment, in the example given here as a NcoI/SacI fragment. The terminus extends from just after the translation stop codon for about 1 kb downstream. The terminus is designed to be flanked by suitable restriction sites such as, for example, SacI at the 5'-end and RsrII/XmaI at the 3'-end.

The complete expression cassette is designed to be mobilized as a suitable restriction fragment, such as a SanDI/RsrII fragment, which can be ligated into the corresponding site located on an *Agrobacterium* binary vector such as the vector given in SEQ ID NO: 80.

All internal restriction sites used in the cloning steps identified above are mutated by single base substitutions to silence them.

Through application of these basic steps a plant expression cassette can be designed that corresponds to the respective probe molecules, particularly probe molecules on the maize chip identified as representing genes that are highly expressed in all tissue samples but essentially not expressed in pollen, particularly probes represented by the DNA sequence as given in SEQ ID NOs: 47 to 56 and those identified as representing genes that are highly expressed in all tissue samples and have essentially no or reduced expression in tassel samples, particularly probes represented by the DNA sequence a given in SEQ ID NOs: 57-79. The former is an expression cassette that should be transcribed in all maize tissues and not in pollen. The latter is an expression cassette that should be transcribed in all maize tissues but not or only moderately transcribed in tassels. This design strategy can be applied to all probes identified in an expression profiling experiment.

In a specific embodiment of the invention, applying the above criteria results in the identification of genes which exhibit the desired expression profile. In particular, a gene is identified which encodes an actin binding protein 3 (ABP3), particularly a actin binding protein 3 of maize (ZmABP3), which is a member of a small gene family that had been previously characterized (Lopez et al., 1996). The gene product has also been called actin depolymerizing factor 3.

It was shown by southern analysis that there are two ABP3 genes in the maize genome (Lopez et al., 1996), designated herein as ZmABP3-A and ZmABP3-B, respectively. The ZmABP3-A and ZmABP3-B cDNAs encode a protein of 139 amino acids that are identical at all residues, except one. The expression profiling data indicate that ZmABP3-B is highly expressed in most tissues of the plant, but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent, whereas. ZmABP3-A is not as highly expressed.

A structural analysis of the ZmABP3-B gene reveals that the ZmABP3-B protein coding region is encoded on 3 exons, which are interrupted by two intervening sequences (introns) flanked by the expected GT...AG border nucleotides.

The regulatory sequence is located in the 5'-region of the ABP3 gene immediately upstream of the coding sequence. The size of the regulatory region is in a range of between about 2 kb to 3 kb, particularly between about 2.3 kb and 2.5 kb, and comprises a 5'-non-transcribed sequence, particularly a 5'-non-transcribed sequence of between about 0.9 kb and 1.3 kb, but especial of about 1.1 kb, and a 5'-UTR, particularly between about of 0.1 kb and 0.3 kb, but especially 0.25 kb of the 5'-UTR and all or part of a nucleotide sequence representing ZmABP3-intron 1, particularly a nucleotide sequence of between about 0.7 kb and 1.2 kb, but especially of about 0.98 kb.

The regulatory sequence according to the invention further comprises part of 3'-sequence that begins just past the ABP3 translation stop codon including transcribed but not translated sequence (UTR) and non-transcribed sequence that functions as the transcriptional terminator and a poly-adenylation signal. In particular, the 3'-sequence is in a range of between about 0.8 kb and about 1.2 kb, particularly between about 0.9 kb and about 1.1 kb, but especially about 1.013 kb. The size of the 3'-UTR is in a range of between about 0.2 kb and about 0.4 kb, but especially about 0.3 kb, and that of the non-transcribed sequence in a range of between about 0.5 kb and about 0.8 kb, but specifically about 0.7 kb.

In a specific embodiment of the invention, the regulatory sequence is modified such that the natural translation start codon is silenced in order to move it to the second exon.

In another embodiment of the invention, candidate probes can be identified on a DNA chip or gene array, particularly a maize DNA chip or gene array such as, for example, the maize Affymetrix™ Chip applying the above criteria, which can be used in the identification of genes or putative genes on the maize genome which exhibit the desired expression profile. Two candidate probes were identified which demonstrate virtually no signal in tassel but a high signal in other tissues. This indicates that the gene represented by said candidate probes is not expressed in tassel, but is highly expressed throughout the rest of the plant. The greatest expression differential, 60-fold higher in non-tassel tissue, was observed in candidate probe Zm033444_S_AT. The other candidate probe (Zm040564_X_AT) showed signal variation depending on the development status of the probed plant material, i.e. a low signal in young tassel that gradually increases to a high or strong signal when the plant becomes older. The signal strength between tassel and non-tassel samples differed by less than 10-fold, but the signal strength in non-tassel samples was nearly 10-fold higher as compared to the other candidate probe. The sequence data indicate that neither probe corresponds to a characterized gene. Both probes identify good candidate genes for development of promoters that deliver high expression in non-tassel tissue and little or no expression in tassels. Given the high signal differential between tassel and non-tassel samples, an expression cassette based on probe Zm033444_S_AT was developed.

Public and proprietary databases can be queried by BLASTN with the candidate probe Zm033444_S_AT sequence to obtain DNA sequence evidence for both transcripts and gDNA corresponding to Zm033444_S_AT. cDNA hits with precise matches to the query sequence fell into two similar contigs. ZmABT1 corresponds to Maize.1482.c47 and Maize.1908.c31, and ZmABT2 corresponds to Maize.1482.c32, Maize.1482.c28, Maize.1482.c53, Maize.1908.c17, Maize.1908.c20, Maize.1908.c37 and AI947567.

The Zm033444_S_AT, ZmABT1 and ZmABT2 sequences can then be used to query maize genomic DNA sequence databases to identify the regulatory sequence(s) that give high expression in non-tassel tissue and little or no expression in tassels. These queries identified three entries, AZM4_12, ZmGSStuc11-12-

04.4740.1 and MAGI_88845, that assemble into a single contig. The ZmABT gDNA sequence is shown in SEQ ID NO: 46. It encodes both the ZmABT1 and ZmABT2 transcript, which suggests that they are alternatively spliced variants of the same transcript.

ZmABT1 is encoded on 5 exons, and ZmABT2 is encoded on 6 exons. The additional exon lies between exon 1 and exon 2 of ZmABT1. The largest open reading frame on ZmABT1 and ZmABT2 can be used to define their translation start and stop codons and further to define the location of each translation start and stop codon. By this analysis both cDNAs use the same translation start and stop codon.

In one important aspect of the present invention the regulatory sequence according to the invention can be used in the development of robust expression cassettes that express recombinant genes in most tissues of the plant but not or substantially not in the tissues of the male reproductive structures, particularly the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In a specific embodiment of the invention a regulatory sequence obtainable from a ABP3 gene, more particularly of regulatory sequence obtainable form a *Zea mays* ABP3 gene, can be used in the development of robust expression cassettes that express recombinant genes in most tissues of the plant but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent.

The transcription initiation region of the regulatory sequence according to the invention, particularly of regulatory sequence obtainable from a ABP3 gene, more particularly of regulatory sequence obtainable from a *Zea mays* ABP3 gene can be obtained in a PCR reaction containing a primer pair involving forward primer P1 (5'-atatatgcatgcggcgcgccgaaagtagcaaacaacaggttcatgtgcac-3') as depicted in SEQ ID NO: 1 and reverse primer P2 (5'-tatataccatggtgggtttgcctgcgaccacaagttca-3') as depicted in SEQ ID NO: 2 through amplification from a gDNA template, particularly a maize gDNA template. In a specific embodiment of the invention a thermocycling program is applied involving amplification at about 95°C for about 15 minutes followed by about 45 cycles at about 94°C for about 1 minute, at about 64°C for about 1 minute and at about 72°C for about 5 minutes. The final extension step is

carried out at about 72°C for about 15 minutes. The reaction product, particularly an about 2.3 kb reaction product, is purified and the DNA extracted using a DNA extraction method known in the art. The DNA is precipitated, recovered and finally cloned into a suitable vector.

The transcription initiation region according to the invention, particularly a transcription initiation region obtainable from an ABP3 gene, more particularly obtainable from a ZmABP3, may be modified in a series of reactions using at least one of the oligonucleotides selected from the group of oligonucleotides depicted in

-SEQ ID NO: 3 (Patg (5'-cagctcgcccgagttggtaaggcccccct-3')),

-SEQ ID NO: 4 ... (Pnco (5'-acagattagtccatcgcccacggt-3')),

-SEQ ID NO: 5... (ADPc-1 (5'-agccctgtccatgacggcccaagcaac-3')),

-SEQ ID NO: 6... (ADPc-2 (5'-agtagcaattcggtaggcacaggcac-3')),

-SEQ ID NO: 7... (ADPc-4 (5'-tctatggtctgcgaggtgcggtggc-3')), and

-SEQ ID NO: 8... (adp3-a (5'-gtccccttcttcgccgcgccagctcgc-3')).

The terminus of the regulatory sequence according to the invention, particularly a terminal sequence obtainable from an ABP3 gene, more particularly a terminal sequence obtainable from a ZmABP3, can be amplified from a gDNA template, particularly a maize gDNA template, in a DNA polymerase reaction using a forward primer (P3 (5'-tatatagagctcgcatcatgatcatgcatcatggact-3')) as depicted in SEQ ID NO: 9 and a reverse primer (P4 (5'-atatatactagtggcgcgccacactttctgtcgcatgtgatttgca-3')) as depicted in SEQ ID NO: 10. A thermocycling program may be applied comprising a first cycle of about 95°C for about 5 minutes followed by about 45 cycles of about 94°C for about 30 seconds, about 50°C for about 1 minute and about 72°C for about 4 minutes. The final extension step may be carried out at about 72°C for about 15 minutes. The about 1 kb reaction product is then purified and the DNA extracted using standard extraction methods. The DNA is precipitated, recovered and cloned into a suitable vector.

The terminus of the regulatory sequence according to the invention, particularly a terminal sequence obtainable from a ABP3 gene, more particularly a terminal sequence obtainable from a ZmABP3, may be modified to remove an internal restriction site, particularly a NcoI restriction site using a suitable primer pair, particularly primer pair Tnco (5'-Pgtaaaaaaaggtcccttggctcccagaaga-3') / T2 (5'-

Pcaatgtgttagactgacgtg-3') as depicted in SEQ ID NO: 11 and SEQ ID NO: 12, respectively, in a DNA polymerase reaction. The thermocycling program employed may comprise a first cycle at about 95°C for about 5 minutes followed by about 30 cycles of about 95°C for about 1 minute, about 50°C for about 1 minute and about 65°C for about 15 minutes. The product may then be processed and sequenced.

The present invention is also directed to expression cassettes that incorporate the regulatory mechanisms of a target gene of interest that shows the desired expression profile, that is high expression in most plant tissues but no expression in pollen tissue, particularly an ABP target gene, more particularly of a ZmABP3 target gene, to control in plants the expression of products of nucleic acid molecules of interest in a manner that mimics the expression profile of the original target gene.

The present invention further includes expression cassettes that incorporate regulatory sequences obtainable from the 5'-region of the target gene, particularly an ABP target gene, more particularly of a ZmABP3 target gene, to express the products of nucleic acid molecules of interest in plant tissues but not or substantially not in pollen tissue, The present invention is also directed to expression cassettes incorporating both regulatory sequences obtainable from the 5'-region and the 3'-region of the target gene, particularly an ABP3 target gene, more particularly of a ZmABP3 target gene.

In another specific embodiment of the invention a regulatory sequence obtainable from maize genomic DNA can be used in the development of robust expression cassettes that transcribe polynucleotides in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent,.

An inclusive gene structure-based design strategy may be used to construct such an expression cassette. To incorporate the known alternative splicing of the putative maize gene identified in a method as described above into the expression cassette, the design strategy can be based on the structure of ZmABT1 transcript as shown in SEQ ID NO: 33.

The transcription initiation region of the regulatory sequence according to the invention, particularly of the ZmABT promoter region can be amplified from a maize

gDNA template in a DNA polymerase reaction containing gDNA and a primer pair involving forward primer ABT P1 forw (5'- CGACCAGCGCGACATGCATGGCA-3') as depicted in SEQ ID NO: 19 and ABT P2 rev (5'- ACCCCAGGGCGTACGACAAG-GCC-3') as depicted in SEQ ID NO: 20. In a specific embodiment of the invention a thermocycling program is applied involving amplification at about 95°C for about 5 minutes followed by about 40 cycles of 94°C for about 30 seconds, about 67°C for about 30 seconds and about 72°C for about 2.5 minutes. The final extension step was done at about 72°C for about 10 minutes.

This amplification reaction leads to an amplification product of about 2.6 kb, which can be purified and the DNA extracted using a standard DNA extraction method. The DNA can than be cloned into a suitable vector such as, for example, the pCR-BluntII -TOPO vector.

The ZmABT promoter can be modified in a series of mutagenesis reactions to silence the endogenous translation start codon, silence a SanDI restriction site and correct point mutations created during amplification. This can be done in a series of reactions using at least one of the oligonucleotides selected from the group of oligonucleotides depicted in

-SEQ ID NO: 21 pABT mut1 (5'-GATGGCCGGATTGGGCTCCCGGGGTGGAG-3')

-SEQ ID NO: 22 pABT mut2 (5'-CTGGGAGGCGCGCAAGGGGCAGTTCCTCG-3')

-SEQ ID NO: 23 pABT mut3 (5'-CCCACCGCCGGAGCACCGAAAGGCCCCGCG-3')

-SEQ ID NO: 24 pABT mut4 (5'-GTCACCCGGGAGCACTTCCCGGCGCCG-3')

-SEQ ID NO: 25 pABT mut5 (5'-CATTGGGCCGAGCACGGCTTCTTCCGC-3')

-SEQ ID NO: 26 pABT mut6 (5'-GGGGTACGGTGTTCTTGAGTCGTGAAGCGAC-3')

The modified ZmABT promoter can the be amplified in another PCR reaction using primers pABT amp1 (5'- GCGTCTAGAGGGACCCCGACCAGCGCGACATGCAT-GGCA-3') as depicted in SEQ ID NO: 27 and pABT amp2 (5'-ACCCCAGGG-CGTACGACAAGGCCCCACCATGGGCGC-3') as depicted in SEQ ID NO: 28. The

PCR product can then be purified and the DNA extracted using standard a DNA extraction method. The DNA can be cloned into a suitable vector such as, for example, the pCR-BluntII -TOPO vector, transformed and sequenced. The ZmABT promoter can then be excised, particularly as an XbaI/NcoI fragment and ligated to a suitable expression vector such as, for example, pNOV6901.

In one embodiment of the invention, an expression cassette is provided comprising a termination sequence which can be obtained form the ZmABT gene identified and described herein above. The ZmABT terminus can be amplified from maize gDNA template in a DNA polymerase reaction containing gDNA and a primer pair involving forward primer ABT P4 (5'-TATATAGAGCTCGAATCGAAGAAGCCACA-CTGTAAATCTGCCGGG-3') as depicted in SEQ ID NO: 29 and reverse primer ABT P5 (5'-AGCAAGGCATATGCAGCAGCTGCTGGTCGGACCGGGCCCTATATA-3') as depicted in SEQ ID NO: 30 resulting in an amplification product of about 1 kb. This reaction product can be purified and the DNA extracted using a standard DNA extraction method. The purified DNA can then be cloned into a suitable vector such as, for example, the pCR4-TOPO-Blunt vector.

In one embodiment of the invention, the ZmABP3 terminus is modified to remove internal NcoI and XhoI restriction sites. This can be done in a series of reactions using at least one of the oligonucleotides selected from the group of oligonucleotides depicted in.

-SEQ ID NO: 31 ABTt m1 (5'- GTCATGCATGGGCATGTGAAGGAGGAGCC-3')

-SEQ ID NO: 32 ABTt m2 (5'- GTTGCATGCATGCTGCATGGCGTCGAGAT-3')

The amplification product can then be processed and sequenced to result in a terminator sequence as shown in SEQ ID NO: 36.

In one embodiment of the invention, an expression cassette is provided that express recombinant genes in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent, comprising both a regulatory sequence at least part of which has a transcription initiation function and a regulatory sequence at least part of which

has a termination function, which regulatory sequences can be obtained form the ZmABT gene identified and described herein above.

In one embodiment of the invention such an expression cassette can be obtained by excising the ZmABT terminus excised and ligating it into a suitable vector already comprising a regulatory sequence at least part of which has a transcription initiation function, particularly the sequence of the ZmABT promoter such as, for example, the pNOV6901-prABT vector as described above.

In one embodiment, the expression cassette according to the invention comprises a regulatory nucleotide sequence comprising approximately 2.6 kb of the 5'-sequence, which consists of approximately 2 kb of 5'-non-transcribed sequence, and about 12 bp of 5'-UTR, approximately 0.6 kb representing exon 1, intron 1 and about 16 bp of exon 2; and approximately 1 kb of the 3'-sequence that begins just past the translation stop codon and includes approximately 0.6 kb of 3'-UTR and about 0.4 kb of non-transcribed sequence, and functions as the transcriptional terminator and poly-adenylation signal.

In one embodiment, an expression cassette according to the invention is provided wherein the natural translation start codon is silenced and moved to the second exon

The complete expression cassette can then be mobilized into a suitable vector for plant transformation and expression such as, for example, an *Agrobacterium* binary vector, particularly *Agrobacterium* binary vector 15289.

The nucleic acid segment of interest can, for example, code for a ribosomal RNA, an antisense RNA or any other type of RNA that is not translated into protein. In another preferred embodiment of the invention, the nucleic acid segment of interest is translated into a protein product. The nucleotide sequence which directs transcription and/or the nucleic acid segment may be of homologous or heterologous origin with respect to the plant to be transformed. A recombinant DNA molecule useful for introduction into plant cells includes that which has been derived or isolated from any source that may be subsequently characterized as to structure size and/or function, chemically altered, and later introduced into plants. Therefore

a useful nucleotide sequence, segment or fragment of interest includes completely synthetic DNA, semi-synthetic DNA, DNA isolated from biological sources, etc. Generally, the introduced DNA is not originally resident in the plant genotype which is the recipient of the DNA, but it is within the scope of the invention to isolate a gene from a given plant genotype, and to subsequently introduce multiple copies of the gene into the same genotype, e.g., to enhance production of a given gene product such as a storage protein or a protein that is involved in carbohydrate metabolism or any other gene of interest as provided in the SEQ ID NOs of the sequence listing.

The introduced recombinant DNA molecule includes but is not limited to, DNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses. The introduced DNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different genotype. The term "chimeric gene" or "chimeric DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not combine DNA under natural conditions, or which DNA sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of untransformed plant.

The introduced recombinant DNA molecule used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of chimeric DNA, such as plasmid DNA,

In one embodiment, the regulatory sequences may be operably associated with an expressible polynucleotide of interest. The expressible polynucleotide may encode a polypeptide or protein of interest.

Such a polypeptide or protein of interest may be one exhibiting a certain biological activity such as, for example, an insecticidal, herbicidal or fungicidal activity or may contribute of an improved performance of a crop plant of agronomic interest in form of improved yield, quality, lodging, biotic and abiotic stress resistance, flowering control, etc.

In one embodiment, the concentration of the polypeptide product expressed from the protein encoding polynucleotide of interest in the tissues of the reproductive structures, particularly in the tissues of the pollen and/or the tassel, is such that no

insecticidal activity can be detected in a standard insect feeding assay. In particular, the concentration of the expression product in the tissues of the male reproductive structures, particularly in the tissues of the pollen and/or the tassel, is below a basic level of about 10 ng/mg soluble protein, particularly of about 5 ng/mg soluble protein, more particularly of about 3 ng/mg soluble protein, but especially of about 2 ng/mg soluble protein or below.

In one specific embodiment of the invention, the polypeptide or protein of interest is an insecticidally active protein or polypeptide, particularly an insecticidally active protein or polypeptide obtainable from *Bacillus thuringiensis*, more particularly a *Bacillus thuringiensis* endotoxin such as, for example, crylA(b) endotoxin. Other endotoxins known to occur in *Bacillus thuringiensis* may likewise be used in association with the regulatory sequence according to the invention to obtain toxin expression in most plant tissues except pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes an endotoxin of *Bacillus thuringiensis* which has at least 80% sequence identity, particularly at least 85% sequence identity, particularly at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence as depicted in SEQ ID NO:15.

In one embodiment, a transgenic plant according to the invention and as described herein is provided, wherein the polypeptide or protein encoding polynucleotide of interest encodes an endotoxin of *Bacillus thuringiensis* which has the nucleotide sequence as depicted in SEQ ID NO: 15.

Once completed, the expression cassette may be mobilized into a suitable vector for plant transformation, such as, for example, a binary vector, which may then be mobilized to maize via *Agrobacterium*-mediated transformation.

Transgenic plants (or plant cells, or plant explants, or plant tissues) incorporating the polynucleotides of the invention and/or expressing a polypeptide of interest such as, for example, a toxin protein of *B. thuringiensis*, can be produced by a variety of well established techniques. Following construction of an expression cassette and a

vector incorporating the regulatory polynucleotide sequence according to the invention and as described herein, standard techniques can be used to introduce the polynucleotide into a plant, a plant cell, a plant explant or a plant tissue of interest. Optionally, the plant cell, explant or tissue can be regenerated to produce a transgenic plant. The plant can be any higher plant, including gymnosperms, monocotyledonous and dicotyledonous plants. Suitable protocols are available for Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip), Cruciferae (cabbage, radish, rapeseed, broccoli, etc.), Curcurbitaceae (melons and cucumber), Gramineae (wheat, corn, rice, barley, millet, etc.), Solanaceae (potato, tomato, tobacco, peppers, etc.), and various other crops. See protocols described in Ammirato et al., eds., (1984) Handbook of Plant Cell Culture--Crop Species, Macmillan Publ. Co., New York, N.Y.; Shimamoto et al. (1989) Nature 338: 274 276; Fromm et al. (1990) Bio/Technol. 8: 833 839; and Vasil et al. (1990) Bio/Technol. 8: 429 434. Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is now routine, and the selection of the most appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods can include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and Agrobacterium tumefaciens mediated transformation.

Transformation of plants can be undertaken with a single DNA molecule or multiple DNA molecules (i.e., co-transformation), and both these techniques are suitable for use with the expression cassettes of the present invention. Numerous transformation vectors are available for plant transformation, and the expression cassettes of this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation.

A variety of techniques are available and known to those skilled in the art for introduction of constructs into a plant cell host. These techniques generally include

transformation with DNA employing *A. tumefaciens* or *A. rhizogenes* as the transforming agent, liposomes, PEG precipitation, electroporation, DNA injection, direct DNA uptake, microprojectile bombardment, particle acceleration, and the like (See, for example, EP 295959 and EP 138341) (see below). However, cells other than plant cells may be transformed with the expression cassettes of the invention. The general descriptions of plant expression vectors and reporter genes, and *Agrobacterium* and *Agrobacterium*-mediated gene transfer, can be found in Gruber et al. (1993).

Expression vectors containing the regulatory polynucleotide sequence according to the invention can be introduced into protoplasts or into intact tissues or isolated cells. Preferably expression vectors are introduced into intact tissue. General methods of culturing plant tissues are provided for example by Maki et al., (1993); and by Phillips et al. (1988). Preferably, expression vectors are introduced into maize or other plant tissues using a direct gene transfer method such as microprojectile-mediated delivery, DNA injection, electroporation and the like. More preferably expression vectors are introduced into plant tissues using the microprojectile media delivery with the biolistic device. See, for example, Tomes et al. (1995). The vectors of the invention can not only be used for expression of structural genes but may also be used in exon-trap cloning, or promoter trap procedures to detect differential gene expression in varieties of tissues, (Lindsey et al., 1993; Auch & Reth et al.).

It is particularly preferred to use the binary type vectors of Ti and Ri plasmids of *Agrobacterium spp.* Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton, rape, tobacco, and rice (Pacciotti et al., 1985: Byrne et al., 1987; Sukhapinda et al., 1987; Lorz et al., 1985; Potrykus, 1985; Park et al., 1985: Hiei et al., 1994). The use of T-DNA to transform plant cells has received extensive study and is amply described (EP 120516; Hoekema, 1985; Knauf, et al., 1983; and An et al., 1985). For introduction into plants, the chimeric genes of the invention can be inserted into binary vectors as described in the examples.

Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e., monocotyledonous or dicotyledonous, targeted for

transformation. Suitable methods of transforming plant cells include, but are not limited to, microinjection (Crossway et al., 1986), electroporation (Riggs et al., 1986), *Agrobacterium*-mediated transformation (Hinchee et al., 1988), direct gene transfer (Paszkowski et al., 1984), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wis. and BioRad, Hercules, Calif. (see, for example, Sanford et al., U.S. Pat. No. 4,945,050; and McCabe et al., 1988). Also see, Weissinger et al., 1988; Sanford et al., 1987 (onion); Christou et al., 1988 (soybean); McCabe et al., 1988 (soybean); Datta et al., 1990 (rice); Klein et al., 1988 (maize); Klein et al., 1988 (maize); Klein et al., 1988 (maize); Fromm et al., 1990 (maize); and Gordon-Kamm et al., 1990 (maize); Svab et al., 1990 (tobacco chloroplast); Koziel et al., 1993 (maize); Shimamoto et al., 1989 (rice); Christou et al., 1991 (rice); European Patent Application EP 0 332 581 (orchardgrass and other Pooideae); Vasil et al., 1993 (wheat); Weeks et al., 1993 (wheat). In one embodiment, the protoplast transformation method for maize is employed (European Patent Application EP 0 292 435, U. S. Pat. No. 5,350,689).

In another embodiment, a nucleotide sequence of the present invention is directly transformed into the plastid genome. Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, and 5,545,818, in PCT application no. WO 95/16783, and in McBride et al., 1994. Following transformation, plants are preferably selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic or herbicide resistance on the transformed plants and selection of transformants can be accomplished by exposing the plants to appropriate concentrations of the antibiotic or herbicide.

After transformed plant cells or plants are selected and grown to maturity, those plants showing the trait of interest are identified. The trait can be any of those traits described above. Additionally, to confirm that the trait of interest is due to the expression of the introduced polynucleotide of interest under control of the regulatory nucleotide according to the invention, expression levels or activity of the polypeptide or polynucleotide of interest can be determined by analyzing mRNA expression using Northern blots, RT-PCR or microarrays, or protein expression using immunoblots or Western blots or enzyme activity assays.

The invention thus relates to plant cells and tissues, to plants derived from such cells and tissues, respectively, to plant material, to the progeny and to seeds derived from such plants, and to agricultural products including processed plant products with improved properties obtainable by, for example, any one of the transformation methods described below.

Once an expression cassette according the present invention and as described herein comprising a regulatory sequence according to the invention in association with a polynucleotide of interest has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques. Preferred plants of the invention include gymnosperms, monocots, and dicots, especially agronomically important crop plants, such as rice, wheat, barley, rye, rape, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane.

The genetic properties engineered into the transgenic plants described above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in progeny plants. Generally said maintenance and propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting. Specialized processes such as hydroponics or greenhouse technologies can also be applied. Use of the advantageous genetic properties of the transgenic plants according to the invention can further be made in plant breeding that aims at the development of plants with improved properties such as tolerance to pests, herbicides, or stress, improved nutritional value, increased yield, or improved structure causing less loss from lodging or shattering. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate progeny plants. Depending on the desired properties different

breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multiline breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant will uniformly obtain properties of both parental lines. Thus, the transgenic plants according to the invention can be used for the breeding of improved plant lines that for example increase the effectiveness of conventional methods such as herbicide or pesticide treatment or allow to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained that, due to their optimized genetic "equipment", yield harvested product of better quality than products that were not able to tolerate comparable adverse developmental conditions.

In one embodiment of the invention, the plant has been transformed with and expresses a polypeptide or protein encoding nucleotide sequence encoding a polypeptide product exhibiting an insecticidal activity, particularly an endotoxin of *Bacillus thuringiensis* in most tissues of the plant but essentially excluding the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent, where the nucleotide sequence is not transcribed to any significant extent. Therefore, essentially no expression occurs in the pollen and/or the tassel tissue and only residual amounts of the expression product, if any, can be detected in said tissues, which is not sufficient for the expression product to fulfil its envisaged biological function in said tissues or to exhibit any toxic effects either towards insects feeding on these tissues or the plant itself.

In particular, the concentration of the polypeptide product expressed from the protein encoding polynucleotide of interest in the tissues of the pollen and/or the tassel is such that no insecticidal activity can be detected in a standard insect feeding assay. In one embodiment of the invention, the concentration of the

expression product in pollen is below a basic level of about 10 ng/mg soluble protein, particularly of about 5 ng/mg soluble protein, more particularly of about 3 ng/mg soluble protein, but especially of about 2 ng/mg soluble protein or below.

The invention also provides methods for preparing expression cassettes comprising the regulatory sequence according to the invention comprising linking an expressible polynucleotide encoding a polypeptide or a protein of interest with the regulatory sequence according to the invention and as described herein to obtain an expression construct, wherein the polynucleotide of interest is operably linked or associated with the regulatory sequence such that expression of the polypeptide or a protein of interest is mediated by the regulatory sequence according to the invention and results in the expression of said polypeptide or a protein of interest in essentially all plant tissues, but essentially excludes expression in the tissues of the reproductive structures of the plant, particularly in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

In one embodiment, the invention relates to a method of producing a transgenic plant expressing a DNA sequence of interest in non-pollen tissue but not or substantially not in the tissues of the pollen and/or the tassel, comprising

a) transforming an expression cassette according to the invention and as described herein into a plant cell which comprises a regulatory nucleotide sequence, at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues but essentially excluding the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent; and

b) regenerating the plant cell transformed in step a) into a plant.

In one embodiment, the invention relates to a method of controlling insect target-pests feeding on vegetative plant tissues such as the leaf, stalk and root and/or on reproductive tissues such as the ear, but protecting non-target pests feeding on pollen comprising

a) growing a plant according to the invention and as described herein in an area that is infested with the target pest;

b) expressing a polypeptide or protein that is capable of controlling said target pest under the control of a regulatory sequence according to the invention and as described herein.

In one embodiment, the invention relates to a method of protecting the reproductive tissues of a plant, particularly the tissues of the pollen and/or the tassel against damage caused by expression in said tissues of a polypeptide or protein of interest comprising

a) growing a plant according to the invention and as described herein;

b) expressing a polypeptide or protein of interest under the control of a regulatory sequence according to the invention and as described herein.

In one embodiment the present invention relates to the use of a regulatory sequence according to the present invention and as disclosed herein for controlling insect target-pests feeding on vegetative plant tissues such as the leaf, stalk and root and/or on reproductive tissues such as the ear, but protecting non-target pests feeding on pollen comprising

a) growing a plant according to the invention and as described herein in an area that is infested with the target pest;

b) expressing a polypeptide or protein that is capable of controlling said target pest under the control of a regulatory sequence according to the invention and as described herein.

In one embodiment the present invention relates to the use of a regulatory sequence according to the present invention and as disclosed herein for protecting the reproductive tissues of a plant, particularly the tissues of the pollen and/or the tassel against damage caused by expression in said tissues of a polypeptide or protein of interest comprising expressing said polypeptide or protein of interest under the control of a regulatory sequence according to the invention and as described herein.

EXAMPLE

The following Examples provide illustrative embodiments. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently claimed subject matter.

All manipulations and techniques necessary to construct and propagate strains described in this invention are known to those skilled in the art. Technical details are described e.g. in Ausubel et al 1995; Sambrook, J, 2001 and Miller, J.H. 1992 and in relevant publications cited within this invention.

Example 1: Non – Pollen Expression

Example 1.1 Identification of ZmABP3

In an expression profiling experiment a maize developmental series was queried on a *Zea mays* (Zm80K) Affymetrix chip for probes that gave strong signals in all samples, but not or substantially not in the pollen sample. All the green tissue and root samples were directly compared to pollen, and probes representing polynucleotides that did not meet the target expression profile were eliminated. The analysis produced two sets of results. The first set contains 36 probes representing polynucleotides that were highly expressed in all the tissue samples, but very low in pollen. The second set contains 10 probes represented polynucleotides that are highly expressed in all tissue samples, but gave no signal in pollen. Alignment of probe sequence with maize cDNA assembly datasets showed that all 46 probes represent *bona fide* maize genes. The top 10 probes are those with the strongest signal across all non-pollen tissues and no signal in pollen (see Table A).

Applying further criteria including determination of the availability of genomic DNA (gDNA) and cDNA sequence for each lead produced Zm07728_s_at as the top candidate that met all promoter development requirements. Literature analysis revealed that this probe represents the gene encoding actin binding protein 3 (ZmABP3) which is a member of a small gene family that had been previously

characterized (Lopez et al., 1996). The gene product has also been called actin depolymerizing factor 3. Lopez et al (1996) confirms in Figure 3 that ZmABP3 is highly expressed in most tissues of the plant examined, except pollen samples.

Lopez et al (1996) also show by southern analysis that there are two ABP3 genes in the maize genome. The ZmABP3 cDNA they report is GenBank Accession X97726, and it corresponds to the TIGR Accession TC248585. This gene was designated ZmABP3-A. Both ZmABP3 genes are represented on the maize (Zm80K) Affymetrix Chip: ZmABP3-A corresponds to probe Zm007595_at and ZmABP3-B corresponds to Zm07728_s_at. The 'Zm07728_s_at' sequence was used to identify the TC248588 in the TIGR database, and MAIZE.974.CB1 in a maize cDNA assembly database. It also identified the MAGI_93606, MAGI_93607, AZM4_39177, ZmGSStuc11-12-04.2725.1, ZmGSStuc11-12-04.2725.2 and CC463190 gDNA sequences. The ZmABP3-A and ZmABP3-B cDNAs encode proteins that are identical at all residues, except one. The expression profiling data indicate that ZmABP3-B is highly expressed in most tissues of the plant, but essentially excluding the tissues of the pollen so that no expression product is present in said tissues to any significant extent. ZmABP3-A is not as highly expressed.

SEQ ID NO: 16 show that the ZmABP3-B mRNA is encoded on 3 exons. The two intervening sequences (introns) are bracketed by the expected GT...AG border nucleotides.

More specifically, SEQ ID NO: 16 discloses the design of the ZmABP3 expression cassette. The ZmABP3 regulatory components to be included in the construct are 2.3 kb of 5'-sequence (prZmABP3-01) which contains 1.1 kb of 5'-non-transcribed sequence, 0.25 kb of 5'-UTR and 0.98 kb representing ZmABP3-B-intron 1; and 1.013 kb of 3'-sequence (tZmZBP3-01) that begins just past the ABP3-B translation stop codon. This includes about 0.3 kb of 3'-UTR and 0.7 kb of non-transcribed sequence.

Table A shows a summary of the top 10 candidate probes representing polynucleotides with a high expression level in all maize tissues and no expression signal in pollen

Probe Name	Description of Reference Gene	Pollen Expression	Average Expression (all tissues)	Zea mays TIGR Hit
AF032370_at	"Zea mays profilin (PRO4) mRNA, complete cds."	absent	4208	TC269677
Ctrl_ZmU45855-3_at	From 808 to 1307 of glyceraldehyde-3-phosphate dehydrogenase GAPC2 (gpc2) mRNA, complete cds.	absent	4275	TC269361
Zm001747_s_at	Similar to CAA63903.1 Pennisetum glaucum; heat shock protein 17.9; P.glaucum mRNA for heat shock protein, HSP 17.9	absent	4945	TC268849
Zm005803_s_at	"Similar to AAB99745.1 Triticum aestivum; HSP70; Triticum aestivum 70 kDa heat shock protein (TaHSP70d) mRNA, complete cds; 70 kDa heat shock protein, molecular chaperone"	absent	4091	TC247918
Zm007728_s_at	Similar to SW:ADF3_MAIZE Q41764 zea mays (maize). actin-depolymerizing factor 3 (adf 3) (zmabp3) (zmadf3).	absent	4805	TC248588
Zm009722_s_at	"Similar to BAC22420.1 Oryza sativa (japonica cultivar-group); ; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7, PAC clone:P0453E03; contains ESTs C96778(C10671),D22278(C10671) unknown	absent	3306	TC248975
Zm015335_s_at	Similar to SW:RS5A_ARATH Q9zut9 arabidopsis thaliana (mouse-ear cress). 40s ribosomal protein s5-1. 2/2003	absent	3598	TC269022
Zm021004_s_at	"Similar to AAD39835.1 Arabidopsis thaliana; Ran-binding protein siRanBP; Arabidopsis thaliana Ran-binding protein (siRanBP) mRNA, complete cds; atranbp1a homolog"	absent	3092	TC259986
Zm058948_s_at	No Description	absent	4337	TC270333
Zm061393_s_a	No Description=sucrose synthase	absent	6509	TC258905

Example 1.2 Cry1AbG6 construction

Cry1AbG6 (2814 bp) is a modified version of the full-length Cry1Ab (pNOV1321, 3546bp) gene. The Geiser sequence (81 bp from 4398-4478 in pNOV1321) and the 3'-end (651bp from 4908-5558 in pNOV1321) were deleted.

The Cry1AbG6 sequence was constructed from pNOV1321 (source vector for the Cry1Ab full-length gene) as follows: pNOV1321 plasmid DNA was cut with BamHI/SacI. The Cry1Ab full-length gene (3546bp, named Michigan) was gel purified and ligated to pTrcHisB expression vector (In vitrogen life technologies, Cat# V36020), which was cut with BamHI/SacI. This construct was named as Michigan-pTrcHisB. The Geiser sequence (81bp) was deleted from Michigan-pTrcHisB by overlapping PCR with the following primers:

5' Bfr1 (5'-cctggtggagtgcttaagcgacgagttctgcctgg-3'), (SEQ ID NO: 83)
3' Xba1 (5'-gggcttctcctccaggaactctagattgcccaggcg-3'), (SEQ ID NO: 84)
5'Gfix (5'-catcggcaagtgccaccacagccaccacttcagcctg-3') (SEQ ID NO: 85) and
3'Gfix (5'-gctgtggtggcacttgccgatggggctggg-3') (SEQ ID NO: 86).

PCR product A was made using high-fidelity PCR with Michigan-pTrcHisB as a template, and the 5' Bfr1 and 3' Gfix primers. PCR product B was made using high-fidelity PCR with Michigan-pTrcHisB as a template, and the 5'Gfix and 3'Xba1 primers. The final PCR used products A and B as templates, and the 5'Bfr1 and 3'Xba1 primers. The final PCR band was digested with AflII/XbaI and gel-purified. This fragment was ligated to Michigan-pTrcHisB that had also been digested with XbaI/AflII. The correct recombinant DNA product was identified by AflII/XbaI digestion analysis. This construct was named as Cry1Ab-G.

A second PCR product was made by high-fidelity PCR using pNOV1321 as a template, the 5'1Ab5Xbal (5'-gcccgcctgggcaatctagagttcctggaggag-3') primer depicted in SEQ ID NO: 87, and the 3'1Ab3d6 (5'-gcgagctcctagatgcggccctcga-gttcctcgaaga-3') primer depicted in SEQ ID NO: 88. The PCR product was digested with XbaI/SacI then ligated to Cry1Ab-G that was also digested with XbaI/SacI. The correct recombinant DNA product was identified using BamHI/SacI restriction analysis. This construct was named as Cry1AbG6.

The Cry1AbG6 sequence was subjected to QuikChange mutagenesis to remove an internal NcoI site. The 25 µL reaction contained
1 µL Cry1AbG6 template,
2.5 µL 10X QuikChange buffer,
1 µL QuikChange dNTP mix,
1 µL of 20 µM cy2' (5'-Pccctgtacggcacgatgggcaacgctgca-3'; SEQ ID NO: 89),
0.75 µL Quik solution and
1 µL QuikChange DNA polymerase.
The thermocycling program was 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 65°C for 20 minutes. The product was processed as described by the manufacturer (Stratagene) and completely sequenced.

The Cry1AbG6 coding sequence was amplified from the mutagenized plasmid template, above, in a 50 µL Pfu turbo (Stratagene) DNA polymerase reaction containing

5 µL template,
5 µL 10X Pfu buffer,
1 µL 10 mM dNTP mix,
1 µL of 20 µM cy1 (5'-atatatccaccatggacaacaaccccaaca-3'; SEQ ID NO: 90),
1 µL of 20 µM cy2 (5'-tatatagagctcctagatgcggccctcgagt-3'; SEQ ID NO: 91) and
1 µL Pfu turbo DNA polymerase.

The thermocycling program was 95°C for 2 minutes followed by 40 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 7 minutes. The final extension step was 72°C for 15 minutes. The 2.8 kb reaction product was gel-purified on 1% TAE agarose, and the DNA was extracted using Qiaprep DNA extraction method. The recovered DNA was digested with NcoI/SacI, then ligated to pNOV6901 vector that was also digested with NcoI/SacI. This operation replaced the GUS coding sequence in pNOV6901 with Cry1AbG6. The Cry1AbG6 sequence is given in SEQ ID NO: 15.

Example 1.3 *Construction of the ZmABP3 Expression Cassette*

An inclusive design strategy was used to develop the ZmABP3 expression cassette. The cassette contains 2.3 kb of 5'-sequence which consists of 1.1 kb of 5'-non-transcribed sequence, 0.25 kb of 5'-UTR and 0.98 kb representing ZmABP3-intron 1. The natural translation start codon was silenced in order to move it to the second exon. The expression cassette also contains 1.013 kb of 3'-sequence that begins just past the ABP3 translation stop codon. This includes about 0.3 kb of 3'-UTR and 0.7 kb of non-transcribed sequence, and functions as the transcriptional terminator and poly-adenylation signal.

The ZmABP3 terminus was amplified from maize gDNA template in a 50 µL Proofstart (Qiagen) DNA polymerase reaction containing

10 µg gDNA,
5 µL 10X Proofstart buffer,

1.5 µL 10 mM dNTP mix,
2.5 µL of 20 µM P3 (5'-tatatagagctcgcatcatgatcatgcatcatggact-3'; SEQ ID NO: 9),
2.5 µL of 20 µM P4 (5'-atatatactagtggcgcgccacactttctgtcgcatgtgatttgca-3'; SEQ ID NO: 10),
10 µL Q solution and
2 µL Proofstart DNA polymerase.
The thermocycling program was 95°C for 5 minutes followed by 45 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 4 minutes. The final extension step was 72°C for 15 minutes. The 1 kb reaction product was gel-purified on 1% TAE agarose, and the DNA was extracted using Qiaprep DNA extraction method. The DNA was ethanol precipitated and recovered in 4 µL ddH_2O, then cloned into the pCR4-TOPO-Blunt vector.
The ZmABP3 terminus was modified to remove an internal NcoI restriction site using the Stratagene QuikChange Multi-site mutagenesis kit. The 25 µL reaction contained
1 µL pCR4-TOPO-ZmABP3-terminus,
2.5 µL 10X QuikChange buffer,
1 µL QuikChange dNTP mix,
1 µL of 20 µM Tnco (5'-Pgtaaaaaaaggtcccttggctcccagaaga-3'; SEQ ID NO: 11),
1 µL of 20 µM T2 (5'-Pcaatgtgttagactgacgtg-3'; SEQ ID NO: 12),
0.75 µL Quik solution and
1 µL QuikChange DNA polymerase.
The thermocycling program was 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 65°C for 15 minutes. The product was processed as described by the manufacturer (Stratagene) and completely sequenced. The ZmABP3-terminus sequence is shown in SEQ ID NO: 14.

The ZmABP3 promoter was amplified from maize gDNA template in a 50 µL Hotstart (Qiagen) DNA polymerase reaction containing
10 µg gDNA,
25 µL 2X Hotstart Master Mix,

1.25 µL of 20 µM P1 (5'-atatatgcatgcggcgcgccgaaagtagcaaacaacaggttcatgtgcac-3'; SEQ ID NO: 1),

1.25 µL of 20 µM P2 (5'-tatataccatggtgggtttgcctgcgaccacaagttca-3'; SEQ ID NO: 2),

10.5 µL Q solution and

2 µL 25 mM $MgCl_2$.

The thermocycling program was 95°C for 15 minutes followed by 45 cycles of 94°C for 1 minute, 64°C for 1 minute and 72°C for 5 minutes. The final extension step was 72°C for 15 minutes. The 2.3 kb reaction product was gel-purified on 1% TAE agarose, and the DNA was extracted using Qiaprep DNA extraction method. The DNA was ethanol precipitated and recovered in 4 µL ddH_2O, then cloned into the pCR4-TOPO vector.

The ZmABP3 promoter was modified in a series of QuikChange reactions as outlined above using the following oligonucleotides:

Patg (5'-cagctcgcccgagttggtaaggcccccct-3'; SEQ ID NO: 3),

Pnco (5'-acagattagtccatcgcccacggt-3'; SEQ ID NO: 4),

ADPc-1 (5'-agccctgtccatgacggcccaagcaac-3'; SEQ ID NO: 5),

ADPc-2 (5'-agtagcaattcggtaggcacaggcac-3'; SEQ ID NO: 6),

ADPc-4 (5'-tctatggtctgcgaggtgcggtggc-3'; SEQ ID NO: 7), and

adp3-a (5'-gtccccttcttcgccgcgccagctcgc-3'; SEQ ID NO: 8).

The ZmABP3 promoter sequence is shown in SEQ ID NO: 13.

The ZmABP3 terminus was ligated to the pNOV6901-Cry1AbG6 vector (from Example 2) as a SacI/SpeI fragment. The ZmABP3 Promoter was subsequently ligated to the vector as a SphI/NcoI fragment. This produced ZmABP3-Cry1AbG6-assembly, shown in SEQ ID NO: 37. The complete ZmABP3-Cry1AbG6 expression cassette was mobilized into a binary vector, pNOV6900, as an AscI fragment. These constructs, ZmABP3-Cry1AbG6-6900 and enhanced ZmABP3-Cry1AbG6-binary, are shown in SEQ ID NOS: 38 and 39, respectively. The only difference between these vectors is the presence of the CaMV-FMV dual enhancer in enhanced ZmABP3-Cry1AbG6-binary. Both were mobilized to maize via Agrobacterium-mediated transformation.

Example 1.4 *Construction of ZmABP3-AmCyan*

The Cry1AbG6 coding sequence was excised from ZmABP3-Cry1AbG6-assembly as an NcoI/SacI fragment. It was replaced with the AmCyan reporter gene coding sequence that was excised from plasmid 13718 as an NcoI/SacI fragment. This produced the ZmABP3-AmCyan-assembly construct shown in SEQ ID NO: 40. The ZmABP3-AmCyan expression cassette was mobilized into a binary vector, pNOV6900, as an AscI fragment. This construct, ZmABP3-AmCyan-binary, is shown in SEQ ID NO: 41. It was mobilized to maize via *Agrobacterium*-mediated transformation.

Example 1.5 *Expression from ZmABP3-AmCyan in transgenic maize*

Several transgenic maize events containing the ZmABP3-AmCyan expression cassette were produced. Those containing a single-copy of the transgene and no un-intended vector sequence were analyzed. All transgenic events accumulated AmCyan transcript in leaf tissue (data not shown). Several tissues from a representative event were examined for AmCyan transcript accumulation. Total RNA was prepared using the Plant RNAeasy total RNA isolation system (Qiagen). Pollen total RNA was prepared using the method described by Shirzadegan et al (1991). Preparation quality was assessed by UV spectrophotometry, and 10 µg of total RNA per sample was resolved on a 1% formaldehyde gel then transferred to Nytran SuPerCharge membrane following the recommended protocol (Schleicher & Schuell). The blot was hybridized to a random-primed ^{32}P-labeled AmCyan DNA probe using high stringency conditions. The results clearly show that ZmABP3 promotes transcription in tassel, leaf, silk, ear and root tissue, but does not promote transcription in pollen.

Example 1.6 *Expression from ZmABP3-Cry1AbG6 in transgenic maize*

Several transgenic maize events containing the ZmABP3-Cry1AbG6 expression cassette were produced. Those containing a single-copy of the transgene and no un-intended vector sequence were analyzed. The T0 events were tested for insecticidal activity against corn earworm twice during the course of development. The first samples were taken at V2-V4, and the second samples were taken at V7-

V9. Leaf discs from lower leaf tips were excised and placed on water-moistened Whatman paper in 47 X 10 mm petri dishes. Ten-to-twenty L1 corn earworm or European corn borer larvae were added to each dish, and they were incubated for 48 hours at 28°C. Leaf discs were then scored for insect damage. Samples with no visible leaf damage and absolute mortality were scored as positive, and those with visible damage were negative. The data obtained show that several transgenic events with activity against both insects were identified.

Cry1AbG6 protein accumulation was also measured in T0 plants using the enzyme-linked immunosorbent assay (ELISA) with a fully-truncated Cry1Ab standard. The first assay was done on seedling leaf tissue, sampled 1-2 weeks after transfer to soil. The second assay was done on leaf tissue from maturing plants, sampled just prior to the transition to reproductive development. The data in TABLE B show the range of Cry1AbG6 protein accumulated in plants with insecticidal activity. The data indicate that plants require nearly 50 ng (or more) Cry1AbG6 protein/mg extractable protein to have insecticidal activity.

TABLE B shows the insect control characteristics of greenhouse grown plants.

Event Number	Cassette Description	Cry1AbG6 (ng/mg extractable protein)		Corn Earworm Activity		ECB Activity
		seedling	adult	V2-V4	V7-V9	V7-V9
1	ABP3-Cry1Abg6	63	79	+	+	+
2	ABP3-Cry1Abg6	54	56	+	+	+
3	ABP3-Cry1Abg6	85	108	+	+	+
4	ABP3-Cry1Abg6	67	94	+	+	+
5	ABP3-Cry1Abg6	45	83	+	+/-	+/-
6	ABP3-Cry1Abg6	68	120	+	+	+
7	ABP3-Cry1Abg6	133	159	+	+	+
8	ABP3-Cry1Abg6	96	46	+	+	+
9	ABP3-Cry1Abg6	138	101	+	+	+
10	ABP3-Cry1Abg6	131	100	+	+	+
11	ABP3-Cry1Abg6	94	65	+	+	+
12	ABP3-Cry1Abg6	111	59	+	+	+
13	ABP3-Cry1Abg6	139	60	+	+	+
14	ABP3-Cry1Abg6	121	81			
15	ABP3-Cry1Abg6	66	55	+	+	+
16	ABP3-Cry1Abg6	130	95	+	+	+

Leaf tissue from T0 plants was assayed for Cry1AbG6 protein by ELISA using truncated Cry1Ab protein as standard, Corn Earworm activity and European Corn Borer (ECB) activity. The plant developmental stage when sampled is indicated at the top of each column. The older (lower) leaf tissue was sampled. For insect assays a (+) indicates no visible leaf damage and complete and absolute insect mortality. Visible leaf damage produced a (-) score.

Example 1.7 *European cornborer efficacy of ZmABP3-Cry1AbG6 events in the field*

The ECB (European corn borer) field efficacy studies were conducted in Stanton, MN (SMN) and Bloomington, IL (BIL) during the 2006 growing season. Near-isogenic hybrids, comprising the ABP3-Cry1AbG6 events listed in TABLE C, Bt11, and a nontransgenic control hybrid were tested. The experimental design was randomized complete block with three replications in each location. A plot consisted of one 5.31 m long row containing 25 plants, with 0.76 m spacing between rows.

TABLE C shows the performance of ZmABP3-Cry1AbG6 maize in field studies.

Trial		MG371				MG331			
Location		BIL				SMN			
Trial Type		ECB				ECB			
Event Number	Cassette Description	ECBLR Leaf Feeding Rating	CEBSN Shank (cm)	ECBKN Ear Feeding (cm)	ECBSN Stalk Feeding (cm)	ECBLR Leaf Feeding Rating	CEBSN Shank (cm)	ECBKN Ear Feeding (cm)	ECBSN Stalk Feeding (cm)
1	ABP3-Cry1Abg6	1,0	0,00	1,42	0,00	1,1	0,00	0,00	0,30
2	ABP3-Cry1Abg6	1,0	0,00	1,42	0,08	1,0	0,00	0,15	0,10
3	ABP3-Cry1Abg6	1,0	0,00	1,25	0,08	1,0	0,00	0,00	0,80
4	ABP3-Cry1Abg6	1,0	0,00	1,57	0,00	1,0	0,10	0,51	1,10
5	ABP3-Cry1Abg6	1,0	0,00	1,25	0,04	1,0	0,00	0,07	0,20
6	ABP3-Cry1Abg6	1,0	0,00	1,08	0,00				
7	ABP3-Cry1Abg6	1,0	0,00	1,31	0,00	1,1	0,10	0,45	0,80
8	ABP3-Cry1Abg6	1,0	0,04	2,00	0,08	1,1	0,00	0,00	0,30
9	ABP3-Cry1Abg6	1,0	0,00	0,92	0,00	1,3	0,00	0,00	0,10
10	ABP3-Cry1Abg6	1,0	0,00	1,42	0,04	1,2	0,00	0,00	0,40
11	ABP3-Cry1Abg6	1,0	0,13	1,17	0,00	1,0	0,00	0,00	0,10
12	ABP3-Cry1Abg6	1,0	0,00	1,62	0,08	1,1	0,00	0,17	0,30
13	ABP3-Cry1Abg6	1,0	0,00	1,29	0,00	1,2	0,00	0,00	0,20
14	ABP3-Cry1Abg6	1,0	0,00	1,10	0,13	1,0	0,00	0,07	0,10
15	ABP3-Cry1Abg6	1,0	0,08	1,33	0,04	1,1	0,00	0,24	0,20
16	ABP3-Cry1Abg6	1,0	0,00	1,33	0,21	1,0	0,00	0,00	0,10
Bt11		1,0	0,00	2,75	0,00	1,3	0,00	0,00	0,00
Negative Check		7,0	0,21	3,00	4,67	4,3	0,40	5,80	13,50
Rep with data		3	3	3	3	3	3	3	3
Loc with data		1	1	1	1	1	1	1	1
Design Used		RCB	RCB	RCB	RCB	RCB	RCB	RCB	RCB
LSD (5%) General EE			0,149	0,923	0,257	0,399	0,200	1,988	0,650
LSD (5%) Excluded Negatives			0,158	0,936	0,255	0,397	0,181	0,505	1,391
CV%			242,21	38,47	72,14	20,10	292,75	138,76	120,87
Probablitiy %			0,90	0,09	0,00	0,00	4,10	0,00	0,00

Two studies were undertaken in Bloomington, IL (BIL) and Stanton, MN (SMN) in 2006. Several ZmABP3-Cry1AG6 events were compared to positive and negative benchmarks represented by Bt11 and Negative Check, respectively.

First-instar ECB larvae were produced from a laboratory colony following procedures outlined in Guthrie (1989) at the Syngenta Seeds, Inc. entomology laboratory in Slater, IA. Eggs were incubated at about 28°C and approximately 80% relative humidity, and neonates were collected from hatching containers

approximately 6 hours after hatch. Larvae were healthy and vigorous when placed on the plants as indicated by movement.

Two ECB application types were performed: ECB1, applied at approximately leaf stage V6–V8 and ECB2, applied at pollen shed. The applications were made with the BioServe Davis Inoculator using 1 ml corn cob grits per application. For ECB1 (first-generation ECB infestation) a total of about 150 larvae were placed into the whorl of each plant, in corn cob grits. Two to four applications were made, with one to six days between each application. The first plant in the row was not treated, and then up to 10 consecutive plants were infested.

For ECB2 (second-generation ECB infestation) a total of about 200 larvae were applied per plant, placed into the ear leaf axil and leaf axils directly above or below the ear, in corn cob grits. Four applications were made, with one to six days between each application. Up to ten consecutive plants on the opposite end of the row from the ECB1 treatment were infested. The last plant in the row was not treated.

The following observations were recorded. For ECB1, up to eight consecutive infested plants in the row were evaluated for foliar ECB damage (ECBLR in TABLE C) at least 14 days after the first infestation. The Guthrie scale of 1-9 (Guthrie et al. (1960) was used and one rating, the average for the evaluated plants, was recorded for each plot. For ECB2, approximately 45 days after the plants were infested, up to eight consecutively infested plants on the opposite end of the row from the ECB1 evaluations were dissected to assess ear shank, ear kernel, and stalk feeding, by measuring feeding tunnel lengths (cm).

ECB2 data were subjected to analyses of variance appropriate for a randomized complete block design. Replications were considered random while all other effects were considered fixed. Mean separation was done using the least significant difference (LSD) procedure, but only if the F-test for entries was significant at the customary 5% significance level. Because there was no variability among the events in the ECB1 data, an analysis of variance was not done for this trait. The data and analysis are summarized in TABLE D. In general, the data show that ZmABP3-Cry1ABG6 affords protection against ECB similar to that observed in Bt11 material.

TABLE D shows the amount of Cry1AbG6 protein in transgenic maize tissue. The youngest developing leaf was tested for Cry1AbG6 by ELISA at 5 developmental stages (V5-V6, V8, V10, R1, R3-R4) for each plant. Cry1AbG6 was also measured in pollen. Events 5, 12, 15 and 16 express the ABP3-Cry1AbG6 construct, and Events A-D express the enhanced ABP3-Cry1Ab construct. Data shown are the mean ± SD (n=8-10).

	Developmental Stage					
	V5-V6	**V8**	**V10**	**R1**	**R3-R4**	**Pollen**
Event 5	39(3.8)	38(2.7)	61(8.2)	75(5.3)	60(3.5)	1.5(0.14)
Event 12	61(5.2)	32(1.9)	50(6.1)	44(5.1)	49(4.4)	1.4(0.39)
Event 15	45(4.5)	45(4.8)	46(4.8)	38(7.4)	55(5.4)	1.0(0.14)
Event 16	58(5.4)	30(2.9)	47(5.3)	53(7.2)	44(4.6)	1.2(0.17)
Event A	260(24)	190(22)	250(18)	200(21)	150(14)	1.3(0.19)
Event B	260(22)	227(29)	240(30)	200(23)	150(76)	1.6(0.30)
Event C	310(31)	210(26)	270(26)	150(15)	160(16)	1.9(0.31)
Event D	310(30)	180(23)	240(15)	170(26)	150(18)	1.4(0.19)

Example 1.8 *Use of ZmABP3 Expression Cassette to improve drought tolerance in maize*

A deregulated form of an Arabidopsis H^+-pyrophosphatase (AtAVP1D) has been shown to improve drought tolerance when over-expressed in several plants (Gaxiola et al., 2001; Park et al., 2005). The improved performance is enabled by high expression throughout the plant. To demonstrate the utility of AtAVP1D to improve drought tolerance in maize, a maize-optimized coding sequence was synthesized. The sequence of the AtAVP1D synthetic gene is shown in SEQ ID NO: 16. It was ligated to the ZmABP3 expression cassette as an NcoI/SacI fragment. The vector map shown in SEQ ID: 42 illustrates the ZmABP3-AtAVP1D expression cassette. The complete ZmABP3-AVP1D expression cassette was excised from the Assembly vector as a SanDI/RsrII fragment and ligated to the RsrII site of the *Agrobacterium* binary vector, 15289. A map of the construct is shown in SEQ ID NO: 43.

Example 1.9 *Measurement of Cry1AbG6 in maize tissue*

Hybrid T1 seed (in the ID5829/AX5707 background) for several ZmABP3-Cry1ABG6 events were produced at a Syngenta field station in Bloomington, IL. Several seed were germinated in 2 inch pots. Seedlings were tested for transgene zygosity, and only hemizygotes were retained. A minimum of 8 plants per event were transplanted to 3 gallon pots and grown in a temperature controlled greenhouse. Leaf tissue from each plant was sampled and assayed for Cry1AbG6 protein at 5 stages of development, V5-V6, V8, V10, R1, and R3-R4 (Ritchie et al., 1997). Pollen was also collected and assayed for Cry1AbG6 protein.

At each stage, leaf tissue (minus the collar, midrib and sheath) was sampled from the youngest expanding leaf. Duplicate samples were pulverized in 96-well blocks. The powder was suspended in 500 µL-1 mL extraction buffer (0.1 M Sodium Borate, 0.5% Tween 20, 0.2% Polyvinylpyrrolidone, 0.05% Sodium Azide, and 1X protease inhibitor cocktail tablets (Roche)). The mixture was clarified by centrifugation and soluble protein quantified using the BCA assay. Fresh pollen was collected in 1.5 mL Eppendorf tubes. Three 3 mm glass beads were added to each tube and the samples were frozen at -80°C. Samples were then pulverized in a horizontal oscillator at 600rpm. Protein was extracted by adding 500 µL-1 mL extraction buffer and incubating at 4°C for 30 minutes. The samples were clarified by centrifugation at 4°C, and the soluble protein in each sample was quantified by BCA Assay.

Samples were normalized for protein content and Cry1AbG6 was quantified by ELISA using fully-truncated Cry1Ab as a standard. Each data point is the mean of duplicate measurements, taken at a different dilution of total protein. Data for each event are reported as the mean ± SD for all siblings.

Results in TABLE D show that the ZmABP3-Cry1AbG6 cassette produces steady Cry1AbG6 protein in leaf tissue throughout development. Some reduction in CryAbG6 protein is evident as the vegetative tissue begins to senesce (R3-R4). Also evident is the 3-5 fold increase in Cry1AbG6 accumulation in events that also have the CaMV-FMV dual-enhancer complex. Finally, the data show virtually no detectable Cry1AbG6 protein in pollen. In all events CryAbG6, on average, accumulates to less than 1.5 ng/mg total soluble protein. Furthermore, the dual-enhancer complex does not influence Cry1AbG6 accumulation in pollen; it is identical between all events. This is consistent with our data showing that ZmABP3

is not transcribed in pollen (Example 5). We conclude that detectable Cry1AbG6 in pollen was likely produced in the microspore mother cells or their progenitors, and carried to pollen through cell division.

EXAMPLE 2 Non-Tassel Expression

Example 2.1 Identification of ZmABT

2.1.1 Expression profiling experiment: A maize developmental series on the Zm80K Affymetrix chip, was queried for probes that gave strong signals in all samples, and a low or no signal in the tassel samples. Twenty-three (23) probes were identified representing polynucleotides that met the expression criteria. To better represent the differential expression signal between the tassel samples and other tissue samples, the ratio of mean signal for other samples and tassel was calculated for each probe. This indicates the expression differential between tassel and other samples. Any signal below 50 is in the experimental noise, which means the gene may not be transcribed or is transcribed at a very low level. To understand the expression level of each gene represented by candidate probes, a second expression profiling study was queried. In this experiment tissues from two maize genotypes were hybridized to the Zm80K Affymetrix chip. In general signals over 1000 indicate high expression and signals over 10,000 indicate very high expression.

2.1.2 Identification of candidate probes: Two top candidate probes were identified. Probe Zm033444_S_AT demonstrates virtually no signal in tassel and a high signal in other tissues. This indicates that the gene represented by Zm033444_S_AT is not expressed in tassel and is highly expressed throughout the rest of the plant. It also demonstrates the greatest expression differential, 60-fold higher in non-tassel tissue. Probe Zm040564_X_AT has a low signal in young tassel that gradually increases to a high or strong signal. The signal strength between tassel and non-tassel samples differs by less than 10-fold. However the signal strength in non-tassel samples is nearly 10-fold higher than Zm033444_S_AT. The sequence data indicate that neither probe corresponds to a characterized gene. Both probes

identify good candidate genes for development of promoters that deliver high expression in non-tassel tissue and little or no expression in tassels. Given the high signal differential between tassel and non-tassel samples, an expression cassette based on probe Zm033444_S_AT was developed.

Table E: shows a summary of the top candidate probes representing polynucleotides with a high expression level in all maize tissues and low expression signal in tassel

Probe	P-Value	BH Q-Value	Mean induction in non-tassel samples	V9 tassel	V12 tassel	V15 tassel
Zm033444_s_at	0,00	0,00	60	16,2	10,2	132
Zm002990_s_at	0,00	0,00	45	32,8	68,7	47,8
Zm006285_at	0,00	0,00	20	37,9	44,1	35,8
Zm000019_at	0,00	0,00	16	117	200	242
Zm006481_s_at	0,00	0,00	14	26,9	32,1	31,5
Zm002987_at	0,00	0,00	14	83,7	80,8	119
Zm004433_at	0,00	0,00	12	53,8	35,3	127
Zm010323_s_at	0,00	0,00	11	45,4	63	71,5
Zm016864_s_at	0,01	0,01	11	89,5	55,6	1280
Zm018791_at	0,01	0,01	11	41,4	34,7	252
Zm028405_s_at	0,00	0,00	10	69	65,1	89
Zm021403_at	0,00	0,00	10	42,2	41,4	71
Zm054116_s_at	0,00	0,00	10	93,3	62,4	219
Zm002990_x_at	0,00	0,00	10	13,6	29,5	29,2
Zm005761_at	0,00	0,00	9,6	33,2	40	46,7
Zm035082_s_at	0,00	0,00	8,5	83	84	143
Zm066342_at	0,00	0,00	8,2	52,9	59,2	199
Zm032921_s_at	0,00	0,00	8,1	57,5	29,8	90,5
Zm040564_x_at	0,01	0,01	7,5	277	143	3710
Zm051284_at	0,01	0,01	6,5	53,2	40	194
Zm011554_at	0,03	0,04	5,4	72,5	64,2	895
Zmmetall_x_at	0,01	0,01	5,3	325	199	2330
Zm011554_x_at	0,04	0,04	4,9	63,5	62,6	664

Example 2.2 Development of an Expression Cassette

DNA sequence evidence to identify cDNAs corresponding to Zm033444_S_AT was collected. Public and proprietary databases were queried by BLASTN with Zm033444_S_AT sequence. cDNA hits with precise matches to the query sequence fell into two similar contigs. ZmABT1 corresponds to Maize.1482.c47 and

Maize.1908.c31, and ZmABT2 corresponds to Maize.1482.c32, Maize.1482.c28, Maize.1482.c53, Maize.1908.c17, Maize.1908.c20, Maize.1908.c37 and AI947567. The Zm033444_S_AT, ZmABT1 and ZmABT2 sequences were used to query maize genomic DNA sequence databases to identify the regulatory sequence(s) that give high expression in non-tassel tissue and little or no expression in tassels. The queries identified three entries, AZM4_12, ZmGSStuc11-12-04.4740.1 and MAGI_88845, that assemble into a single contig. The ZmABT gDNA sequence is shown in SEQ ID NO: 46. It encodes both ZmABT1 and ZmABT2 (SEQ ID NO: 33 and 34, respectively). They are alternatively spliced variants of the same transcript.

ZmABT1 is encoded on 5 exons, and ZmABT2 is encoded on 6 exons. The additional exon lies between exon 1 and exon 2 of ZmABT1. The largest open reading frame on ZmABT1 and ZmABT2 was used to define their translation start and stop codons. Both cDNAs used the same translation start and stop codon. This information enabled the design of a ZmABT-based expression cassette.

Example 3: Construction of a ZmABT-GUS Expression Cassette

An inclusive, gene structure-based design strategy was used to construct the ZmABT expression cassette. To incorporate the known alternative splicing of this gene into the expression cassette, the design strategy was based on the structure of ZmABT1. The cassette contains 2.615 kb of 5'-sequence, which consists of 2.020 kb of 5'-non-transcribed sequence, 12 bp of 5'-UTR and 0.58 kb representing exon 1, intron 1 and 16 bp of exon 2. The natural translation start codon was silenced in order to move it to the second exon. The expression cassette also contains 1.039 kb of 3'-sequence that begins just past the translation stop codon. This includes 0.603 kb of 3'-UTR and 0.436 kb of non-transcribed sequence, and functions as the transcriptional terminator and poly-adenylation signal.

The ZmABT promoter was amplified from maize gDNA template in a 50 µL Proofstart (Qiagen) DNA polymerase reaction containing 10 µg gDNA, 5 µL 10X Proofstart Buffer, 1.0 µL 10 mM dNTP mix, 1.0 µL of 20 µM ABT P1 forw (5'-

CGACCAGCGCGACATGCATGGCA-3'; SEQ ID NO: 19), 1.0 µL of 20 µM ABT P2 rev (5'- ACCCCAGGGCGTACGACAAGGCC-3'; SEQ ID NO: 20), and 10.0 µL 5X Q solution. The thermocycling program was 95°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 67°C for 30 seconds and 72°C for 2.5 minutes. The final extension step was 72°C for 10 minutes. The 2.6 kb reaction product was gel-purified on 1% TBE agarose and the DNA was extracted using Qiaprep DNA extraction method. The DNA was cloned into the pCR-BluntII -TOPO vector.

The ZmABT promoter was modified in a series of mutagenesis reactions to silence the endogenous START codon, silence a SanDI restriction site and correct point mutations created during amplification. This was done using the Stratagene QuikChange Multi-site mutagenesis kit. The 25 µL reaction contained 1 µL pCR4-TOPO-ZmABT-promoter, 2.5 µL 10X QuikChange buffer, 1 µL QuikChange dNTP mix, 0.75 µL Quik solution, 1 µL QuikChange DNA polymerase and 1 µL of 20 µM of at least one of the following oligonucleotides:

pABT mut1 (5'-GATGGCCGGATTGGGCTCCCGGGGTGGAG-3') (SEQ ID NO: 21)

pABT mut2 (5'-CTGGGAGGCGCGCAAGGGGCAGTTCCTCG-3') (SEQ ID NO: 22)

pABT mut3 (5'-CCCACCGCCGGAGCACCGAAAGGCCCCGCG-3') (SEQ ID NO: 23)

pABT mut4 (5'-GTCACCCGGGAGCACTTCCCGGCGCCG-3') (SEQ ID NO: 24)

pABT mut5 (5'-CATTGGGCCGAGCACGGCTTCTTCCGC-3') (SEQ ID NO: 25)

pABT mut6 (5'-GGGGTACGGTGTTCTTGAGTCGTGAAGCGAC-3') (SEQ ID NO: 26)

The thermocycling program was 95°C for 1 minute followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 65°C for 12 minutes. The product was processed as described by the manufacturer (Stratagene) and completely sequenced. The ZmABT promoter sequence is shown in SEQ ID NO: 35.

The corrected ZmABT promoter was PCR amplified from the TOPO vector in a 50 µL Proofstart (Qiagen) DNA polymerase reaction as above using primers pABT amp1 (5'- GCGTCTAGAGGGACCCCGACCAGCGCGACATGCATGGCA-3'),

depicted in SEQ ID NO: 27 and pABT amp2 (5'-ACCCCAGGGCGTACGACAA-GGCCCCACCATGGGCGC-3'), depicted in SEQ ID NO: 28. The PCR product was gel-purified on 1% TBE agarose and the DNA was extracted using Qiaprep DNA extraction method. The DNA was cloned into the pCR-BluntII -TOPO vector, transformed and sequenced. The ZmABT promoter was excised as an XbaI/NcoI fragment and ligated to pNOV6901.

The ZmABT terminus was amplified from maize gDNA template in a 50 µL Extensor (ABgene) DNA polymerase reaction containing 10 µg gDNA, 5 µL 10X Extensor buffer #1, 2.0 µL 10 mM dNTP mix, 2.0 µL of 20 µM ABT P4 (5'-TATATAGAGCTCGAATCGAAGAAGCCACACTGTAAATCTGCCGGG-3'; SEQ ID NO: 29), 2.0 µL of 20 µM ABT P5 (5'-AGCAAGGCATATGCAGCAGCTGCT-GGTCGGACCGGGCCCTATATA-3'; SEQ ID NO: 30), 10 µL 5X Q solution, 0.5 µL Extensor DNA polymerase and 0.5 µL Amplitaq DNA polymerase. The reactions were overlaid with mineral oil and the thermocycling program was 95°C for 2 minutes followed by 40 cycles of 98°C for 2 seconds, 63°C for 1 minute and 68°C for 4 minutes. The final extension step was 68°C for 7 minutes. The 1 kb reaction product was gel-purified on 1% TAE agarose, and the DNA was extracted using Qiaprep DNA extraction method. The DNA was ethanol precipitated and recovered in 4 µL ddH_2O, then cloned into the pCR4-TOPO-Blunt vector.

The ZmABT terminus was modified to remove internal NcoI and XhoI restriction sites using the Stratagene QuikChange Multi-site mutagenesis kit, as above. The 25 µL reaction contained 1 µL pCR4-TOPO-ZmABT-promoter, 2.5 µL 10X QuikChange buffer, 1 µL QuikChange dNTP mix, 0.75 µL Quik solution, 1 µL QuikChange DNA polymerase and 1 µL of 20 µM of at least one of the following oligonucleotides:

ABTt m1 (5'- GTCATGCATGGGCATGTGAAGGAGGAGCC-3') (SEQ ID NO: 31)

ABTt m2 (5'- GTTGCATGCATGCTGCATGGCGTCGAGAT-3') (SEQ ID NO: 32)

The thermocycling program was 95°C for 1 minute followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 65°C for 13 minutes. The product was processed as described by the manufacturer (Stratagene) and completely sequenced. The ZmABT terminator sequence is shown in SEQ ID NO: 36.

The ZmABT terminus was excised as a SacI/ApaI fragment and ligated to pNOV6901-prABT vector (above). This produced plasmid 15772 (ZmABT Assembly), and a plasmid map is shown in SEQ ID NO: 44. The complete ZmABT expression cassette was mobilized as a SanDI/RsrII fragment into the RsrII site of the *Agrobacterium* binary vector 15289. A plasmid map of this construct, 15773, is shown in SEQ ID NO: 45.

Example 4: Extension of DNA Probe Sequences to Designed Expression Cassettes

DNA sequence representing probes on the maize chip can easily be extended to designed expression cassettes following the steps outlined above. The DNA sequence for probes identified as representing genes that are highly expressed in all tissue samples and not expressed in pollen (Table A) and those that are highly expressed in all tissue samples and have reduced expression in tassel samples (Table E) is reported as SEQ ID NOs: 47-79.

An additional probe candidate from the expression profiling analysis for each expression category was selected to demonstrate progression from this DNA sequence to a finished binary vector with the designed expression cassette linked to the GUS reporter gene. The method used is identical to that for ZmABP3 and ZmABT. In summary the process steps to be applied are as follows:

1. Flank each expression cassette with SanDI/RsrII sites and report as cloned into the RsrII site of 15289 (SEQ ID NO: 80).
2. Promoter consists of 1000-1500 bp of sequence upstream of the transcription start site and extends 10 bases into the second exon, or to the natural translation start codon if it is not on the first exon. It terminates with the maize optimized Kozak sequence 'gtaaaccatgg'. The engineered translation start codon is now embedded in the NcoI restriction endonuclease site 'ccatgg'. Mutate all translation start codons in the theoretical transcript that are upstream of the engineered NcoI site. Ensure at least one stop codon is in each reading frame upstream of the engineered NcoI site.

The promoter is designed to be flanked by XhoI/SanDI at the 5'-end and NcoI at the 3'-end.

3. The Gene Of Interest (GOI) is represented by the GUS reporter gene as an NcoI/SacI fragment.

4. The terminus extends from just after the translation stop codon for 1 kb downstream. The terminus is designed to be flanked by SacI at the 5'-end and RsrII/XmaI at the 3'-end.

5. The complete expression cassette is designed to be mobilized as a SanDI/RsrII fragment, which can be ligated into an RsrII site located on an *Agrobacterium* binary vector such as 15289 (SEQ ID NO: 80).

6. Mutate all internal SanDI, RsrII, NcoI, SacI, XhoI and XmaI sites by single base subsitution to silence them.

Through application of these basic steps a plant expression cassette (SEQ ID NO: 81) can be designed that corresponds to probe Zm058948_s_at (SEQ ID NO: 55) and a plant expression cassette (SEQ ID NO: 82) that corresponds to probe Zm002990_s_at (SEQ ID NO: 62). The former is an expression cassette that should be transcribed in all maize tissues and not in pollen. The latter is an expression cassette that should be transcribed in all maize tissues and have reduced transcription in tassels. This design strategy applies to all probes identified in Tables A and E.

Further details of how to make such expression cassettes are described in US2005235311, which is incorporated herein by reference in its entirety.

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WO 95/16783

Claims

1. A transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct comprising at least one protein encoding polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function directing expression of said polynucleotide of interest to most plant tissues, but essentially excluding the tissues of the reproductive plant structures.

2. A transgenic plant according to claim 1, wherein expression of said polynucleotide of interest is directed to the tissues target insects normally feed on.

3. A transgenic plant according to claim 1, wherein said tissues of the reproductive plant structures are the tissues of the pollen and/or the tassel.

4. .A transgenic plant according to claim 1, wherein the protein encoding polynucleotide of interest is not or substantially not transcribed in the tissues of the pollen and/or the tassel so that no expression product is present in said tissues to any significant extent.

5. A transgenic plant according to claim 1, wherein the chimeric DNA construct comprises a protein encoding polynucleotide of interest operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function obtainable from a gene present in the maize genome.

6. A transgenic plant according to claim 1, wherein the chimeric DNA construct comprises a protein encoding polynucleotide of interest operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function obtainable from a gene that is expressed in all tissue samples and not or substantially not expressed in the

tissues of the pollen and/or the tassel, which gene is represented by a proble molecule, particularly a DNA probe molecule exhibiting a DNA sequence as depicted in SEQ ID NOs: 47 to 79.

7. A transgenic plant according to claim 6, wherein the chimeric DNA construct comprises a protein encoding polynucleotide of interest operably associated with and/or under operable control of a regulatory nucleotide sequence, at least part of which has a transcription initiation function obtainable from a gene encoding an actin depolymerizing factor 3 (ABP3), which is expressed in most tissues of the plant, but not or substantially not in the tissues of the pollen so that no expression product is present in said tissues to any significant extent.

8. A transgenic plant according to claim 7, wherein said actin depolymerizing factor 3 (ABP3) gene is from maize.

9. A transgenic plant according to claim 8, wherein the nucleotide sequence providing the transcription initiation function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the pollen so that no or substantially no expression product is present in said tissues to any significant extent.

10. A transgenic plant according to claim 8, wherein the complementary strand of the nucleotide sequence providing the transcription initiation function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 13 and wherein said regulatory sequence controls transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the pollen.

11. A transgenic plant according to claim 8, wherein the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 13.

12. A transgenic plant according to claim 1, wherein said polynucleotide of interest further provides a transcription termination function, wherein the sequence providing said function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 14.

13. A transgenic plant according to claim 1, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the complementary strand of the nucleotide sequence providing said function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 14.

14. A transgenic plant according to claim 12, wherein the nucleotide sequence providing the transcription termination function is the sequence depicted in SEQ ID NO: 14.

15. A transgenic plant according to claim 1, wherein the nucleotide sequence providing the transcription initiation function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

16. A transgenic plant according to claim 1, wherein the complementary strand of the nucleotide sequence providing the transcription initiation function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 35 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but essentially excluding the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

17. A transgenic plant according to claim 15, wherein the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 35.

18. A transgenic plant according to claim 1, wherein said polynucleotide of interest further provides a transcription termination function, wherein the sequence providing said function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 36.

19. A transgenic plant according to claim 1, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the complementary strand of the nucleotide sequence providing said function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 36.

20. A transgenic plant according to claim 18, wherein the regulatory nucleotide sequence providing the transcription termination function is the sequence depicted in SEQ ID NO: 36.

21. A transgenic plant according to claims 1, wherein said protein encoding polynucleotide of interest is a polypeptide or protein encoding nucleotide sequence.

22. A transgenic plant according to claim 21, wherein polynucleotide of interest encodes a polypeptide product exhibiting an insecticidal activity.

23. A transgenic plant according to claim 22, wherein the gene of interest encodes an endotoxin of *Bacillus thuringiensis*.

24. A transgenic plant according to claim 22, wherein the concentration of the polypeptide product expressed from the protein encoding polynucleotide of interest in plant reproductive structures, particularly in the tissues of the pollen and/or the tassel, is such that no insecticidal activity can be detected in a standard insect feeding assay and also no toxic effects towards the plant reproductive structures, particularly the tissues of the pollen and/or the tassel.

25. A transgenic pant according to claim 24, wherein the polypeptide concentration in the tissues of the plant reproductive structures, particularly in the tissues of the pollen and/or the tassel, is below a basic level of 10 ng/mg soluble protein.

26. A transgenic plant according to claim 1, wherein the gene of interest encodes a polypeptide product contributing to the enhancement of drought tolerance.

27. A transgenic plant according to claim 26, wherein the gene of interest encodes a deregulated form of an H^+-pyrophosphatase.

28. A transgenic plant according to any of the preceding claims, which plant is a *Zea mays* plant.

29. A regulatory nucleotide sequence, at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues, but essentially excluding the tissues of the male reproductive plant structures.

30. A regulatory nucleotide sequence according to claim 29, wherein the tissues of the male reproductive plant structures are the tissues of the pollen and/or the tassel.

31. A regulatory nucleotide sequence according to claim 29, wherein the expression in the tissues of the pollen and/or the tassel is such that no expression product is present in said tissues to any significant extent.

32. A regulatory nucleotide sequence according to claim 29 which nucleotide sequence can be obtained from a gene present in the maize genome.

33. A regulatory nucleotide sequence according to claim 32, which nucleotide sequence is obtainable from a gene that is expressed in all tissue samples and not or substantially not expressed in the tissues of the pollen and/or the tassel, which gene is represented by a probe molecule, particularly a DNA probe molecule exhibiting a DNA sequence as depicted in SEQ ID NOs: 47 to 79.

34. A regulatory nucleotide sequence according to claim 33, which nucleotide sequence is obtainable from a gene encoding an actin depolymerizing factor 3, which is expressed in most tissues of the plant, but essentially excluding the tissues of the pollen so that no or substantially no expression occurs in the tissues of the pollen and no or substantially no expression product is present in said tissues to any significant extent.

35. A regulatory nucleotide sequence according to claim 34 obtainable from a genomic *Zea mays* DNA template using a forward primer of SEQ ID NO: 1 and a reverse primer of SEQ ID NO: 2 in a PCR reaction.

36. A regulatory nucleotide sequence according to claim 35 which is modified using one or more oligonucleotides selected from the group of oligonucleotides depicted in SEQ ID NO:3 , SEQ ID NO:4 , SEQ ID NO:5 , SEQ ID NO:6 , SEQ ID NO:7 and SEQ ID NO:8.

37. A regulatory nucleotide sequence according to claim 34, wherein the nucleotide sequence providing the transcription initiation function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 13 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the pollen so that no or substantially no expression product is present in said tissues to any significant extent..

38. A regulatory nucleotide sequence according to claim 34, wherein the complementary strand of the nucleotide sequence providing the transcription initiation function hybridizes to a nucleotide sequence depicted in SEQ ID NO:13 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the pollen so that no or substantially no expression product is present in said tissues to any significant extent..

39. A regulatory nucleotide sequence according to claim 38, wherein hybridization occurs under stringent hybridization conditions.

40. A regulatory nucleotide sequence according to claim 37, wherein the nucleotide sequence providing the transcription initiation function is the nucleotide sequence depicted in SEQ ID NO: 13.

41. A regulatory nucleotide sequence according to claim 34, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the sequence providing said function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 14.

42. A regulatory nucleotide sequence according to claim 34, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the complementary strand of the nucleotide sequence providing said function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 14.

43. A regulatory nucleotide sequence according to claim 41, wherein the nucleotide sequence providing the transcription termination function is the sequence depicted in SEQ ID NO: 14.

44. A regulatory nucleotide sequence according to claim 43 which comprises a transcriptional terminator and poly-adenylation signal.

45. A regulatory nucleotide sequence according to claim 29, at least part of which has a transcription initiation function which mediates expression of an operably associated protein encoding polynucleotide of interest in most plant tissues but essentially excludes the tissues of the tassel so that no or substantially no expression occurs in the tissues of the tassel and no or substantially no expression product is present in said tissues to any significant extent..

46. A regulatory nucleotide sequence according to claim 32 obtainable from a genomic *Zea mays* DNA template using a forward primer of SEQ ID NO: 19 and a reverse primer of SEQ ID NO: 20 in a PCR reaction.

47. A regulatory nucleotide sequence according to claim 46, which is modified using one or more oligonucleotides selected from the group of oligonucleotides depicted in SEQ ID NO:21 , SEQ ID NO:22 , SEQ ID NO:23 , SEQ ID NO:24 , SEQ ID NO:25 and SEQ ID NO:26.

48. A regulatory nucleotide sequence according to claim 45, wherein the nucleotide sequence providing the transcription initiation function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 35 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially

not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent.

49. A regulatory nucleotide sequence according to claim 45, wherein the complementary strand of the nucleotide sequence providing the transcription initiation function hybridizes to a nucleotide sequence depicted in SEQ ID NO:35 and wherein said regulatory sequence mediates transcription of an operably associated protein encoding polynucleotide of interest such that said associated polynucleotide is transcribed in most tissues of the plant but not or substantially not in the tissues of the tassel so that no expression product is present in said tissues to any significant extent..

50. A regulatory nucleotide sequence according to claim 49, wherein hybridization occurs under stringent hybridization conditions.

51. A regulatory nucleotide sequence according to claim 48, wherein the nucleotide sequence providing the transcription initiation function is the sequence depicted in SEQ ID NO: 35.

52. A regulatory nucleotide sequence according to claim 45, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the sequence providing said function has at least 80% sequence identity with a nucleotide sequence depicted in SEQ ID NO: 36.

53. A regulatory nucleotide sequence according to claim 45, wherein said regulatory nucleotide sequence further provides a transcription termination function and wherein the complementary strand of the nucleotide sequence providing said function is capable of hybridizing with a nucleotide sequence depicted in SEQ ID NO: 36.

54. A regulatory nucleotide sequence according to claim 52, wherein the nucleotide sequence providing the transcription termination function is the sequence depicted in SEQ ID NO: 36.

55. A transgenic plant according to claim 1, wherein said polynucleotide of interest is operably associated with a regulatory nucleotide sequence according to any

of claims 29 to 40 and 45 to 51 that comprises a nucleotide sequence providing a transcription initiation function.

56. A transgenic plant according to claim 55, wherein said polynucleotide of interest is operably associated with a regulatory nucleotide sequence according to claims 41 to 44 and 52 to 54 that further comprises a nucleotide sequence providing a transcription termination function.

57. An expression cassette comprising a regulatory nucleotide sequence according to any of claims 29 to 40 and 45 to 51 that comprises a nucleotide sequence providing a transcription initiation function.

58. An expression cassette according to claim 57, comprising a regulatory nucleotide sequence according to any of claims 41 to 44 and 52 to 54 that further comprises a nucleotide sequence providing a transcription termination function.

59. A vector molecule comprising an expression cassette according to claim 57.

60. A transgenic plant comprising an expression cassette of claim 57.

61. A transgenic plant comprising a vector molecule of claim 59.

62. A method of producing a transgenic plant expressing a DNA sequence of interest in non-pollen tissue but not or substantially not in pollen comprising

a) transforming an expression cassette according to claim 57 or a vector molecule of claim 59 into a plant cell or plant tissue; and

b) regenerating the plant cell transformed in step a) into a plant.

63. A method according to claim 62, wherein an expression cassette is transformed into a whole plant.

64. A method of controlling insect target-pests feeding on vegetative plant tissues such as the leaf, stalk and root and/or on reproductive tissues such as the ear, but protecting non-target pests feeding on pollen comprising

a) growing a plant according to any of the preceding claims in an area that is infested with the target pest;

b) expressing a polypeptide or protein that is capable of controlling said target pest under the control of a regulatory sequence according to any of the preceding claims.

65. A method of protecting the reproductive tissues of a plant, particularly the tissues of the pollen and/or the tassel against damage caused by expression in said tissues of a polypeptide or protein of interest comprising

a) growing a plant according to any of the preceding claims; and

b) expressing in said plant a polypeptide or protein of interest under the control of a regulatory sequence according to any of the preceding claims.

INTERNATIONAL SEARCH REPORT

PCT/US 09/47873

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01H 1/00; A01H 9/00 (2009.01)
USPC - 800/278; 800/295
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)-A01H 1/00; A01H 9/00 (2009.01)
USPC-800/278; 800/295

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 536/23.1, 24.2; 800/289

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar: pollen, silk, tassel, tisue specific, expression, syngenta, thurigiensis, bt toxin, endotoxin, corn, maize, Zea mays, pyrophosphatase, actin depolymerizing factor, abp3, regulatory nucleotide sequence, pollinator, bee, honey bee, drought tolerance, recombinant. GenCore 6.3: SEQ ID N

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 6,320,100 B1 (KOZIEL et al.) 20 November 2001 (20.11.2001) col 4 ln 1-10; col 13 ln 45-60; col 15 ln 45-67; col 17 ln 5-20; col 53 ln 45-60	1-5, 21-25, 28-32, 45, 57, 59-61 ---------- 6-14, 26-27, 33-34, and 37-44
Y	US 2004/0214272 A1 (LA Rosa et al.) 28 October 2004 (28.10.2004) para [0007]; SEQ ID NO: 179676	6-10, 33-34, 37-39, 41-42
Y	LOPEZ et al. Pollen specific expression of maize genes encoding actin depolymerizing factor-like proteins. Proc. Natl. Acad. Sci. USA July 1996, 93:7415-7420; pg 7415 col 2 para 2	7-10, 34, 37-39, 41-42
Y	WILSON. Genbank ACC# AC211477.2. 29 September 2007 (29.09.2007). [Retrieved from the Internet 06 Sep 2009: <http://www.ncbi.nlm.nih.gov/nuccore/166158565>])	9-10,12-13, 37-39, 41-42
Y	PARK et al. Up-regulation of a H+-pyrophosphatase(H+-PPase) as a strategy to engineer drought-resistant crop plants. PNAS 27 December 2005, 102(52):18830-18835; abstract	26-27

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2009 (16.11.2009)	04 DEC 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

PCT/US 09/47873

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore 6.3: SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:47

INTERNATIONAL SEARCH REPORT

PCT/US 09/47873

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 55-56, 58, 62-65
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-14, 21-34, 37- 45, 57, 59-61, drawn to a transcription initiation nucleotide sequence at least 80% identical to SEQ ID NO:13, and transcription termination nucleotide sequence at least 80% identical to SEQ ID NO:14, and a transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct comprising at least one polynucleotide of interest, operably associated with and/or under operable control of said transcription initiation nucleotide sequence, wherein said polynucleotide of interest is not expressed in the tissues of the reproductive plant structures, limited to a probe of SEQ ID NO:47.

********** Please See Supplemental Sheet **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14, 21-34, 37- 45, 57, 59-61, restricted to SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:47

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

********** Supplemental Box **********

Continuation of Box No. III. Lack of Unity:

Group II+: claims 1-6, 15-36, 45-54, 57, 59-61, drawn to a transcription initiation nucleotide sequence and transcription termination nucleotide sequence, and a transgenic plant comprising said sequences and at least one polynucleotide of interest, wherein a protein encoded by said polynucleotide of interest is essentially excluded from the tissues of thereproductive plant structures. Should an additional fee(s) be paid, Applicant is invited to elect specific transcription initiation and transcription termination nucleotide sequences, and a probe to be searched. The exact claims searched will depend on the specifically SEQ ID NOs.

The inventions listed as Groups I-II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I and II+ share a technical feature of a transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct comprising at least one polynucleotide of interest, operably associated with and/or under operable control of a regulatory nucleotide sequence, such that said polynucleotide of interest is not expressed in the tissues of the reproductive plant structures. However, this shared technical feature is obvious over an article entitled "Pollen specific expression of maize genes encoding actin depolymerizing factor-like proteins" by LOPEZ, et al. (Proc. Natl. Acad. Sci. USA Jul 1996, 93:7415-7420) that discloses "the differential expression of the three ZmABP genes" (pg 7418, Fig 3, legend) and that ZmABP3 is not expressed in pollen" (pg 7418, Fig 3, and col 1, "ZmABP3 is only suppressed in pollen"). It would have been obvious to one of ordinary skill in the art how to create a transgenic plant comprising stably integrated in its genome a chimeric polynucleotide construct, by cloning the ZmABP3 and its regulatory sequences, and substituting said gene by a gene of interest.

As to the claimed regulatory nucleotide sequences that flank the ZmABP3 gene, i.e. the claimed SEQ ID NO: 13 and 14, they both we known at the time of the invention: Direct Submission to GenBank AC211477 (7-JUN-2008. [Retrieved from the Internet 06 Sep 2009: <http://www.ncbi.nlm.nih.gov/nuccore/166158565>]) discloses 192055 bp long DNA discloses a polynucleotide comprising a fragment that is 98.7% identical to the claimed transcription initiation nucleotide sequence of SEQ ID NO: 13 (nucleotides 36196-38514), and also discloses a polynucleotide comprising a fragment that is 97.9 % identical to the claimed transcription termination nucleotide sequence of SEQ ID NO: 14 (nucleotides 3901540025). While there is no prior art on record disclosing that said nucleotide sequences are expression regulatory sequences, one of ordinary skill in the art would have been able to locate regulatory sequence for the ZmABP3 gene via routine experimentation.

Finally and with regard to the claimed probe of SEQ ID NO: 47, US 2004/0214272 A1 to La Rosa et al. discloses a polynucleotide that is 100% identical to SEQ ID NO: 47 (SEQ ID NO:17676, nucleotides 1088-1526). As the above nucleotide sequences were known at the time of the invention, and said transgenic plant comprising said sequences was obvious at the time of the invention, neither the sequences or the plant cannot be considered a special technical feature that would otherwise unify the groups.

Groups I and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.