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(54) Title: IMPROVED PLANT OIL PRODUCTION

(57) Abstract: The peroxisome located isoform of monodehydroascorbate reductase (MDAR4) is discovered to have a key role in the accumulation and breakdown of oil in plant seeds. MDAR4 forms part of the membrane-associated APX/MDAR system which protects membrane lipids and integral membrane proteins from oxidative damage by H2O2. The APX/MDAR system acts as a cordon to limit the escape of H2O2 from the peroxisome. Also discovered is the close association of oil bodies of seeds with peroxisomes. Transformed plants in which MDAR4 activity is suppressed, whether by mutation of the gene encoding MDAR4, or by sense or antisense suppression for example, have an oil content which is greater than unmodified or wild type plants. Transfected plants or plant cells which overexpress MDAR4 are useful for the screening of inhibitors or antagonists of MDAR4 which in turn are used to suppress MDAR4 activity during seed development and generated higher oil content in seeds.

IMPROVED PLANT OIL PRODUCTION

TECHNICAL FIELD

The present invention relates to plant oils and methods of increasing the amount of oil in plants, including plant parts, e.g. seeds.

BACKGROUND ART

The seeds of many plants contain oil that serves as an essential source of carbon to drive post-germinative growth and allow photosynthetic establishment (Hayashi et al., 1998). The breakdown of this oil is accompanied by the generation of massive amounts of H_2O_2 within the peroxisome because H_2O_2 is formed as a by-product of the acyl-CoA oxidase step of fatty acid β-oxidation (Graham and Eastmond, 2002).

The primary seed storage reserve of many higher plants is triacylglycerol (TAG), which is found in membrane-bound oil bodies. During germination, TAG reserves are broken down and the carbon skeletons used to support post-germinative growth. The initial step in the process is catalysed by a TAG lipase, which hydrolyses TAG at the oil/water interface to yield free fatty acids and glycerol. In most seeds TAG lipase activity is only detectable upon germination and increases concomitantly with the disappearance of TAG. The free fatty acids released by TAG lipase are subsequently converted to sucrose via the sequential action of β-oxidation, the glyoxylate cycle and gluconeogenesis.

Following germination, oil-bearing plant seeds, including for example, *Arabidopsis,* rely on storage oil breakdown to supply carbon skeletons and energy for early seedling growth. Hydrogen peroxide (H_2O_2) is generated within the peroxisome as a by-product of fatty acid β-oxidation.

In WO2004/113543 there is disclosed plant lipase polypeptides which are neutral or acid lipases that have activity toward triacylglycerol. These enzymes are associated with oil bodies via a conserved membrane localisation domain.

WO2006/131750 discloses a lipase with activity towards triacylglycerol and which has no homology with the lipases disclosed in WO2004/113543. The lipase gene is termed Reserve Deposition/Mobilisation 1 (RDM-1). RDM-1 mutants are unable to hydrolyze triacylglycerol indicating an essential role for this lipase in lipid metabolism. The RDM-1 lipase protein is located in the oil body membrane.

In eukaryotes the oxidative metabolism that takes place in peroxisomes generates H_2O_2, which must be detoxified in order to prevent damage to proteins, lipids and DNA (van den Bosch et al., 1992; Singh, 1996; Willekens et al., 1997). This important task is performed universally by catalase, which catalyses the decomposition of H_2O_2 into molecular oxygen and water within the peroxisomal matrix. Defects in catalase cause peroxisome dysfunction and are detrimental in mammals, plants and yeast (Sheikh et al., 1998; Willekens et al., 1997; Zhang et al., 1993; Horiguchi et al., 2001).

Uniquely therefore, in addition to catalase, higher plant peroxisomes possess a membrane bound ascorbate-dependent electron transfer system which is also able to remove H_2O_2 (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996; Mullen and Trelease, 1996; Karyotou and Donaldson, 2005). This system is thought to rely on the cooperative action of ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDAR). APX initiates electron transfer from two molecules of ascorbate to convert H_2O_2 to water and the monodehydroascorbate is then recycled to reduced ascorbate by MDAR via electron transfer from NADH. Monodehydroascorbate can also spontaneously disproportionate to ascorbate and dehydroascorbate and the latter can be reduced back to ascorbate by glutathione-dependent dehydroascorbate reductase (Jiménez et al., 1997; del Rio et al., 1998). Although the importance of catalase is well established, the physiological requirement for a component of the APX/MDAR system has yet to be demonstrated.

Although catalase is highly active in plant peroxisomes, it has a much lower affinity for H_2O_2 than APX, suggesting that at low concentrations H_2O_2 is likely to be preferentially scavenged by the APX/MDAR system (Bunkelmann and Trelease, 1996; Mullen and Trelease, 1996). One hypothesis is that the membrane-association of APX/MDAR allows the system to protect membrane lipids and integral proteins from oxidative damage, and act as a cordon to limit the escape of H_2O_2 into the cytosol (Yamaguchi et al., 1995; Mullen and Trelease, 1996; Karyotou and Donaldson, 2005). MDAR may also play a role in reductant balance within the peroxisome by recycling NAD^+ (Bowditch and Donaldson, 1990; Mullen and Trelease, 1996).

The physiological importance of catalase in maintaining redox balance in plant peroxisomes has been demonstrated in several studies using mutants or antisense suppression (Kendall et al., 1983; Willekens et al., 1997; Takahashi et al., 1997; Vandenabeele et al., 2004).

Over-expression of *APX3*, encoding a peroxisomal isoform from *Arabidopsis thaliana*, has been reported to increase protection against oxidative stress (Wang et al., 1999). However, Narendra et al., (2006) have shown that a null mutant in *APX3* is healthy under normal growth conditions. The role of the peroxisomal APX/MDAR system in plant growth and development is unclear. There is also uncertainty about the role of the APX/MDAR system in relation to catalase.

It has previously been reported that during periods of rapid β-oxidation mammalian peroxisomes can leak H_2O_2 (Mueller et al., 2002).

A number of studies have previously presented electron micrographic evidence to suggest that associations exist in oilseeds cells between oil bodies and other sub-cellular compartments, principally peroxisomes (e.g. Wanner and Theimer, 1978; Hayashi et al., 2001). Chapman and Trelease (1991) provide biochemical evidence that in cotton seedlings neutral lipids are transferred directly from the oil body into the peroxisomal membrane.

In yeast, a close association between oil bodies and peroxisomes has also recently been reported and evidence has been provided that proteins involved in β-oxidation (e.g. acyl-CoA oxidase) are localized close to the inner surface of the peroxisomal membrane at sites of contact with the oil body (Binns et al., 2006).

Proteomic analysis by Job et al., (2005) has established that in wild type *Arabidopsis* seeds and seedlings, proteins from many sub-cellular compartments exhibit a significant level of oxidation. However, no oxidised oil body proteins have been identified.

DISCLOSURE OF THE INVENTION

The inventors have surprisingly found that a conditional seedling-lethal *sugar-dependent2* (*sdp2*) mutant of *Arabidopsis thaliana* is deficient in the peroxisomal membrane isoform of monodehydroascorbate reductase MDAR (MDAR4). The inventors have also surprisingly discovered that the MDAR4 component of the ascorbate-dependent electron transfer system is responsible for detoxifying H_2O_2 which escapes the peroxisome. The inventors have found that this function is necessary to protect oil bodies that are in close proximity to peroxisomes. Without protection from oxidative damage, the triacylglycerol lipase of the oil body membrane is inactivated and this cuts off the supply of carbon for seedling establishment.

The invention therefore provides a method of increasing the oil content of a plant cell comprising reducing or eliminating MDAR4 activity. The method is therefore applicable to increasing the oil content of plant cells, whether in culture or *in vivo* in the form of plant tissues, whole plants, plant parts or seeds.

The reduction or elimination of MDAR4 activity preferably takes place in the peroxisome of the plant cell.

The reduction or elimination of MDAR4 activity preferably takes place during seed development and/or seed maturation, including desiccation of seeds. In plants where MDAR4 activity is reduced or eliminated, a greater accumulation of oil takes place than would otherwise be expected in the case of plants where MDAR4 activity is at a normal (unmodified) level.

Figure 1 shows a general pattern for levels of storage oil and other components during the plant life cycle where MDAR4 activity is not modified. During the oil synthesis phase and prior to desiccation of the seed, oil accumulates to a maximum level, but then generally falls back to a lesser level in the mature seed following desiccation. Breakdown of oil therefore starts to take place during desiccation of the seed and not just during germination. Without wishing to be bound by any particular theory, the inventors believe that an increase in seed oil arising as a result of reducing or eliminating MDAR4 activity takes place because the rate of oil breakdown is reduced compared to when MDAR4 operates at normal levels of activity. In other words, the ratio of oil synthesis rate to oil breakdown rate during seed development and/or desiccation is greater (compared to normal) when MDAR4 activity is reduced or eliminated.

The reduction or elimination of MDAR4 activity may comprise supression of MDAR4 expression. In preferred aspects, the plant is preferably transformed or transfected with a nucleic acid or vector capable of suppressing MDAR4 expression. Such transfection or transformation is preferably stable to the extent that the phenotype may be passed to the next generation.

The reduction or elimination of MDAR4 activity may comprise antisense suppression of MDAR4 expression. Alternatively or in addition, the reduction or elimination of MDAR4 activity may comprise sense suppression of MDAR4 expression.

The method of the invention therefore includes the making of plant cells which are null for MDAR4. Such cells and resultant plants and tissues, include a non-functional copy of the nucleic acid sequence for MDAR4, wherein the activity of the polypeptide encoded

by said nucleic acid is ablated. Methods to provide such a cell are well known in the art and include the use of antisense genes to regulate the expression of specific targets; insertional mutagenesis using T-DNA; the introduction of point mutations and small deletions into open reading frames and regulatory sequences; and double stranded inhibitory RNA (RNAi).

A number of techniques are well known to the average skilled person for specifically ablating genes and/or gene products. One is the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell that results in the destruction of mRNA complementary to the sequence included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The RNAi molecule is typically derived from exonic or coding sequence of the gene which is to be ablated. Surprisingly, only a few molecules of RNAi are required to block gene expression that implies the mechanism is catalytic. The site of action appears to be nuclear as little if any RNAi is detectable in the cytoplasm of cells indicating that RNAi exerts its effect during mRNA synthesis or processing.

RNAi molecules are typically as small as 18 mers, although lengths in the range 16 mers to 50 mers are possible. Lengths of RNAi molecules include 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45 or 49 mers.

Another embodiment of RNAi involves the synthesis of so called "stem loop RNAi" molecules that are synthesised from expression cassettes carried in vectors. The DNA molecule encoding the stem-loop RNA is constructed in two parts, a first part that is derived from a gene the regulation of which is desired. The second part is provided with a DNA sequence that is complementary to the sequence of the first part. The cassette is typically under the control of a promoter that transcribes the DNA into RNA. The complementary nature of the first and second parts of the RNA molecule results in base pairing over at least part of the length of the RNA molecule to form a double stranded hairpin RNA structure or stem-loop. The first and second parts can be provided with a linker sequence. Stem loop RNAi has been successfully used in plants to ablate specific

mRNAs and thereby affect the phenotype of the plant (for example, see Smith *et al* (2000) Nature 407, 319-320).

An effective suppression of MDAR4 expression is a combination of both the sense and the antisense technique. (See Waterhouse *et al* (1998) P.N.A.S. 95: 13959=13964).

In a preferred embodiment of the invention a cassette is provided with at least two promoters adapted to transcribe sense and antisense strands of a nucleic acid molecule encoding MDAR4.

Rapidly hybridizing RNA molecules can be used.

Rapidly hybridizing RNA molecules may be used. The efficiency of antisense RNA molecules which have a size of more than 50 nucleotides will depend on the annealing kinetics in vitro. Thus, e.g., rapidly annealing antisense RNA molecules exhibit a greater inhibition of protein expression than slowly hybridizing RNA molecules (Wagner et al 1994) Annu. Rev. Microbiol., 48: 713-742; Rittner et al. (1993) Nucl. Acids Res. 21:1381-1387). Such rapidly hybridizing antisense RNA molecules particularly comprise a large number of external bases (free ends and connecting sequences), a large number of structural subdomains (components) as well as a low degree of loops (Patzel et al (1998) Nature Biotechnology 16: 64-68) . The hypothetical secondary structures of the antisense RNA molecule may, e.g., be determined by aid of a computer program, according to which a suitable antisense RNA DNA sequence is chosen.

Different sequence regions of the DNA molecule may be inserted into the vector. One possibility consists, e.g., in inserting into the vector only that part which is responsible for ribosome annealing. Blocking in this region of the mRNA will suffice to stop the entire translation. A particularly high efficiency of the antisense molecules also results for the 5'- and 3'-nontranslated regions of the gene.

Preferably the DNA molecules used to transfect according to the invention include a sequence which comprises a deletion, insertion and/or substitution mutation of the MDAR4 gene. The number of mutant nucleotides is variable and varies from a single one to several deleted, inserted or substitutes nucleotides. The reading frame may be shifted by the mutation. In such "knock out" genes it is important that the expression of MDAR4 is disturbed, and the formation of an active, functional protein is prevented. In doing so, the site of the mutation is variable, as long as expression of an active protein is prevented. Preferably, the mutation is in the catalytic region of the protein. The method of introducing mutations in DNA sequences are well known to the skilled person, as are the various possibilities of mutagenesis. Coincidental mutageneses as well as, in particular, directed mutageses, e.g. the site-directed mutagenesis, oligonucleotide-controlled mutagenesis or mutageneses by aid of restriction enzymes may be employed.

The invention may also provide a DNA molecule which codes for a ribozyme which comprises two sequence portions of at least 10 to 15 base pairs each, which are complementary to sequence portions of an inventive DNA molecule as described above so that the ribozyme complexes and cleaves the mRNA which is transcribed from a natural MDAR4 DNA molecule. The ribozyme will recognize the MRNA of the MDAR4 by complementary base pairing with the mRNA. Subsequently, the ribozyme will cleave and destroy the RNA in a sequence- specific manner, before the enzyme is translated. After dissociation from the cleaved substrate, the ribozyme will repeatedly hybridize with RNA molecules and act as specific endonuclease. In general, ribozymes may specifically be produced for inactivation of a certain mRNA, even if not the entire DNA sequence which codes for the protein is known. Ribozymes are particularly efficient if the ribosomes move slowly along the mRNA. In that case it is easier for the ribozyme to find a ribosome-free site on the mRNA. For this reason, slow ribosome mutants are also suitable as a system for ribozymes (J. Burke, 1997, Nature Biotechnology; 15, 414-415). This DNA molecule is particularly advantageous for the downregulation and inhibition, respectively, of the expression of plant MDAR4.

One possible way is also to use a varied form of a ribozyme, i.e. a minizyme. Minizymes are efficient particularly for cleaving larger mRNA molecules. A minizyme is a hammerhead ribozyme which has a short oligonucleotide linker instead of the stem/loop II. Dimer-minizymes are particularly efficient (Kuwabara *et al.*, 1998, Nature Biotechnology, 16; 961-965).

In other ways of operating the invention, the activity of MDAR4 may be reduced or eliminated by an inhibitor or antagonist of MDAR4.

The MDAR4 inhibitor or antagonist is preferably expressed from an heterologous nucleic acid molecule or vector introduced into the plant or is an expression product of a gene endogenous to the genome of the plant.

When an heterologous nucleic acid molecule is introduced into the plant then it may integrated into the host plant genome. The nucleic acid may integrate into a chromosomal location in the the plant genome by a process of homologous recombination.

When a vector is introduced into the plant it may be an epigenetic element or an artificial chromosome.

Preferably, the desired nucleic acid in a vector is operably linked to an appropriate promoter or other regulatory elements for transcription in the host cell. Any workable promoter can be employed. The vector may be a bi-functional expression vector which functions in multiple hosts. In the example of nucleic acids encoding polypeptides according to the invention this may contain its native promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

A promoter is the nucleotide sequence upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Suitable promoters include constitutive, tissue-specific, inducible, developmental or other promoters for expression in plant cells comprised in plants, depending on design. Such

promoters include viral, fungal, bacterial, animal and plant-derived promoters capable of functioning in plant cells.

Constitutive promoters include, for example CaMV 35S promoter (Odell *et al* (1985) Nature 313, 9810-812); rice actin (McElroy *et al* (1990) Plant Cell 2: 163-171); ubiquitin (Christian *et al* . (1989) Plant Mol. Biol. 18 (675-689); pEMU (Last *et al* (1991) Theor Appl. Genet. 81: 581-588); MAS (Velten *et al* (1984) EMBO J. 3. 2723-2730); ALS promoter (U.S. Application Seriel No. 08/409,297), and the like. Other constitutive promoters include those in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680, 5,268,463; and 5,608,142.

The nucleic acid or vector capable of suppressing MDAR4 expression may be a transposable element. Generally, any suitable *Arabidopsis* transposable element may be used. In crop plants, a preferred transposable element is Tilling.

When an MDAR4 inhibitor or antagonist is employed it is preferably applied to the plant. Usually, such inhibitors or antagonists may be applied in aqueous solution in the form of a spray, dip or paste.

When the plant is treated with a nucleic acid or vector, or an inhibitor or an antagonist of MDAR4, then the treatment may be during or at a stage selected from flowering, fertilization, seed setting, desiccation of the seed or during seed storage. In particularly preferred embodiments the inhibition of MDAR4 is specific to MDAR4 and not any of the other MDAR4 isoforms. Nucleic acids which suppress MDAR4 expression may consist of all or part of the unique peroxisomal membrane target motif. Also, the nucleic acids may target the 5' and/or 3' UTRs. Advantageously, inhibition of just the MDAR4 isoform is desirable when increasing plant oil content, particularly with respect to seeds.

The nucleic acid preferably comprises: (a) a nucleotide sequence of SEQ ID NO: 1 or a sequence having at least 50% identity thereto,

(b) a fragment of at least 18 contiguous nucleic acids of (a), or

(c) the complement of (a) or (b).

In preferred embodiments, the nucleotide sequence has at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 99.5% identity with SEQ ID NO: 1.

The nucleic acid preferably comprises a nucleotide sequence encoding:

(a) an amino acid sequence selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6, or an amino acid sequence having at least 50% identity thereto,

(b) a polypeptide of at least 6 contiguous amino acids of (a),

or a strand complementary to (a) or (b).

In preferred embodiments, the amino acid has a sequence of at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 99.5% identity with SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 , SEQ ID NO: 5 or SEQ ID NO:6.

The nucleic acids described above will usually comprise a first stand (as defined) and a second complementary strand. The first and second strands being capable of hybridization with one another and other related strands, depending on the degree of sequence identity.

Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY, 2001); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization:	5x SSC at 65°C for 16 hours
Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization:	5x-6x SSC at 65°C-70°C for 16-20 hours
Wash twice:	2x SSC at RT for 5-20 minutes each
Wash twice:	1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (allows sequences that share at least 50% identity to hybridize)

Hybridization:	6x SSC at RT to 55°C for 16-20 hours
Wash at least twice:	2x-3x SSC at RT to 55°C for 20-30 minutes each.

There are a variety of equations and computer algorithms that can be used to calculate the Melting Temperature (Tm) of a nucleic acid duplex. This is another suitable measure for defining the range of nucleic acid sequences homologous to SEQ ID NO:1, or fragments of such sequences and their complements, that form part of the present invention.

For example, two standard approximation calculations can be used. For sequences less than 14 nucleotides the formula is:

$$T_m = (wA+xT) * 2 + (yG+zC) * 4$$

where w,x,y,z are the number of the bases A,T,G,C in the sequence, respectively (from Marmur, J., and Doty, P. (1962) *J Mol Biol* **5**:109-118).

For sequences longer than 13 nucleotides, the equation used is:

$$T_m = 64.9 + 41*(yG+zC-16.4)/(wA+xT+yG+zC)$$

See Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T., and Itakura, K. (1979) Nucleic Acids Res 6:3543-3557 (Abstract) and Sambrook, J., and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY. (CHSL Press)

The assumptions made for both of the above equations is that the annealing occurs under the standard conditions of 50 nM nucleic acid, 50 mM Na^+, and pH 7.0.

The nucleic acid sequence may be of greater identity than 50% as described above. The nucleic acid sequence may have an identity of at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or at least 99% identity with the nucleic acid sequence of SEQ ID NO:1, or the nucleic acid sequence encoding the protein of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6.

The amino acid sequences of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 also include amino acid sequences of at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or at least 99% identity therewith.

The polypeptides encoded by SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 may be modified by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are

considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and aspartic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants which retain or enhance the same biological function and activity as the reference polypeptide from which it varies.

The nucleic acid sequence preferably encodes a deficient or at least partially inactive monodehydroascorbate reductase 4 (MDAR4).

The way in which a DNA construct or vector is introduced into a plant host is not critical to the invention. Various methods for plant cell transformation include the use of Ti- or Ri- plasmids, microinjection, electroporation, DNA particle bombardment, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of oils. The germination of seeds deficient or reduced in MDAR4 activity may require exogenously supplied sucrose and/or other growth promoting substances to overcome the effect of a lack of oil breakdown and lack of seedling establishment.

Once a transgenic plant is obtained which is capable of producing seed having increased

amounts of oil compared to the unmodified plant, traditional plant breeding techniques, including methods of mutagensis, may be employed to further manipulate the fatty acid composition. Additional foreign fatty acid modifying DNA sequences may be introduced via genetic engineering to further manipulate the fatty acid composition.

The invention therefore provides the products of the method of the invention for increasing oil content of a plant cell; namely plant cells, plant tissues, plant organs and parts, as well as whole plants and their propagative materials, particularly seeds. All of the aforementioned plant cell characteristics described in connection with the method of the invention apply equally to the products of the invention.

The invention also provides a plant cell transformed or transfected with nucleic acid capable of reducing or eliminating monodehydroascorbate reductase (MDAR4) activity. In such a plant cell the reduction or elimination of MDAR4 activity is preferably in the peroxisome.

The reduction or elimination of MDAR4 activity in the plants and plant cells preferably comprises supression of MDAR4 expression.

The plant cell is preferably transformed or transfected with a nucleic acid or vector capable of suppressing MDAR4 expression.

The vector preferably comprises a cell or tissue specific promoter.

The promoter is preferably an inducible promoter or a developmentally regulated promoter.

Chemical-regulated promoters may be used to modulate the expression of the desired nucleic acid sequence in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induced gene expression, or a chemical-

repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and may include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al* (1991) Proc. Natl. Acad. Sci. USA 88: 10421-10425 and McNellie *et al.* (1998) Plant J. 14(2): 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) Mol. Gen. Genet. 227: 229-237, and US Patent Nos. 5,814,618 and 5,789,156.

Where enhanced expression in particular tissues is desired, tissue-specific promoters may be utilised. Tissue-specific promoters include those described by Yamamoto *et al.* (1997) Plant J. 12(2): 255-265; Kawamata *et al* (1997) Plant Cell Physiol. 38(7): 792-803; Hansen *et al* (1997) Mol. Gen. Genet. 254(3): 337-343; Russell *et al.* (1997) Transgenic Res. 6(2): 157-168; Rinehart *et al* (1996) Plant Physiol. 112(3): 1331-1341; Van Camp *et al* (1996) Plant Physiol. 112(2): 525-535; Canevascni *et al* (1996) Plant Physiol. 112(2): 513-524; Yamamoto *et al* (1994) Plant Cell Physiol. 35(5): 773-778; Lam (1994) Results Probl. Cell Differ. 20: 181-196; Orozco *et al* (1993) Plant Mol. Biol. 23(6): 1129-1138; Mutsuoka *et al* (1993) Proc. Natl. Acad. Sci. USA 90(20): 9586-9590; and Guevara-Garcia *et al* (1993) Plant J. 4(3): 495-50.

In a preferred embodiment of the invention said tissue specific promoter is a promoter which is active during the accumulation of oil in developing oil seeds, (for example see Broun *et al.* (1998) Plant J. 13(2): 201-210).

When a particular nucleic acid sequence is comprised in a vector or construct so as to be operably linked then it is linked and part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA

operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Particular vectors are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809).

Vectors may also include selectable genetic marker such as those that confer selectable phenotypes such as resistance to herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

The methods of the invention may be used in relation to all kinds of plants including Gymnosperms, Pteridophytes and Bryophytes. Of particular industrial utility are algae, including both freshwater and marine algae, single or multicellular. Included within the scope of the invention are algal cells for the production of oils, whether in cell culture or in whole plant form.

For all aspects of the invention, the plant cell may be comprised in a plant selected from a monocot or a dicot.

The plant cell of all aspects of the invention may be comprised in a plant selected from the families Brassicaceae or Compositae, or as listed in table 5.

In preferred embodiments of all aspects of the invention, the cell, tissue or plant is selected from: corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Tritium aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet

potato (*Iopmoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (Citrus spp.) cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avacado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

Preferably, the plants are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea), and other root, tuber or seed crops. Important seed crops are oil-seed rape, sugar beet, maize, sunflower, soybean, sorghum, and flax (linseed). Horticultural plants to which the present invention may be applied may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper.

Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava been, lentils, chickpea, etc.

The invention also provides a plant, plant organ or plant tissue comprising a plant cell of the invention as herein defined. In preferred embodiments, the invention provides a plant seed comprising a plant cell as defined herein.

The invention further provides a method of identifying an agent which increases the oil content of a seed, comprising contacting a plant deficient in monodehydroascorbate reductase (MDAR4), harvesting the mature or developing seed and determining the oil

content of the seed. The oil content of a seed is preferably determined by measuring fatty acid content of the seed.

The invention therefore provides a plant cell overexpressing monodehydroascorbate reductase (MDAR4).

The invention includes plant tissue or a plant comprising a plant cell which overexpresses monodehydroascorbate reductase (MDAR4). The overexpression is measured in relation to a native, wild-type or non-transgenic plant of the same species, variety or cultivar.

The overexpression of MDAR4 may be least 2-fold above basal level expression, optionally at least 5-fold; 10-fold, 20-fold, 30-fold, 40-fold, or 50-fold. The cell may overexpress the MDAR4 gene by at least 100-fold above basal level expression when compared to a non-transgenic cell of the same species.

It will be apparent that means to increase the activity of a polypeptide encoded by a nucleic acid molecule are known to the skilled person. For example, and not by limitation, increasing the gene dosage by providing a cell with multiple copies of said gene. Alternatively or in addition, a gene(s) may be placed under the control of a powerful promoter sequence or an inducible promoter sequence to elevate expression of mRNA encoded by said gene. The modulation of mRNA stability is also a mechanism used to alter the steady state levels of an mRNA molecule, typically via alteration to the 5' or 3' untranslated regions of the mRNA.

The overexpression of MDAR4 may be engineered to take place during specific phases of seed development, e.g. post-fertilization and/or seed setting.

The invention also provides a method of identifying an agent which increases the oil content of a plant cell, tissue or seed, comprising contacting a plant tissue or plant which overexpresses monodehydroascorbate reductase (MDAR4) with a candidate agent and

then determining the oil content of the cell, tissue or seed. The oil content of a seed is preferably determined by measuring the fatty acid content of the seed.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

The invention will now be described in more detail and by way of example having regard to the drawings in which:

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows in schematic form the patterns of storage oil synthesis and breakdown during the plant life cycle of *Arabidopsis*.

Figure 2 shows post-germinative growth and fatty acid breakdown in *sdp2*.

(A) Images of 5 d old *sdp2* and wild type seedlings grown on medium with and without 1% (w/v) sucrose. The scale bar is 1 cm.

(B) Total fatty acid and (C) eicosenoic acid (20:1) content of *sdp2* and wild type seeds and 5 d old seedlings grown on medium with 1% (w/v) sucrose. Values are the mean ± SE of measurements on five batches of 20 seeds or seedlings.

Figure 3 shows the molecular characterization of *SDP2*.

(A) Mapping *SDP2*. PCR based SSLP, CAPS and SNAP markers were used to map *SDP2* to an 80 kb region on the top arm of Chromosome 3 near GL1. The

positions of markers a to f (listed in Table 1) are denoted by bars and the number of recombination events / total number of chromosomes (380) are listed below each.

(B) Schematic diagram of the *SDP2* locus showing mutations in At3g27820 (*MDAR4*). Black bars are exons and the white bar and arrow represent the 5' and 3' UTRs, respectively. Insertion / substitution positions are numbered relative to the ATG. A full-length cDNA clone of *SDP2* has previously been published by the SSP consortium (GenBank accession number AY039980).

(C) Complementation of *sdp2-4* by transformation with a genomic copy of *MDAR4*. Images of 5 d old *sdp2-4* (left) and *sdp2-4 MDAR4* (right) seedlings grown on medium without sucrose. The scale bar is 1 cm.

(D) *SDP2* transcript levels in 2 d old wild type and *sdp2* seedlings detected using RT-PCR. The PCR primers amplify a 568 bp product from the 3' end of the cDNA.

(E) SDP2 protein in peroxisomes purified from wild type and *sdp2-4* seedlings detected using Western blotting. *The larger polypeptide band corresponding to MDAR4 is 54 kDa.

Figure 4 shows peroxisome clustering and proximity to oil bodies in *sdp2* seedlings.

(A) Confocal images of epidermal cells.

(B) EM images of parenchyma cells of cotyledons from 5 d old *sdp2-4* and wild type seedlings grown on medium containing 1% (w/v) sucrose. For confocal microscopy peroxisomes were visualized using a genetic background containing PTS1 targeted GFP driven by the 35S promoter (Cutler et al., 2000). P is peroxisome, OB is oil body and C is chloroplast. In (A) and (B) scale bars are 10 μm and 1 μm, respectively.

Figure 5 shows β-oxidation-dependent oxidative damage to oil bodies in *sdp2* seedlings.

(A) Rate of [^{14}C]triolein hydrolysis.

(B) lipid hydroperoxides (LOOHs) levels.

(C) protein carbonyl levels in oil bodies purified from 2 d old *sdp2-4*, *pxa1*, *sdp2-4 pxa1* and wild type seedlings. The concentration of triolein was equivalent to 10 mM. Values are the mean ± SE of measurements on five separate preparations.

Figure 6 shows carbonylation and inactivation of SDP1.

(A) Effect of H_2O_2 concentration on recombinant SDP1 activity and level of carbonylation. Purified recombinant SDP1 was pre-incubated with H_2O_2 for 2 h at 22°C before the protein was assayed for either lipase activity or carbonyl levels. Lipase assays were performed using 10 mM [^{14}C]triolein as a substrate. Prior to the addition of the substrate H_2O_2 was removed from the samples by adding 10 units ml^{-1} of catalase and incubating for 10 min at 22°C. Catalase assays confirmed that essentially all the H_2O_2 is removed by this treatment. Values are the mean ± SE of measurements from four separate incubations.

(B) Activity of recombinant SDP1 on oil bodies purified from 2 d old *sdp2-4* and wild type seedlings. Values are the mean ± SE of measurements from four separate incubations.

(C) Detection using anti-DNP antibodies of carbonyl groups in SDP1 immunoprecipitated from oil body membranes of 2 d old *sdp2-4* and wild type seedlings.

(D) Detection of SDP1 protein in purified oil body membranes from 2 d old *sdp2-4* and wild type seedlings, using anti-SDP1 antibodies.

Figure 7 shows metabolite levels in germinating *sdp2* seedlings.

(A) H_2O_2.

(B) total ascorbate (ascorbate + dehydroascorbate).

(C) reduced ascorbate as a % of total in 1 d old and 3 d old *sdp2-4*, *pxa1*, *sdp2-4 pxa1* and wild type seedlings germinated on medium containing 1% (w/v) sucrose. nd, not determined. Values are the mean ± SE of measurements on five separate extracts.

Figure 8 shows β-Oxidation of 2,4-dichlorophenoxybutyric acid (2,4-DB) by *sdp2* seedlings.

(A) Effect of 2,4-DB concentration.

(B) 2,4-D concentration on root length of 5 d old *sdp2-4*, *pxa1*, *sdp2-4 pxa1* and wild type seedlings grown on medium containing 1% (w/v) sucrose. Values are the mean ± SE of measurements on five batches of 20 seedlings.

Figure 9 shows the effect of catalase deficiency on fatty acid breakdown during post-germinative growth.

(A) Total fatty acid.

(B) eicosenoic acid (20:1) content of seeds and 5 d old seedlings grown on medium with 1% (w/v) sucrose. Col4 is wild type, Pthw is empty vector control and CAT2AS, CAT2HP1 and CAT2HP2 are catalase deficient lines (Vandenabeele et al., 2004). Values are the mean ± SE of measurements on five batches of 20 seeds or seedlings.

(C) Catalase activities in extracts from 2 d old seedlings of Col4, Pthw, CAT2AS, CAT2HP1 and CAT2HP2. Values are the mean ± SE of measurements on three separate extracts.

Figure 10 shows a schematic diagram illustrating the proposed role of MDAR4 in storage oil breakdown in germinating *Arabidopsis* seeds. ASC is ascorbate, MDA is monodehydroascorbate, SDP1 is a lipase, PXA1 is an ABC transporter, ACX is acyl-CoA oxidase, CAT is catalase, APX is ascorbate peroxidase and SDP2 or MDAR4 is monodehydroascorbate reductase.

Figure 11 is the cDNA sequence of SDP2/MDAR4 (At3g27820) SEQ ID NO:1

Figure 12 is the amino acid sequence of SDP2/MDAR4 (At3g27820) SEQ ID NO:2

Figure 13 is an amino acid sequence alignment of MDAR isoforms from *Arabidopsis* with SDP2/MDAR4 (SEQ ID NO:2) generated using ClustalX (version 1.83). The

isoform sequences are At5g03630 (SEQ ID NO:3); At3g09940 (SEQ ID NO:4); At3g52880 (SEQ ID NO: 5) and A1g63940 (SEQ ID NO: 6). $Gly^{11,}$ Val^{14} and Gly^{386} from SDP2/MDAR4 (At3g27820) are marked in red.

EXAMPLES

Plant material and growth conditions

Wild type *Arabidopsis thaliana* (ecotype Colombia 0 and Landsberg *erecta*) were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). The *sdp2-4* mutant (SALK_068667) was obtained from T-DNA express (Alonso et al., 2003). The PTS1 targeted GFP line A5 (Cutler et al., 2000), the *pxa1* mutant (Zolman et al., 2001) and catalase deficient lines (Vandenabeele et al., 2004) were kindly provided by Prof. Chris Somerville (Carnegie Institution, Stanford University, CA, USA), Prof. Bonnie Bartel (Rice University, Houston, Texas, USA) and Dr. Frank Van Breusegem (Ghent University, VIB, Belgium), respectively. Seeds were surface sterilised, applied to agar plates containing ½ strength MS salts (Sigma-Aldrich, Poole, Dorset, UK) and imbibed in the dark for 4 d at 4°C. The plates were then transferred to a growth chamber set to 21°C (16h light/8h dark; *PPFD* = 150 μmol m^{-2} s^{-1}). In some experiments 1% (w/v) sucrose, 2,4-dichlorophenoxybutyric acid (2,4-DB) or 2,4-dichlorophenoxyacetic acid (2,4-D) were added to the agar medium.

Metabolite analysis

The fatty acid composition of seed and seedling lipids was measured by CG analysis after combined digestion and fatty acid methyl ester formation from frozen tissue using the method of Browse et al., (1986). H_2O_2 and ascorbate (ascorbate and dehydroascorbate) were extracted from 0.1g FW of seedlings and measured spectophotometrically using the methods described by Huang et al., (2005).

Mapping

The *sdp2-1* mutant was out-crossed to wild type ecotype Landsberg *erecta*. F1 plants were allowed to self fertilise and the F2 progeny were screened for the sugar-dependent phenotype. Genomic DNA was isolated from 190 F2 *sdp2-1* lines using the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, Poole, Dorset, UK). Mapping was carried out using simple sequence polymorphisms (Bell and Ecker, 1994), cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993) and single-nucleotide amplified polymorphisms (Drenkard et al., 2000) utilizing information from the Monsanto *Arabidopsis* polymorphism collection (Jander et al., 2002). New markers used in this study are listed in Table 1 below. Candidate genes within the mapping interval were amplified from *sdp2-1*, *sdp2-2* and *sdp2-3* genomic DNA by PCR and sequenced to identify mutations.

Table 1. New SSLP and CAPS markers used to map *SDP2*.

BAC	Name	type	Primers	Col0	Ler	Enzyme
KIG2	a	SSLP	AGGGACAGTTTTCCAAAGCATA – SEQ ID NO: 7 CAAAGTTACTTAATTTCCATAACCAA – SEQ ID NO: 8	173bp	159bp	
MGF10	b	CAPS	CTAAATCATCACTTTTTGGATTCGTA – SEQ ID NO: 9 TGTGGTTAAACTCTTAGGTTGTTGTC – SEQ ID NO: 10	895bp	-	XbaI
K16N12	c	SSLP	GAGGAAATGTGGTGATGTAGCA – SEQ ID NO: 11 CTCTTCCTCGACGACAAACTCT – SEQ ID NO: 12	302bp	278bp	
K16N12	d	SSLP	AAACAATTGTGAAGATGCAAGG – SEQ ID NO: 13 GCATCTAGAGGGGATTTGCTTA – SEQ ID NO: 14	143bp	125bp	
K24A2	e	SSLP	CCATCAAACCATACATGTCACC – SEQ ID NO: 15 GATGAGGAGAGTCCTGAGCTTG – SEQ ID NO: 16	333bp	-	HinfI

MIG10	f	CAPS	TGATTCCGGGTTTCAATTATTC CAATGCCTTGATCCTCCATACT	500bp	-	HapII

Transcript and protein analysis

DNAse-treated total RNA was isolated from *Arabidopsis* seedlings using the RNeasy kit from Qiagen Ltd. (Crawley, West Sussex, UK). The synthesis of single stranded cDNA was carried out using SuperScript™ II RNase H⁻ reverse transcriptase from Invitrogen Ltd. (Paisley, UK). *SDP2* transcripts were detected by PCR using primers SDP2F (5'-CAAAGACGGGAGCCACTTAC-3'- SEQ ID NO: 16) and SDP2R (5'-CTGCTGACTCACAACCGTGT-3 – SEQ ID NO: 17'). Measurement of total protein levels, SDS-polyacrylamide gel electrophoresis and Western blot analysis were carried out as described previously (Eastmond, 2004). Rabbit anti-cucumber MDAR antiserum (Sano et al., 1995), anti-DNP antibody (Millipore) and anti-SDP1 antibody were used as primary antibodies, at dilutions of 1 in 1000, 1 in 30 and 1 in 500, respectively. The SDP1 antibody was raised against the SDP1 specific peptide SSEDSGLQEPVSGSVC by Eurogentec (Belgium) and affinity purified.

Microscopy

TEM was carried out as described previously (Eastmond, 2006). Five d old *Arabidopsis* seedlings were fixed for 2 h in 2.5 % (v/v) glutaraldehyde, 4 % (v/v) formaldehyde in 100 mM phosphate buffer (pH 7.0), with a secondary fixation of 1 % (w/v) osmium tetroxide in phosphate buffer for 1 h. The tissue was embedded in Spurrs resin, sectioned and stained with uranyl acetate and Reynolds lead citrate. Confocal microscopy was performed using a Zeiss LSM 510 Meta on an Axioplan 2M, fitted with a 63 x PlanApo lens (NA 1.4). The sample was excited with a 488 nm Argon laser and GFP emission collected via a 505-530 nm BP filter. Bright field images were captured simultaneously with the transmission detector.

Organelle purification, protein purification and measurement of oxidative damage

Oil bodies and oil body membranes were purified from 2 d old *Arabidopsis* seedlings, and recombinant N-terminal His_6-tagged SDP1 was expressed and purified using protocols that were described previously (Eastmond, 2006). Peroxisomal fractions were obtained from homogenates of 5 d old etiolated *Arabidopsis* seedlings using sucrose density gradient centrifugation as described previously (Eastmond et al., 2000b). The levels of LOOHs in purified oil body lipids were estimated using the FOX (ferrous oxidation xylenol orange) assay following the protocol described in Sattler et al., (2004). The only modification to the method was that the lipids were extracted from oil bodies in 1.5 ml tubes without homogenization. The levels of protein carbonyls in oil body membranes and purified recombinant SDP1 was determined using the spectrophotometric quantification method described by Nguyen and Donaldson (2005). SDP1 was immunoprecipitated from oil body membranes using the IP50 Protein G Immunoprecipitation Kit (Sigma) and carbonyl groups were detected using the OxyBlot™ Protein Oxidation Detection Kit (Millipore) as described by Nguyen and Donaldson (2005).

Enzyme assays

Triacylglycerol lipase activity was measured in purified oil body membranes and recombinant SDP1 using an emulsion of [^{14}C]triolein as described previously (Eastmond, 2006). Purified oil bodies were also used as a substrate for recombinant SDP1 using the assay procedure described in Eastmond, (2006). For assays of peroxisomal enzymes 2 d old seedlings grown on medium containing 1% (w/v) sucrose were ground in a pestle and mortar with 1 ml of buffer (150 mM Tris/HCl pH 7.5, 10 mM KCl, 1 mM EDTA, 10 mM FAD, 10 % (v/v) glycerol). The extract was clarified by centrifugation at 15,000 *g* for 30 min at 4°C and the supernatant was desalted using a Sephadex G-50 spin column. The extract was assayed spectrophotometrically for acyl-CoA oxidase, enoyl-CoA hydratase,

L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, isocitrate lyase, malate synthase, catalase and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase activitities according to methods described previously (Eastmond et al., 2000a; Rylott et al., 2006; Takahashi et al., 1997; Hancock et al., 2005).

Complementation of *sdp2-4*

A region of genomic DNA containing *MDAR4* was amplified from *Arabidopsis* using primers 5'-atctcatgattgagtgggtgattggttg-3'- (SEQ ID NO: 18) and 5'-agcttcttcgagggttagggatgagatt-3' (SEQ ID NO: 19) and the product was cloned into the pCR2.1-TOPO vector from Invitrogen. Using standard molecular-biology techniques, *MDAR4* was excised and cloned into the pGREENII vector (Hellens et al., 2000). The *MDAR4* construct was transformed into *Agrobacterium tumefaciens* strain GV3101 containing the pSOUP vector (Hellens et al., 2000) by electroporation and into *Arabidopsis sdp2-4* ecotype Columbia by the floral dip method (Clough and Bent, 1998). Transformants containing the T-DNA were selected by screening for loss of a sugar-dependent phenotype and the presence of wild type *MDAR4* transcripts was confirmed by RT-PCR.

Example 1 - SDP2 is required for storage oil hydrolysis in germinating *Arabidopsis* seeds

In *Arabidopsis* seeds the breakdown of stored triacylglycerol (TAG) following germination is essential to drive the initial phase of seedling growth and allow photosynthetic establishment (Hayashi et al., 1998). To investigate this process, a forward genetic screen was used to isolate a collection of *Arabidopsis* mutants that require an alternate exogenous source of carbon (sucrose) to support post-germinative growth (Eastmond, 2006). Screening these *sugar-dependent* (*sdp*) mutants for defects in TAG hydrolysis revealed that *sdp1*, *sdp2* and *sdp3* are defective in the lipase activity that is associated with the oil body membranes. The patatin domain TAG lipase encoded by *SDP1* has been characterized previously (Eastmond, 2006).

The *sdp2* mutant germinates normally (see Table 2 below) but the cotyledons fail to expand or green and seedling growth arrests (see Figure 2A).

Table 2. Frequency of *sdp2* germination.

Time (DAI)	Germination (%)				
	Wild type	*sdp2-1*	*sdp2-2*	*sdp2-3*	*sdp2-4*
0	0	0	0	0	0
1	14	6	4	8	11
2	89	66	59	70	81
3	99	79	71	82	89
4	99	85	78	93	98

Seeds were sterilized and imbibed on agar plates containing ½ MS (pH 5.7) for 4 d in the dark at 4°C and germinated in light (16h light/8h dark, *PPFD* = 150 μmol m^{-2} s^{-1}) at 21°C. Germination was scored as radicle emergence and is expresses as a percentage of 500 seeds. DAI is days after imbibition.

Providing sucrose rescues the growth phenotype (Figure 2A) and once *sdp2* seedlings establish photosynthetic competence they grow normally and complete their life cycle. Analysis of the fatty acid content of *sdp2* seeds germinated in the presence of sucrose revealed that the mutant is severely impaired in its ability to breakdown TAG (see Figures 2B and 2C). Eicosinoic acid is specifically found in TAG in *Arabidopsis* (Lemieux et al., 1990) and therefore it can be used as a convenient marker to monitor TAG breakdown (Eastmond et al., 2000a). The levels of both total fatty acids and eicosinoic acid (20:1) decline by less than 20 % over the course of the first 5 d of post-germinative growth in *sdp2*, while in wild type seedlings they fall by 60% and 95%, respectively.

Example 2 - SDP2 encodes a component of the peroxisomal antioxidant system

The *SDP2* locus was mapped to a region on Chromosome 3 near *GLABRA1,* and between PCR-based markers ciw11 and snap77 (see Figure 3A). Further mapping reduced the

interval to a ~80 kb region, containing 22 open reading frames. Sequencing candidate genes within this region revealed that three independent ethyl methanesulphonate (EMS) *sdp2* alleles contained mutations in At3g27820 (Figure 3B). This gene encodes an isoform of monodehydroascorbate reductase (MDAR4) that is associated with the peroxisomal membrane (Lisenbee et al., 2005).

In the *sdp2-1* mutant allele a T to C mutation causes a Val to Ala amino acid substitution at position +14 in the polypeptide sequence (Figure 3B). In the *sdp2-2* and *sdp2-3* mutant alleles A to G mutations give rise to Gly to Glu amino acid substitutions in the polypeptide sequence at positions +11 and +386, respectively (Figure 3B). An amino acid sequence alignment suggests that the Gly residues at positions +11 and +386 in MDAR4 are conserved among all *Arabidopsis* MDAR isoforms (Obara et al., 2002). The Val residue at position +14 is also conserved in MDAR1, 2, 3 and 4, but not in MDAR5 and 6. These last two isoforms are encoded by At1g63940 and they contain N-terminal targeting signals for either the chloroplast or the mitochondrion, depending on the transcriptional start site (Obara et al., 2002). A T-DNA allele of *MDAR4* (*sdp2-4*) that contains an insertion in exon 6 (Figure 3B) was also obtained from the SALK collection (Alonso et al., 2003). This line exhibits the same phenotype as the EMS alleles (Figure 2A). To confirm that the phenotype of *sdp2-4* is caused by a defect in *MDAR4* the mutant was complemented by transformation with a construct carrying a genomic copy of the gene (Figure 3C).

RT-PCR performed on RNA from 2 d old seedlings showed that *MDAR4* transcripts are present in wild type, *sdp2-1*, *sdp2-2* and *sdp2-3* but are absent from *sdp2-4* (Figure 3D).

To investigate whether MDAR4 protein is present in plants having the *sdp2-4* allele Western blots were carried out on peroxisome enriched fractions obtained by sucrose gradient centrifugation of crude seedling extracts (Eastmond et al., 2000b). A polyclonal antibody was used that was raised against a 47 kDa MDAR from cucumber (Sano et al., 1995). This antibody has been shown previously to recognize two polypeptides in purified *Arabidopsis* peroxisomes (Lisenbee et al., 2005). The 47 kDa band corresponds

to the matrix isoform MDAR1 and the 54 kDa band corresponds to the membrane isoform MDAR4. Both bands were present in peroxisomal fractions from wild type but the 54 kDa band was specifically absent in peroxisomal fractions from *sdp2-4* (Figure 3E).

Example 3 - Peroxisomes and oil bodies cluster together in *sdp2* seedlings

Confocal microscopy was used to investigate peroxisome morphology and proximity to oil bodies in living cells of *sdp2* seedlings. In epidermal cells from the cotyledons of 5 d old *sdp2-4* seedlings GFP-labelled peroxisomes formed large (5 to 10 μm) clusters that are associated with groups of transparent spherical un-degraded oil bodies (Figure 4A). In contrast, in wild type, essentially no oil bodies remained by this stage of development and the individual peroxisomes were dispersed throughout the cytosol (Figure 4A). EM images of sections through parenchyma cells from the cotyledons of 5 d old seedlings also suggest an association between oil bodies and peroxisomes (Figure 4B). The striking peroxisome clustering phenotype in *sdp2-4* was only observed in seedling tissues that contained un-degraded oil bodies and was not obvious in tissues from other stages in plant development under the growth conditions used in this study (data not shown).

Example 4 - β-oxidation causes oxidative damage to oil bodies in *sdp2* seedlings

During the post-germinative growth of oilseeds acyl-CoA oxidase, which is the first enzyme of peroxisomal fatty acid β-oxidation, is likely to be the major source of H_2O_2 production in the cell (Graham and Eastmond, 2002). In order to determine whether fatty acid β-oxidation inhibits oil body lipase activity in *sdp2* seedlings the *sdp2-4* mutant was crossed into the fatty acid catabolism deficient mutant *pxa1*. The PXA1/CTS/PED3 protein is an ABC transporter, which is required to import substrate (fatty acids or acyl-CoAs) into the peroxisome for β-oxidation (Zolman et al., 2001). TAG lipase activity was almost undetectable in oil body membranes prepared from 2 d old *sdp2-4* seedlings (Figure 5A). However, lipase activity was recovered to wild type levels in the *sdp2-4 pxa1* double mutant (Figure 5A). To investigate whether oil bodies from *sdp2* incur

oxidative damage lipid peroxidation was estimated using the FOX (ferrous oxidation xylenol orange) assay (Griffiths et al., 2000) and protein oxidation was monitored by detecting protein carbonyls (Levine et al., 1990). Both lipid hydroperoxides (LOOHs) and protein carbonyl levels were significantly elevated in oil bodies purified from 2 d old *sdp2-4* seedlings verses wild type and in both cases these increased levels were suppressed in the *pxa1* background (Figure 5B,C).

Example 5 - SDP1 is a target of oxidative damage in *sdp2* seedlings

In vitro incubation of purified recombinant SDP1 with H_2O_2 showed that the protein can be inactivated by concentrations greater than 0.5 mM (Figure 6A). The loss of catalytic activity caused by H_2O_2 was accompanied by oxidation of the protein, as indicated by a 20-fold increase in the level of carbonylation (Figure 6A). Enzyme assays showed that recombinant SDP1 is still able to hydrolyse oil bodies purified from 2 d old *sdp2* seedlings, but at a significantly lower rate than from wild type (Figure 6B). This suggests that peroxidation of substrate and/or oxidation of additional oil body proteins also forms a partial barrier to oil hydrolysis by SDP1.

To investigate whether SDP1 suffers oxidative damage in *sdp2-4* seedlings the protein was purified from oil body membranes by immunoprecipitation using affinity purified polyclonal antibodies raised against an SDP1 peptide. The purified protein was then reacted with 2,4-dintrophenylhydrazine (DNP) to derivatise carbonyl groups and the carbonyl groups were detected with anti-DNP antibodies. Carbonyls could only be detected in SDP1 purified from *sdp2-4* oil body proteins and not from wild type (Figure 6C). The polyclonal antibody raised against the SDP1 peptide detected similar amounts of SDP1 in oil body protein samples from *sdp2-4* and wild type (Figure 6D). Together these data suggest that *SDP1* is transcribed, translated and targeted to oil bodies in *sdp2* mutant seedlings, but that it is then inactivated by oxidative damage.

Example 6 - An initial increase in H_2O_2 levels in *sdp2* is likely to result from a failure to recycle ascorbate

H_2O_2, the total ascorbate pool (ascorbate + dehydroascorbate) and the percentage of the ascorbate pool that is reduced were all measured in 1 d old and 3 d old *sdp2-4* seedlings grown on medium with sucrose (Huang et al., 2005). At d 1 the total ascorbate pool in *sdp2-4* was similar to wild type but the percentage of reduced ascorbate was lower and the level of H_2O_2 was increased (Figure 7). In contrast, at d 3 H_2O_2 levels in *sdp2-4* were lower than those of wild type (Figure 7A). The production of H_2O_2 in *sdp2-4* and wild type seedlings was strongly suppressed in a *pxa1* background at both d 1 and d 3 (Figure 7A). Without wishing to be bound by any particular theory, the inventors propose a model in which, immediately following *sdp2* germination, the production of H_2O_2 by fatty acid β-oxidation leads to a negative feedback loop whereby the H_2O_2 inactivates SDP1 and progressively cuts off the supply of substrate for β-oxidation, which in turn reduces H_2O_2 production.

Example 7 - Seedlings of *sdp2* retain the capacity to β-oxidize 2,4-dichlorophenoxybutyric acid

The *sdp2* mutant was grown on medium containing 2,4-dichlorophenoxybutyric acid (2,4-DB). This compound is converted to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by a single cycle of β-oxidation (Hayashi et al., 1998). *Arabidopsis* mutants in many genes that are either directly required for fatty acid β-oxidation or for peroxisome function in general exhibit a 2,4-DB resistant phenotype (see Baker et al., 2006). To date this list of mutants includes *pxa1/cts/ped3, acx1, acx3, acx4, aim1, ped1/kat2, cys2 cys3, chy1, ped2/pex14, pex4, pex5* and *pex6*. However, unlike these mutants, root growth in *sdp2-4* seedlings does not exhibit increased resistance to 2,4-DB (Figure 8), indicating that many peroxisomal membrane and matrix proteins remain at least partially functional. *In vitro* assays performed on extracts of 2 d old *sdp2-4* seedlings also show that the activities of peroxisomal marker enzymes, acyl-CoA oxidase, enoyl-CoA hydratase, L-3-

hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, isocitrate lyase, malate synthase and catalase are all similar to wild type (see Table 3 below).

Table 3. Peroxisomal and oil body membrane enzyme activities in wild type, *sdp2* and CAT2HP2 seedlings.

Enzyme	Activity (nmol mg^{-1} protein min^{-1})			
	WT (Col0)	*sdp2-4*	WT (Col4)	CAT2HP2
(Peroxisome)				
ACX (16:0)	4.1 ± 0.7	3.6 ± 0.5	4.6 ± 0.3	4.0 ± 0.6
ACX (10:0)	10.7 ± 1.7	8.9 ± 0.9	11.9 ± 2.0	7.9 ± 3.3
ACX (4:0)	42.9 ± 3.8	40.1 ± 4.2	36.7 ± 2.9	34.8 ± 4.8
ECH (4:0)	46.3 ± 3.7	47.5 ± 4.9	48.6 ± 5.2	43.1 ± 5.0
HAD (4:0)	36.5 ± 3.8	39.0 ± 2.7	41.7 ± 4.4	37.6 ± 3.6
KAT (4:0)	54.9 ± 7.2	58.4 ± 9.4	57.9 ± 7.0	44.1 ± 11
ICL	79.6 ± 6.6	81.7 ± 5.2	87.3 ± 7.9	16.5 ± 2.0*
MLS	47.2 ± 2.0	52.6 ± 3.7	50.0 ± 5.3	20.5 ± 1.9*
CAT	2302 ± 245	2459 ± 319	2511± 304	211 ± 29*
(Oil body)				
Lipase	21.9 ±3.9	0.4 ±0.1*	23.9 ±4.0	17.8 ±3.5
(Cytosol)				
GAPDH	179 ±24	166 ±31	nd	nd

Seeds were germinated on medium containing 1% (w/v) sucrose. Peroxisomal enzyme activities were measured in whole extracts from 2 d old seedlings. Acyl-CoA oxidase (ACX) activity was assayed using palmitoyl-CoA, decanoyl-CoA and butyryl-CoA. Enoyl-CoA hydratase (ECH) was assayed using crotonyl-CoA. L-hydroxyacyl-CoA dehydrogenase (HAD) and 3-ketoacyl-CoA thiolase (KAT) activities were assayed using acetoacetyl-CoA. ICL, MLS and CAT are isocitrate lyase, malate synthase and catalase, respectively. Lipase activity was measured in purified oil body membranes using 10 mM [^{14}C]triolein as a substrate. Values are the mean ± SE of measurements on three separate extracts. *Activity significantly different from WT ($P < 0.001$). nd, not determined.

The activity of the cytosolic marker enzyme NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured in 2 d old seedlings of *sdp2-4* grown on medium containing sucrose (see Table 3 above). This enzyme is an indicator of oxidative damage caused by H_2O_2 in *Arabidopsis* (Hancock et al., 2005, Job et al., 2005). The activity of GAPDH was not affected, suggesting that the deficiency in

MDAR4 is unlikely to have caused a general increase in oxidative damage to cytosolic proteins.

Example 8 - Catalase deficiency has a comparatively small effect on storage oil breakdown

A series of *Arabidopsis CATALASE2* anti-sense lines (Vandenabeele et al., 2004) were screened for their ability to breakdown storage oil following germination. Surprisingly, unlike *sdp2*, none of these lines were strongly impaired in total fatty acid or eicosinoic acid breakdown when grown on medium containing sucrose (see Figure 9). Enzyme assays confirmed that the lines had reduced catalase activity following germination, with CAT2HP2 exhibiting the lowest level (only 10% of wild type; Figure 9C). Further analysis of CAT2HP2 seedlings revealed that the activities of specific peroxisomal enzymes (isocitrate lyase and malate synthase) are significantly reduced in comparison with wild type, while oil body membrane lipase activity is not affected (see Table 3 above). This indicates that a 90% reduction in catalase activity does impact negatively on peroxisome function albeit insufficiently to prevent fatty acid β-oxidation from occurring when seedlings are provided with sucrose.

Table 4 Total fatty acid content of seeds from *Arabidopsis* MDAR4 mutants

Genotype	Fatty acid content (μg seed^{-1})	**(% increase)**
SDP1-1	8.73 ±0.15	
sdp1-1	9.50 ±0.21*	**(+9%)**
SDP1-5	8.54 ±0.46	
sdp1-5	9.48 ±0.27*	**(+11%)**
SDP2-4	9.13 ±0.19	
sdp2-4	10.09 ±0.33*	**(+10%)**
SDP2-5	8.76 ±0.20	
sdp2-5	10.12 ±0.41*	**(+15%)**
SDP3-1	8.45 ±0.31	

sdp3-1	8.71 ±0.27	
SDP3-2	8.21±0.23	
sdp3-2	8.70 ±0.19*	**(+6%)**

Wild type and MDAR4 mutant plants were grown in the glasshouse in P24 trays containing F2 compost. Wild types are in capital letters and mutants in lower case. Table 4 shows the seed fatty acid content. The total fatty acid content of batches of seeds was measured by gas chromatography following direct extraction/methylation (see Browse *et al* (1986) Anal. Biochem. 152: 141). Values are the mean ±SE of measurements on seeds from 8 to 10 individual plants. *=significantly different from wild type ($P < 0.05$). The *sdp2-4* and *sdp2-5* alleles are SALK_068667 and SALK_030775 (Alonso et al., Science 301, 653-657. The other mutant alleles are described in Eastmond, (2006) Plant Cell 18, 665-675. Compared to wild type, the mutants show significantly higher seed oil content due to the suppression of MDAR4. Compared to the other mutants, *sdp2* mutants have a surprisingly higher seed fatty acid content.

The inventors have found that the phenotype of *sdp2* lacks the APX/MDAR system and consequently some of the H_2O_2 produced by acyl-CoA oxidase following seed germination escapes from the peroxisome and causes oxidative damage to oil bodies, inactivating SDP1 (Figure 10). Analysis of *sdp2* seedlings immediately following germination confirmed that they have elevated levels of H_2O_2 and that their oil body proteins and lipids become oxidized. Furthermore, when *sdp2* was introduced into a *pxa1* background, which cannot β-oxidize fatty acids (Zolman et al., 2001), H_2O_2 levels, oxidative damage to oil bodies and loss of lipase activity were all suppressed. The activity of SDP1 can be inhibited by H_2O_2 in vitro. Finally oxidised SDP1 can be detected in oil bodies from *sdp2* seedlings but not from wild type. Inactivation of SDP1 is sufficient to account for much of the sugar-dependent phenotype of *sdp2* (Eastmond, 2006). However, it cannot be discounted that additional proteins, which are necessary for oil hydrolysis and utilization might also be damaged.

Unlike oil bodies, peroxisomes do not appear to depend so greatly on the APX/MDAR system for protection against H_2O_2. Seedlings of *sdp2* are able to β-oxidize 2,4-DB. This capability implies that numerous proteins in the peroxisomal membrane and matrix retain at least part of their normal capacity (see Baker et al., 2006). The activities of seven peroxisomal marker enzymes are also not adversely affected in *sdp2*. It is possible that a defect in the APX/MDAR system is not severely detrimental to peroxisomes because catalase remains active within the matrix. However, the possibility exists that peroxisomes in *sdp2* are damaged. Analysis of catalase anti-sense lines (Vandenabeele et al., 2004) showed that following *Arabidopsis* seed germination certain peroxisomal enzyme activities are reduced, while lipase activity on oil body membranes is unaffected. In particular the activity of the key glyoxylate cycle enzyme isocitrate lyase (Eastmond et al., 2000a) is reduced by 80% in the strongest catalase anti-sense line. This observation is consistent with *in vitro* data, which shows that isocitrate lyase from castor bean endosperm is readily inactivated by H_2O_2 and that this enzyme physically associates with catalase in the peroxisome (Yanik and Donaldson, 2005; Nguyen and Donaldson, 2005).

Using confocal microscopy the inventors have shown that peroxisomes and oil bodies cluster together in the cotyledon cells of living *sdp2* seedlings and that this association persists as long as the oil bodies remain undegraded. Without wishing to be bound by any particular theory, the inventors have found that physical contact may play a role in storage oil breakdown in oilseeds by facilitating the transfer of fatty acids from oil bodies to peroxisomes so that they can be β-oxidized.

The concentration of H_2O_2 required to inhibit SDP1 in vitro (~0.5 mM) is 16-fold higher than the estimated cytosolic concentration in 1 d old *sdp2* seedlings (~0.03 mM). This concentration was calculated using the data from Figure 7A, assuming that the cytosol constitutes about 20% of the seedlings volume. However, H_2O_2 concentrations are unlikely to be uniform and could be heightened at, or near, the peroxisomal membrane, particularly if acyl-CoA oxidases are situated there. Again, without wishing to be bound by any particular theory, the inventors observe that an association between oil bodies and peroxisomes explains at least in part why the deficiency in MDAR4 is so detrimental to

storage oil hydrolysis since it would result in a close proximity between SDP1 and acyl-CoA oxidases, which generate H_2O_2.

The *sdp2* mutant is defective in the second enzyme in the APX/MDAR system (MDAR4). Metabolite measurements suggest that in *sdp2,* the monodehydroascorbate produced at the peroxisomal membrane cannot be recycled efficiently causing the availability of ascorbate to diminish and the APX/MDAR system to collapse. There are several alternative ways in which ascorbate could still be replenished for APX in the absence of MDAR4. In addition to MDAR4, *Arabidopsis* peroxisomes also contain the soluble matrix isoform MDAR1 (Lisenbee et al. 2005). Monodehydroascorbate can also disproportionate to ascorbate and dehydroascorbate and biochemical studies have suggested that glutathione-dependent dehydroascorbate reductase activity is present in plant peroxisomes and therefore could convert dehydroascorbate back to ascorbate (Jiménez et al., 1997; del Rio et al., 1998). Ascorbate and monodehydroascorbate are likely to be shuttled across the peroxisomal membrane since the catalytic site of MDAR4 is situated on the matrix side of the peroxisomal membrane, while the catalytic site of APX3 is on the cytosolic side (Lisenbee et al., 2005). The phenotype of *sdp2* suggests that nothing can complement the function of MDAR4 in protecting oil bodies against β-oxidation-dependent oxidative damage.

The predominant APX isoform from the peroxisomal membranes of *Arabidopsis* is APX3 (At4g35000). Narendra et al., (2006) have recently reported that the *APX3* gene is dispensable for growth and development. Therefore, a deficiency in APX activity might not give rise to the same phenotype as *sdp2*. Redundancy cannot be dismissed as an explanation for the apparent disparity considering that there are nine APX genes in the *Arabidopsis* genome (Lisenbee et al., 2003). Specifically there is a homologue of APX3 (APX5; At4g35970) with sequence similarity throughout the polypeptide sequence, including the C-terminal transmembrane domain and targeting motif. However, the level of expression of *APX5* is very low relative to *APX3* (Narendra et al., 2006).

The inventors do not preclude the possibility that MDAR4 functions independently of APX. Sattler et al., (2004) have shown that a deficiency in the lipid soluble antioxidants tocopherols (vitamin E) also leads to lipid peroxidation after the germination of *Arabidopsis* seeds and that this adversely effects storage oil breakdown and seedling growth. Furthermore, sucrose can partially relieve the post-germinative growth defect. Tocopherols can scavenge lipid peroxy radicals yielding a tocopheroxyl radical that could be recycled by reacting with ascorbate to produce monodehydroascorbate (Liebler, 1993). MDAR4 may function in a one-electron redox cycle that regenerates tocopherol from the tocopheroxyl radical at the peroxisomal membrane. However, it is unlikely that MDAR4 can operate solely by this mechanism since the *sdp2* mutant has a more severe post-germinative growth arrest phenotype than the tocopherol deficient *vte2* mutant under normal growth conditions (Sattler et al., 2004). It is also possible that ascorbate could scavenge reactive oxygen species directly, or that MDAR4 might be capable of recycling the oxidation products of other powerful antioxidants, such as phenolics (Sakihama et al., 2000). Indeed MDAR is unique in that it is the only enzyme known to use organic radicals as substrates (Hossain et al., 1984).

In addition to its role in H_2O_2-detoxification, peroxisomal MDAR has also been implicated in fatty acid catabolism through the provision of NAD^+ cofactor for L-3-hydroxyacyl-CoA dehydrogenase and malate dehydrogenase (Bowditch and Donaldson, 1990; Mullen and Trelease, 1996). These enzymes are required for β-oxidation and glyoxylate cycle function, respectively and theoretically if H_2O_2 was detoxified entirely by APX/MDAR the system could recycle sufficient NAD^+ for both pathways (Mullen and Trelease, 1996). However, Mettler and Beevers (1980) have proposed an alternative scheme in which peroxisomal malate dehydrogenase operates in 'reverse' to supply NAD^+ for L-3-hydroxyacyl-CoA dehydrogenase via a metabolite shuttle, coupled to the mitochondrial electron transport chain. Malate dehydrogenase has since been shown to perform this role in yeast (van Roermund et al., 1995). Analysis of *sdp2* suggests that MDAR4 is not essential for providing all the NAD^+ in *Arabidopsis* peroxisomes since the mutant can β-oxidize 2,4-DB. In contrast, it has recently been reported that *Arabidopsis* seedlings that lack peroxisomal malate dehydrogenase activity are also impaired in oil

breakdown and cannot β-oxidize 2,4-DB (see Baker et al., 2006). This provides evidence to suggest that the scheme proposed by Mettler and Beevers (1980) supplies the majority of NAD^+ *in vivo*, although MDAR4 (and MDAR1) activity must also contribute some NAD^+.

In addition to fatty acid β-oxidation, the photo-respiratory pathway also generates large quantities of H_2O_2 in plant peroxisomes as a result of the activity of glycolate oxidase (Willekens et al., 1997). Catalase has been shown to play a major role in detoxifying this H_2O_2. Anti-sense suppression of catalase results in oxidative damage and triggers cell death in tobacco and *Arabidopsis* plants that are subjected to high light treatment (Willekens et al., 1997; Vandenabeele et al., 2004). Although public micro array data shows that *MDAR4* is expressed in leaves, *sdp2* plants do not exhibit more obvious phenotypic symptoms of oxidative damage than wild type when they are subjected to high light (data not shown). Further studies will be required to determine if MDAR4 has a role associated with photo-respiration. Reactive oxygen species also play important signalling roles in plants (Hancock et al., 2005) and it is conceivable that inhibition of SDP1 activity might be a significant mechanism in the regulation of oil hydrolysis during post-germinative growth.

Catalase and the APX/MDAR system are both important parts of the peroxisomal antioxidant machinery during the post-germinative growth of *Arabidopsis* seedlings. The inventors have found that their roles are physiologically different and that neither can fully compensate for the loss of the other. Catalase protects constituents of the peroxisomal matrix from oxidative damage while the main role of MDAR4 is proposed by the inventors to be the prevention of H_2O_2 from escaping beyond the outer surface of the peroxisomal membrane. The consequences of H_2O_2 escape are fatal primarily because inactivation of triacylglycerol hydrolysis on closely associated oil bodies prevents the seedling from releasing the carbon skeletons and energy that it needs for initial post-germinative growth.

Accession Numbers

The GenBank accession number for an *SDP2* (*MDAR4*) cDNA is AY039980.

Table 5

Acanthaceae
Aceraceae
Achariaceae
Achatocarpaceae
Actinidiaceae
Adoxaceae
Aextoxicaceae
Agdestidaceae
Aizoaceae
Akaniaceae
Alangiaceae
Alseuosmiaceae
Alzateaceae
Amaranthaceae
Amborellaceae
Anacardiaceae
Ancistrocladaceae
Anisophylleaceae
Annonaceae
Apocynaceae
Aquifoliaceae
Araliaceae
Aralidiaceae
Aristolochiaceae
Asclepiadaceae
Asteropeiaceae
Aucubaceae
Austrobaileyaceae
Avicenniaceae
Balanitaceae
Balanopaceae
Balanophoraceae
Balsaminaceae
Barbeuiaceae
Barbeyaceae
Basellaceae
Bataceae
Begoniaceae
Berberidaceae
Betulaceae
Bignoniaceae
Bixaceae
Bombacaceae
Boraginaceae
Bretschneideraceae
Brunelliaceae
Bruniaceae
Buddlejaceae
Burseraceae
Buxaceae
Byblidaceae
Cabombaceae
Cactaceae
Callitrichaceae
Calycanthaceae
Calyceraceae
Campanulaceae
Canellaceae
Cannabaceae
Canotiaceae
Capparaceae
Caprifoliaceae
Cardiopteridaceae
Caricaceae
Carlemanniaceae
Caryocaraceae
Caryophyllaceae
Casuarinaceae
Cecropiaceae

Celastraceae
Cephalotaceae
Ceratophyllaceae
Cercidiphyllaceae
Chenopodiaceae
Chloranthaceae
Chrysobalanaceae
Circaeasteraceae
Cistaceae
Clethraceae
Cneoraceae
Cobaeaceae
Cochlospermaceae
Columelliaceae
Combretaceae
Compositae
Connaraceae
Convolvulaceae
Coriariaceae
Cornaceae
Corylaceae
Corynocarpaceae
Crassulaceae
Crossosomataceae
Cruciferae
Crypteroniaceae
Ctenolophonaceae
Cucurbitaceae
Cunoniaceae
Cyclocheilaceae
Cynomoriaceae
Cyrillaceae
Daphniphyllaceae
Datiscaceae
Davidsoniaceae
Degeneriaceae
Dialypetalanthaceae
Diapensiaceae
Dichapetalaceae
Didiereaceae
Didymelaceae
Diegodendraceae
Dilleniaceae
Dioncophyllaceae
Dipentodontaceae
Dipsacaceae
Dipterocarpaceae
Droseraceae
Duckeodendraceae
Ebenaceae
Elaeagnaceae
Elaeocarpaceae
Elatinaceae
Emblingiaceae
Empetraceae
Epacridaceae
Eremolepidaceae
Eremosynaceae
Ericaceae
Erythroxylaceae
Escalloniaceae
Eucommiaceae
Eucryphiaceae
Euphorbiaceae
Euphroniaceae
Eupomatiaceae
Eupteleaceae
Fagaceae
Flacourtiaceae
Fouquieriaceae
Frankeniaceae
Garryaceae
Geissolomataceae
Gentianaceae
Geraniaceae
Gesneriaceae
Gisekiaceae
Glaucidiaceae
Globulariaceae
Goetzeaceae
Gomortegaceae
Goodeniaceae
Goupiaceae
Greyiaceae

Griseliniaceae
Grossulariaceae
Grubbiaceae
Gunneraceae
Guttiferae
Gyrostemonaceae
Halophytaceae
Haloragaceae
Hamamelidaceae
Hectorellaceae
Helwingiaceae
Hernandiaceae
Himantandraceae
Hippocastanaceae
Hippuridaceae
Hoplestigmataceae
Huaceae
Humiriaceae
Hydnoraceae
Hydrangeaceae
Hydrophyllaceae
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Idiospermaceae
Illecebraceae
Illiciaceae
Irvingiaceae
Ixonanthaceae
Juglandaceae
Krameriaceae
Labiatae
Lacistemataceae
Lactoridaceae
Lardizabalaceae
Lauraceae
Lecythidaceae
Leeaceae
Leguminosae-caesalpinioideae
Leguminosae-mimosoideae
Leguminosae-papilionoideae
Leitneriaceae
Lennoaceae
Lentibulariaceae
Lepidobotryaceae
Limnanthaceae
Linaceae
Lissocarpaceae
Loasaceae
Loganiaceae
Lophopyxidaceae
Loranthaceae
Lythraceae
Magnoliaceae
Malesherbiaceae
Malpighiaceae
Malvaceae
Marcgraviaceae
Medusagynaceae
Medusandraceae
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Menispermaceae
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Misodendraceae
Molluginaceae
Monimiaceae
Montiniaceae
Moraceae
Morinaceae
Moringaceae
Myoporaceae
Myricaceae
Myristicaceae
Myrothamnaceae
Myrsinaceae
Myrtaceae
Nelumbonaceae
Nepenthaceae
Nesogenaceae
Neuradaceae

Nyctaginaceae
Nymphaeaceae
Ochnaceae
Olacaceae
Oleaceae
Oliniaceae
Onagraceae
Oncothecaceae
Opiliaceae
Oxalidaceae
Paeoniaceae
Pandaceae
Papaveraceae
Paracryphiaceae
Parnassiaceae
Passifloraceae
Pedaliaceae
Pellicieraceae
Penaeaceae
Pentaphragmataceae
Pentaphylacaceae
Penthoraceae
Peridiscaceae
Phellinaceae
Phrymaceae
Physenaceae
Phytolaccaceae
Piperaceae
Pittosporaceae
Plagiopteraceae
Plantaginaceae
Platanaceae
Plumbaginaceae
Podoaceae
Podostemaceae
Polemoniaceae
Polygalaceae
Polygonaceae
Portulacaceae
Primulaceae
Proteaceae
Ptaeroxylaceae
Pterostemonaceae
Quiinaceae
Rafflesiaceae
Ranunculaceae
Resedaceae
Retziaceae
Rhabdodendraceae
Rhamnaceae
Rhizophoraceae
Rhoipteleaceae
Rhynchocalycaceae
Roridulaceae
Rosaceae
Rubiaceae
Rutaceae
Sabiaceae
Saccifoliaceae
Salicaceae
Salvadoraceae
Santalaceae
Sapindaceae
Sapotaceae
Sarcolaenaceae
Sargentodoxaceae
Sarraceniaceae
Saururaceae
Saxifragaceae
Schisandraceae
Scrophulariaceae
Scyphostegiaceae
Scytopetalaceae
Simaroubaceae
Simmondsiaceae
Solanaceae
Sphaerosepalaceae
Sphenostemonaceae
Stachyuraceae
Stackhousiaceae
Staphyleaceae
Stegnospermataceae
Sterculiaceae
Stilbaceae

Strasburgeriaceae
Stylidiaceae
Stylobasiaceae
Styracaceae
Surianaceae
Symplocaceae
Tamaricaceae
Tepuianthaceae
Tetracentraceae
Tetrachondraceae
Tetrameristaceae
Theaceae
Theligonaceae
Theophrastaceae
Thymelaeaceae
Ticodendraceae
Tiliaceae
Torricelliaceae
Tovariaceae
Trapaceae
Tremandraceae
Trigoniaceae
Trimeniaceae
Triplostegiaceae
Trochodendraceae
Tropaeolaceae
Turneraceae
Ulmaceae
Umbelliferae
Urticaceae
Vahliaceae
Valerianaceae
Verbenaceae
Violaceae
Viscaceae
Vitaceae
Vochysiaceae
Winteraceae
Zygophyllaceae
Acoraceae
Agavaceae
Alismataceae
Alliaceae
Aloaceae
Alstroemeriaceae
Amaryllidaceae
Anarthriaceae
Anthericaceae
Aphyllanthaceae
Aponogetonaceae
Araceae
Asparagaceae
Asphodelaceae
Asteliaceae
Blandfordiaceae
Bromeliaceae
Burmanniaceae
Butomaceae
Calectasiaceae
Cannaceae
Centrolepidaceae
Colchicaceae
Commelinaceae
Convallariaceae
Corsiaceae
Costaceae
Cyanastraceae
Cyclanthaceae
Cymodoceaceae
Cyperaceae
Dasypogonaceae
Dioscoreaceae
Doryanthaceae
Dracaenaceae
Ecdeiocoleaceae
Eriocaulaceae
Eriospermaceae
Flagellariaceae
Gramineae
Haemodoraceae
Hanguanaceae
Heliconiaceae
Hemerocallidaceae
Herreriaceae

Hostaceae
Hyacinthaceae
Hydatellaceae
Hydrocharitaceae
Hypoxidaceae
Iridaceae
Ixioliriaceae
Joinvilleaceae
Juncaceae
Juncaginaceae
Lanariaceae
Lemnaceae
Lilaeaceae
Liliaceae
Limnocharitaceae
Lomandraceae
Lowiaceae
Marantaceae
Mayacaceae
Melanthiaceae
Musaceae
Orchidaceae
Palmae
Pandanaceae
Petermanniaceae
Philesiaceae
Philydraceae
Phormiaceae
Pontederiaceae
Posidoniaceae
Potamogetonaceae
Rapateaceae
Restionaceae
Rhipogonaceae
Ruscaceae
Scheuchzeriaceae
Smilacaceae
Stemonaceae
Strelitziaceae
Taccaceae
Tecophilaeaceae
Thurniaceae
Trichopodaceae
Trilliaceae
Triuridaceae
Typhaceae
Velloziaceae
Xanthorrhoeaceae
Xyridaceae
Zannichelliaceae
Zingiberaceae
Zosteraceae

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CLAIMS

1. A method of increasing the oil content of a plant cell comprising reducing or eliminating monodehydroascorbate reductase (MDAR4) activity.

2. A method as claimed in claim 1, wherein the reduction or elimination of MDAR4 activity is in the peroxisome.

3. A method as claimed in any of claims 1 to 3, wherein the reduction or elimination of MDAR4 activity takes place during seed development and/or maturation.

4. A method as claimed in any preceding claim, wherein the reduction or elimination of MDAR4 activity comprises supression of MDAR4 expression.

5. A method as claimed in claim 4, wherein the plant is transformed or transfected with a nucleic acid or vector capable of suppressing MDAR4 expression.

6. A method as claimed in any preceding claim, wherein the reduction or elimination of MDAR4 activity comprises antisense suppression of MDAR4 expression.

7. A method as claimed in any of claims 1 to 5, wherein the reduction or elimination of MDAR4 activity comprises sense suppression of MDAR4 expression.

8. A method as claimed in claimed in any preceding claim, wherein the activity of MDAR4 is reduced or eliminated by an inhibitor or antagonist of MDAR4.

9. A method as claimed in claim 8, wherein the MDAR4 inhibitor or antagonist is expressed from heterologous nucleic acid molecule or vector introduced into the plant or is an expression product of a gene endogenous to the genome of the plant.

10. A method as claimed in claim 9, wherein the heterologous nucleic acid molecule introduced into the plant has integrated into the host plant genome.

11. A method as claimed in claim 9, wherein the vector introduced into the plant is an epigenetic element or an artificial chromosome.

12. A method as claimed in any of claims 5 or 8 to 11, wherein the nucleic acid or vector capable of suppressing MDAR4 expression is a transposable element.

13. A method as claimed in any preceding claim, wherein the MDAR4 inhibitor or antagonist is applied to the plant.

14. A method as claimed in any preceding claim, wherein the plant is treated with a nucleic acid or vector, an inhibitor or an antagonist of MDAR4, at or during a stage selected from flowering, fertilization, seed setting, seed desiccation or during seed storage.

15. A method as claimed in any preceding claim, wherein the nucleic acid comprises:
(a) a nucleotide sequence of SEQ ID NO: 1 or a sequence having at least 50% identity thereto,
(b) a fragment of at least 18 contiguous nucleic acids of (a), or
(c) the complement of (a) or (b).

16. A method as claimed in claims 1 to 14, wherein the nucleic acid comprises a nucleotide sequence encoding:

(a) an amino acid sequence selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6, or an amino acid sequence having at least 50% identity thereto,

(b) a polypeptide of at least 6 contiguous amino acids of (a).

17. A method as claimed in claim 15 or claim 16, wherein the nucleic acid sequence encodes a monodehydroascorbate reductase 4 (MDAR4).

18. A method as claimed in any of claims 5 to 17, wherein the vector is *Agrobacterium*.

19. A method as claimed in any preceding claim, wherein the plant is a monocot or a dicot.

20. A method as claimed in any preceding claim, wherein the plant is a member of the Cruciferae or the Compositae.

21. A method as claimed in any preceding claim, wherein the plant is selected from corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Tritium aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Iopmoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (Citrus spp.) cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avacado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

22. A plant cell transformed or transfected with nucleic acid capable of reducing or eliminating monodehydroascorbate reductase (MDAR4) activity.

23. A plant cell as claimed in claim 22, wherein the reduction or elimination of MDAR4 activity is in the peroxisome.

24. A plant cell as claimed in claim 22 or claim 23, wherein the reduction or elimination of MDAR4 activity comprises supression of MDAR4 expression.

25. A plant cell as claimed in any of claims 22 to 24 transformed or transfected with a nucleic acid or vector capable of suppressing MDAR4 expression.

26. A plant cell as claimed in claim 25, wherein the vector comprises a cell or tissue specific promoter.

27. A plant cell as claimed in claim 26, wherein the promoter is an inducible promoter or a developmentally regulated promoter.

28. A plant cell as claimed in any of claims 22 to 27, wherein the reduction or elimination of MDAR4 activity comprises antisense suppression of MDAR4 expression.

29. A plant cell as claimed in any of claims 22 to 28, wherein the reduction or elimination of MDAR4 activity comprises sense suppression of MDAR4 expression.

30. A plant cell as claimed in claimed in any of claims 22 to 29, wherein the activity of MDAR4 is reduced or eliminated by an inhibitor or antagonist of MDAR4 expressed by an heterologous nucleic acid molecules or vector, or an inhibitor or antagonist of MDAR4 expressed from a gene endogenous to the genome of the cell.

31. A plant cell as claimed in claim 30, wherein the heterologous nucleic acid molecule or vector is integrated into the host cell genome.

32. A plant cell as claimed in claim 30 or claim 31, wherein the heterologous nucleic acid or vector is an epigenetic element or an artificial chromosome.

33. A plant cell as claimed in any of claims 25 to 32, wherein the nucleic acid or vector capable of suppressing MDAR4 expression is a transposable element.

34. A plant cell as claimed in any of claims 22 to 33, wherein the nucleic acid comprises: (a) a nucleotide sequence of SEQ ID NO: 1 or a sequence having at least 50% identity thereto,

(b) a fragment of at least 18 contiguous nucleic acids of (a), or

(c) the complement of (a) or (b).

35. A plant cell as claimed in any of claims 22 to 34, wherein the nucleic acid comprises a nucleotide sequence encoding:

(a) an amino acid sequence selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6, or an amino acid sequence having at least 50% identity thereto,

(b) a polypeptide of at least 6 contiguous amino acids of (a).

36. A plant cell as claimed in claim 34 or claim 35, wherein the nucleic acid sequence encodes monodehydroascorbate reductase 4 (MDAR4).

37. A cell as claimed in any of claims 22 to 36, wherein the vector is *Agrobacterium*.

38. A plant cell as claimed in any of claims 22 to 37 comprised in a plant selected from a monocot or a dicot.

39. A plant cell as claimed in any of claims 22 to 37 comprised in a plant selected from the Cruciferae or the Compositae.

40. A plant cell as claimed in any of claims 22 to 37 comprised in a plant selected from corn (*Zea mays*), canola (*Brassica napus, Brassica rapa* ssp.), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), sunflower (*Helianthus annus*), wheat

(*Tritium aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Iopmoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (Citrus spp.) cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avacado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

41. A plant, plant organ or plant tissue comprising a plant cell as claimed in any of claims 23 to 40.

42. A plant seed comprising a plant cell as claimed in any of claims 23 to 40.

43. A method of identifying an agent which increases the oil content of a seed, comprising contacting a plant deficient in monodehydroascorbate reductase (MDAR4), harvesting the mature or developing seed and determining the oil content of the seed.

44. A method as claimed in claim 43, wherein the oil content of a seed is determined by measuring fatty acid content of the seed.

45. A plant cell overexpressing monodehydroascorbate reductase (MDAR4).

46. A plant tissue or plant comprising a plant cell of claim 45.

47. A method of identifying an agent which increases the oil content of a plant cell, tissue or seed, comprising contacting a plant cell of claim 45 or plant tissue or plant of claim 46 with a candidate agent and then determining the oil content of the cell, tissue or seed.

48. A method as claimed in claim 47, wherein the oil content of a seed is determined by measuring the fatty acid content of the seed.

49. An antibody specifically recative to MDAR4.

50. An antibody as claimed in claim 49 being a monoclonal antibody.

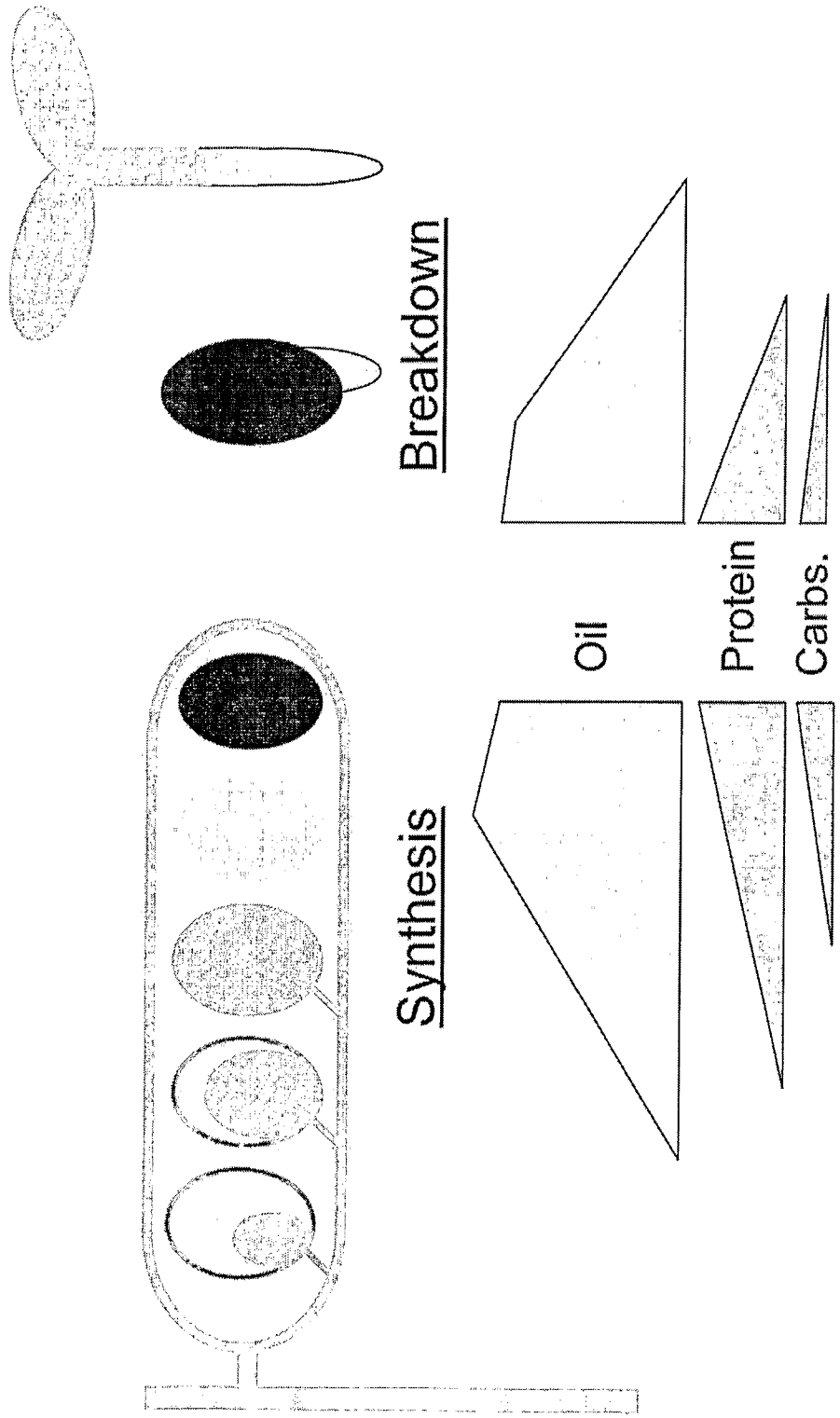
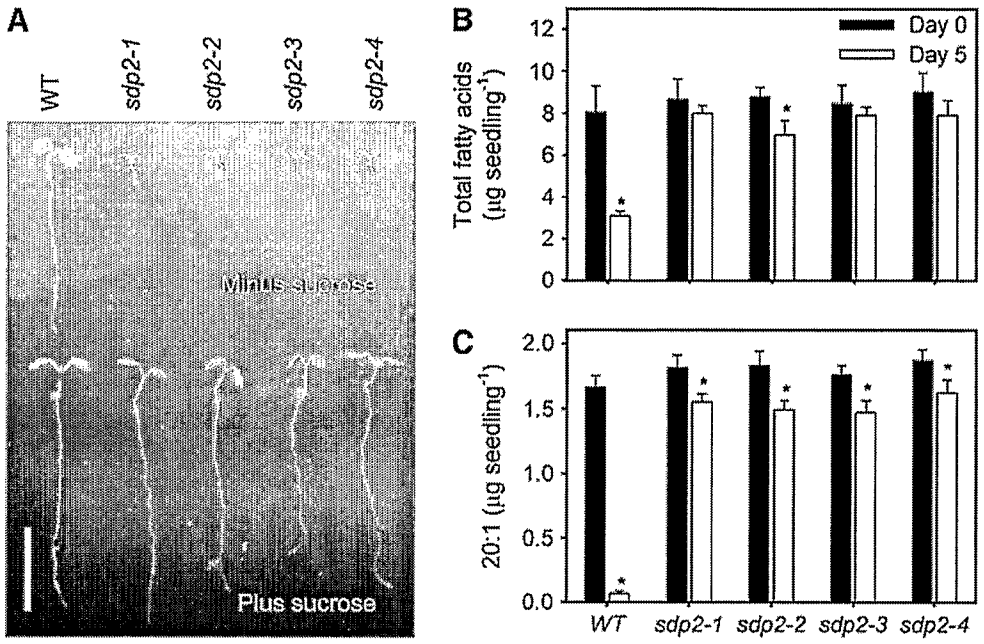


Fig. 1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WT
sdp2-1
sdp2-2
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Minus sucrose
Plus sucrose
B
Total fatty acids (µg seedling-1)
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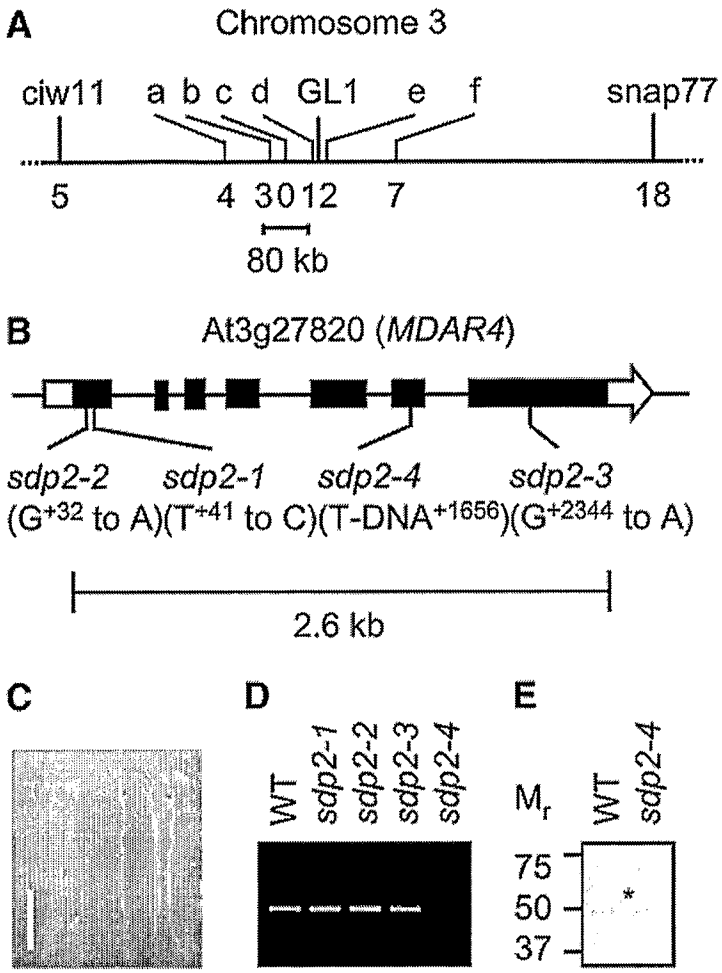
sdp2-2
sdp2-3
sdp2-4

Fig. 2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sdp2-2
sdp2-1
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sdp2-3
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2.6 kb
C
D
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Fig. 3

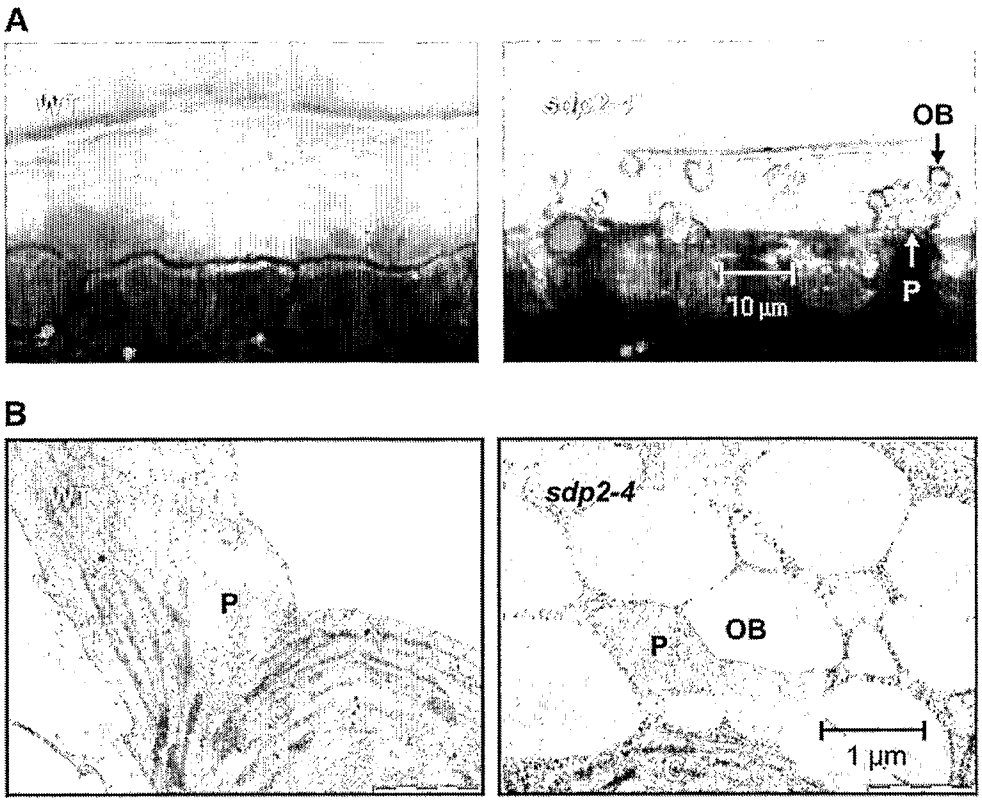
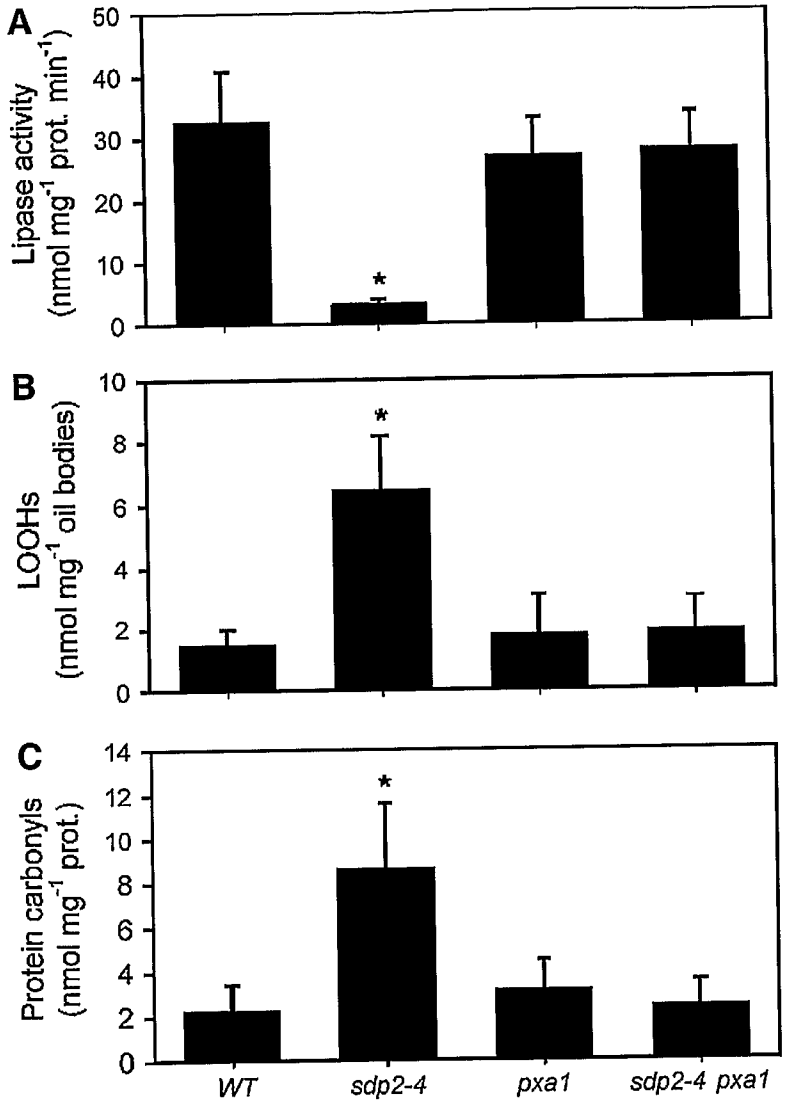


Fig. 4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Lipase activity
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B
LOOHs
(nmol mg-1 oil bodies)
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C
Protein carbonyls
(nmol mg-1 prot.)
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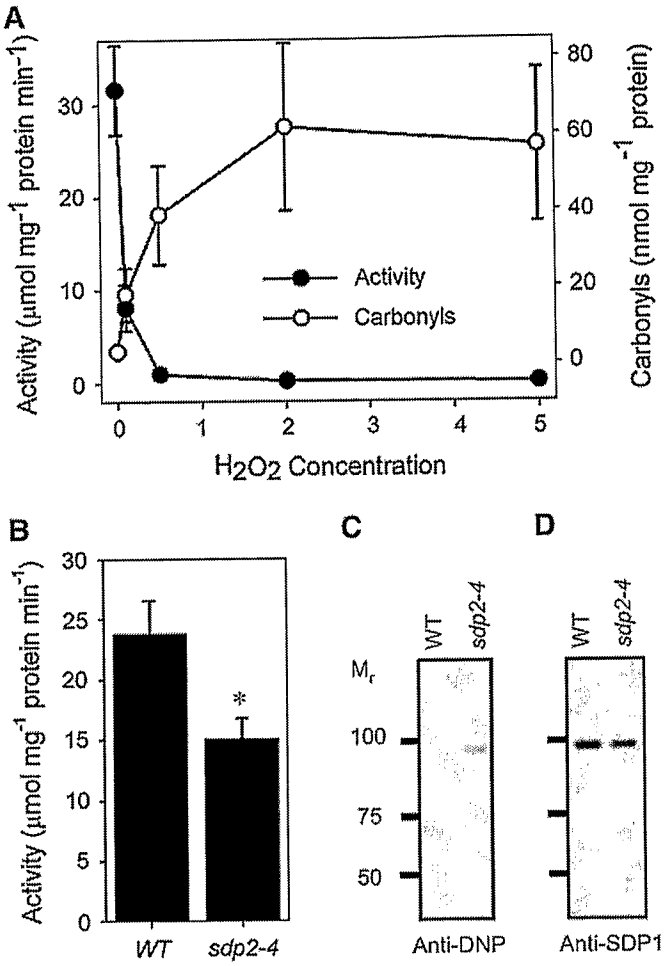
Fig. 5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Activity
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WT
sdp2-4
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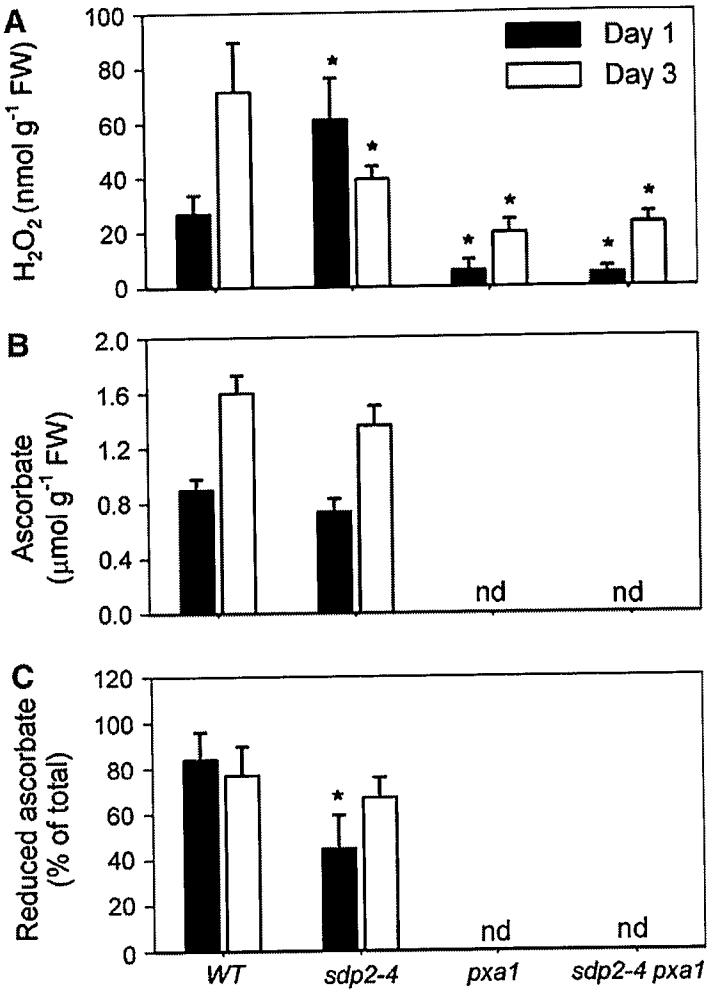
Fig. 6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Day 3
*
B
Ascorbate (µmol g^{-1} FW)
2.0
1.6
1.2
0.8
0.4
0.0
nd
nd
C
Reduced ascorbate (% of total)
120
100
80
60
40
20
0
nd
nd
WT
sdp2-4
pxa1
sdp2-4 pxa1

Fig. 7

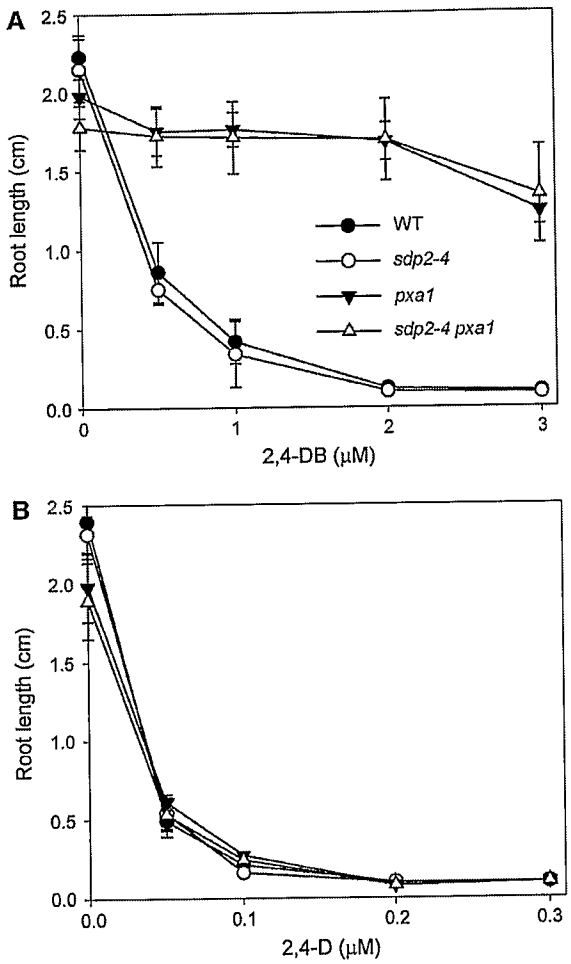
A
Root length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
2,4-DB (µM)
WT
sdp2-4
pxa1
sdp2-4 pxa1
B
Root length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.1
0.2
0.3
2,4-D (µM)

Fig. 8

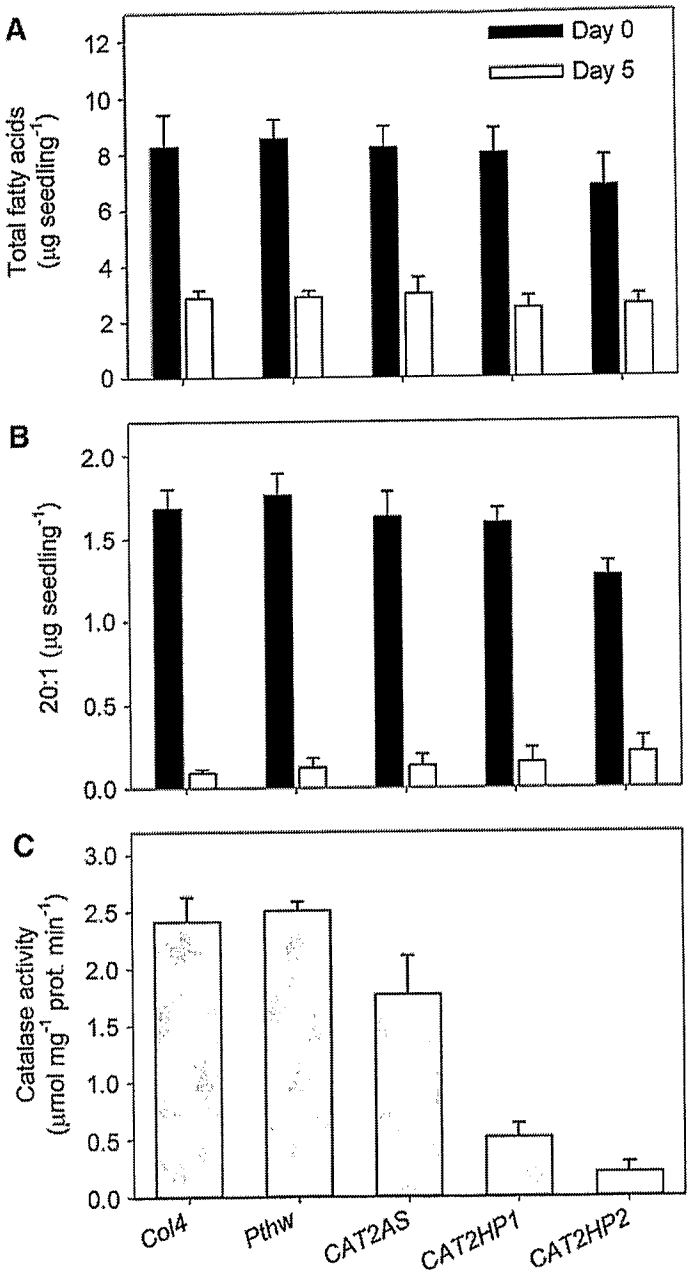
A
Day 0
Day 5
Total fatty acids (µg seedling^{-1})
0
2
4
6
8
10
12
B
20:1 (µg seedling^{-1})
0.0
0.5
1.0
1.5
2.0
C
Catalase activity (µmol mg^{-1} prot. min^{-1})
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Col4
Pthw
CAT2AS
CAT2HP1
CAT2HP2

Fig. 9

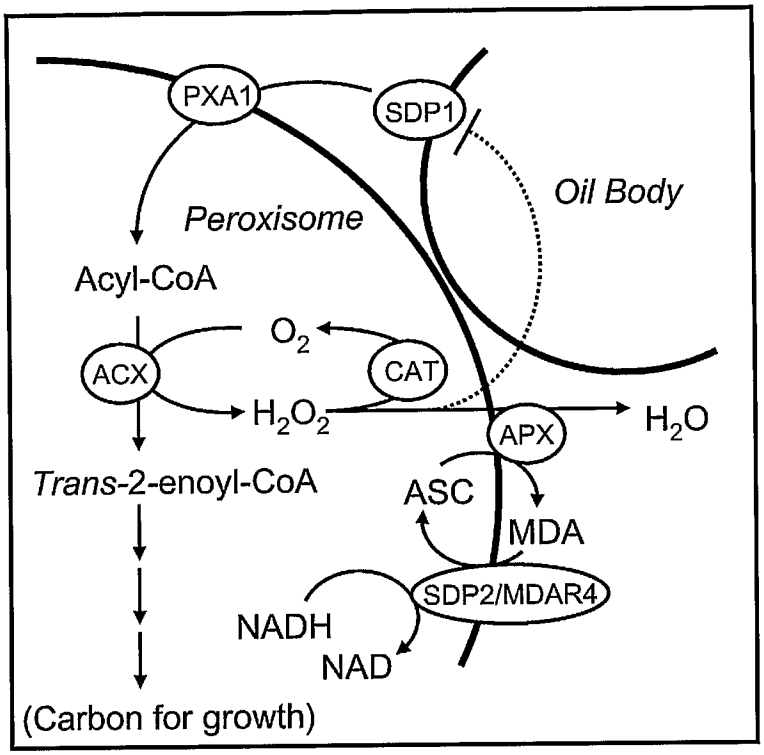
PXA1
SDP1
Oil Body
Peroxisome
Acyl-CoA
O_2
ACX
CAT
H_2O_2
APX
H_2O
Trans-2-enoyl-CoA
ASC
MDA
SDP2/MDAR4
NADH
NAD
(Carbon for growth)

Fig. 10

```
   1 GATCCGATAA CAGCATTTTC TATTTTCTTC TGTGCCTCAA AAAATCATAA
  51 TTTTCTCGGA AAATTAAAAA TCATGTTTAA GATTTTCTCG AGAAAGTAAA
 101 AAAAGGAAGA AGAAGAAGAA GAAACCATGG GAAGAGCTTT CGTGTATGTA
 151 ATTCTCGGAG GAGGTGTTGC TGCTGGTTAC GCAGCTCTCG AATTCACCCG
 201 TCGAGGTGTC TCCGATGGCG AACTCTGTAT CATTTCCGAA GAACCCGTTG
 251 CACCGTATGA GAGGCCTGCC TTAAGCAAAG GTTTTCTGCT GCCAGAAGCT
 301 CCTGCACGGC TTCCTTCTTT CCACACTTGT GTTGGAGCTA ATGATGAGAA
 351 GCTTACACCG AAATGGTACA AGGATCATGG AATTGAATTG GTACTTGGAA
 401 CTCGTGTTAA GTCCGTTGAT GTGAGGAGGA AGACTCTTTT ATCGAGCACA
 451 GGGGAGACTA TAAGTTACAA ATTCTTGATC ATTGCAACAG GTGCCCGGGC
 501 GTTGAAGCTT GAAGAATTTG GGGTGGAAGG CTCAGATGCT GAAAATGTTT
 551 GTTACTTACG AGATCTAGCT GATGCAAATA GGCTTGCCAC TGTGATTCAA
 601 TCAAGCTCCA ACGGGAATGC GGTTGTTATC GGCGGTGGCT ATATCGGTAT
 651 GGAGTGTGCT GCATCTTTAG TGATCAACAA AATCAATGTG ACGATGGTTT
 701 TCCCAGAGGC TCACTGCATG GCACGTCTCT TTACGCCCAA GATTGCAAGT
 751 TTGTATGAAG ACTACTACAG GGCTAAAGGC GTAAAATTTA TAAAAGGAAC
 801 GGTTTTGACA TCATTTGAGT TTGACTCGAA CAAAAAGGTT ACTGCAGTTA
 851 ATCTCAAAGA CGGGAGCCAC TTACCAGCCG ACTTGGTTGT GGTTGGTATC
 901 GGGATACGAC CAAACACAAG CTTGTTTGAA GGACAGTTAA CAATAGAGAA
 951 AGGAGGGATA AAAGTGAACA GCAGAATGCA GTCAAGTGAC AGCTCAGTGT
1001 ATGCAATAGG CGATGTAGCT ACATTTCCAG TGAAACTATT TGGCGAAATG
1051 AGGAGGCTTG AGCACGTGGA CTCAGCTAGG AAATCTGCGC GACACGCAGT
1101 TTCAGCCATC ATGGATCCAA TAAAGACCGG AGACTTTGAT TACTTACCAT
1151 TTTTCTACTC AAGGGTCTTC GCATTCTCAT GGCAATTCTA TGGAGACCCT
1201 ACAGGTGATG TTGTGCACTT TGGAGAGTAT GAAGACGGGA AATCATTTGG
1251 AGCGTATTGG GTTAAAAAGG GTCACCTTGT TGGATCTTTT CTTGAAGGAG
1301 GCACTAAAGA AGAATATGAA ACTATCTCAA AGGCGACACA ATTGAAACCA
1351 GCTGTGACTA TTGATTTGGA AGAATTGGAA AGAGAAGGAC TCGGGTTTGC
1401 TCACACGGTT GTGAGTCAGC AGAAAGTTCC TGAAGTTAAG GATATTCCGA
1451 GCGCTGAGAT GGTGAAGCAA TCTGCAAGTG TGGTCATGAT AAAGAAGCCT
1501 TTGTATGTAT GGCACGCAGC TACTGGAGTC GTTGTGGCTG CATCTGTGGC
1551 TGCGTTCGCG TTTTGGTATG GGAGGAGACG CCGTAGATGG TGAGGAAAAA
1601 AAAAACTCTT CAAGTGTTGG ATTCATTGGA AATGGAACTT GCTGTTGTTT
1651 GTAGCATTGG TGGGAGAAAT ATAGAAGGGA GAGTCCAAAC TACTCAATGT
1701 GATTACATTG GTATACGATT ATTATATGCT TCTTTTAAAA TGCATAAACG
1751 TTTCCACTAC AAACTTTG
```

Fig. 11

```
  1 MGRAFVYVIL GGGVAAGYAA LEFTRRGVSD GELCIISEEP VAPYERPALS
 51 KGFLLPEAPA RLPSFHTCVG ANDEKLTPKW YKDHGIELVL GTRVKSVDVR
101 RKTLLSSTGE TISYKFLIIA TGARALKLEE FGVEGSDAEN VCYLRDLADA
151 NRLATVIQSS SNGNAVVIGG GYIGMECAAS LVINKINVTM VFPEAHCMAR
201 LFTPKIASLY EDYYRAKGVK FIKGTVLTSF EFDSNKKVTA VNLKDGSHLP
251 ADLVVVGIGI RPNTSLFEGQ LTIEKGGIKV NSRMQSSDSS VYAIGDVATF
301 PVKLFGEMRR LEHVDSARKS ARHAVSAIMD PIKTGDFDYL PFFYSRVFAF
351 SWQFYGDPTG DVVHFGEYED GKSFGAYWVK KGHLVGSFLE GGTKEEYETI
401 SKATQLKPAV TIDLEELERE GLGFAHTVVS QQKVPEVKDI PSAEMVKQSA
451 SVVMIKKPLY VWHAATGVVV AASVAAFAFW YGRRRRRW
```

Fig. 12

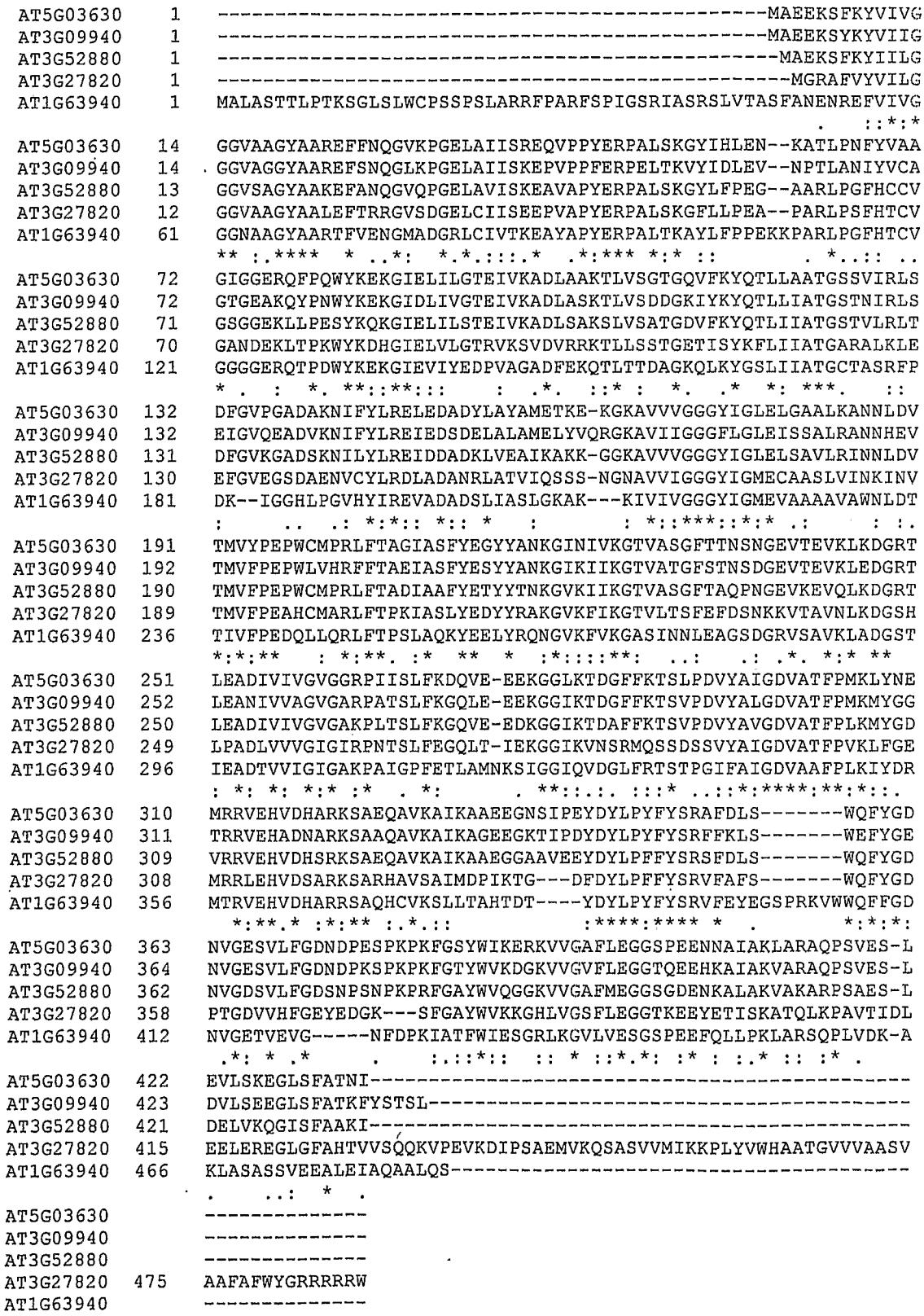
AT5G03630 1 ----------MAEEKSFKYVIVG
AT3G09940 1 ----------MAEEKSYKYVIIG
AT3G52880 1 ----------MAEKSFKYIILG
AT3G27820 1 ----------MGRAFVYVILG
AT1G63940 1 MALASTTLPTKSGLSLWCPSSPSLARRFPARFSPIGSRIASRSLVTASFANENREFVIVG
AT5G03630 14 GGVAAGYAAREFFNQGVKPGELAIISREQVPPYERPALSKGYIHLEN--KATLPNFYVAA
AT3G09940 14 GGVAGGYAAREFSNQGLKPGELAIISKEPVPPFERPELTKVYIDLEV--NPTLANIYVCA
AT3G52880 13 GGVSAGYAAKEFANQGVQPGELAVISKEAVAPYERPALSKGYLFPEG--AARLPGFHCCV
AT3G27820 12 GGVAAGYAALEFTRRGVSDGELCIISEEPVAPYERPALSKGFLLPEA--PARLPSFHTCV
AT1G63940 61 GGNAAGYAARTFVENGMADGRLCIVTKEAYAPYERPALTKAYLFPPEKKPARLPGFHTCV
AT5G03630 72 GIGGERQFPQWYKEKGIELILGTEIVKADLAAKTLVSGTGQVFKYQTLLAATGSSVIRLS
AT3G09940 72 GTGEAKQYPNWYKEKGIDLIVGTEIVKADLASKTLVSDDGKIYKYQTLLIATGSTNIRLS
AT3G52880 71 GSGGEKLLPESYKQKGIELILSTEIVKADLSAKSLVSATGDVFKYQTLIIATGSTVLRLT
AT3G27820 70 GANDEKLTPKWYKDHGIELVLGTRVKSVDVRRKTLLSSTGETISYKFLIIATGARALKLE
AT1G63940 121 GGGGERQTPDWYKEKGIEVIYEDPVAGADFEKQTLTTDAGKQLKYGSLIIATGCTASRFP
AT5G03630 132 DFGVPGADAKNIFYLRELEDADYLAYAMETKE-KGKAVVVGGGYIGLELGAALKANNLDV
AT3G09940 132 EIGVQEADVKNIFYLREIEDSDELALAMELYVQRGKAVIIGGGFLGLEISSALRANNHEV
AT3G52880 131 DFGVKGADSKNILYLREIDDADKLVEAIKAKK-GGKAVVVGGGYIGLELSAVLRINNLDV
AT3G27820 130 EFGVEGSDAENVCYLRDLADANRLATVIQSSS-NGNAVVIGGGYIGMECAASLVINKINV
AT1G63940 181 DK--IGGHLPGVHYIREVADADSLIASLGKAK---KIVIVGGGYIGMEVAAAAVAWNLDT
AT5G03630 191 TMVYPEPWCMPRLFTAGIASFYEGYYANKGINIVKGTVASGFTTNSNGEVTEVKLKDGRT
AT3G09940 192 TMVFPEPWLVHRFFTAEIASFYESYYANKGIKIIKGTVATGFSTNSDGEVTEVKLEDGRT
AT3G52880 190 TMVFPEPWCMPRLFTADIAAFYETYYTNKGVKIIKGTVASGFTAQPNGEVKEVQLKDGRT
AT3G27820 189 TMVFPEAHCMARLFTPKIASLYEDYYRAKGVKFIKGTVLTSFEFDSNKKVTAVNLKDGSH
AT1G63940 236 TIVFPEDQLLQRLFTPSLAQKYEELYRQNGVKFVKGASINNLEAGSDGRVSAVKLADGST
AT5G03630 251 LEADIVIVGVGGRPIISLFKDQVE-EEKGGLKTDGFFKTSLPDVYAIGDVATFPMKLYNE
AT3G09940 252 LEANIVVAGVGARPATSLFKGQLE-EEKGGIKTDGFFKTSVPDVYALGDVATFPMKMYGG
AT3G52880 250 LEADIVIVGVGAKPLTSLFKGQVE-EDKGGIKTDAFFKTSVPDVYAVGDVATFPLKMYGD
AT3G27820 249 LPADLVVVGIGIRPNTSLFEGQLT-IEKGGIKVNSRMQSSDSSVYAIGDVATFPVKLFGE
AT1G63940 296 IEADTVVIGIGAKPAIGPFETLAMNKSIGGIQVDGLFRTSTPGIFAIGDVAAFPLKIYDR
AT5G03630 310 MRRVEHVDHARKSAEQAVKAIKAAEEGNSIPEYDYLPYFYSRAFDLS-------WQFYGD
AT3G09940 311 TRRVEHADNARKSAAQAVKAIKAGEEGKTIPDYDYLPYFYSRFFKLS-------WEFYGE
AT3G52880 309 VRRVEHVDHSRKSAEQAVKAIKAAEGGAAVEEYDYLPFFYSRSFDLS-------WQFYGD
AT3G27820 308 MRRLEHVDSARKSARHAVSAIMDPIKTG---DFDYLPFFYSRVFAFS-------WQFYGD
AT1G63940 356 MTRVEHVDHARRSAQHCVKSLLTAHTDT----YDYLPYFYSRVFEYEGSPRKVWWQFFGD
AT5G03630 363 NVGESVLFGDNDPESPKPKFGSYWIKERKVVGAFLEGGSPEENNAIAKLARAQPSVES-L
AT3G09940 364 NVGESVLFGDNDPKSPKPKFGTYWVKDGKVVGVFLEGGTQEEHKAIAKVARAQPSVES-L
AT3G52880 362 NVGDSVLFGDSNPSNPKPRFGAYWVQGGKVVGAFMEGGSGDENKALAKVAKARPSAES-L
AT3G27820 358 PTGDVVHFGEYEDGK---SFGAYWVKKGHLVGSFLEGGTKEEYETISKATQLKPAVTIDL
AT1G63940 412 NVGETVEVG-----NFDPKIATFWIESGRLKGVLVESGSPEEFQLLPKLARSQPLVDK-A
AT5G03630 422 EVLSKEGLSFATNI----------
AT3G09940 423 DVLSEEGLSFATKFYSTSL----------
AT3G52880 421 DELVKQGISFAAKI----------
AT3G27820 415 EELEREGLGFAHTVVSQQKVPEVKDIPSAEMVKQSASVVMIKKPLYVWHAATGVVVAASV
AT1G63940 466 KLASASSVEEALEIAQAALQS----------
AT5G03630 ----------
AT3G09940 ----------
AT3G52880 ----------
AT3G27820 475 AAFAFWYGRRRRRW
AT1G63940 ----------

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/001461

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82

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)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MURTHY SIVA A ET AL: "Suppression of monodehydroascorbate reductase expression in transgenic tobacco confers tolerance to paraquat-mediated oxidative stress" PLANT PHYSIOLOGY (ROCKVILLE), vol. 114, no. 3 SUPPL., 1997, page 298, XP001537845 & PLANT BIOLOGY '97: 1997 ANNUAL MEETINGS OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS AND THE CANAD; VANCOUVER, BRITISH COLUMBIA, CANADA; AUGUST 2-6, 1997 ISSN: 0032-0889 abstract ----- -/--	22-25, 29-31, 34,35, 37,38, 40-42

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 2008	15/02/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, Thomas

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/19994 A (DU PONT [US]; BUTLER KARLENE H [US]; CAHOON REBECCA E [US]; KLEIN THEO) 22 March 2001 (2001-03-22) e.g. SEQ ID NO:3, 4 the whole document	22-42, 45,46
X	LISENBEE CAYLE S ET AL: "Arabidopsis peroxisomes possess functionally redundant membrane and matrix isoforms of monodehydroascorbate reductase" PLANT JOURNAL, vol. 43, no. 6, September 2005 (2005-09), pages 900-914, XP002466826 ISSN: 0960-7412 cited in the application page 907, left-hand column	45,46,49
Y	EASTMOND PETER J: "MONODEHYROASCORBATE REDUCTASE4 is required for seed storage oil hydrolysis and postgerminative growth in Arabidopsis" PLANT CELL, vol. 19, no. 4, 20 April 2007 (2007-04-20), pages 1376-1387, XP002466827 ISSN: 1040-4651 the whole document	1-50
Y	EASTMOND PETER J: "SUGAR-DEPENDENT1 encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating Arabidopsis seeds" PLANT CELL, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, US, vol. 18, no. 3, March 2006 (2006-03), pages 665-675, XP002415336 ISSN: 1040-4651 page 672, left-hand column, line 23, paragraph 1 - line 27 page 672, left-hand column, line 12, paragraph 2 - line 14	1-50
A	RIZHSKY LUDMILA ET AL: "Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase" PLANT JOURNAL, vol. 32, no. 3, November 2002 (2002-11), pages 329-342, XP002466828 ISSN: 0960-7412	

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2007/001461

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ELTAYEB AMIN ELSADIG ET AL: "Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses" PLANTA (BERLIN), [Online] vol. 225, no. 5, 17 October 2006 (2006-10-17), pages 1255-1264, XP002466829 ISSN: 0032-0935 Retrieved from the Internet: URL:http://www.springerlink.com/content/100484/> [retrieved on 2008-01-28] the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2007/001461

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0119994 A	22-03-2001	AU 7367900 A	17-04-2001