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(54) **Title:** PLANTS WITH INCREASED TOLERANCE TO WATER DEFICIT

(57) **Abstract:** The invention relates to a method for increasing the tolerance of a plant to water deficit, by overexpression in said plant of a protein, designated ABA4, which is involved in the conversion of violaxanthin to neoxanthin in the biosynthesis of abscisic acid.

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PLANTS WITH INCREASED TOLERANCE TO WATER DEFICIT.

The present invention relates to the identification of proteins useful for improving tolerance to water deficit in plants, to nucleic acid sequences encoding said proteins, and to their use for increasing the tolerance of plants to water deficit.

"Water deficit" relates to a situation where a plant has insufficient water for optimal functioning of its physiological processes.

Water deficit is one of the most important abiotic stresses affecting plants, and is one of the main factors responsible for yield loss in crops. Water deficit can severely affect plant yield, growth and reproduction. Therefore, it is important to identify genes that have the capacity to improve plant tolerance to water deficit.

The plant hormone abscisic acid (ABA) is renowned for being a stress hormone. Its levels rise in response to abiotic stress, in particular upon water deficit, and it has been shown to be necessary for the induction of a variety of adaptive responses from stomatal closure to osmotic adjustment (NAMBARA and MARION-POLL, Annu Rev Plant Biol, 56, 165-85, 2005).

ABA is a sesquiterpenoid (C_{15}) that is synthesized in plants via an indirect pathway using carotenoid precursors. In plants carotenoids are essential components of the photosynthetic apparatus and are the red, orange and yellow pigments found in many flowers and fruit. Early ABA biosynthesis reactions occur, therefore, in plastids. The direct precursors of ABA are the xanthophylls zeaxanthin, violaxanthin and neoxanthin with the first ABA biosynthesis specific reaction being the oxidative cleavage of 9-*cis*-epoxycarotenoids (Figure 1). The cleavage product xanthoxin is then transited to the cytoplasm where it undergoes conversion to abscisic aldehyde followed by oxidation to yield ABA. The biosynthetic pathway of ABA is schematized on Figure 1.

Genes encoding the enzymes for most of the steps of the ABA biosynthesis pathway have been cloned or their

function confirmed using ABA-deficient mutants. The zeaxanthin epoxidase (*ZEP*) gene was first cloned from a *Nicotiana plumbaginifolia* insertion mutant and is defective in the Arabidopsis *aba1* mutant (MARIN et al., Embo J, 15, 2331-42, 1996). The ZEP enzyme catalyses the conversion of zeaxanthin to violaxanthin via antheraxanthin in two successive epoxidation reactions (Figure 1). The maize *VP14* mutant is mutated in a 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) gene required for oxidative cleavage (SCHWARTZ et al., Science, 276, 1872-4, 1997; TAN et al., Proc Natl Acad Sci U S A, 94, 12235-40, 1997) and *in vitro* activity assays suggested that either violaxanthin or neoxanthin *cis*-isomers could be substrates. A short chain dehydrogenase/reductase (SDR) catalyses the oxidation of xanthoxin to abscisic aldehyde (Figure 1) and was identified using Arabidopsis *aba2* mutants (CHENG et al., Plant Cell, 14, 2723-43, 2002; GONZALEZ-GUZMAN et al., Plant Cell, 14, 1833-46, 2002). Defects in two genes affect the last step of ABA biosynthesis (Figure 1) one encoding the abscisic aldehyde oxidase apoprotein and the second an enzyme required for the sulfuration of the molybdenum cofactor. The *aba3* mutant was shown to have a lesion in a gene encoding a molybdenum cofactor sulfurase (SCHWARTZ et al., Plant Physiol, 114, 161-6, 1997), and the corresponding gene was identified by positional cloning (BITTNER et al., J. Biol. Chem. 276, 40381-4, 2001; XIONG et al. , Plant Cell 13, 2063-83, 2001), whereas the role of an aldehyde oxidase in the last step of ABA biosynthesis was confirmed, thanks to the *Arabidopsis aldehyde oxidase3* (*aao3*) mutant (SEO et al., Proc Natl Acad Sci U S A, 97, 12908-13, 2000).

Most of the Arabidopsis ABA-deficient mutants identified to date have been demonstrated to present germination that is resistant to paclobutrazol, or equivalent gibberellic acid (GA) biosynthesis inhibitors. This phenotype is related to the antagonistic effects of ABA and GA on germination making the balance between the levels of the two hormones determinant as to whether a seed germinates or not. In an ABA-deficient mutant the GA requirement for germination

is thus less than in a wild-type seed and paclobutrazol has been used effectively as a screening method to identify mutants affected in ABA biosynthesis or signalling (JACOBSEN and OLSZEWSKI, Plant Cell, 5, 887-96, 1993; LEON-KLOOSTERZIEL et al., Plant J, 10, 655-61, 1996; NAMBARA et al., Plant Cell Physiol, 39, 853-8, 1998). In accordance with the role of ABA in responses to water deficit, ABA-deficient mutants also often show phenotypes related to defects in stomatal closure; increased water loss and a wilty phenotype on water deficit (SEO et al., Proc Natl Acad Sci U S A, 97, 12908-13, 2000; KOORNNEEF et al., Theor. Appl. Genet. 61, 385-93, 1982; LEON-KLOOSTERZIEL et al., Plant Physiol, 110, 233-40, 1996). It has been shown that overexpression of Arabidopsis *NCED* genes, *AtNCED3* and *AtNCED6*, increases endogenous ABA levels, and improves tolerance to water deficit (IUCHI et al., Plant J, 27, 325-33, 2001; LEFEBVRE et al., Plant J, 45, 309-319, 2006).

The inventors have now identified in *Arabidopsis thaliana* a protein involved in the conversion of violaxanthin to neoxanthin, which is hereinafter referred to as ABA4. Mutants of the *ABA4* gene wherein the ABA4 protein is no longer functional are affected in ABA biosynthesis and in response to water deficit.

The inventors have further found orthologs of *ABA4* from *Arabidopsis thaliana* in other plants such as maize, wheat and rice.

The nucleotide sequence of an *ABA4* gene of *Arabidopsis thaliana* (*AtABA4*) is available under accession number At1g67080 in the TAIR Arabidopsis database. It is reproduced herein as SEQ ID NO: 1, and the corresponding polypeptide sequence is indicated as SEQ ID NO: 2.

Nucleotide sequences of two *ABA4* genes from maize (*ZmABA4a* and *ZmABA4b*) are indicated herein respectively as SEQ ID NO: 3 (*ZmABA4a*) and SEQ ID NO: 5 (*ZmABA4b*), and the corresponding polypeptide sequences are respectively indicated as SEQ ID NO: 4 (ZmABA4a) and SEQ ID NO: 6 (ZmABA4b).

The nucleotide sequence of an *ABA4* gene from rice (*OsABA4*) is indicated herein as SEQ ID NO: 7 and the

corresponding polypeptide sequence is indicated as SEQ ID NO: 8.

The nucleotide sequence encoding an ABA4 protein from wheat (*TaABA4*) is indicated herein as SEQ ID NO: 9 and the corresponding polypeptide sequence is indicated as SEQ ID NO: 10.

AtABA4 from *Arabidopsis thaliana* is predicted to be 220 amino acids in length with the first 68 amino acids presenting the features of a chloroplast signal peptide as predicted by the Chloro P programme (EMANELSSON et al., Protein Sci. 8, 978-84, 1999); rich in hydroxylated amino acids, few acidic amino acids and an alanine residue at position 69 in agreement with the chloroplast transit peptide cleavage-site motif (KEEGSTRA et al., Annu. Rev. Plant Physiol. Mol. Biol. 40, 471-501, 1989; GAVEL and von HEIJNE, FEBS Lett. 261, 455-458, 1990). The mature ABA4 protein has a predicted molecular mass of 17.0 kDa and a theoretical pI of 7.91.

ZmABA4a from maize is predicted to be 235 amino acids in length with the first 82 amino acids presenting the features of a chloroplast signal peptide as predicted by the Chloro P programme (cited above). This maize mature ABA4 protein has a predicted molecular mass of 16.7 kDa and a theoretical pI of 8.53.

ZmABA4b from maize is predicted to be 232 amino acids in length with the first 79 amino acids presenting the features of a chloroplast signal peptide as predicted by the Chloro P programme. This mature ZmABA4b protein has a predicted molecular mass of 16.7 kDa and a theoretical pI of 8.54.

OsABA4 from rice is predicted to be 228 amino acids in length with the first 75 amino acids presenting the features of a chloroplast signal peptide as predicted by the Chloro P programme. This mature OsABA4 protein has a predicted molecular mass of 16.2 kDa and a theoretical pI of 8.03.

TaABA4 from wheat is predicted to be 227 amino acids in length with the first 74 amino acids presenting the features of a chloroplast signal peptide as predicted by the

Chloro P programme. This mature TaABA4b protein has a predicted molecular mass of 16.8 kDa and a theoretical pI of 9.01.

Hydropathy analyses of the ABA4 proteins of *Arabidopsis*, maize, rice and wheat indicate the presence of 4 helical transmembrane domains, but no domains of known function were identified.

The present invention provides a method for increasing the tolerance of a plant to water deficit, wherein said method comprises overexpressing in said plant an ABA4 polypeptide comprising the following regions:

a) a chloroplast signal peptide;

b) a region comprising a sequence having at least 60%, and preferably at least 65%, and by order of increasing preference, at least 70%, 75%, 80%, 85%, 90%, 95% ,98 ,or 99% identity with the region 69-220 of the polypeptide SEQ ID NO: 2, or with the region 83-235 of the polypeptide SEQ ID NO: 4, or with the region 80-232 of the polypeptide SEQ ID NO: 6 or with the region 76-228 of the polypeptide SEQ ID NO: 8, with the region 75-227 of the polypeptide SEQ ID NO: 10.

Unless otherwise specified, the sequence identity values provided herein are calculated using the algorithm of NEEDLEMAN and WUNSCH (J Mol Biol. 48, 443-53, 1970) under default parameters.

According to a preferred embodiment of the invention, region b) of said polypeptide comprises:

i) four domains defined by the following sequences (one letter code):

- htm 1 :$ASX_1X_2Fx_3X_4gtX_5AVLPFYTLMX_6X_2A$ (SEQ ID NO: 11),
- htm 2 :$X_7x_2PYX_6x_8LGX_9LYX_2YLLYx_{10}SW$ (SEQ ID NO: 12),
- htm 3 :$MTX_{11}ASAWIHLLX_2VDLFAA$ (SEQ ID NO: 13),
- htm 4 :$SVSLCLLFCPX_6GIX_{12}X_{13}HX_{14}$ (SEQ ID NO: 14),

wherein X_1 to X_{14} represent a non-hydrophilic amino acid (i.e any amino acid except R, K, N, Q, or E), and preferably, X_1 = S or C, X_2 = V or A, X_3 = A or T, X_4 = V, L or W, X_5 = T, V or I, X_6 = V or I, X_7 = S,G or T, X_8 = I or A, X_9 = V, L or I, X_{10} = I or L, X_{11} = L or V, X_{12} = V, L or A, X_{13} = A,

T, or S, X_{14} =V, A or F; these domains represent predicted transmembrane domains;

ii) a block of five amino acids $SKYX_{15}L$ (SEQ ID NO: 15), wherein X_{15} = M or W, between domains htm2 and htm3;

iii) a protein kinase C motif (Prosite PDOC00005) defined by the sequence TKX_{16} wherein X_{16} = R or K, between domains htm1 and htm2.

Advantageously, region b) comprises the following sequence:

$QIASX_1X_2FX_3X_4GTX_5AVLPFYTLMX_6X_2APX_{17}AX_{18}X_6TKX_{16}X_{19}X_{20}X_{21}SX_7X_2PYX_6X_8LGX_9L$
$YX_2YLLYX_{10}SWTPX_{21}TX_{10}X_{16}X_{22}MFX_{23}SKYX_{15}LPELX_{24}GIX_2X_{16}MFX_{23}SEMTX_{11}ASAWIH$
$LLX_2VDLFAARQVYX_{25}DGX_{10}X_{26}NX_{27}X_6ETRHSVSLCLLFCPX_6GIX_{12}X_{13}HX_{14}X_{11}TK$
(SEQ ID NO: 16)

wherein X_1 to X_{16} are as defined above and X_{17} = K or N, X_{18} = E, D, or S, X_{19} = C, T, or A X_{20} = M or V, X_{21} = E or D, X_{22} = A or Y, X_{23} = A or S, X_{24} = S, P, A, or T, X_{25} = H, N, or Q, X_{26} = K, R, or E, X_{27} = Q or N.

The chloroplast signal peptide a) can be a signal peptide of an ABA4 polypeptide. For instance, it can be selected among:

- a peptide having the following sequence:
MGFSSFISQPLSSSLSVMKRNVSAKRSELCLDSSKIRLDHRWSFIGGSRISVQSNSYTVVHK
KFSGVR (SEQ ID NO: 17)

- a peptide having the following sequence:
MAPCASPSALALSASTRVSSFPLTLRPRPRPEARVPRAPGGAQLRPATACSWPRPLLPELAP
AFPRAGARSAGRPQPLFRPR (SEQ ID NO: 18)

- a peptide having the following sequence:
MAPCASPSALALSASTRVSILRLPLALRQRAEARVPGAQFRPSTACSWARPLLPELAGAVPR
AGARGTGRRTQPLFRPR (SEQ ID NO: 19)

- a peptide having the following sequence:
MAALLLLSSAARVGVAAPLALRQQRPVVLPGGQLRTGSGAGAASAWAARPLRPELAAVSRPA
VPARGRAPLFRPR (SEQ ID NO: 20)

- a peptide having the following sequence:
MAASSPSALALSPSTRVVAGPSLLLAVKRTPATRVAAAPSGQLPACSWGPLRPELAPAPGPC
AARCRAPLLRPR (SEQ ID NO: 21)

The chloroplast signal peptide a) can also be any transit peptide for a polypeptide localized in the chloroplast such as those identified in proteomic analyses (FERRO et al.

Mol. Cell. Proteomics 2, 325-345, 2003), or those disclosed in PCT WO2004/001050.

Preferably, said ABA4 polypeptide is selected among AtABA4 (SEQ ID NO: 2), ZmABA4a (SEQ ID NO: 4), ZmABA4b (SEQ ID NO: 6), OsABA4 (SEQ ID NO: 8) and TaABA4 (SEQ ID NO: 10). Other ABA4 polypeptides suitable for carrying out the invention include in particular those encoded by genes that are orthologs of *AtABA4, ZmABA4a, ZmABA4b, OsABA4 or TaABA4*. They can be identified for instance by screening plant EST databases to select candidate ESTs encoding polypeptides having the above-defined percent of identity with SEQ ID NO: 2, 4, 6, 8 or 10 or by screening libraries of plant cDNAs with degenerate nucleic acid probes which can be derived from any of SEQ ID NO: 2, 4, 6, 8 or 10, or from regions which are conserved between these sequences. The selected ESTs, or the selected cDNAs can then be checked for the presence of the sequences encoding the 4 transmembrane domains and the protein kinase C motif defined above and the conserved sequence SEQ ID NO: 15.

The term "plant" as used herein, include dicotyledons as well as monocotyledons, and in particular those of agronomical interest, as crop plants (for instance : rice, maize, wheat, barley, rapeseed, soybean, peas, sunflower, etc...) as fruit, vegetables or ornamental plants (for instance solanaceaous or rosaceous plant).

"Overexpressing" a polypeptide refers either to artificially expressing said polypeptide in plants which do not naturally express it, or to artificially increasing its expression (for instance by adding at least one additional copy of a sequence encoding said polypeptide) in plants which naturally express it.

The invention also provides means for carrying out said overexpression.

This includes in particular recombinant DNA constructs for expressing an ABA4 polypeptide in a host-cell, or a host organism, in particular a plant cell or a plant. These DNA constructs can be obtained and introduced into said

host cell or organism by well-known techniques of recombinant DNA and genetic engineering.

Recombinant DNA constructs of the invention include in particular expression cassettes, comprising a polynucleotide encoding an ABA4 polypeptide as defined above, under the control of an appropriate promoter.

Said promoter can be any promoter functional in a plant cell. The choice of the more appropriate promoter may depend in particular on the chosen host plant, on the organ(s) or tissue(s) targeted for expression, and on the type of expression (i.e. constitutive or inducible) that one wishes to obtain.

A large choice of promoters suitable for expression of heterologous genes in plants is available in the art. They can be obtained for instance from plants, plant viruses, or bacteria such as *Agrobacterium*. They include constitutive promoters, i.e. promoters which are active in most tissues and cells and under most environmental conditions, tissue or cell specific promoters which are active only or mainly in certain tissues or certain cell types, and inducible promoters that are activated by physical or chemical stimuli, such as those resulting from water deficit.

Non-limitative examples of constitutive promoters that are commonly used in plant cells are the cauliflower mosaic virus (CaMV) 35S promoter, the Nos promoter, the rubisco promoter, the Cassava vein Mosaic Virus (CsVMV) promoter, the rice actin promoter, followed by the rice actin intron (RAP-RAI) contained in the plasmid pAct1-F4 (MCELROY et al., Molecular and General Genetics, 231(1), 150-160, 1991).

Non-limitative examples of organ or tissue specific promoters that can be used in the present invention include for instance High Molecular Weight Glutenin (HMWG) promoter which is kernel specific (THOMAS and FLAVELL, Plant Cell., 2, 1171-80, 1990), or the leaf specific pPEPc promoter (JEANNEAU et al, Biochimie, 84, 1127-1135, 2002).

Inducible promoters that can be used in the present invention include stress responsive promoters which can be induced by drought stress. By way of example, one can mention

promoters comprising a dehydration-responsive element (DRE), such as the rd29A promoter (KASUGA et al. Nature Biotech., 17, 287-291, 1999, NARUSAKA et al., Plant J. 34, 137-48, 2003.)

The expression cassette generally also includes a transcriptional terminator, such as the 35S transcriptional terminator. They may also include other regulatory sequences, such as transcription enhancer sequences or introns (for example the FAD2 intron described in WO 2006/003186, or the actin intron (MCELROY et al., 1991 cited above). Among the terminators which can be used in the constructs of the invention, mention may be made in particular of the 3' end of the Agrobacterium tumefaciens nopaline synthase gene (DEPICKER et al., J Mol Appl Genet. 1(4):361-370, 1982). Mention may also be made of the 35S polyA terminator of the cauliflower mosaic virus (CaMV), described by FRANCK et al. (Cell. 21(1):285-94, 1980).

The invention also includes recombinant vectors containing an expression cassette comprising a polynucleotide encoding an ABA4 polypeptide as defined above, under transcriptional control of a suitable promoter. Said expression cassette may be a recombinant expression cassette as defined above, or an expression cassette wherein the polynucleotide encoding an ABA4 polypeptide is under control of its endogenous promoter. Classically, said recombinant vectors also include one or more marker genes, which allow for selection of transformed hosts. As non-limitative examples of marker genes, mention may be made of genes which confers resistance to an antibiotic, for example to hygromycin (HERRERA-ESTRELLA et al., EMBO J. 2(6): 987-995 1983) or resistance to an herbicide such as the sulfonamide asulam (WO 98/49316).

The selection of suitable vectors and the methods for inserting DNA constructs therein are well known to persons of ordinary skill in the art. The choice of the vector depends on the intended host and on the intended method of transformation of said host. A variety of methods for genetic transformation of plant cells or plants are available in the art for many plant species, dicotyledons or monocotyledons. By

way of non-limitative examples, one can mention virus mediated transformation, transformation by microinjection, by electroporation, microprojectile mediated transformation, *Agrobacterium* mediated transformation, and the like.

For instance, in the case of monocotyledons, one can advantageously use the method described by ISHIDA et al. (Nature Biotech. 14, 745-750, 1996).

The invention also provides a host cell comprising a recombinant DNA construct of the invention. Said host cell can be a prokaryotic cell, for instance an Agrobacterium cell, or a eukaryotic cell, for instance a plant cell genetically transformed by a DNA construct of the invention.

The invention also comprises plants genetically transformed by a DNA construct of the invention expressing an ABA4 polypeptide. Preferably, said plants are transgenic plants, wherein said construct is contained in a transgene integrated in the plant genome, so that it is passed onto successive plant generations.

The invention also provides a method for producing a transgenic plant having an increased tolerance to water deficit, when compared to a non-transgenic plant, said method comprising the steps consisting of:

- transforming at least one plant cell with a vector containing an expression cassette expressing an ABA4 polypeptide, as defined above;
- cultivating said transformed plant cell in order to regenerate a plant having in its genome a transgene containing said expression cassette.

The transgenic plants of the present invention includes not only those obtainable by the above-mentioned method, but also the descendents thereof (including hybrid transgenic plants, obtained by crossing at least one transgenic plant of the invention with another plant devoid of the transgene), provided that they comprise in their genome one or more copies of a transgene containing an expression cassette expressing an ABA4 polypeptide.

The expression of the ABA4 polypeptide in said transgenic plants provides them with an increased tolerance to

water deficit, when compared to a plant devoid of said transgene.

The invention also encompasses isolated organs or tissues (such as fruits, seeds, leafs, pollen, flowers, roots, tubers) of transgenic plants of the invention.

The invention also provides a method for obtaining plants that have an increased tolerance to water deficit, due to a mutation in the *ABA4* gene.

Such a mutation can be localized in the coding regions of the gene or in its *cis* regulatory regions. It can be a mutation resulting in an increased level of expression of the ABA4 mRNA, and/or of the ABA4 polypeptide, or in a higher stability of said mRNA or polypeptide, or in an increased activity of the ABA4 polypeptide.

The method of the invention includes inducing random mutations in a plant of interest, for instance through EMS mutagenesis, selecting the mutants which have a mutation in the *ABA4* gene, and selecting among them those that have an increased tolerance to water deficit, resulting from said mutation.

Selection of mutants which have a mutation in the *ABA4* gene can easily be done using methods of high throughput mutagenesis and screening, such as TILLING (Targeting Induced Local Lesions IN Genomes, described by McCALLUM et al, Plant Physiol., 123, 439-442, 2000).

The mutants that have an increased tolerance to water deficit can be identified with various morphological, physiological and/or biochemical assays. By way of example, one can measure water loss rates under conditions of water deficit (for instance through the visualization of leaf temperature by thermal images as described in the examples below), and select the mutants which have a lower water loss rate than the wild type plants. Tolerance to water deficit can also be evaluated by observation of phenotypic characteristics of the plants, such as growth (for instance length and width of the leaves and/or final height of the plant), or yield (number, filling or weight of a predefined number of grains)

under conditions of water deficit compared with normal conditions.

The invention also provides means for identifying and selecting plants which are tolerant to water deficit.

The invention thus provides:

- a method for identifying an allele of an *ABA4* gene associated with a given phenotype of tolerance to water deficit, wherein said method comprises isolating a nucleic acid fragment comprising said *ABA4* gene or a portion thereof from at least one plant expressing said phenotype, and sequencing said fragment.

The invention further provides:

- a method for identifying polymorphisms associated with tolerance to water deficit, in an *ABA4* gene, wherein said method comprises identifying, as described above, at least two different alleles of said *ABA4* gene associated with different phenotypes of tolerance to water deficit, and comparing the sequences of said alleles.

Once a polymorphism has been identified, reagents and kits allowing the routine detection of said polymorphism can be designed. Commonly used reagents are nucleic acid probes, or restriction enzymes, or PCR primers, or combinations thereof. The choice of a reagent or of a combination of reagents depends of the nature of the polymorphism.

Preferred kits and reagents are those comprising a set of primers allowing specific PCR amplification of a DNA segment spanning the polymorphic locus. For microsatellites and insertion/deletion polymorphisms, PCR primers may be sufficient, since the allelic forms of the polymorphism may be differentiated by the size of the amplification product. In the case of single nucleotide polymorphisms (SNP), one will generally also use a restriction enzyme, which allows the differentiation of allelic forms by the presence or size of restriction fragments.

The invention also provides a method for testing a plant for its tolerance to water deficit, wherein said method comprises detecting whether an allele of an *ABA4* gene

associated with a given phenotype of tolerance to water deficit is present in said plant.

For this purpose, it is also possible to use a nucleic acid encoding an ABA4 polypeptide, or a fragment thereof, as a probe or a target for amplification, for selecting plants naturally overexpressing an ABA4 and therefore exhibiting better tolerance to water deficit. Preferably, the amplified fragment has a length of about 500 pb, more preferably, of about 500 to 1000 pb.

By way of example, one can use the following pair of primers: GGCGATTTTATTCACCACTG (SEQ ID NO: 35) and GACCATGAGCGTGTAGAAGG (SEQ ID NO: 36) to amplify a fragment from ZmABA4a, and the following pair of primers: GGCGAGAATTCGCCACTACC (SEQ ID NO:37) and GACCATGAGGGTGTAGAAGG (SEQ ID NO:38) to amplify a fragment from ZmABA4b.

Foregoing and other objects and advantages of the invention will become more apparent from the following detailed description and accompanying drawings. It is to be understood however that this foregoing detailed description is exemplary only and does not restrict the invention.

Figure 1: ABA biosynthesis pathway: ABA is synthesized from C_{40} carotenoid precursors and the pathway shown is from the xanthophyll zeaxanthin onwards. Enzymes responsible for the different reactions are boxed. ZEP, zeaxanthin epoxidase, NSY, neoxanthin synthase, NCED, 9-*cis*-epoxycarotenoid dioxygenase, SDR, short chain dehydrogenase/reductase, AAO, abscisic aldehyde oxidase, MCSU, molybdenum cofactor sulfurase. Question marks indicate steps for which enzymes remain to be identified.

Figure 2: HPLC profile of wild-type and aba4-1 leaf pigments: tN, *trans*-neoxanthin, cN, *cis*-neoxanthin, tV, *trans*-violaxanthin, L, lutein, Chlb, chlorophyll b.

Figure 3: Structure of the *ABA4* gene and putative protein, and characterization of the *aba4* mutants.

(A) Schematic representation of *ABA4* gene structure and the sites of the aba4 mutant insertions. Rectangles represent the positions of open reading frames (B) Effect of *aba4* mutations on *ABA4* gene expression. (C) Alignment of the ABA4 predicted

amino acid sequences from Arabidopsis thaliana (SEQ ID NO:2), Maize ABA4 proteins : sequences ZmABA4a (SEQ ID NO:4) and ZmABA4b (SEQ ID NO:6), Wheat (SEQ ID NO:10), Rice (SEQ ID NO:8) protein corresponding to annotated gene Os01g03750. Completely conserved residues across 3 or more sequences are shaded black and similar residues conserved across three or more sequences are shaded grey. The arrow indicates the ABA4 putative transit peptide cleavage site, * indicates the site of putative protein kinase C phosphorylation and the lines indicate the position of predicted helical transmembrane (htm) domains identified using PHDhtm (ROST et al., Protein Science 7, 1704-1718, 1996). (D) Analysis and comparison of the hydrophobicity profile of the predicted ABA4 mature protein and freshwater eel rhodopsin (Q90215).

Figure 4: Tissue specific expression patterns of the *ABA4* gene.

(A) Quantitative RT-PCR analysis of *ABA4* gene expression in : rL, rosette leaves, cL, cauline leaves; St, inflorescence stem; Fl; flowers; y, young siliques, m, mature siliques. Steady state mRNA levels are presented as a percentage of the constitutive *EF1αA4* abundance. Error bars represent S.E. values (n=4).

Figure 5: Effect of dehydration on leaf ABA contents and ABA4 steady state transcript levels.

(A) Quantitative RT-PCR analysis of *ABA4* gene expression in leaves and roots of wild-type with (t2) or without a 2 hours dehydration stress (t0). Steady state mRNA levels are presented as a percentage of the constitutive *EF1αA4* abundance. Error bars represent S.E. values (n=4). (B, C) ABA levels accumulated in rosettes of 4 week-old plants in response to dehydration for wild-type (Wt) and *aba4-1*, *aba4-2*, *aba4-3*, *aba1-5* and *aba1-13* mutants from either Ws-2 (B) or Col-0 (C) accessions. ABA content was determined before (t0) and after dehydration for 2 hours (t2). Error bars represent S.E. values (n=≥6).

Figure 6: Phenotype of *aba4* mutants on water deficit as compared to *aba1* mutant alleles and wild-type.

(A, B) Rapid dehydration phenotypes of *aba4-1*, *aba4-2*, *aba4-3*, *aba1-5*, *aba1-13* mutants and wild-type (Wt). The rate of water loss was determined for mutant and wild-type rosettes from either (A) Ws-2 or (B) Col-0 accessions. Error bars represent S.E. values (n=4).

Figure 7: Role and regulation of *ABA4* in the post-germination developmental arrest of seedling growth in response to high sucrose concentrations.

(A, B) Seedlings were grown on media containing increasing concentrations of sucrose for 3 weeks. The number of seedlings with first leaves was scored and compared to the total number of seeds sown for wild-type (Wt) and *aba4-1*, *aba4-2*, *aba4-3*, *aba1-5* and *aba1-13* mutants from either Ws-2 (A, C) or Col-0 (B, D) accessions. (C) Quantitative RT-PCR analysis of *ABA4* gene expression in seedlings of wild-type, *aba4-1* or *aba1-13* grown for 3 weeks on media containing 30 mM, 200 mM or 300 mM sucrose. Steady state mRNA levels are presented as a percentage of the constitutive *EF1αA4* abundance. Error bars represent S.E. values (n=3).

Figure 8: Seed phenotypes associated with ABA4 deficiency.

(A, B) Paclobutrazol resistance of germinating seeds and (C) germination of freshly harvested seeds. The number of seedlings with green cotyledons (A, B) or seeds with protruding radicle (C) was scored and compared to the total number of seeds sown for wild-type (Wt) and *aba4-1*, *aba4-2*, *aba4-3*, *aba1-5* and *aba1-13* mutants from either Ws-2 (A, C) or Col-0 (B) accessions. Error bars represent S.E. values (n=3).

Figure 9: Effect of water deficit on transgenic plants expressing the *ABA4* gene under the control of the 35S promoter. Rapid dehydration of homozogous transgenic plants, selected for hygromycin resistance from the progeny of independent primary transformants in Ws-2 (A) and Col-0 (B) accessions. Error bars represent S.E values (n=4)

Figure 10: Schematic representation of the vector pBIOS1290.

Figure 11: Carotenoids content of transgenic maize lines overexpressing ABA4.

Figure 11a represents the total carotenoid content; Figure 11b represents the t-Violaxanthin content, Figure 11c represents

the c-Neoxanthin content; Figure 11d represents the c-Violaxanthin content.

HPLC Analysis was performed on 14 transgenic lines (# 1-14). For each transgenic line, one to five plants were tested; the number of copies of the construct for each tested plant is indicated above the number of the transgenic line. The graph (-♦-) represents the mean of three independent HPLC measures (A, B and C).

Figure 12: Loss of water under dessication conditions in transgenic T: -♦-) maize lines overexpressing ABA4 compared to non-transgenic (NT: -■-) plants. Each point represents the mean of three plants.

EXAMPLE 1: IDENTIFICATION OF THE *aba4-1* MUTANT

The *aba4-1* mutant was isolated from the Institut National de la Recherche Agronomique Versailles T-DNA collection (BECHTOLD et al., C.R. Acad.Sci., 316, 1194-1199, 1993) in the Ws-2 background. The plants were screened for their ability to germinate and develop on the GA biosynthesis inhibitor paclobutrazol. Pools of 100 T3 or T4 lines were sown on 0.5% agarose plates containing 100 µM paclobutrazol and incubated in a growth cabinet for 10 days, 16h light at 20°C /8h dark at 15°C. Resistant seedlings developing green cotyledons were transferred to solid Gamborg B5 media (Duchefa, The Netherlands) containing 29 mM sucrose and 1µg/mL GA_3 and cultured for a further 6 days in the same environmental conditions before transplanting to soil and growth for seed production in a glasshouse with a minimum photoperiod of 13 hours assured by supplementary lighting. Three successive backcrosses to wild-type were performed with selection for a line lacking the non-linked T-DNA insertion at the first back-cross. This screen allowed the selection of eleven mutants that were able to germinate and develop on paclobutrazol.

Six of these mutants were found to correspond to *aba3* mutant alleles.

The remaining 5 mutants were analyzed for ABA content and carotenoid composition.

ABA Content Determination

Rosettes were detached from plants grown in soil in the glasshouse (22°C, minimum 13 hours photoperiod) for 3 weeks and placed for 2 hours under a lamina flow hood for stressed samples then harvested and frozen in liquid nitrogen at the same time as equivalent unstressed rosettes. ABA was extracted from ground freeze-dried rosettes or dry seeds as described previously (NORTH et al., Plant Sci., 169, 115-124, 2005). In each experiment measurements were carried out using tissue from at least three individual plants with five independent assays of each sample; each experiment was repeated three times.

Analysis of Carotenoid Composition

Extractions were carried out using rosette leaves from 3 week old plants grown in soil in the glasshouse (22°C, minimum 13 hours photoperiod). Pigments were extracted in acetone from 6 mm leaf discs and separated by HPLC as described previously (NORTH et al., Plant Sci., 169, 115-124, 2005) Pigments were detected with a photodiode-array detector (Beckman-Coulter, Villepinte, France). Peak identification was based on comparison of retention times and absorption spectra to commercially available standards; zeaxanthin, lutein, ß-carotene (Extrasynthèse, Genay, France), chlorophyll a and b (Fluka, Sigma-Aldrich Chimie, St. Quentin Fallavier, France) or published values (BRITTON, UV/Visible spectroscopy. In Carotenoids. Vol 1B: Spectroscopy. G Britton, S Liaaen-Jensen, H Pfander, eds, (Basel: Birkhäuser Verlag), pp. 13-62, 1995).

2 of the 5 mutants showed altered carotenoid compositions on HPLC analysis of leaf extracts. Analysis of the ABA contents of the two mutants confirmed them to be affected in ABA biosynthesis as ABA levels were reduced in both leaves and seeds.

One mutant presented an accumulation of zeaxanthin characteristic of *aba1* mutants defective for the zeaxanthin epoxidase gene. Sequencing of the *ABA1* gene in this mutant identified the insertion of an A 2851 bp into the zeaxanthin epoxidase coding sequence, thus altering the coding sequence

for the following 45 amino acids before generating a premature STOP codon; this mutant was thus named *aba1-13*.

The HPLC profile of leaf extracts of the second mutant is shown in Figure 2. Compared to wild-type, extracts from leaves of this mutant accumulated *trans*-violaxanthin and lacked neoxanthin isomers, indicating that neoxanthin synthesis was affected. No ABA-deficient mutant affected in neoxanthin synthesis has been described to date so the novel locus was called *aba4-1*.

EXAMPLE 2: ISOLATION AND CHARACTERIZATION OF *aba4-2* AND *aba4-3* MUTANTS

Genetic analysis of the *aba4-1* mutant found that it was inherited as a single recessive locus, but that no T-DNA insertion co-segregated with the mutant phenotype (data not shown). Mapping of the mutation using linkage analysis to molecular markers localized the gene to a region on the lower arm of chromosome 1 between nga280 and nga111. Fine mapping reduced the region to a 136kb interval containing 21 annotated genes. A search of the SIGnAL database (ALONSO et al., Science, 301, 653-7, 2003) was carried out for Salk T-DNA insertion lines within these genes. Homozygous insertion lines corresponding to 9 of the predicted genes were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and were analyzed for their leaf carotenoid composition. One line (Salk line N637455, Columbia-0 accession) with an insertion in At1g67080 exhibited the same carotenoid profile as the *aba4-1* mutant, as shown in Table I below. This mutant was called *aba4-3*.

Table I

	Carotenoids (mmol/mol chlorophyll a ± SE).						
Genotype	Zeaxanthin	Antheraxanthin	t-Violaxanthin	c-Violaxanthin	t-Neoxanthin	c-Neoxanthin	Lutein
Wt Ws	5.3 ± 0.2	7.0 ± 0.05	37.5 ± 0.8	1.0 ± 0.05	4.1 ± 0.2	35.9 ± 0.7	115.7 ± 1.4
aba4-1	4.1 ±0.6	8.5 ± 0.7	69.7 ± 0.3	6.9 ±0.2	ND	ND	122.3 ± 5.8
Wt Col-0	10.5 ± 2.8	7.7 ± 0.3	24.6 ± 1.2	1.6 ± 0.1	3.3 ± 0.1	31.7 ± 0.6	120.6 ± 2.7
aba4-3	8.1 ± 0.4	10.5 ± 0.8	50.3 ± 2.0	5.6 ± 0.1	ND	ND	122.9 ± 4.1

Values shown are means of 3 individual measurements. ND, not detectable

A further homozygous insertion line in At1g67080 was obtained from the Versailles T-DNA mutant collection FlagDB/FST (SAMSON et al., Nucleic Acids Res, 30, 94-7, 2002) and this also showed the modified carotenoid profile on HPLC analysis (data not shown) and was therefore called *aba4-2*.

The ABA levels of well watered rosette leaves and dry seeds of *aba4-1*, *aba4-2* and *aba4-3* mutants compared to wild-type and *aba1* mutants were analyzed. The results are shown in Table II below.

Table II

	ABA content (pmol g^{-1} DW).	
Genotype	Well-watered rosettes	Dry seeds
Wt Ws	287 ± 26	595 ± 24
aba4-1	15 ± 12	353 ± 14
aba4-2	53 ± 30	368 ± 17
aba1-13	82 ± 28	153 ± 12
Wt Col-0	260 ± 32	491 ± 27
aba4-3	272 ± 21	211 ± 17
aba1-5	84 ± 33	57 ± 8

Values are means from ≥6 individual measurements ± SE

Both T-DNA insertion mutants showed similar reductions in ABA levels in seeds as the *aba4-1* mutant, but only the mutants in the Ws-2 accession showed reduced ABA contents in well watered rosette leaves.

Crosses between the three different mutant lines did not result in genetic complementation, thus confirming that the *ABA4* gene corresponded to At1g67080.

EXAMPLE 3: CHARACTERIZATION OF THE *ABA4* GENE AND OF THE CORRESPONDING PROTEIN.

Comparison of the *ABA4* genomic sequence with that obtained for a full-length *ABA4* cDNA available in the RIKEN Arabidopsis full-length clone collection allowed the gene structure to be predicted as having six exons and five introns. The deduced polypeptide is predicted to be 220 amino acids in length with the first 68 amino acids presenting the features of a chloroplast signal peptide; rich in hydroxylated amino acids, an alanine residue at position 69 in agreement with the chloroplast transit peptide cleavage-site motif (KEEGSTRA, Cell, 56, 247-53, 1989; GAVEL and VON HEIJNE, FEBS Lett, 261, 455-8, 1990). Furthermore, this protein was identified in a proteomic analysis of proteins present in chloroplast envelope membrane extracts of Arabidopsis (FERRO et al., Mol Cell Proteomics, 2, 325-45, 2003). The mature ABA4 protein thus has a predicted molecular mass of 17.0kDa and a theoretical pI of 7.91. Hydropathy analyses of the putative protein indicate the presence of 4 helical transmembrane domains, but no domains of known function were identified.

Sequencing of the At1g67080 gene in the *aba4-1* mutant revealed the insertion of an A in the open reading frame at position 985. The frameshift induced by this insertion changes the following 4 amino acids of the predicted protein, before introducing a STOP codon.

A schematic representation of *ABA4* gene structure and of the relative positions of the *aba4* mutant insertions is shown in Figure 3A. Rectangles indicate the positions of open reading frames.

RT-PCR analysis was performed using primers in exons 3 and 5 of *ABA4* (forward primer 5'-GGTTTTACCCTTCTATACTC-3' (SEQ ID NO: 22) and reverse primer 5'-CATTTTAGCTATTCCAGAC-3'(SEQ ID NO: 23). A control experiment was performed with primers for the *EF1α-4a* gene transcript (forward primer 5'-ATGCCCCAGGACATCGTGATTTCAT-3'(SEQ ID NO: 24) and reverse primer 5'-TTGGCGGCACCCTTAGCTGGATCA-3'(SEQ ID NO: 25).

The results are shown in Figure 3B. In agreement with the mutations they contained transcripts were not detectable in *aba4-2* and *aba4-3*, but were present in the *aba4-1* mutant.

A BLASTP search identified similar sequences in maize, wheat, rice and cyanobacteria, but the protein appears to be unique in Arabidopsis implying that ABA4 is a single copy gene in the genome.

Figure 3C shows the alignment of the ABA4 predicted amino acid sequences from *Arabidopsis thaliana* (SEQ ID NO: 2), maize ZmABA4a (SEQ ID NO: 4) and ZmABA4b (SEQ ID NO: 6), wheat (SEQ ID NO: 10), and rice (SEQ IN NO: 8).

Sequence alignment was performed using the MultAlin programme (CORPET, Nucl. Acids Res., 16, 10881-10890, 1988) using the Blosum-62-12-2 symbol comparison table. The figure was drawn using Boxshade version 3.21 (HOFMANN, K. and BARON, M.D., http://www.ch.embnet.org/software/BOX_form.html) with a similarity threshold of 50% and the default similarities (FYW, IVLM, RK, DE, GA, TS, NQ).

The higher plants proteins contain a N-terminal region which is not present in the cyanobacterial sequences. The Arabidopsis, maize, wheat, and rice proteins do not show

any primary sequence similarity over this region, which corresponds to a predicted chloroplast signal peptide.

All of the protein sequences were predicted to contain 4 transmembrane domains. In the higher plant sequences an additional, block of 5 amino acids, four of which are conserved, is found in the putative loop between transmembrane domains 2 and 3, suggesting a function specific to the higher plant protein activity. In addition, a protein kinase C motif (Prosite PDOC00005) was conserved in all the sequences that would result in the phosphorylation of a conserved threonine (T105 in ABA4).

Searches carried out for proteins with a similar hydropathic profile to ABA4 (found that the region corresponding to transmembrane helices 1 to 4 of the rhodopsin apoprotein shares significant structural similarity (Figure 3D).

Analysis and comparison of the hydrophobicity profiles of the predicted ABA4 mature protein and freshwater eel rhodopsin (Q90215) was performed using the method of KYTE and DOOLITTLE (J Mol Biol, 157, 105-32, 1982). Hydropathy was calculated over a window length of 11 amino acids (DNA strider program, version 1.4 f2).

EXAMPLE 4: EXPRESSION PATTERN OF THE *ABA4* GENE

ABA4 gene expression in the main tissue types was analyzed by quantitative reverse transcription PCR (QRT-PCR).

Total RNA was prepared from various frozen plant tissues using Sigma mammalian total RNA kit (Sigma-Aldrich, St. Quentin Fallavier, France) following the manufacturer's protocol and including an on column DNase I treatment (RNase-free DNase set, Qiagen, Courtaboeuf, France). Total RNA (2µg) was used as a template to synthesize first strand cDNA using an oligo(dT) 18 mer primer and the SuperScript first-strand synthesis kit (Invitrogen, Cergy Pontoise, France) according to manufacturer's instructions.

Quantitative real-time PCR reactions were performed using the LightCycler FastStart DNA master SYBR green I kit in a Roche LightCycler 1.0 (Roche Diagnostic, Penzberg, Germany). Reactions used 5µl of 1:50 diluted sscDNAs in a total volume

of 20µl. Gene specific primers that had been tested for their efficiency rates and sensitivity on dilution series of cDNAs were as follows: *ABA4*, forward primer 5'-AATGACTCTTGCTTCTGCTTGGAT-3'(SEQ ID NO: 26), reverse primer 5'-GCTTTGGTTACGAAATGCGAAACGAT-3'(SEQ ID NO: 27); *EF1αA4*, forward primer 5'-CTTCTTGCTTTCACCCTTGGTGT-3'(SEQ ID NO: 28), reverse primer 5'-TGTCAGGGTTGTATCCGACCTT-3'(SEQ ID NO: 29). The efficiencies of the two primer sets used were almost identical. The reactions were incubated as follows, denaturation of cDNAs and hot start of recombinant Taq DNA polymerase - 95°C for 8 min. then 45 cycles of 95°C for 10 sec., 59°C for 4 sec., 72°C for 9 sec. After the final PCR cycle a fusion curve was obtained to verify the specificity of the PCR amplification by heating at 94°C for 1 sec, before cooling to 65°C for 30 sec. Followed by an increase to 94°C with a temperature transition rate of 0.1°C S-1.

The results are shown in Figure 4. Significant levels of transcripts of *ABA4* were found in all tissues examined, with slightly higher levels of expression being detected in flowers.

In order to obtain more detailed information about the cell types expressing *ABA4*, constructs were generated with a 1.7 kb region upstream of the ATG start codon fused to the ß-glucuronidase (*GUS*) reporter gene and transformed into wild-type Arabidopsis.

Several independent transformants presented similar *GUS* transgene expression patterns, although staining intensities varied. Consistent with the QRT-PCR analyses, histochemical staining of transformants found expression in all tissues types examined. In leaves of both plantlets and mature plants staining was extensive with clear expression in vascular tissue as well as trichome cytoplasm. *ABA4*promoter::*GUS* expression in roots was more restricted with clear staining of vascular tissue and root hairs. GUS staining in flowers was limited to the sepals, stamens (excluding the pollen) and the stigma below the papillae. In young siliques the staining below the stigmatic papillae was more intense and staining of the pedicel was also observed. As siliques matured

the valves became entirely stained with the vascular tissue showing more intense colouring. *ABA4*promoter::*GUS* was also expressed throughout the embryo although the future root appeared to be more weakly stained.

EXAMPLE 5: ROLE OF *ABA4* IN STRESS RESPONSES

QRT-PCR analysis of *ABA4* expression was carried out on leaf and root tissue from wild-type plants that had been subjected to a rapid dehydration.

For dehydrated tissue, 28 day old plants that had been grown in the greenhouse in a 50:50 mix of standard compost and sand were used. The root system was washed free of growth media and whole plants were placed under a lamina flow hood for 2 hours. Control non-stressed plant material was harvested at the end of the dehydration period. The rosettes and root system of both stressed and control plants were separated and frozen in liquid N_2. QRT-PCR was performed as described in Example 4, before dehydration and after dehydration for 2 hours.

The results are shown in Figure 5A.

Water deficit did not induce any significant change in transcript levels in either roots or leaves.

To determine whether *ABA4* expression was nonetheless required for the increase in ABA biosynthesis in response to dehydration, ABA measurements were carried out on dehydrated rosettes of *aba4* mutants. ABA content was determined before and after dehydration for 2 hours.

The results are shown in Figures 5B and 5C.

In a similar manner to the *aba1* mutants, all 3 *aba4* mutant alleles showed a marked reduction in ABA accumulation as compared to wild-type (Figure 5B and C). This indicates that *ABA4* gene expression is important for ABA production. In addition, although the basal ABA levels observed for the *aba4-3* mutant were similar to wild-type, the induction of ABA biosynthesis by water deficit was affected suggesting that the absence of effect on basal levels is due to factors related to the Col-0 accession rather than a particularity of the *aba4-3* mutation.

To verify that the reduced ABA levels observed in *aba4* mutants were indeed important for their response to water deficit, their water loss phenotypes were examined.

Rapid dehydration assays were carried out using 3 week old plants grown in soil in the glasshouse (22°C, minimum 13 hours photoperiod). Four rosettes per genotype were cut from the root system and water loss measured as described previously (NORTH et al., 2005).

The results are shown in Figures 6A and 6B.

On rapid dehydration water loss rates in *aba4* mutants were higher than those of wild-type, although water loss was less than that of the *aba1-5* mutant.

Nine seeds from each genotype were sown on 9 cm x 9 cm pots containing a 50:50 (v/v) mixture of sand (2-3 mm particles) and horticultural compost and cultured as described previously (MERLOT et al., Plant J, 30, 601-9, 2002). When plants were 16 days old and watering had been withheld for 3 days.

Thermal images were obtained using a Thermacam PM250 infrared camera (Inframetrics, FLIR Systems, North Billerica, MA, USA) equipped with a 16° lens exactly as described by MERLOT et al., (2002) cited above.

Visualisation of leaf temperature by infrared thermography permits differences in water loss on progressive dehydration to be distinguished, due to the cooling effect of transpiration on leaf temperature (MERLOT et al., 2002, cited above).

After 3 days of water deficit the leaf temperature of *aba4* and *aba1* mutants was clearly lower than that of wild-type plants.

These results indicate that the reduced ABA contents found in *aba4* mutants are such that responses to both rapid and progressive water deficit are affected.

Mutants affected in ABA biosynthesis in vegetative tissues display sucrose insensitive seedling development (ARENAS-HUERTERO et al., Genes Dev, 14, 2085-96, 2000). This phenotype is related to the involvement of ABA in sugar signalling which induces a postgerminative arrest in wild-type

seedlings grown on high sugar concentrations (LOPEZ-MOLINA et al., Proc Natl Acad Sci U S A, 98, 4782-7, 2001).

Sucrose sensitivity of seedling development was assayed by sowing surface sterilised seed onto solid Gamborg B5 media (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with sucrose in a range of concentrations from 30 mM to 300 mM. Fifty seeds were sown using a sterile scalpel so that spacing between seeds was identical, with 3 repetitions at each concentration. Seeds were stratified at 4°C for 3 days and then incubated at 20°C with a 16: 8 hours light/dark photoperiod and a light intensity of 50 µmol m-2 s-1 for 3 weeks. Seedlings that had formed true first leaves were scored as sucrose resistant.

The results are shown in Figures 7A and 7B.

In agreement with the reduced levels of ABA in stressed rosette leaves, *aba4* mutants showed sucrose resistant development (Figure 7A and B). Interestingly, the *aba4-3* knock-out mutant in the Col-0 background showed a higher level of resistance than an equivalent knock-out, the *aba4-2* mutant in the Ws-2 accession.

It has previously been demonstrated that the expression of the ABA biosynthesis genes *AtZEP1*, *ABA2*, *AAO3* and *ABA3* is induced in seedlings grown on high concentrations of glucose in an ABA dependent manner (CHENG et al., Plant Cell, 14, 2723-43, 2002). To investigate whether similar regulation occurs for the *ABA4* gene, QRT-PCR analyses were carried out on seedlings grown on high concentrations of sucrose.

The results are shown in Figure 7C.

These results indicate that expression of the *ABA4* gene is repressed by sucrose in a concentration dependent manner in wild-type of both Ws-2 and Col-0 accessions. Furthermore, in the *aba1-13* and *aba4-1* mutants repression occurred in a similar manner, although the degree of repression at high sucrose concentrations was less, indicating that although ABA was involved, it was not essential for repression. In addition, in non-stressed seedlings of *aba1-13* and *aba4-1* the *ABA4* transcript levels were lower suggesting

that basal levels of ABA have a positive effect on *ABA4* gene regulation in the absence of stress or signalling.

EXAMPLE 6: GERMINATION CHARACTERISTICS OF *ABA4* MUTANTS

As described above the *aba4-1* mutant was isolated due to its increased paclobutrazol resistance compared to wild-type. Paclobutrazol resistance has been associated with reduced seed dormancy in other ABA-deficient mutants (LEFEBVRE et al., Plant J, 45, 309-19, 2006).

In order to compare the characteristics of the *aba4-1* mutant with those of the *aba4* knock-out mutant alleles, paclobutrazol resistant germination was examined over a range of concentrations.

Paclobutrazol resistance and dormancy analyses were carried out as previously described (LEFEBVRE et al., 2006).

The results are shown in Figures 8A to 8C.

All of the *aba4* mutants showed similar paclobutrazol resistance phenotypes compared to wild-type, although they are less resistant than *aba1* mutants. These differences corresponded well with the differences observed in seed ABA content (cf. Table II above).

Analysis of the germination of freshly harvested seeds showed that although *aba4-1* mutants are less dormant than wild-type, the effect is much milder than that observed in the *aba1-13* mutant (Figure 8C). Similar differences in paclobutrazol resistance and dormancy phenotypes have been observed for the *nced6* and *nced9* mutants (LEFEBVRE et al., 2006) and indicate that the reduction of ABA levels in *aba4* mutants is close to the threshold required for dormancy induction, whereas the levels are sufficiently low to counteract the effect of paclobutrazol inhibition of GA biosynthesis.

EXAMPLE 7: OVEREXPRESSION OF *ABA4* IN *ARABIDOPSIS*

A genomic fragment containing the *ABA4* gene from 9 bp before the ATG to 183 bp after the STOP (corresponding to position 170 to the end of SEQ ID NO: 1) was amplified using the proofreading *Pfu* Ultra DNA polymerase (Stratagene, Amsterdam, The Netherlands) and the following primers containing partial GATEWAY™ (Invitrogen, Cergy Pontoise,

France) B1 or B2 recombination site sequences, forward 5'-AAAAAAGCAGGCTATTTGAATCAGAGATGGG-3'(SEQ ID NO: 30), reverse 5'-AAGAAAGCTGGGTCAGGAGGTTTTCAAGTTGC-3'(SEQ ID NO: 31). A second PCR was then carried out using primers B1, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'(SEQ ID NO: 32), and B2, 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'(SEQ ID NO: 33) and *Taq* DNA polymerase (Eurobio, Les Ulis, France). The resulting PCR product was then recombined into vector pDONR207 (Invitrogen, Cergy Pontoise, France) using BP clonase according to the manufacturer's instructions and transformed into *E. coli* strain DH10B. After confirming the integrity of the *ABA4* fragment by sequencing, transfer to the binary vector pMDC32 (CURTIS and GROSSNIKLAUS, Plant Physiol, 133, 462-9, 2003), containing a dual *35S* promoter, was carried out using LR clonase (Invitrogen, Cergy Pontoise, France) according to manufacturer's instructions. The resulting plasmid was introduced into *A. tumefaciens* C58C1pMP90 by triparental mating as previously described (MARIN et al. 1996).

Stable transformation of wild type Col-0 and Ws-2 accessions was carried out using the floral dip method (CLOUGH and BENT, Plant J, 16, 735-43, 1998) and selecting hygromycin resistant transformants. Homozygous hygromycin resistant plants were selected in the progeny of primary transformants, which exhibited a 3:1 segregation for hygromycin resistance. Compared to wild type, water loss was reduced in detached rosettes for 5 transgenic lines among 9 tested in the Ws-2 accession (Fig. 9A) and in all 4 lines tested in the Col-0 accession (Fig. 9B). This indicates that constitutive expression of *ABA4* gene enhances plant tolerance to water deficit. Furthermore, increased tolerance to water deficit correlated with higher neoxanthin contents in leaves of transgenic plants, as shown in Table III below.

Table III

	Total (Z + A + V + N)	Carotenoid % (Z + A + V + N)					
Genotype	mmol/mol chlorophyll a	Zeaxanthin	Antheraxanthin	t-Violaxanthin	c-Violaxanthin	t-Neoxanthin	c-Neoxanthin
Wt Col-0	79.9 ± 1.6	13.3 ± 3.3	9.7 ± 0.4	31.0 ± 1.8	2.0 ± 0.2	4.2 ± 0.1	40.0 ± 1.4
35S::ABA4 #7	80.7 ± 2.6	9.9 ± 2.9	6.2 ± 0.2	31.0 ± 1.2	3.7 ± 0.2	7.0 ± 0.1	42.2 ± 1.6
35S::ABA4 #11	79.1 ± 0.7	5.7 ± 0.5	6.7 ± 0.3	33. 4 ± 1.4	3.3 ± 0.2	6.2 ± 0.2	44.6 ± 0.8
35S::ABA4 #15	85.6 ± 2.1	9.6 ± 2.0	5.7 ± 0.1	33.3 ± 1.7	3.0 ± 0.2	5.9 ± 0.1	42.4 ± 0.2
35S::ABA4 #16	79.4 ± 1.7	5.1 ± 1.3	5.4 ± 0.4	32.9 ± 1.1	4.3 ± 0.2	7.9 ± 0.1	44.4 ± 0.4

Values shown are means and standard errors of 3 individual measurements

This indicates that constitutive expression of *ABA4* gene results in increased neoxanthin synthesis.

EXAMPLE 8: OVEREXPRESSION OF *ABA4* IN MAIZE

Maize plants were transformed with a sequence encoding an ABA4 polypeptide in order to increase their tolerance to water deficit.

A synthetic ZmABA4 sequence (SynABA4-ZmMod) codon optimised for a higher expression in maize was constructed. This sequence is shown under SEQ ID NO: 34. It encodes a ZmABA4 protein with 100% identity to the native ZmABA4a protein (SEQ ID NO: 4). The SynABA4-ZmMod coding sequence was cloned as an *Nco*I/*Not*I fragment, into the entry vector pENTR4 (Invitrogen), cut by *Nco*I and *Not*I. The resulting entry clone is named pBIOS1286. The fragment of pBIOS1286 containing SynABA4-ZmMod was then transferred into the plant binary GATEWAY® Destination vector pBIOS886 by *in vitro* recombination using the Gateway LR Clonase™ II enzyme mix (Invitrogen). The destination vector pBIOS886 is a derivative of pSB11 (KOMARI *et al.*, Plant J. 10, 165-174, 1996) containing selectable marker genes for selection of maize transformants, a constitutive CsVMV promoter (VERDAGUER et al. Plant Mol Biol 6, 1129-1139, 1996) followed a rice Actin intron (MCELROY et al., Plant Cell, (2) 163, 1990), a GATEWAY® cassette, and a Sac66 polyadenylation sequence (JENKINS et al., Plant Cell Environ. 22, 159-167, 1999). The resulting expression vector, named pBIOS1290, is shown in Figure 10. It expresses SynABA4-ZmMod under the control of the CsVMV promoter.

pBIOS 1290 was transferred into Agrobacteria LBA4404 (pSB1) according to KOMARI *et al.*(1996, cited above) and the maize cultivar A188 was subsequently transformed with this agrobacterial strain as described by ISHIDA *et al.*(1996, cited above).

It is also possible to obtain a similar construct encoding ZmABA4a by use of a coding sequence derived from SEQ ID NO:3, and a similar construct encoding ZmABA4b by use of a coding sequence derived from SEQ ID NO:5, and to use any of these constructs to transform maize plants, as described above.

Transgenic lines were characterized by Southern Blot and their progeny by Q-PCR to confirm the copy number. The transgene expression was confirmed by RT-PCR. Transgenic lines were cultivated in greenhouse, under irrigation conditions.

Several transgenic lines were tested for their carotenoid content by HPLC analysis, and for their loss of water under dessication conditions.

The results of HPLC analysis are shown in Figure 11. Some of the transgenic lines have a high content in carotenoids (see transgenic lines 2 and 11 on Figure 11a), particularly in trans-violaxanthin (Figure 11b) and in cis-neoxanthin (Figure 11c); the quantity of these carotenoids appears to depend on the number of copies of the transgene. The cis-violaxanthin content is constant (Figure 11d).

Dessication experiments were performed on plants presenting five leaves. The mature (ligulated) leaf number 3 was cut, weighed and placed under laminar bench flow, then weighed regularly to evaluate the water loss.

The results observed on one of the transgenic lines tested are shown on Figure 12. These results show that the transgenic plants overexpressing ABA4 lose less water that the non transgenic plants.

These results show that overexpression of ABA4 results in a higher content in trans-violaxanthin and cis-neoxanthin, and in a better resistance to dehydration.

CLAIMS

1) A method for increasing the tolerance of a plant to water deficit, wherein said method comprises overexpressing in said plant an ABA4 polypeptide comprising the following regions:

a) a chloroplast signal peptide;

b) a region comprising a sequence having at least 60% identity with the region 69-220 of the polypeptide SEQ ID NO: 2, or with the region 83-235 of the polypeptide SEQ ID NO: 4, or with the region 80-232 of the polypeptide SEQ ID NO: 6 or with the region 76-228 of the polypeptide SEQ ID NO: 8, or with the region 75-227 of the polypeptide SEQ ID NO: 10.

2) A method of claim 1, wherein region b) of said ABA4 polypeptide comprises:

i) four domains defined by the following sequences (one letter code):

- htm 1 :$ASX_1X_2FX_3X_4GTX_5AVLPFYTLMX_6X_2A$ (SEQ ID NO: 11),
- htm 2 :$X_7X_2PYX_6X_8LGX_9LYX_2YLLYX_{10}SW$ (SEQ ID NO: 12),
- htm 3 :$MTX_{11}ASAWIHLLX_2VDLFAA$ (SEQ ID NO: 13),
- htm 4 :$SVSLCLLFCPX_6GIX_{12}X_{13}HX_{14}$ (SEQ ID NO: 14),

wherein X_1 to X_{14} represent a non-hydrophilic amino acid;

ii) a conserved sequence of 5 amino acids $SKYX_{15}L$ (SEQ ID NO: 11) wherein X_{15} = M or W, between domains htm2 and htm3;

iii) a protein kinase C motif (Prosite PDOC00005) defined by the sequence TKX_{14} wherein X_{14} = R or K, between domains htm1 and htm2.

3) A method of claim 2, wherein region b) of said ABA4 polypeptide comprises the following sequence:
$QIASX_1X_2FX_3X_4GTX_5AVLPFYTLMX_6X_2APX_{17}AX_{18}X_6TKX_{16}X_{19}X_{20}X_{21}SX_7X_2PYX_6X_8LGX_9LYX_2YLLYX_{10}SWTPX_{21}TX_{10}X_{16}X_{22}MFX_{23}SKYX_{15}LPELX_{24}GIX_2X_{16}MFX_{23}SEMTX_{11}ASAWIHLLX_2VDLFAARQVYX_{25}DGX_{10}X_{26}NX_{27}X_6ETRHSVSLCLLFCPX_6GIX_{12}X_{13}HX_{14}X_{11}TK$ (SEQ ID NO: 16) wherein X_1 = S or C, X_2 = V or A, X_3 = A or T, X_4 = V, L or W, X_5 = T, V or I, X_6 = V or I, X_7 = S,G or T, X_8 = I or A, X_9 = V, L or I, X_{10} = I or L, X_{11} = L or V, X_{12} = V, L or

A, X_{13} = A, T, or S, X_{14} =V, A or F, X_{15} = M or W, X_{16} = R or K, X_{17} = K or N, X_{18} = E, D, or S, X_{19} = C, T, or A X_{20} = M or V, X_{21} = E or D, X_{22} = A or Y, X_{23} = A or S, X_{24} = S, P, A, or T, X_{25} = H, N, or Q, X_{26} = K, R, or E, X_{27} = Q or N.

4) A method of any of claims 1 to 3 wherein the chloroplast signal peptide of said ABA4 polypeptide is selected among:

- a peptide having the following sequence: MGFSSFISQPLSSSLSVMKRNVSAKRSELCLDSSKIRLDHRWSFIGGSRISVQSNSYTVVHK KFSGVR (SEQ ID NO: 17)
- a peptide having the following sequence: MAPCASPSALALSASTRVSSFPLTLRPRPRPEARVPRAPGGAQLRPATACSWPRPLLPELAP AFPRAGARSAGRPQPLFRPR (SEQ ID NO: 18)
- a peptide having the following sequence: MAPCASPSALALSASTRVSILRLPLALRQRAEARVPGAQFRPSTACSWARPLLPELAGAVPR AGARGTGRRTQPLFRPR (SEQ ID NO: 19)
- a peptide having the following sequence: MAALLLLSSAARVGVAAPLALRQQRPVVLPGGQLRTGSGAGAASAWAARPLRPELAAVSRPA VPARGRAPLFRPR (SEQ ID NO: 20)
- a peptide having the following sequence: MAASSPSALALSPSTRVVAGPSLLLAVKRTPATRVAAAPSGQLPACSWGPLRPELAPAPGPC AARCRAPLLRPR (SEQ ID NO: 21)

5) A method of any of claims 1 to 4, wherein said ABA4 polypeptide is selected among SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10.

6) A recombinant DNA construct comprising a polynucleotide encoding an ABA4 polypeptide, as defined in any of claims 1 to 5, under control of an appropriate promoter.

7) An expression cassette comprising a DNA construct of claim 6.

8) An expression vector comprising a DNA construct of claim 6.

9) A host cell comprising a recombinant DNA construct of claim 6 or an expression cassette of claim 7, or an expression vector of claim 8.

10) A host cell of claim 9 which is a plant cell.

11) A transgenic plant containing a transgene comprising a DNA construct of claim 6 or an expression cassette of claim 7.

12) A method for producing a transgenic plant having an increased tolerance to water deficit, when compared to a non transgenic plant, said method comprising the steps consisting of:

- transforming at least one plant cell with a vector containing an expression cassette of claim 7;
- cultivating said transformed plant cell in order to regenerate a plant containing in its genome a transgene comprising said expression cassette.

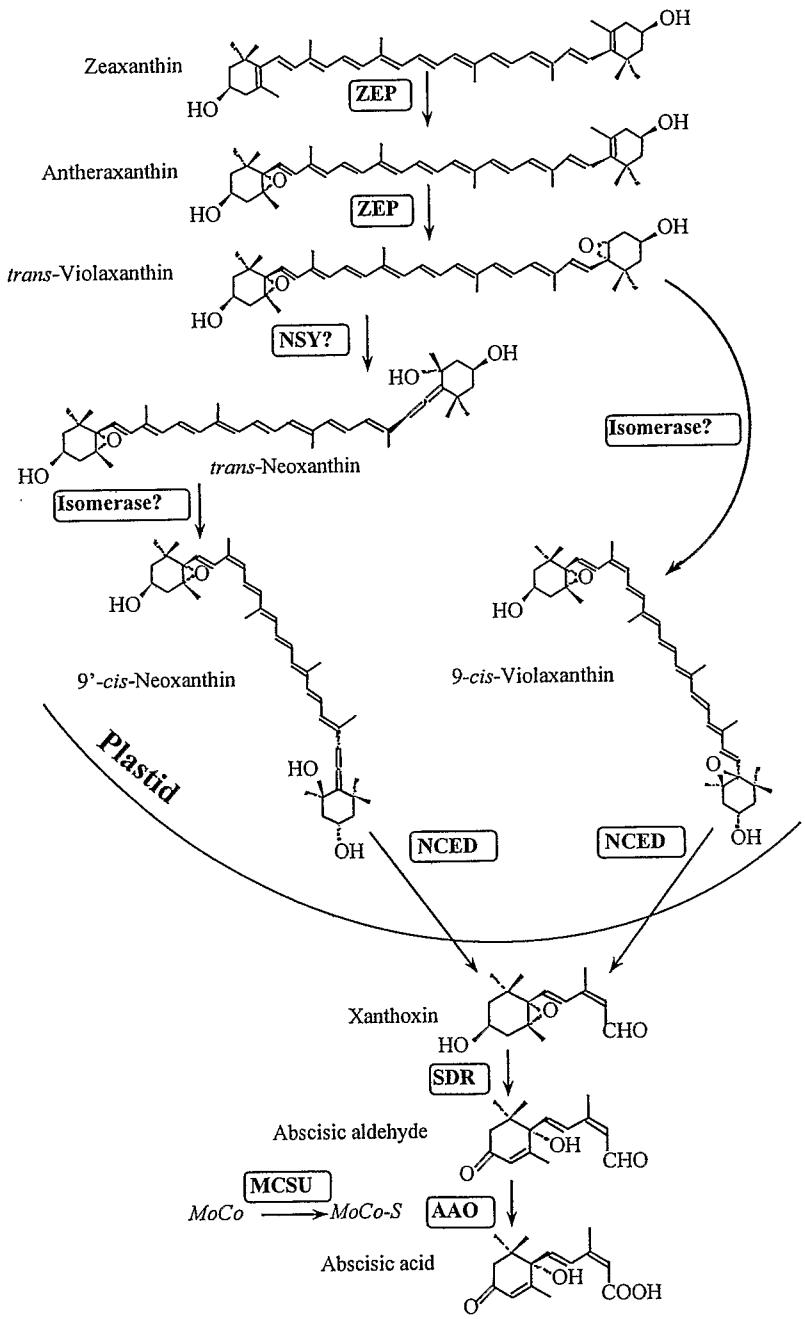

Zeaxanthin
ZEP
Antheraxanthin
ZEP
trans-Violaxanthin
NSY?
trans-Neoxanthin
Isomerase?
Isomerase?
9'-cis-Neoxanthin
9-cis-Violaxanthin
Plastid
NCED
NCED
Xanthoxin
SDR
Abscisic aldehyde
MCSU
MoCo
MoCo-S
AAO
Abscisic acid

Figure 1

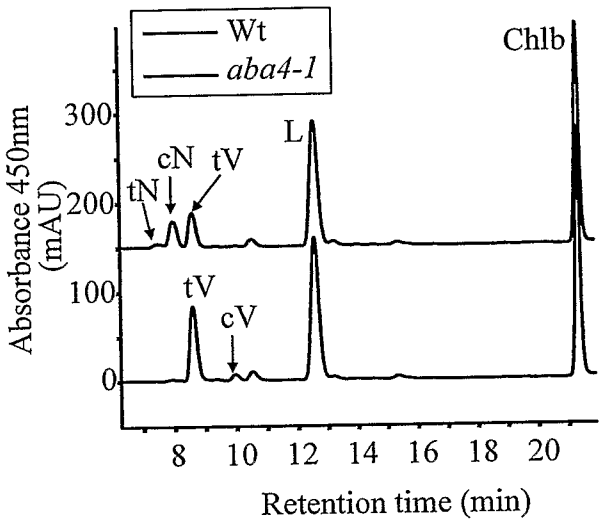

Wt
aba4-1
Chlb
L
cN
tV
tN
tV
cV
Absorbance 450nm (mAU)
300
200
100
0
8
10
12
14
16
18
20
Retention time (min)

Figure 2

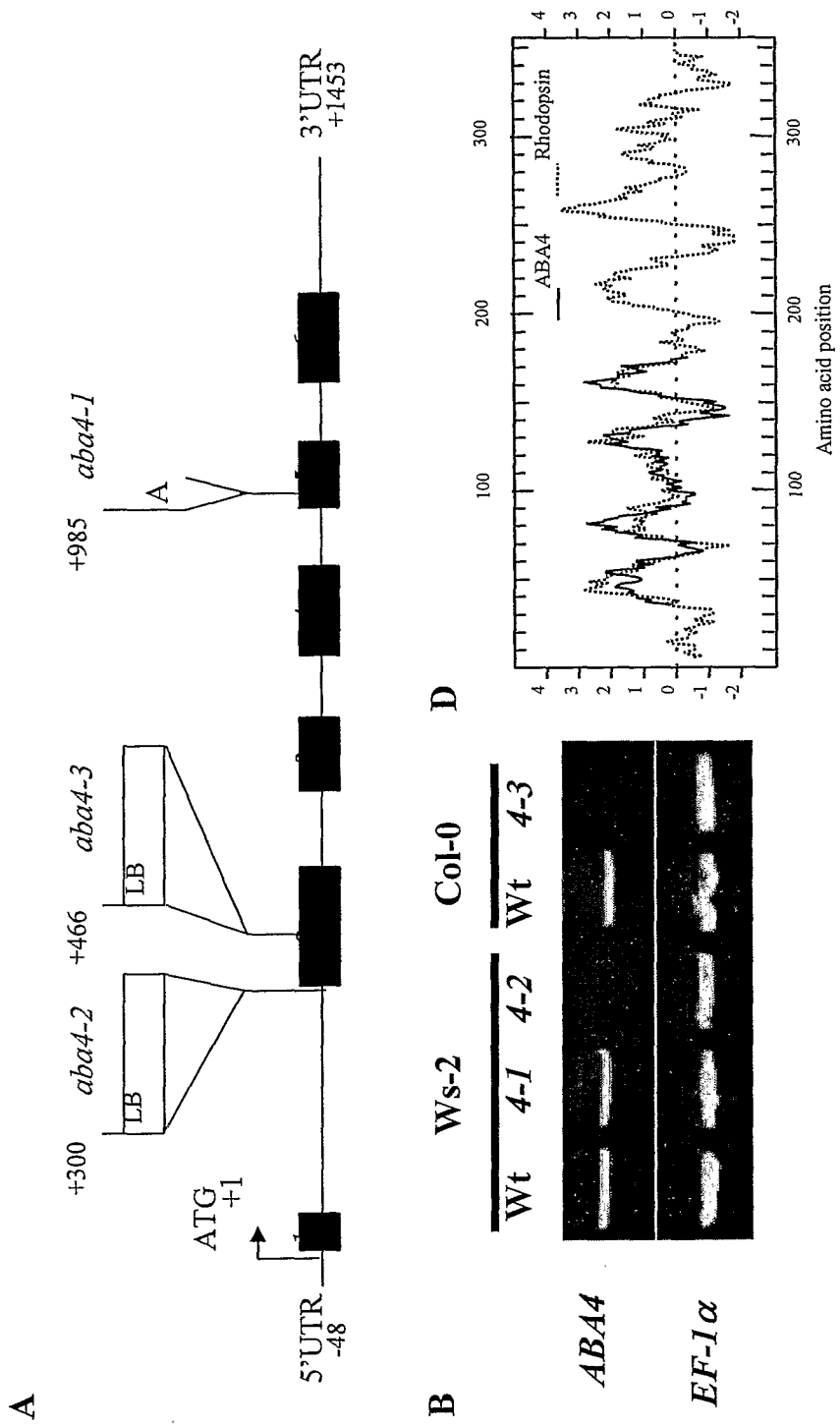

A
5'UTR
-48
ATG
+1
+300
aba4-2
LB
+466
aba4-3
LB
+985
aba4-1
A
3'UTR
+1453
B
Ws-2
Wt
4-1
4-2
Col-0
Wt
4-3
ABA4
EF-1α
D
ABA4
Rhodopsin
Amino acid position
100
200
300
4
3
2
1
0
-1
-2

Figure 3

C

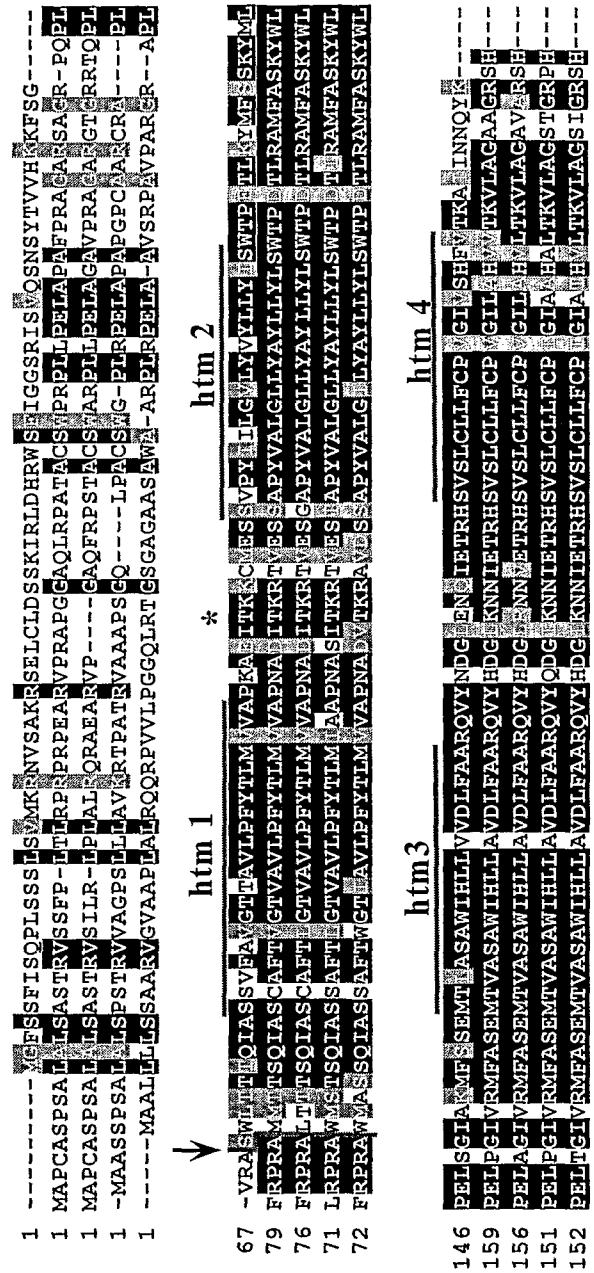


Figure 3 (followed)

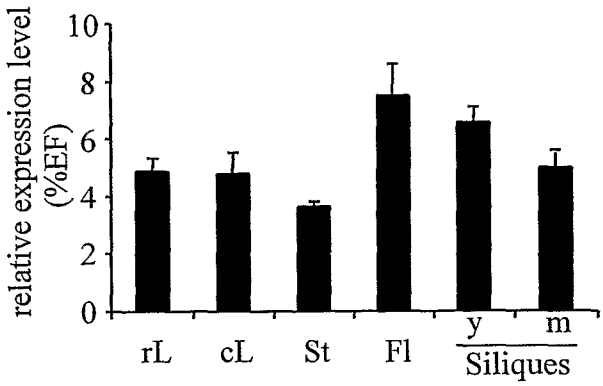

relative expression level (%EF)
10
8
6
4
2
0
rL
cL
St
Fl
y
m
Siliques

Figure 4

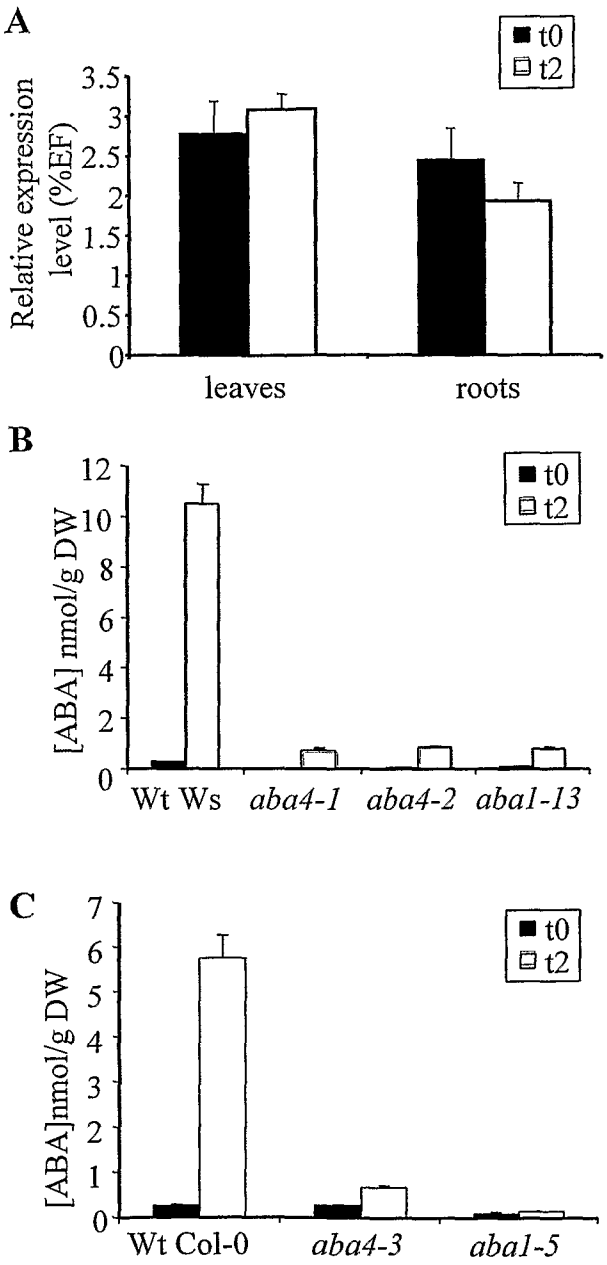

A
t0
t2
Relative expression level (%EF)
3.5
3
2.5
2
1.5
1
0.5
0
leaves
roots
B
t0
t2
[ABA] nmol/g DW
12
10
8
6
4
2
0
Wt Ws
aba4-1
aba4-2
aba1-13
C
t0
t2
[ABA]nmol/g DW
7
6
5
4
3
2
1
0
Wt Col-0
aba4-3
aba1-5

Figure 5

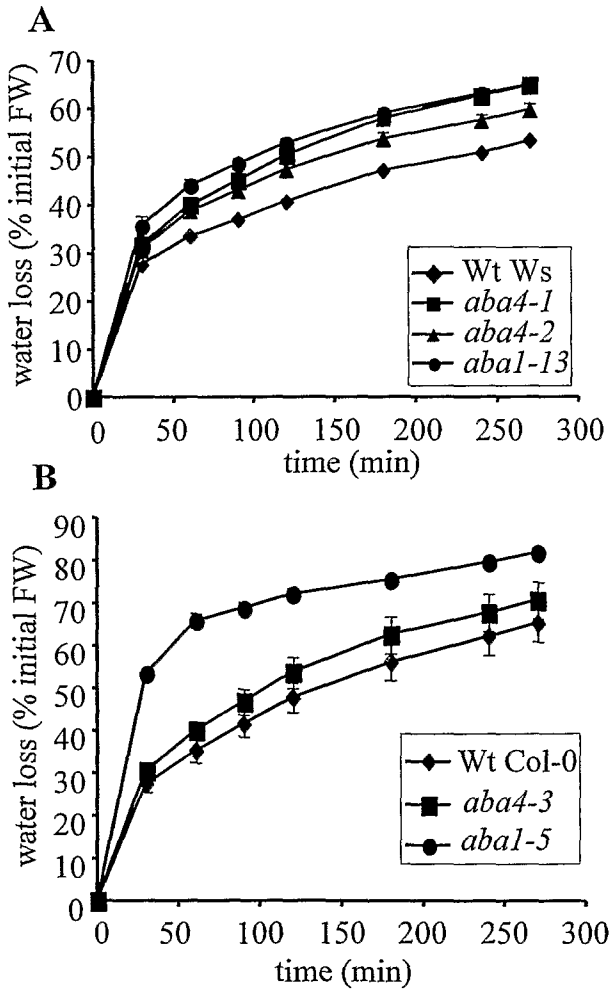

A
water loss (% initial FW)
70
60
50
40
30
20
10
0
0 50 100 150 200 250 300
time (min)
Wt Ws
aba4-1
aba4-2
aba1-13
B
water loss (% initial FW)
90
80
70
60
50
40
30
20
10
0
0 50 100 150 200 250 300
time (min)
Wt Col-0
aba4-3
aba1-5

Figure 6

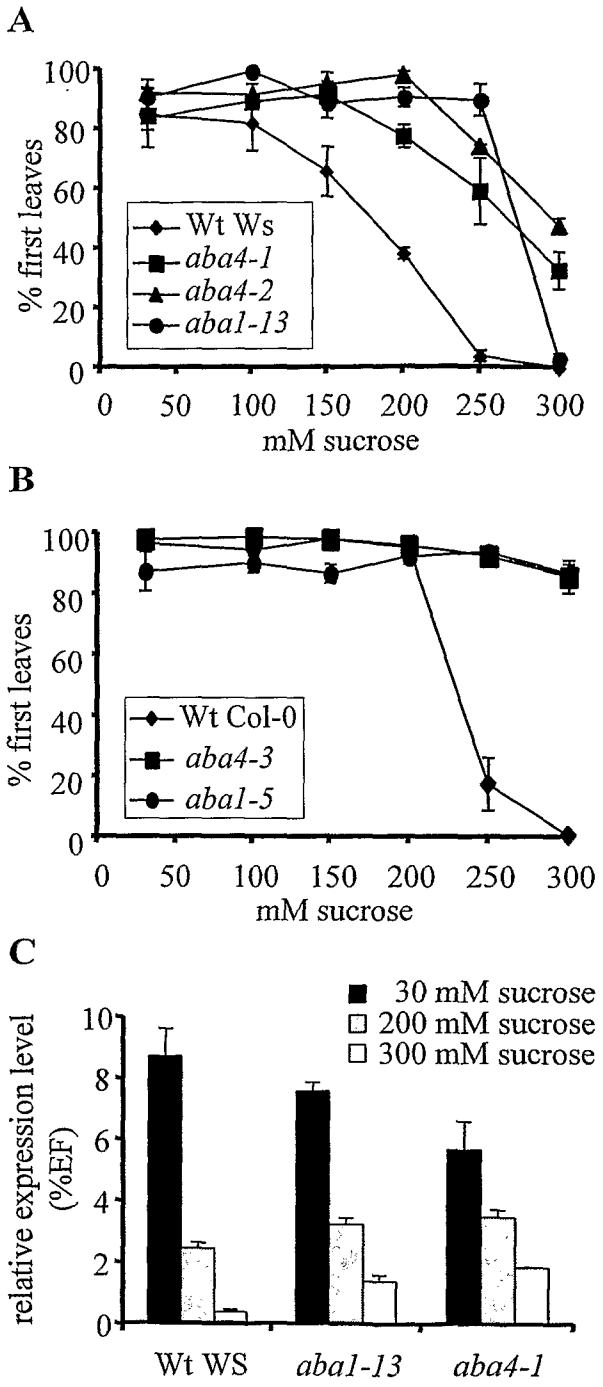

A
100
80
60
40
20
0
% first leaves
Wt Ws
aba4-1
aba4-2
aba1-13
0 50 100 150 200 250 300
mM sucrose
B
100
80
60
40
20
0
% first leaves
Wt Col-0
aba4-3
aba1-5
0 50 100 150 200 250 300
mM sucrose
C
30 mM sucrose
200 mM sucrose
300 mM sucrose
relative expression level (%EF)
10
8
6
4
2
0
Wt WS
aba1-13
aba4-1

Figure 7

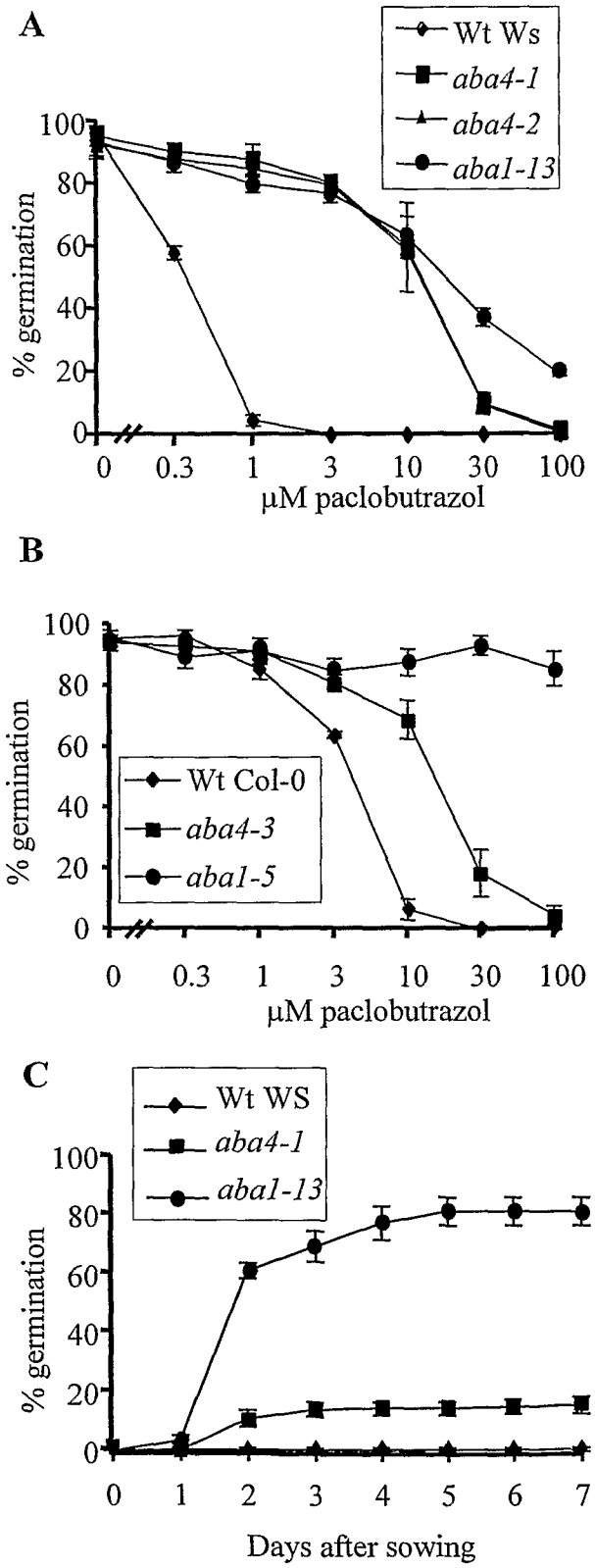

A
Wt Ws
aba4-1
aba4-2
aba1-13
% germination
100
80
60
40
20
0
0
0.3
1
3
10
30
100
µM paclobutrazol
B
Wt Col-0
aba4-3
aba1-5
% germination
µM paclobutrazol
C
Wt WS
aba4-1
aba1-13
% germination
0
1
2
3
4
5
6
7
Days after sowing

Figure 8

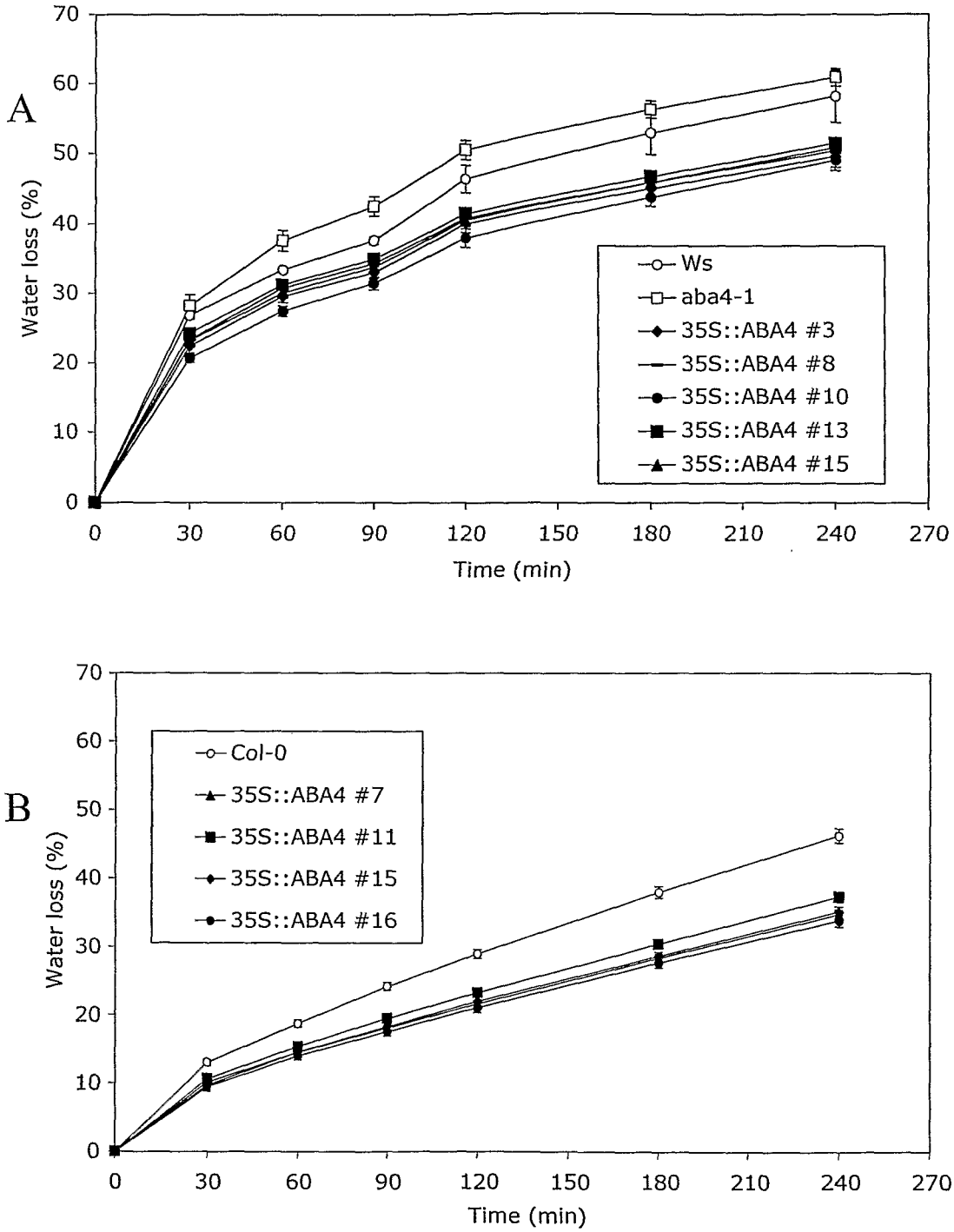

A
Water loss (%)
Time (min)
Ws
aba4-1
35S::ABA4 #3
35S::ABA4 #8
35S::ABA4 #10
35S::ABA4 #13
35S::ABA4 #15
B
Water loss (%)
Time (min)
Col-0
35S::ABA4 #7
35S::ABA4 #11
35S::ABA4 #15
35S::ABA4 #16

Figure 9

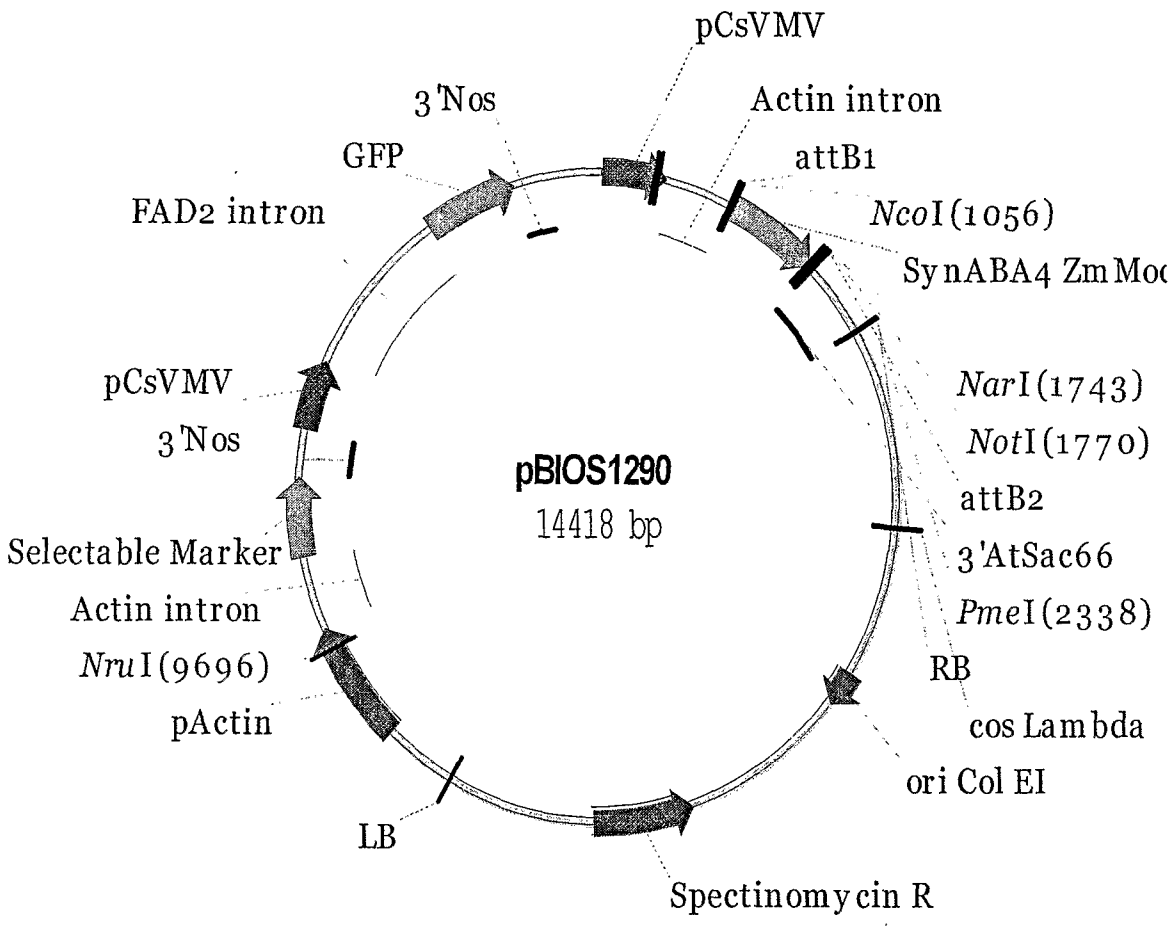
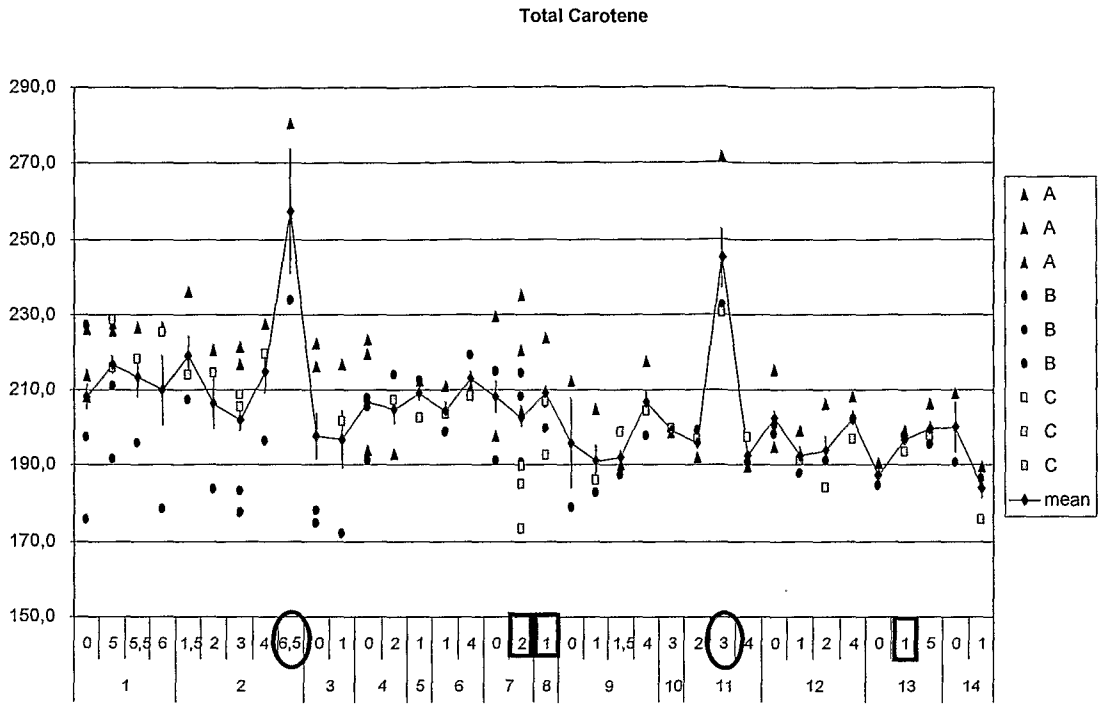

pCsVMV
3'Nos
Actin intron
GFP
attB1
FAD2 intron
NcoI(1056)
SynABA4 ZmMoc
pCsVMV
NarI(1743)
3'Nos
NotI(1770)
pBIOS1290
14418 bp
attB2
Selectable Marker
3'AtSac66
Actin intron
PmeI(2338)
NruI(9696)
RB
pActin
cos Lambda
ori Col EI
LB
Spectinomycin R

Figure 10

A


Total Carotene
290,0
270,0
250,0
230,0
210,0
190,0
170,0
150,0
A
A
A
B
B
B
C
C
C
mean

B

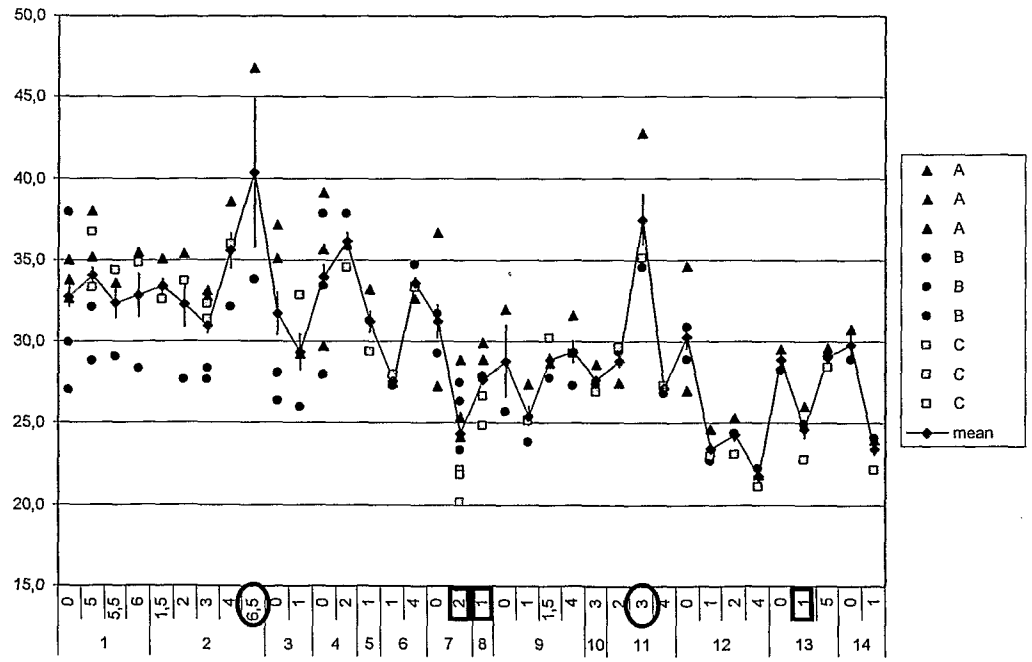
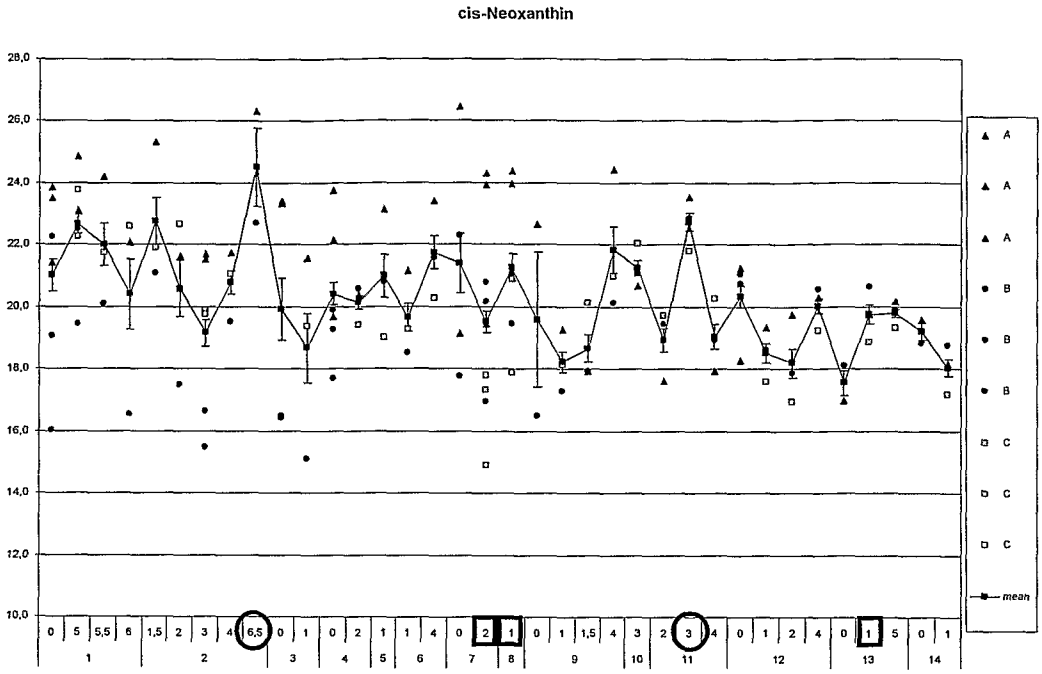

trans-violaxanthin
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
A
A
A
B
B
B
C
C
C
mean

Figure 11

C



D

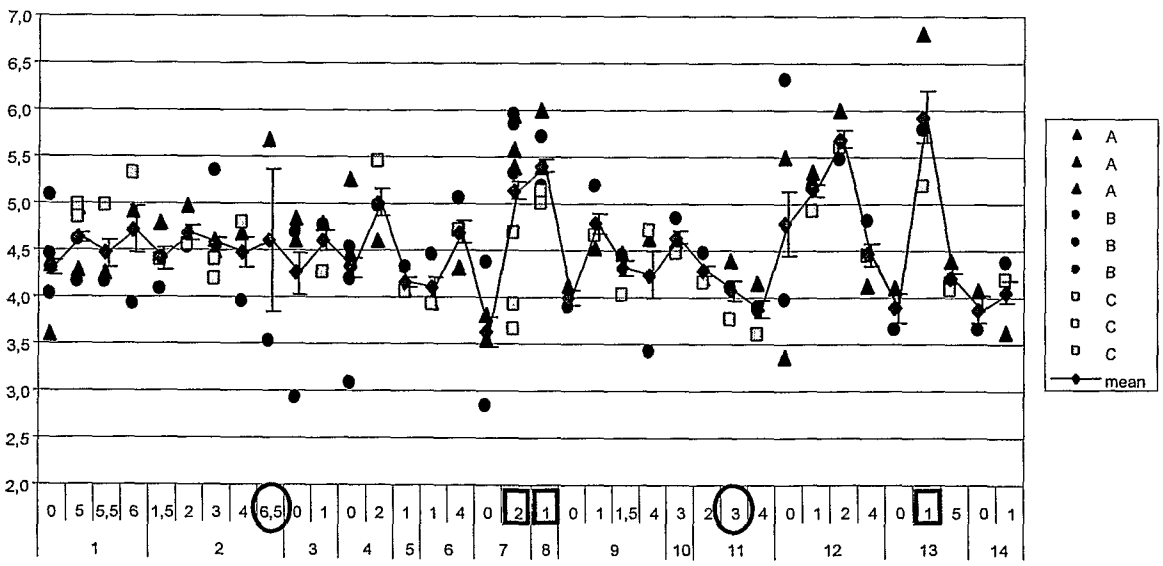


Figure 11 (followed)

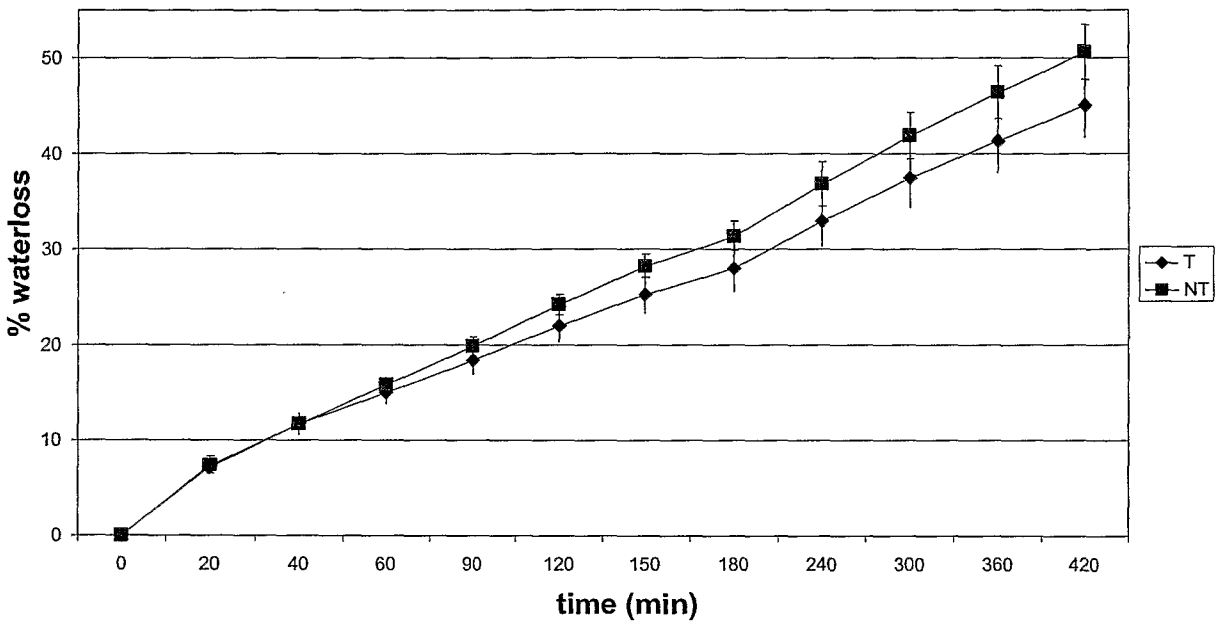

RAA-002b
% waterloss
0
10
20
30
40
50
0
20
40
60
90
120
150
180
240
300
360
420
time (min)
T
NT

Figure 12