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(71) Applicant *(for all designated States except US)*: **CARGILL, INCORPORATED** [US/US]; P.O. Box 5624, Minneapolis, MN 55440-5624 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SHORROSH, Basil, S.** [US/US]; 3311 Laredo Lane, Ft. Collins, CO 80526 (US). **HALLIER, Sonia** [FR/US]; 460 Maroon Bells Circle, Livermore, CO 80536 (US).

(74) Agent: ELLINGER, Mark, S.; Fish & Richardson P.C., P.A., Suite 3300, 60 South Sixth Street, Minneapolis, MN 55402-1104 (US).

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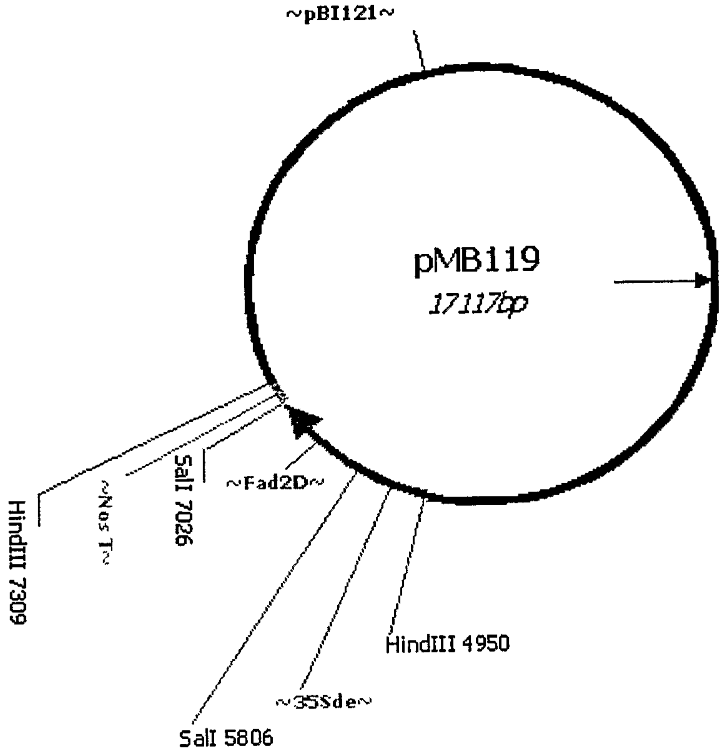
(54) Title: METHODS OF MAKING PLANTS THAT EXHIBIT ENHANCED DISEASE RESISTANCE

(57) Abstract: Methods for producing plants that exhibit enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* are described.



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METHODS OF MAKING PLANTS THAT EXHIBIT ENHANCED DISEASE RESISTANCE

TECHNICAL FIELD

This invention relates to fungal resistance in plants, and more particularly to methods of making plants that exhibit enhanced resistance to fungal infections.

BACKGROUND

Fungal diseases are responsible for damage to many cultivated species. The amount of damage varies each year, depending on temperature, amount of rain, and quantity of inoculum present in fields. In some instances, fungal diseases can completely destroy fields, leading to an estimated average loss of yield of 20% of crops worldwide. Blackleg, one of the predominant fungal diseases in rape plants, typically results in losses of tens of millions of dollars annually. *Sclerotinia*, another predominant fungal disease of *Cruciferae* plants, which includes *Brassica* plants as well as 400 other species of plants including *Compositae* plants such as sunflower and leguminous plants such as pea, also can result in significant economic losses.

Blackleg disease is caused by an *Ascomycetes* fungus whose perfect or sexual form is known as *Leptosphaeria maculans* and whose imperfect or asexual form is known as *Phoma lingam*. The sexual form provides the primary inoculum each year and is responsible for the high variability of the fungus. *L. maculans* is in fact a complex of species, of which two main groups have been identified, TOX^+ and TOX°. The TOX^+ species is aggressive and produces two toxins, sirodesmin and phomalide. Within the TOX^+ species, several strains or pathogenicity groups (PG) exist. In Europe, Australia, and Eastern Canada, PG3 and PG4 are the predominant strains of *L. maculans*, while in western Canada, PG2 is the predominant strain of *L. maculans*.

Ascospores on infested canola stubble are the main source of infection by the virulent (*i.e.,* TOX^+) *L. maculans*. Ascospores are released into the air and can infect cotyledons and younger leaves of plant seedling via stomata or wounds. The initial infection is biotrophic, but then becomes necrotrophic and produces asexual bodies (pycnidia) in the dead tissue. The spores produced from the pycnidia bodies, called pycnidiospores, are believed to spread and infect other leaves of neighboring plants after being released by rain splash or other mechanical stress. The consequence of fungal invasion of canola plants is the formation of blackened canker in the stem, hence the name "blackleg." The blackleg disease can lead to plant death prior to producing seeds, and therefore has a major impact on seed yield.

Sclerotinia sclerotiorum, an *Ascomycetes* fungus, produces white rot on the plants it infects. *S. sclerotiorum* has broad ecological distribution, but is most common in temperate regions. *S. sclerotiorum* produces a fluffy white mass of mycelia on the surface of the host and on adjacent soil surfaces. Dense white bodies start forming within the mycelia, which becomes black and hard as the fungus matures. The hardened mycelia are called sclerotia, which allow the fungus to survive for several years in soil. When conditions are suitable, the sclerotia will germinate, and produce either mycelium, which can infect plants that are in direct contact with the sclerotia, or spore-producing apothecia. Most infections in canola result from air-borne spores produced by apothecia at the soil surface. Spores often infect, germinate, and grow in petals and eventually fall on other plant tissue. The fungus eventually leads to leaf, stem, and fruit rot. In the stem, the fungus will form new sclerotia, which fall to the soil at harvest, thereby completing the disease cycle.

Strategies for limiting fungal damage include prophylactic measures such as crop rotations or burying of crop debris, fungicide use, and genetic improvement. Prophylactic measures, however, are not very effective as fungi can survive for many years in the soil. Fungicides can be effective when applied at the appropriate time, but cost often is high compared with any gain in yield. Furthermore, genetic improvement in plants for resistance to *S. sclerotiorum* has been limited since only plant species that exhibit low tolerance to the fungus have been identified. Thus, a need exists for plants that are resistant to such fungi, as well as for methods of improving plant resistance to fungi.

SUMMARY

The invention is based on the surprising discovery that decreasing expression of the gene encoding delta 12 fatty acid desaturase (fad2) or omega 6 fatty acid desaturase (fad6) in plants provides such plants with a significantly enhanced ability to resist infection by *L. maculans* and/or *S. sclerotiorum* compared with existing natural resistance. Expression of fad2 or fad6 can be decreased such that Fad2 or Fad6 proteins, respectively, are reduced at particular locations (*e.g.*, the leaves), at a particular stage of development, or upon stimulation by the appropriate environmental conditions.

In one aspect, the invention provides a method for producing a dicotyledonous plant. Such a method includes introducing a nucleic acid construct into cells of a plant, wherein the nucleic acid construct comprises a regulatory element operably linked to a delta-12 fatty acid desaturase (fad2) nucleic acid molecule; generating one or more progeny plants from the cells; and identifying at least one of the progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* relative to a corresponding control plant.

Representative plants include *Brassica* plants, *Helianthus annuus* plants, and *Glycine max* plants. Methods of the invention also can be used to make hybrid plants (*e.g., Brassica* or *Helianthus annuus* hybrid plants). Generally, the regulatory element is a promoter such as a cruciferin promoter and/or a constitutive promoter. In some embodiments, the fad2 nucleic acid molecule includes both fad2F and fad2D nucleic acid sequences.

In some embodiments, the fad2 nucleic acid molecule is operably linked to the regulatory element in sense orientation. Such a molecule can result in co-suppression of fad2 nucleic acid sequences in the plant. In another embodiment, a construct for use in the methods of the invention can encode a self-splicing ribozyme such as the negative strand of the satellite RNA from Barley Yellow Dwarf Virus operably linked to the fad2 nucleic acid molecule.

In another embodiment, the fad2 nucleic acid molecule is operably linked to the regulatory element in antisense orientation. In an embodiment, the fad2 nucleic acid molecule comprises fad2 nucleic acid sequences in sense orientation operably linked to fad2 nucleic acid sequences in antisense orientation, wherein the fad2 nucleic acid sequences in sense orientation and the fad2 nucleic acid sequences in antisense orientation are on the same strand, wherein the fad2 nucleic acid sequences in sense orientation and the fad2 nucleic acid sequences in antisense orientation are complementary. Such a construct can further include a spacer nucleic acid sequence, wherein the spacer nucleic acid sequences operably link the fad2 nucleic acid sequences in sense orientation and the fad2 nucleic acid sequences in antisense orientation, wherein the spacer nucleic acid sequences are between the fad2 nucleic acid sequences in sense orientation and the fad2 nucleic acid sequences in antisense orientation.

Typically, a plant made by a method of the invention produces seeds that exhibit an altered oleic acid to linoleic acid ratio relative to seeds produced by the corresponding control plant. It is desirable that leaves of the plant exhibit an altered oleic acid to linoleic acid ratio relative to leaves from the corresponding control plant.

Methods of the invention can further include producing a plant line from one or more of the progeny plants. Methods of the invention also can include contacting the progeny plants with a compound selected from the group consisting of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), wherein the contacting is prior to or after the identifying.

In some embodiments, the nucleic acid construct further comprises a regulatory element operably linked to a nucleic acid molecule encoding a KASII polypeptide, a nucleic acid molecule encoding a thioesterase polypeptide, or a regulatory element operably linked to a nucleic acid molecule encoding an omega 6 fatty acid desaturase (fad6). In other embodiments, the methods of the invention further include introducing a second nucleic acid construct into the cells, wherein the second nucleic acid construct comprises a regulatory element operably linked to a nucleic acid molecule encoding a polypeptide selected from the group consisting of thioesterase, 3-ketoacyl synthase II (KASII), chitinase, β-1,3-glucanase, PR5, and PR1.

In another aspect, the invention provides a method for producing a *Brassica* plant. Such a method includes introducing a nucleic acid construct into *Brassica* cells, wherein the nucleic acid construct comprises a regulatory element operably linked to a delta-12 fatty acid desaturase (fad2) nucleic acid molecule; generating one or more progeny plants from the cells; and identifying at least one of the progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

In another aspect, the invention provides a method for producing a *Brassica* plant. Such a method includes providing plant cells comprising a mutation in a fad2 and/or fad6 nucleic acid; generating one or more progeny plants from the cells; and identifying at least one of the progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

In yet another aspect, the invention provides a method for producing a *Brassica* plant. Such a method includes introducing a nucleic acid construct into *Brassica* cells, wherein the nucleic acid construct comprises a regulatory element operably linked to an omega 6 fatty acid desaturase (fad6) nucleic acid molecule; generating one or more progeny plants from the cells; and identifying at least one of the progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

The invention also features a transgenic plant comprising a nucleic acid construct. The construct comprises a regulatory element operably linked to an omega 6 fatty acid desaturase nucleic acid. The regulatory element confers expression in at least one vegetative tissue of the plant, and the plant has enhanced resistance to *S. sclerotiorum* and/or *L. maculans* in at least that tissue, relative to the corresponding resistance in that tissue of a corresponding control plant. The plant can have enhanced resistance to *S. sclerotiorum* and the regulatory element can confer expression in leaf tissue. The plant can be a *Brassica* plant, e.g., a *Brassica napus* plant. The plant can have enhanced resistance to *L. maculans* and the regulatory element can confer expression in stem tissue.

The invention also features a transgenic plant comprising a nucleic acid construct. The construct comprises a regulatory element operably linked to a delta-12 fatty acid desaturase nucleic acid, and the regulatory element confers expression in at least one vegetative tissue of the plant. The plant has enhanced resistance to *S. sclerotiorum* and/or *L. maculans* in at least that tissue, relative to the corresponding resistance in that tissue of a corresponding control plant. The plant can have enhanced resistance to *S. sclerotiorum* and the regulatory element can confer expression in leaf tissue. The plant can be a

Brassica plant, e.g., a *Brassica napus* plant. The plant can have enhanced resistance to *L. maculans* and the regulatory element can confer expression in stem tissue.

In still another aspect, the invention provides a method for producing a *Brassica* plant. Such a method includes increasing the ratio of oleic acid to linoleic acid in *Brassica* cells; generating one or more progeny plants from the cells; and identifying at least one of the progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans*. The oleic acid and the linoleic acid can be cytosolic or chloroplastic.

In another aspect, the invention provides the use of a nucleic acid construct comprising a regulatory element operably linked to a fad2 and/or fad6 nucleic acid molecule for the enhanced resistance in a plant to *S. sclerotiorum* or *L. maculans*. The regulatory element can be a promoter, for example, a cruciferin promoter. In certain embodiments, the fad2 and/or fad6 nucleic acids are in sense orientation; in other embodiments, the fad2 and/or fad6 nucleic acids are in antisense orientation.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic of the pMB119 construct containing fad2.

DETAILED DESCRIPTION

The invention features methods of making plants that exhibit enhanced resistance to fungi, in particular, to *S. sclerotiorum* and *L. maculans*. Plants and plant lines made using methods of the invention exhibit an altered ratio of oleic acid (18:1) to linoleic acid (18:2), which can be achieved, for example, by decreasing the expression of the enzymes involved in the conversion of 18:1 to 18:2 such as fad2 and/or fad6.

Fad2 is also known as omega-6 fatty acid desaturase, cytoplasmic oleic desaturase, or oleate desaturase. Fad2 catalyzes the desaturation of cytosolic oleic acid (18:1) to linoleic acid (18:2). Desaturation refers to the removal of two hydrogen atoms from a molecule, resulting in the formation of a carbon-carbon double bond, and generally occurs in plastids and in the endoplasmic reticulum. Specifically, Fad2 catalyzes the formation of a double bond between carbon positions 6 and 7 (numbered from the methyl end), *i.e.,* carbons that correspond to positions 12 and 13 (numbered from the carbonyl carbon) of an 18 carbon-long fatty acyl chain. A microsomal fad2 has been cloned and characterized (see, for example, Okuley et al., 1994, *Plant Cell,* 6:147-58). The nucleotide sequences of higher plant genes encoding microsomal fad2 and mutants thereof are described in U.S. Patent Nos. 6,372,965 and 6,342,658.

In *Brassica,* two major isoforms of Fad2 have been characterized, Fad2D and Fad2F. *Brassica* plants having a point mutation in fad2D (*e.g.,* IMC129) exhibit increased oleic acid content in the seeds, but not in the roots or leaves. Such plants do not show any morphological abnormalities. In addition, *Brassica* plants containing a point mutation in both fad2D and fad2F (*e.g., B. napus* lines Q508 and 5Q4275) exhibit increased oleic acid in the seeds and roots.

Omega-6 fatty acid desaturase (fad6) is the chloroplastic fad2. Fad6 catalyzes the desaturation of chloroplast 16:1 and 18:1 to 16:2 and 18:2, respectively. Plants deficient in Fad6 have increased levels of monounsaturated fatty acids and are deficient in trienoic fatty acids. Plants carrying a mutation in fad6 are characterized, at low temperatures, by leaf chlorosis, a reduced growth rate, and changes in chloroplast morphology such as a decrease in size and appressed regions of thylakoid membranes. Fad6 is thought to be a transmembrane protein. See, for example, Hitz et al., 1994, *Plant Physiol.,* 105:635-641 and GenBank Accession Nos. AF229391, AF229392, and X78311.

In conjunction with decreasing expression of fad2 or fad6, the expression of other genes such as those encoding products involved in fatty acid synthesis or host defense can be manipulated to further enhance the observed disease resistance. Genes encoding products involved in fatty acid synthesis include, for example, 3-ketoacyl synthase II (KASII), and thioesterase. Representative host defense genes include, but are not limited to, those encoding chitinase, β-1,3-glucanase, PR5 (*e.g.*, osmotin or osmotin-like proteins such as AP24), and PR1. See, for example, PCT Publication No. WO 02/061043. Additional host defense genes that can be used in the methods of the invention include those that encode peroxidases, ribosome inactivating proteins, protease inhibitors, defensins, thionins, ribonucleases, polygalacturonase inhibitor proteins, lipid transfer proteins, glycine-rich proteins, and extensin or hydroxyproline rich proteins.

3-ketoacyl synthase II (KASII) is involved in the biosynthesis of saturated fatty acids in plant chloroplasts. C2 units from acyl thioesters are linked sequentially, beginning with the condensation of acetyl Co-enzyme A (CoA) and malonyl-acyl carrier protein (malonyl-ACP) to form a C4 acyl fatty acid. This condensation reaction is catalyzed by a 3-ketoacyl synthase III (KASIII). The enzyme 3-ketoacyl synthase I (KASI) catalyzes the stepwise condensation of a fatty acyl moiety (C4 to C14) with C2 groups and malonyl-ACP to produce a 3-ketoacyl-ACP product that is 2 carbons longer than the original substrate (C6 to C16). The last condensation reaction in the chloroplast, converting C16 to C18, is catalyzed by KASII. 3-ketoacyl moieties are also referred to as ß-ketoacyl moieties. Representative KASII sequences include those shown in GenBank Accession Nos. AF026149 and U39441, and in Domergue & Post-Beittenmiller, 2000, *Biochem. Soc. Trans.*, 28:610-3.

A group of enzymes that may regulate the characteristics of plant lipids are the acyl-CoA thioesterases (ACHs). Thioesterases hydrolyze an ester bond in long chain fatty acyl-CoAs, yielding free fatty acid and CoA. Thus, thioesterases can thus influence intracellular levels of free fatty acids and their CoA esters. For example, when a fatty acyl group becomes 16 carbons long, a thioesterase enzyme hydrolyzes the fatty acyl group, thereby forming free palmitate (palmitoyl-ACP + H_2O → palmitate + ACP-SH). The sequences of representative thioesterases can be found, for example, in GenBank Accession Nos. X87842 and AJ488493, as well as U.S. Patent Nos. 5,955,650 and 6,331,664.

Chitinases are endohydrolases that hydrolyze the β-1,4 bond between the N-acetylglucosamine residues of chitin, a component of the wall of many pathogenic fungi.

Chitinases have been cloned from *Arabidopsis*, tobacco, bean, cucumber, tomato, and bacteria, and the sequences deposited in GenBank (see, for example, GenBank Accession No. A16119 for tobacco intracellular chitinase, AB015996 for chiA of *Serratia marcescens*, GenBank Accession No. AB026636 for an *Arabidopsis thaliana* chitinase, and GenBank Accession No. AJ301671 for a *Nicotinia sylvestris* endochitinase). See also, U.S. Patent 5,993,808.

β-1,3-glucanases are a family of endohydrolases that degrade fragments of glucan, another component of the wall of pathogenic fungi. β-1,3-glucanases have been cloned from *Arabidopsis*, pea, soybean, tobacco, bean, cucumber, tomato, rice, *Hevea* (para rubber), and bacteria. See GenBank Accession Nos. A16121, AB025632, AL353822, and D76437 for the nucleic acid and amino acid sequences of glucanases from tobacco, *A. thaliana*, *Neurospora crassa*, and rice, respectively. See also, U.S. Patent Nos. 6,087,560 and 6,066,491.

PR5 proteins are thaumatin-like proteins that are part of the osmotin family of proteins. Osmotins have an external surface with highly basic residues. Osmotins are thought to permeabilize the membrane surface of fungi, resulting in a modification of the pH gradient and destabilization of pressure gradients that maintain the tip of the hyphae in a tensed state. Consequently, cytoplasmic material is leaked and the hyphae rupture, or in the case of spores, the spores lyse. Osmotin or osmotin-like polypeptides have been cloned from tobacco, soybean, carrot, cotton, potato, and bean. GenBank Accession Nos. X65701, AL049500, and D76437 provide the nucleic acid and amino acid sequences of osmotin or osmotin-like proteins from tobacco, *A. thaliana*, and *Nicotiana sylvestris*. See also, U.S. Patent No. 6,087,161.

Endogenous PR1 proteins are highly induced during infection with pathogenic agents. PR1 proteins have been cloned from tobacco, *Arabidopsis*, and parsley. See GenBank Accession Nos. AL031394, X12572, and AI352904 for the nucleic acid and amino acid sequences of PR1 proteins from *A. thaliana*, parsley, and *B. napus*, respectively.

Decreasing expression of fad2 and/or fad6 alone or in combination with increasing the expression of one or more genes involved in fatty acid synthesis, or of host defense genes provides enhanced resistance to *S. sclerotiorum* and/or *L. maculans* in plants. For example, decreasing expression of fad2 and/or fad6 and increasing the expression of genes encoding PR5 and chitinase can provide enhanced resistance to blackleg in a transgenic plant relative to a control plant in which the expression of fad2 is not decreased and the amount of such polypeptides is not increased. In addition, decreasing expression of fad2 and/or fad6 while increasing the expression of genes encoding chitinase, glucanase, and PR5 can provide enhanced resistance to *S. sclerotiorum* in a plant relative to a control plant.

Nucleic Acid Constructs

Nucleic acid constructs suitable for use in the methods of the invention include nucleic acids encoding fad2 or fad6 operably linked to one or more regulatory elements such as a promoter. In conjunction with constructs containing fad2 or fad6, constructs containing nucleic acids encoding proteins involved in fatty acid synthesis or host defense proteins can be used in the methods of the invention to enhance resistance to *S. sclerotiorum* and/or *L. maculans*. Standard molecular biology techniques can be used to generate nucleic acid constructs.

The expression of fad2 or fad6 can be decreased using co-suppression technology. Co-suppression is a reduction in expression of a target gene upon introduction into a cell of a nucleic acid that is ultimately transcribed into an mRNA that has homology with the target gene's transcript. Therefore, co-suppression of fad2 or fad6 can be achieved using a construct that contains a fad2 or fad6 nucleic acid, respectively, operably linked in sense orientation to a regulatory element. It is not necessary that the fad2 or fad6 nucleic acid in the construct be full-length or have 100% homology with the target fad2 or fad6 nucleic acid, respectively, to be co-suppressed. See, for example, U.S. Patent Nos. 5,034,323 and 5,231,020 for a description of co-suppression technology.

Expression of fad2 or fad6 also can be decreased using antisense technology. The specific hybridization of a fad2 or fad6 antisense molecule with endogenous fad2 or fad6 nucleic acids, respectively, can interfere with the normal function of the endogenous nucleic acid. When the endogenous nucleic acid is DNA, antisense technology can disrupt replication and transcription. When the endogenous nucleic acid is RNA, antisense technology can disrupt, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity of the RNA. Antisense technology can also facilitate nucleolytic degradation of an endogenous RNA. See, for example, Brantl, 2002, *Biochim. Biophys. Acta*, 1575:15-25 and Sazani et al., 2002, *Curr. Opin. Botechnol.*, 13:468-72. Antisense molecules can be directed at regions encompassing the translation initiation or termination codon of fad2 or fad6. Antisense molecules also can be directed at the fad2 or fad6 open reading frame (ORF), at the 5' and 3' untranslated region of fad2 or fad6, and at intron regions and intron-exon junction regions. The effectiveness of an antisense molecule to decrease expression of fad2 or fad6 can be evaluated by measuring levels of the fad2 or fad6 mRNA or protein, respectively (*e.g.,* by Northern blotting, RT-PCR, Western blotting, ELISA, or immunohistochemical staining).

The term "hybridization," as used herein with respect to antisense technology, means hydrogen bonding, which can be Watson-Crick, Hoogsteen, or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. It is understood in the art that the sequence of a fad2 or fad6 antisense molecule need not be 100% complementary to that of its endogenous fad2 or fad6 nucleic acid, respectively, to be able to hybridize. A fad2 or fad6 antisense molecule specifically hybridizes to an endogenous fad2 or fad6 nucleic acid, respectively, when (a) binding of the antisense molecule to the fad2 or fad6 DNA or RNA molecule, respectively, interferes with the normal function of the fad2 or fad6 DNA or RNA, respectively, and (b) there is sufficient complementarity to avoid non-specific binding of the antisense molecule to non-fad2 or non-fad6 sequences, respectively, under conditions in which specific binding is desired, *i.e.,* under conditions in which *in vitro* assays are performed or under physiological conditions for *in vivo* assays. In some embodiments, it may be useful to design multiple antisense molecules that each hybridize to a different region of fad2 or fad6. In such embodiments, multiple antisense molecules can be on the same construct or on different constructs.

RNA interference (RNAi) technology also can be used to decrease expression of fad2 or fad6. RNAi technology utilizes constructs that produce aberrant RNA transcripts, which disrupt transcription and/or translation of the endogenous fad2 or fad6, respectively. See, for example, U.S. Patent No. 6,506,559; and PCT Publication Nos.

WO 99/53050; WO 01/12824; and WO 01/29058 for a description of RNAi technology and its use in decreasing expression of an endogenous nucleic acid.

An RNAi co-suppression construct can include a promoter, a fad2 or fad6 sequence operably linked to the promoter in sense orientation, a nucleic acid molecule encoding a self-splicing ribozyme operably linked to the fad2 or fad6 sequence, respectively, and a terminator sequence, operably linked to the self-splicing ribozyme nucleic acid. The nucleic acid molecule encoding the self-splicing ribozyme can be the negative strand from the satellite RNA of Barley Yellow Dwarf Virus, for example. Alternatively, an RNAi hairpin construct can contain a promoter, a region of the fad2 or fad6 sequence operably linked to the promoter in sense orientation, a spacer nucleic acid molecule operably linked to the sense fad2 or fad6 sequence, respectively, the complement of the same region of the fad2 or fad6 sequence, respectively, operably linked to the spacer nucleic acid in antisense orientation, and a terminator sequence operably linked to the antisense fad2 or fad6 sequence, respectively. Following transcription of an RNAi hairpin construct, a double-stranded duplex RNA (*e.g.*, a hairpin) is formed between the fad2 or fad6 complementary regions.

The expression of genes that produce proteins involved in fatty acid synthesis (e.g., KASII, or thioesterases) or host defense (*e.g.*, chitinase, β-1,3-glucanase, PR5, and/or PR1) can be manipulated to further enhance a plant's resistance to pathogens. For example, an exogenous nucleic acid encoding a host defense protein can be operably linked to a regulatory element and introduced into a plant using transgenic methods such as those described herein. The exogenous nucleic acid can be expressed to produce an amount of a host defense protein that is in addition to that produced by the endogenous nucleic acids. Further by way of example, an exogenous nucleic acid encoding a protein involved in fatty acid synthesis operably linked to a regulatory element can be introduced into a plant. Expression in plant tissue of such an exogenous nucleic acid can result in altered levels of particular fatty acids, thereby enhancing disease resistance. Altered levels of fatty acids in the plant tissue can be due to, for example, an increase in the amount of such protein (*e.g.*, expression of an additional copy of a nucleic acid) or a decrease in the amount of such protein (*e.g.*, by co-suppression of the exogenous nucleic acid).

Regulatory elements typically do not themselves code for a gene product. Instead, regulatory elements affect the expression level of the coding sequence. Suitable regulatory elements include promoters and enhancers. Regulatory elements can be constitutive or inducible, and can be tissue specific (*e.g.,* roots, seeds, veins, or the like) or developmental stage specific (*e.g., Brassica* developmental stages 1, 2, 3, 4, or 5). As used herein, "constitutive promoter" refers to a promoter that facilitates expression of a nucleic acid molecule without significant tissue- or temporal-specificity. An inducible promoter refers to a promoter that facilitates expression of a nucleic acid molecule in response to a stimulus such as a chemical, or light. Suitable promoters are known (*e.g.,* Weising et al., *Ann. Rev. Genetics* 22:421-478 (1988)). The following are representative examples of promoters suitable for use herein: regulatory sequences from fatty acid desaturase genes (*e.g., Brassica fad2D* or *fad2F*, see PCT Publication No. WO 00/07430); alcohol dehydrogenase promoter from corn; light inducible promoters such as the ribulose bisphosphate carboxylase (Rubisco) small subunit gene promoters from a variety of species; major chlorophyll a/b binding protein gene promoters; the 19S or 35S promoters of cauliflower mosaic virus (CaMV); hsr203j promoter from tobacco (Pontier et al., 1994, *Plant J.*, 5:507-21); as well as synthetic or other natural promoters that are either inducible or constitutive. See, *e.g.,* U.S. Patent No. 6,087,560. Particularly useful are regulatory elements that facilitate expression in vegetative tissue(s) but result in little or no expression in seeds. Such regulatory elements can be operative in vegetative tissues such as leaves, both immature and mature, stems, or both leaves and stems. Such regulatory elements can confer constitutive expression in a vegetative tissue(s) or inducible expression in a vegetative tissue(s). An example of a vegetative tissue-specific regulatory element is the FD *rold* promoter from *Agrobacterium.*

In some embodiments, the regulatory element is a promoter of plastid gene expression. Non-limiting examples of such a promoter include a 16S ribosomal RNA promoter, promoters of the photosynthetic genes *rbcL* and *psbA*, as well as the light-regulated promoter of the *psbD* operon. See, U.S. Patent No. 5,877,402 for constructs suitable for stable transformation of plastids.

In other embodiments, regulatory sequences are seed-specific, *i.e.,* the particular gene product is preferentially expressed in developing seeds and expressed at low levels or not at all in the remaining tissues of the plant. Non-limiting examples of seed-specific promoters include napin, phaseolin, oleosin, and cruciferin promoters. Further examples of suitable regulatory sequences for expression of fad2, fad6, or host defense genes are known in the art.

Promoters that result in expression of proteins in different tissues (*e.g.*, stems vs. leaves) may be especially useful in different geographical regions. Sub-strains of *L. maculans* having different degrees of virulence may be present in different regions of the world, *e.g.*, PG3 strains in Europe and Australia may have different degrees of virulence compared to strains in other geographic regions. In addition, the plant tissue in which fungal disease is most commonly observed may vary between different geographic regions. Thus, promoters can be used that result in expression in those tissues.

Additional regulatory elements may be useful in the nucleic acid constructs of the present invention, including, but not limited to, polyadenylation sequences, translation control sequences (*e.g.*, a ribosome binding site), enhancers, introns, and targeting sequences (*i.e.*, a sequence targeting to a particular organelle, such as a plastid). Such additional regulatory elements may not be necessary for sufficient expression of fad2 or fad6 nucleic acids, although they may increase expression by affecting transcription, stability of the mRNA, translational efficiency, or the like. Such elements can be included in a nucleic acid construct as desired to obtain optimal expression of fad2 or fad6 nucleic acids, respectively, in the host cell(s).

An example of a nucleic acid construct encoding fad2 in sense orientation is shown in FIG. 1. The 35S double enhancer promoter (35Sde) was cloned 5' of the fad2 sequences, and a Nos terminator was cloned into the construct 3' of the fad2 sequence. One or more additional constructs (*e.g.*, co-suppression, antisense, or RNAi) can be generated that contain fad6 nucleic acids, or that contain genes encoding proteins involved in fatty acid synthesis or host defense. Nucleic acids encoding such proteins can be together on a single construct or each can be on a separate construct.

Decreasing expression of fad2 and/or fad6

Methods of enhancing disease resistance in a plant can be accomplished by decreasing expression of fad2 and/or fad6. In the context of the present invention, "decreasing expression" means a decrease in the transcription or translation of a fad2 or fad6 gene. Expression of fad2 or fad6 can be decreased by mutagenesis, by transgenics, by combinations thereof, or by using existing germplasm.

Mutagenesis

Expression of fad2 and/or fad6 also can be decreased by mutagenesis. Mutagenesis is the introduction of mutations into nucleic acids, either *in vitro* or *in vivo*. Mutations can be induced in living organisms or in cultured cells by a variety of mutagens, including ionizing radiation, ultraviolet radiation, or chemical mutagens, by infection with certain viruses which integrate into the host genome, or by the introduction of nucleic acids previously mutagenized *in vitro*. For example, benzo[a]pyrene, *N*-acetoxy-2-acetylaminofluorene and aflatoxin B1 cause GC to TA transversions in bacteria and mammalian cells, although benzo[a]pyrene in particular can produce base substitutions such as AT to TA. *N*-nitroso compounds cause GC to AT transitions, while alkylation of the O4 position of thymine results in TA to CG transitions.

Plant cells from *Brassica* tissue can be mutagenized, for example, by exposing the cells to a chemical mutagen, and plants can be regenerated from such cells. The plant cells or the regenerated plant can be screened for fatty acid content, particularly for the levels of oleic acid and linoleic acid, to identify fad2 or fad6 mutants. Alternatively, a mutation can be introduced into a fad2 or fad6 nucleic acid molecule and introduced transgenically into a plant.

Transgenic cells and plants

Transgenic cells and plants in which expression of fad2 or fad6 has been decreased can be obtained by introducing an appropriate nucleic acid construct (*e.g.*, a co-suppression construct, an antisense construct, or an RNAi construct as described above) into one or more plant cells and regenerating a plant. A nucleic acid construct can be introduced into a plant cell by well-known transformation methods.

Leaves, seeds, or other tissue can be analyzed to identify those plants containing the construct or having the desired level of expression of the construct. For example, the polymerase chain reaction (PCR) and/or Southern blotting can be used to determine if seeds or other tissue contains the nucleic acid construct. Northern and/or Western blots can be used to examine expression of the construct.

Plants used in the methods of the invention can be any dicotyledonous species, including, for example, *Cruciferae* plants such as *Brassica* spp. (both low erucic and high erucic acid rapeseed). Suitable *Brassica* species include *B. napus*, *B. juncea*, *B. nigra*, *B. carinata*, *B. oleracea*, and *B. rapa*. The *Brassica* variety designated Westar transformed with wild type fad2 (Westar/fad2) also can be used in the methods of the invention. Other suitable species includes sunflower (*Helianthus annuus*), soybean (*Glycine max*), castor bean (*Ricinus communis*), peanut (*Arachis hypogaea*), tomato (*Lycopersicon esculentum*), and flax (*Linum usitatissimum*). In some species, plant lines that exhibit some degree of natural disease resistance can be used. In such lines, resistance to blackleg and/or *Sclerotinia* can be improved by decreasing expression of fad2 and/or fad6.

Transformation techniques for use in the invention include, without limitation, *Agrobacterium*-mediated transformation, polyethylene glycol treatment of protoplasts, electroporation, and particle gun transformation. Illustrative examples of transformation techniques are described in PCT Publication No. WO 99/43202 and U.S. Patent No. 5,204,253 (particle gun), and U.S. Patent No. 5,188,958 (*Agrobacterium*). Transformation methods utilizing the Ti and Ri plasmids of *Agrobacterium* spp. typically use binary type vectors. Walkerpeach et al., in *Plant Molecular Biology Manual*, Gelvin & Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994). If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art. In addition, various plant species can be transformed using the pollen tube pathway technique.

A plant described herein may be used as a parent to develop a plant line, or may itself be a member of a plant line, *i.e.,* it is one of a group of plants that display little or no genetic variation between individuals for a particular trait. Such lines can be created by several generations of self-pollination and selection for those species amenable to self-pollination. Vegetative propagation from a single parent using tissue or cell culture techniques also can be used. In some embodiments, cytoplasmic male sterility breeding systems are used to develop homozygous lines and also can be used to develop hybrids. Methods for producing hybrids can be found, for example, in U.S. Patent No. 6,323,392, and PCT Publication Nos. WO 92/05251 and WO 98/027806. In addition, Fick (1978, *Agronomy*, 19:279-337) and Vranceanu & Stoenescu (1971, *Euphytica*, 20:536-41) describe protocols for hybrid production in sunflower. Additional breeding techniques to create plant lines are known in the art.

Transgenic plants can be entered into a breeding program, *e.g.*, to increase seed, to introgress the novel construct(s) into other lines or species, or for further selection of other desirable traits. Desirable traits also can be fixed using di-haploid methods for screening and selecting embryos. Additional transgenic plants can be obtained by vegetative propagation of a single transgenic plant, for those species amenable to such techniques.

Progeny of transgenic plants are included within the scope of the invention, provided that such progeny exhibit resistance to *S. sclerotiorum* and/or *L. maculans*. Progeny of an instant plant include, for example, seeds formed on F1, F2, F3, and subsequent generation plants, or seeds formed on BC1, BC2, BC3, and subsequent generation plants. Seeds produced by a transgenic plant can be planted and the resulting plants can be bred or otherwise propagated (*e.g.*, selfed) to obtain plants homozygous for the construct. Di-haploid plants exhibiting decreased expression of fad2 also are within the scope of the invention, and can be generated by methods known in the art.

In some embodiments, a transgenic plant expressing fad2 or fad6 from a first nucleic acid construct can be crossed or mated with a second transgenic plant expressing a gene involved in fatty acid synthesis or host defense from a second nucleic acid construct to obtain plants having the desired combination of polypeptides and, thus, exhibiting the desired level of disease resistance.

Analyzing Plants

Plants that have enhanced resistance to *S. sclerotiorum* and/or *L. maculans* can be identified by known techniques, including *in vitro* tests on plant tissue or field, growth chamber, or greenhouse tests. For *S. sclerotiorum*, tests for resistance can be performed by inoculating stems or detached leaves of plants with *S. sclerotiorum* mycelium, then evaluating any resulting necrosis (*e.g.*, measuring length of necrosis) after a period of time sufficient for infection to develop. Blackleg resistance can be evaluated, for example, by inoculating cotyledons or stems with *L. maculans* and measuring the length of necrosis or mean disease severity (MDS) after a period of time sufficient for infection to develop. MDS can be measured on a scale from 0 to 5, with 5 being the worst.

Susceptible lines and highly resistant lines are used to obtain control values. For such inoculations and evaluations, it is desirable to use a substrain of *L. maculans* that is prevalent in the geographic region in which the resulting plants are to be utilized.

In some embodiments, plants that have enhanced fungal resistance can be identified by an increased amount of oleic acid in leaf tissue from such plants. In the case of *Brassica* plants, the amount of oleic acid can constitute from about 10% to about 25% of the total fatty acid content of the leaf tissue, e.g., about 10% to about 20%, about 11% to about 20%, about 12% to about 20%, about 13% to about 20%, about 14% to about 20%, about 12% to about 18%, or about 15% to about 18%.

In some embodiments, plants that have enhanced fungal resistance can be identified by an increased ratio of oleic acid/linoleic acid in leaf tissue from such plants. In the case, of *Brassica* plants, the ratio of oleic acid/linoleic acid can be from about 1.1 to about 5.0, e.g., about 1.1 to about 4.0, about 1.1 to about 4.0, about 1.2 to about 4.0, about 1.3 to about 4.0, about 1.4 to about 3.0, or about 1.5 to about 2.5.

In some embodiments, plants that have enhanced fungal resistance can be identified by an increased amount of oleic acid in stem tissue from such plants. In the case of *Brassica* plants, the amount of oleic acid can constitute from about 20% to about 35% of the total fatty acid content of the stem tissue, e.g., about 20% to about 30%, about 21% to about 35%, about 22% to about 30%, about 23% to about 30%, about 24% to about 30%, about 22% to about 25%, or about 15% to about 25%.

A phenotype is considered enhanced when the phenotype of a particular cell, plant, plant line, or plant population is statistically significantly different from a corresponding cell, plant, plant line, or plant population that is a suitable control, at a $p \leq 0.05$ with an appropriate parametric or non-parametric statistic. A suitable parametric or non-parametric statistic includes a Chi-square test, a Student's t-test, a Mann-Whitney test, or an F-test. In some embodiments, a difference in phenotype is statistically significant at $p < 0.01$, $p < 0.005$, or $p < 0.001$.

In some embodiments, plants that have enhanced fungal resistance can be identified by *in vitro* analysis of protein expression levels, evaluation of *in vitro* resistance in plant tissue, and/or *in vivo* resistance of whole plants in the field.

Additional Resistance Factors

In addition to the methods described above for decreasing expression of fad2 and/or fad6 using transgenic techniques or mutagenesis, expression of one or more host defense genes (exogenous or endogenous) can be induced by compounds such as salicylic acid, jasmonic acid, and ethylene to further enhance resistance. Salicylic acid, jasmonic acid, and ethylene are endogenous signal molecules that have been implicated in plant defense signaling pathways against pathogen infection.

Salicylic acid levels increase locally and systematically in plant tissue following pathogen infection, and exogenous application of salicylic acid results in enhanced resistance to a broad range of pathogens. Studies have shown that salicylic acid is required for the rapid localized activation of the expression of defense response genes to produce, for example, PR proteins. Salicylic acid also induced systemic expression of defense response genes to produce, for example, PR proteins, which results in systemic acquired resistance. An extensive review of the role of salicylic acid in plant disease resistance can be found in Dempsey et al. (1999, *Crit. Rev. Plant Sci.*, 18:547-75). Jasmonic acid, a fatty-acid-derived signaling molecule, is involved in several aspects of plant biology including pollen and seed development, and defense against wounding, ozone, insect pests and microbial pathogens. Jasmonic acid and/or ethylene can induce the production of several PR proteins, including Plant Defensin1.2 (PDF1.2), Thionin2.1 (THI2.1), Hevein-Like proteins (HEL) and ChitinaseB (CHIB). Experiments indicated that nearly half of the genes induced by ethylene were also induced by jasmonic acid, but further indicated that jasmonic acid and ethylene were able to independently regulate separate sets of genes.

Therefore, plants in which the expression of fad2 and/or fad6 is decreased can be treated with one or more of the above-indicated compounds to increase expression of genes involved in host defense, thereby further enhancing the resistance to *S. sclerotiorum* and/or *L. maculans*. In addition, nucleic acids encoding enzymes involved in the biosynthesis of such signaling molecules can be introduced into plants using the methods and materials described above to increase the amount of salicylic acid in the plant and thereby increase expression of genes involved in host defense. See, for example, Verberne et al., 2000, *Nat. Biotech.*, 18:779-83, and Yalpani et al., 2001, *The Plant Cell*, 13:1401-9.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Materials and Methods

Plant Lines

Westar variety is wild type for both fad2D and fad2F. The IMC129 variety contains a point mutation in fad2D. The Q508 and 5Q4275 lines were derived from IMC129 (*i.e.,* both lines contain the point mutation in fad2D) and each contains a different point mutation in fad2F. Q508/fad2 is the Q508 line transformed with wild type fad2D under control of the 35S double enhancer promoter (35Sde) (see, for example, Li et al., 2001, *Plant Sci.*, 160:877-87 and references therein). The expression of wild type fad2D in Q508 plants likely complements both the fad2D and fad2F mutations.

Evaluation of *S. sclerotiorum* (*Sclerotinia*) Resistance

To test Westar (a susceptible control), IMC129, Q508, 5Q4275, and Q508/fad2 lines for *Sclerotinia* resistance *in vitro*, fully expanded young leaves having a length of about 10 cm were removed from plants grown in a greenhouse environment and deposited in a round Petri dish (14 cm diameter) containing agar (7.5 g of technical grade agar per liter, 75 ml per dish). A *S. sclerotiorum* mycelial implant (7 mm in diameter) was deposited at the tip of the leaf and the dishes were kept at 16°C in an air-conditioned room. Humidity was maintained by wrapping the plates in plastic bags. The length of necrosis (in cm) along the midrib of the tested leaf was measured four days later and compared to the length of necrosis on a control (Westar) leaf. Mycelial implants were prepared by growing the fungus on agar-containing water for four days then sub-culturing onto potato dextrose agar (PDA) medium for three days. For every line, at least 4 sets of 5 plants were evaluated using one leaf from each plant (Table II).

Evaluation of Blackleg Resistance

The Williams test (phoma test on cotyledons) was performed on the Westar (a susceptible control), IMC129, Q508, 5Q4275, Q508/fad2, and Dunkeld (a spring *B.*

napus line that is resistant to the PG3 strain of *L. maculans*) lines, to evaluate blackleg resistance. See Williams & Delwiche, 1979 Cruciferae Conference, *Eucarpia,* 1980, page 164. Nine plants per line were evaluated for blackleg resistance. The rape plants were sown in a flat containing a greenhouse soil mixture and maintained at 25°C until the test was ready to be performed. The plants were watered and fertilized. Young leaves were systematically removed up to the day of the inoculation, which was 10 days after sowing. A small hole was made in each cotyledon lobe before inoculation and 10 μl of a suspension of pycnidiospores (500,000 spores/ml) were deposited in each hole. The flats were placed in cloches for two days. Necrosis diameter was measured two weeks later.

The blackleg test on stem was performed on 1 month-old plants. A hole was created in the stem at about 8 cm from the bottom of the plant. Ten μl of a suspension of pregerminated pycnidiospores (500,000 spores/ml) were deposited in each hole. Pycnidiospores were obtained by growing the *L. maculans* PG2 strain on V8 medium under near-ultraviolet light, recovering the pycnidiospores in sterile water, and filtering to remove impurities. Pregerminated pycnidiospores were made by adding glucose to the spore suspension at a final concentration of 10 mM. The spores were then pre-germinated for 3 days at room temperature. The wound was then covered by an adhesive bandage onto which 15μl of the spore suspension had been applied. The plants were kept at least 80% humidity for at least the first 4 days after inoculation. The scoring was performed one month after inoculation by measuring the length of necrosis on the stem.

Statistical Analysis

The Spectral Analysis Software program (Release 8.02, SAS Institute, Inc., Cary, NC) was used to perform statistical analysis using ANOVA with Duncan grouping at 95% confidence.

Preparation of Fatty Acid Methyl Esters and Analysis by Capillary GLC

Plant tissue was placed into a 15 ml polypropylene centrifuge tube, dried overnight at 135°C, minced by shaking with beads, treated with 0.6 ml of methanol/KOH solution, mixed on a vortex mixer for 30 sec, and then incubated in a water bath at 60°C for 60 seconds. About 4.0 ml of saturated NaCl was added to the tube followed by 1.0 ml of iso-octane, mixed on a vortex mixer for 30 sec, and then centrifuged for 5 minutes to separate and purify the organic layer. Approximately 700 μl of the organic layer containing the fatty acid methyl esters was removed from the tube and placed into a GC auto-sampler vial. The vial was purged with nitrogen gas to remove the oxygen and preserve the sample.

Fatty acid esters were analyzed using American Oil Chemists' Society (AOCS) Method Ce 1e-91. One µl of each sample was injected into a Hewlett Packard 6890 gas chromatograph by means of an auto-sampler. A normalized percentage was calculated and reported for each fatty acid in the sample.

GC conditions were as follows: Column: 5 m x 0.32 mm DB-Wax (0.5 µm film thickness); Detector: FID; Inlet temperature: 250°C; Detector temperature: 250°C; Split ratio: 100:1; Carrier gas: helium at 30.0 ml/min; and Oven program: 1.0 min at 220°C; 10.0°C/min to 245°C; 3.0 min at 245°C.

pMB119 Construct

A full-length oleoyl-fatty acid desaturase isoform "D" (fad2D) was amplified from *Brassica napus* genomic DNA (Westar variety) by PCR using primers Fad2D-Exp5' having the sequence: 5'-cau cau cau cau aaa aaa aac aac cat ggg tgc agg tgg aag aat-3' (SEQ ID NO:1) and Fad2D-Exp3' having the sequence: 5'-acu acu acu acu gtc gac ata gaa gag aaa ggt tca g-3' (SEQ ID NO:2). The amplified DNA was ligated into the pAMP1 vector (GIBCO). Subsequently, both strands of the fad2D gene was sequenced to confirm fidelity, and then released with *Sal*I digestion. The *Sal*I fragment containing the fad2D gene was cloned 3' to the 35Sde promoter at the *Sal*I site in the p1079 vector. Next, the DNA region containing the 35Sde-fad2D-Nos terminator cassette was released from p1079 by *Hind*III and subcloned at the *Hind*III site in pBI121 (Clontech, Palo Alto, CA), thereby generating pMB119. Figure 1 shows a schematic of pMB119.

Complementation of fad2 Mutations in Q508 Canola Line

The Q508 line contains a point mutation in the fad2D gene and another different point mutation in the fad2F gene. This line was transformed with the pMB119 binary construct using the *Agrobacterium*-mediated transformation method. Transgenic plants were evaluated for their fatty acid composition (Table I). Results confirmed that the wild type fad2D gene was able to complement the fad2 mutation in Q508.

Table I. Leaf Fatty Acid Composition of Canola Lines

Canola Lines	Leaf Fatty Acid Composition (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Westar	13.46	0.41	2.81	1.49	13.25	67.56
IMC129	12.54	0.31	1.75	1.39	13.11	70.28
Q508	10.82	0.52	3.42	16.73	6.77	59.69
5Q4275	11.78	0.44	1.24	11.48	6.88	67.14
Q508/fad2	12.8	0.46	4.43	3.81	15.67	59.44

Example 2 - Non-Transgenic Approach To Enhancing Resistance To *Sclerotinia* in *Brassica*

IMC129 plants were similar to Westar plants in their resistance to *S. sclerotiorum* infection based on the detached leaf assay (Table II, Table III). Q508 and 5Q4275 lines displayed enhanced resistance to *S. sclerotiorum* infection in detached leaves (Table II, Table III).

To determine whether the mutations in fad2D and fad2F genes are directly responsible for the enhanced plant resistance to *S. sclerotiorum*, the resistance of Q508/fad2 to *Sclerotinia* infection was examined. Q508/fad2 plants show normal root growth at low temperatures, and the fatty acid compositions of their seeds, roots, and leaves are similar to that of the Westar variety. Q508/fad2 plants, similar to Westar variety, did not display enhanced resistance to *S. sclerotiorum* infection in leaves (Table II, Table III). Therefore, the fad2 mutations in Q508 plant are directly responsible for the enhanced resistance to *S. sclerotiorum* infection in leaves.

fad2 mutations directly influence the cytosolic levels of 18:1, 18:2, and 18:3 fatty acids in plant tissues. Thus, the observed plant resistance to *S. sclerotiorum* in leaves is linked to the altered levels of these fatty acids. The most significant change is in the 18:1 and 18:2 fatty acid levels and their ratio. See Table IV. The high levels of 18:1 fatty acid and the low levels of 18:2 fatty acid in Q508 and 5Q4275 canola lines correlate with enhanced disease resistance to *S. sclerotiorum* infection in leaves (Table II, Table III). Conversely, the low levels of 18:1 fatty acid and high levels of 18:2 fatty acid in IMC129 and Q508/fad2 correlate with a level of *S. sclerotiorum* resistance in leaves that is similar to the resistance exhibited by the Westar variety (Table II, Table III).

Table II. Plant Leaf Resistance to *Sclerotinia* Infection

Canola Lines	Lesion Length Relative to Westar
Westar	100
IMC129	91
Q508	76
5Q4275	75
Q508/fad2	97

Table III. Statistical Analysis of Plant Leaf Resistance to *S. Sclerotiorum* Infection Using ANOVA With Duncan Grouping at 95% Confidence

Duncan Grouping[a]	Lesion Length Relative to Westar	Leaf #	Canola Lines
A	100	20	Westar
B	76	24	Q508
B	72	23	5Q4275

[a] Duncan, 1955, *Biometrics*, 11:1-42

Table IV. The level of 18:1 and 18:2 fatty acid and their ratio

	18:1	18:2	18:1/18:2
Westar	1.5%	13%	0.12
IMC129	1.4%	13%	0.11
Q508	17%	7%	2.47
5Q4275	11.5%	7%	1.67
Q508/fad2D	3.8%	16%	0.24

Based on these observations, it is possible to enhance the disease resistance of other plant species to necrotrophic pathogen infection, by altering their 18:1 and 18:2 fatty acids level. For example, sunflower leaves are low in oleic acid (~ 3%) and linoleic acid (~16%). Therefore, mutagenesis or transformation of sunflower such that the expression of fad2 is decreased in leaf tissue can enhance the resistance of sunflower plants to *Sclerotinia* infections.

Example 3 - Transgenic Approach To Enhancing Resistance To *Sclerotinia* in *Brassica*

Canola tissue (Westar variety) was transformed with a construct containing fad2 in sense orientation under control of the Cruciferin promoter, a *Brassica napus* seed specific promoter, and regenerated into plants (Westar/fad2). Seeds from Westar/fad2 exhibited co-suppressed fad2 expression as evidenced by the following. The 18:1 and 18:2 fatty acid level in the seeds from Westar/fad2 plants was 85% and 2% of total fatty acid content, respectively. Interestingly, when compared to Westar variety, Westar/fad2 plants also showed an increase in 18:1 content from about 1.5% in Westar leaves to about 15% in Westar/fad2 leaves, and a decrease in 18:2 content from about 13% in Westar leaves to about 8% in Westar/fad2 leaves (Table V). Westar/fad2 plants also exhibited enhanced resistance to *S. sclerotiorum* similar to that exhibited by the Q508 line (Table II, Table VI).

Table V. Leaf Fatty Acid Composition of Canola Lines

Canola Lines	Leaf Fatty Acid Composition					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Westar/fad2	11.17	0.53	4.01	14.92	8.21	58.89
Westar	13.46	0.41	2.81	1.49	13.25	67.56

Table VI. Leaf Resistance to *S. Sclerotiorum* Infection

Canola Lines	Lesion Length Relative to Westar
Westar	100
Westar/fad2	79
Q508	76

Example 4 - Non-Transgenic Approach To Enhancing Resistance To Blackleg in *Brassica*

Example 2 describes the creation of several canola lines with mutations in fad2 genes, and their fatty acid composition in leaves and seeds. These lines were also tested for their resistance to *L. maculans* pathogen, the causal agent of Blackleg disease using the cotyledon assay. Plants with enhanced resistance to *S. sclerotiorum* infection (Table II) were also slightly more resistant to *L. maculans* infection (Table VII).

Table VII. Cotyledons Resistance to *L. maculans* Infection

Canola Lines	Severity of Lesion[a]
Westar	4
IMC129	4
Q508	3
5Q4275	3

[a] 1 = 1 mm lesion, 2 = 3 mm lesion, 3 = 6 mm lesion, and 4 = 10 mm lesion

Example 5 - Transgenic Approach To Enhancing Resistance To Blackleg in *Brassica*

Example 3 describes the development of Westar/fad2 transgenic plants. Westar/fad2 plants also were tested for resistance to *L. maculans,* the causal agent of blackleg, using the stem assay. The 18:1 and 18:2 fatty acid levels in Westar/fad2 seeds was 85% and 2% of total fatty acid content, respectively. Compared to the Westar variety, Westar/fad2 plants showed an increase in 18:1 content of about 6.3% in Westar stems to about 26.4% in Westar/fad2 stems, and a decrease in 18:2 content from about 17.5% in Westar stems to about 7.6% in Westar/fad2 stems (Table VIII).

Interestingly, Westar/fad2 plants with enhanced resistance to *S. sclerotiorum* infection on the leaf (Table VI) were also slightly more resistant to *L. maculans* infection on the stem (Table IX). The *L. maculans* resistance observed in Westar/fad2 plants was statistically significant when compared to the *L. maculans* resistance observed in the wild type Westar plants (LSD = 0.05).

Table VIII. Stem Fatty Acid Composition of Canola Lines

Canola Lines	Stem Fatty Acid Composition					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Westar/fad2	13.64	0.86	2.02	26.43	7.55	48.40
Westar	17.21	0.85	4.43	6.29	17.46	52.94

Table IX. Stem Resistance to *L. maculans* Infection

Canola Lines	Length of Necrosis Relative to Westar
Westar	100
Westar/fad2	82

Example 6 – Transgenic Approach to Enhancing Resistance to *Sclerotinia* in Sunflower

Sunflower (*Helianthus annuus*) lines are available that exhibit high oleic acid in the seeds (see, for example, Sperling et al., 1990, *Z. Naturforsch*, 45:166-72). The lines that exhibit high oleic acid in the seeds exhibit an oleic acid content in the leaves that is similar to wild type sunflower (see Table X and, for example, Sperling et al., 1990, *Z. Naturforsch*, 45:166-72). To generate sunflower plants exhibiting high oleic acid in their leaves and/or stem, sunflower plants are transformed with a binary construct containing a fad2 nucleic acid under control of the ribulose 1,5-bisphosphate carboxylase (rbcS) promoter using a protocol involving a combination of particle bombardment followed by *Agrobacterium*-mediated transformation (Bidney et al., 1992, *Plant Mol. Biol.*, 18:301-13, and Malone-Schoneberg et al., 1994, *Plant Sci.*, 103:199-207).

The resulting plants are analyzed for fatty acid composition as described in Example 1, and compared with the fatty acid composition of wild type sunflower (Table X). Those plants exhibiting an increase in 18:1 and a decrease in 18:2 levels are evaluated for their resistance to infection by *Sclerotinia* as described in Example 1. *Sclerotinia* resistance in the transgenic sunflower plants is compared to *Sclerotinia* resistance in wild type sunflower plants.

Table X. Leaf Fatty Acid Composition of Sunflower

Plant Variety	Leaf Fatty Acid Composition (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Wild Type	12.7	0.2	0.99	3.37	15.57	63.85

Example 7 – Transgenic Approach to Enhancing Resistance to *Sclerotinia* in Soybean

Soybean (*Glycine max*) plants are transformed with a binary construct containing a fad2 nucleic acid coding region under control of the rbcS promoter using *Agrobacterium*-mediated transformation (see, for example, U.S. Patent No. 5,416,011).

The resulting plants are analyzed for fatty acid composition as described in Example 1. Those plants exhibiting an increase in 18:1 and a decrease in 18:2 levels are evaluated for their resistance to infection by *Sclerotinia* as described in Example 1. *Sclerotinia* resistance in the transgenic soybean plants is compared to the *Sclerotinia* resistance exhibited by the wild type soybean plants.

Example 8 – Non-transgenic Approach to Enhancing Resistance to *Sclerotinia* in Sunflower or Soybean

Mutagenesis, for example, can be used to enhance resistance to *Sclerotinia* in sunflower or soybean. Mutagenesis of sunflower or soybean is performed essentially as described in U.S. Patent No. 5,668,299 or WO 92/03919. The resulting plants are analyzed for fatty acid composition as described in Example 1, and compared with the fatty acid composition of wild type sunflower (Table X) or wild type soybean. Those plants exhibiting an increase in 18:1 and a decrease in 18:2 levels are evaluated for their resistance to infection by *Sclerotinia* as described in Example 1. *Sclerotinia* resistance in the mutagenized sunflower or soybean plants is compared to *Sclerotinia* resistance in wild type sunflower or soybean plants.

Example 9—High Oleic Dihaploid Lines and *Sclerotinia* Resistance

Leaves were detached from a number of different high-oleic dihaploid canola lines at a time when the plants were flowering and treated with *Sclerotinia* as described in

Example 1. The dihaploid canola lines were generated by crossing a high oleic canola line (~85%) with a canola line containing typical levels of oleic acid (~55-60%), and flowers from the F1 plants were made dihaploid using colchicines. Experiments were repeated four times with two pathological tests each. The sizes of the resulting necrotic lesions were measured in millimeters (mm) (Table XI). Leaves from the same plants near the end of their flowering stage were analyzed for fatty acid composition (Table XI). IMC304 and Westar were used as positive and negative controls, respectively.

The coefficient of correlation of *Sclerotinia* resistance with oleic acid was $R^2=0.3114$, and the coefficient of correlation of *Sclerotinia* resistance with the ratio of oleic acid to linoleic acid was $R^2=0.2407$.

Table XI. *Sclerotinia* Resistance and Fatty Acid Composition in Dihaploid Lines

	sclerotinia leaf necrosis (mm)	C16:0	C16:1	C18:0	C18:1	C18:2	18:1/18:2	C18:3
01ZG.109	24	11.3	0.4	1.2	13.4	7.9	1.7	65.0
Z630022	24	12.2	0.4	1.6	11.5	9.7	1.2	63.6
IMC304	26	11.3	0.4	1.3	11.1	9.3	1.2	65.5
Z630039	27	10.9	0.4	1.0	12.9	9.1	1.4	60.7
GA134RT181	28	11.3	0.2	1.5	10.3	9.4	1.1	65.9
Z629834	28	12.1	0.3	1.3	10.6	9.3	1.1	65.8
Z629845	29	12.9	0.4	1.5	13.5	11.2	1.2	59.0
Z630042	31	11.4	0.4	1.2	13.0	9.8	1.3	62.8
GA134RT67	33	12.6	0.4	1.5	10.7	8.0	1.3	65.9
Z630025	33	11.8	0.4	1.3	12.2	8.7	1.4	64.0
GA134RT-43	34	12.4	0.4	1.3	12.4	7.7	1.6	63.8
Z630030	34	11.2	0.4	1.1	13.2	10.5	1.3	62.2
Westar	36	13.5	0.4	2.8	1.5	13.3	0.1	67.6
Z630125	36	12.2	0.3	1.6	8.7	8.1	1.1	64.7
Z630138	40	12.1	0.3	1.4	10.1	10.0	1.0	63.7
Z630041	43	12.4	0.3	1.5	8.1	9.3	0.9	67.3

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for producing a dicotyledonous plant, said method comprising:

introducing a nucleic acid construct into cells of said plant, wherein said nucleic acid construct comprises a regulatory element operably linked to a delta-12 fatty acid desaturase (fad2) nucleic acid molecule;

generating one or more progeny plants from said cells; and

identifying at least one of said progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* relative to a corresponding control plant.

2. The method of claim 1, wherein said plant is a *Brassica* plant.

3. The method of claim 1, wherein said plant is a *Helianthus annuus* plant.

4. The method of claim 1, wherein said plant is a *Glycine max* plant.

5. The method of claim 1, wherein said plant is a hybrid plant.

6. The method of claim 1, wherein said regulatory element is a promoter.

7. The method of claim 6, wherein said promoter is a cruciferin promoter.

8. The method of claim 6, wherein said promoter is a constitutive promoter.

9. The method of claim 1, wherein said fad2 nucleic acid molecule is operably linked to said regulatory element in sense orientation.

10. The method of claim 9, wherein expression of said fad2 nucleic acid molecule results in co-suppression of fad2 nucleic acid sequences in said plant.

11. The method of claim 1, wherein said construct further comprises a nucleic acid molecule encoding a self splicing ribozyme operably linked to said fad2 nucleic acid molecule.

12. The method of claim 11, wherein said nucleic acid molecule encoding said self splicing ribozyme is a negative strand satellite RNA from Barley Yellow Dwarf Virus.

13. The method of claim 1, wherein said fad2 nucleic acid molecule is operably linked to said regulatory element in antisense orientation.

14. The method of claim 1, wherein said fad2 nucleic acid molecule comprises fad2 nucleic acid sequences in sense orientation operably linked to fad2 nucleic acid sequences in antisense orientation, wherein said fad2 nucleic acid sequences in sense orientation and said fad2 nucleic acid sequences in antisense orientation are on the same strand, wherein said fad2 nucleic acid sequences in sense orientation and said fad2 nucleic acid sequences in antisense orientation are complementary.

15. The method of claim 14, further comprising a spacer nucleic acid sequence, wherein said spacer nucleic acid sequences operably link said fad2 nucleic acid sequences in sense orientation and said fad2 nucleic acid sequences in antisense orientation, wherein said spacer nucleic acid sequences are between said fad2 nucleic acid sequences in sense orientation and said fad2 nucleic acid sequences in antisense orientation.

16. The method of claim 1, wherein said fad2 nucleic acid molecule comprises fad2F and fad2D nucleic acid sequences.

17. The method of claim 1, wherein said plant produces seeds that exhibit an altered oleic acid to linoleic acid ratio relative to seeds produced by said corresponding control plant.

18. The method of claim 1, wherein leaves of said plant exhibit an altered oleic acid to linoleic acid ratio relative to leaves from said corresponding control plant.

19. The method of claim 1, further comprising:

producing a plant line from one or more of said progeny plants.

20. The method of claim 1, further comprising:

contacting said progeny plants with a compound selected from the group consisting of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), wherein said contacting is prior to or after said identifying.

21. The method of claim 1, further comprising:

introducing a second nucleic acid construct into said cells, wherein said second nucleic acid construct comprises a regulatory element operably linked to a nucleic acid molecule encoding a polypeptide selected from the group consisting of thioesterase, 3-ketoacyl synthase II (KASII), chitinase, β-1,3-glucanase, PR5, and PR1.

22. The method of claim 1, wherein said nucleic acid construct further comprises a regulatory element operably linked to a nucleic acid molecule encoding a KASII polypeptide or a nucleic acid molecule encoding a thioesterase polypeptide.

23. The method of claim 1, wherein said nucleic acid construct further comprises a regulatory element operably linked to a nucleic acid molecule encoding an omega 6 fatty acid desaturase (fad6).

24. A method for producing a *Brassica* plant, said method comprising:

introducing a nucleic acid construct into *Brassica* cells, wherein said nucleic acid construct comprises a regulatory element operably linked to a delta-12 fatty acid desaturase (fad2) nucleic acid molecule;

generating one or more progeny plants from said cells; and identifying at least one of said progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

25. A method for producing a *Brassica* plant, said method comprising:

providing plant cells comprising a mutation in a fad2 and/or fad6 nucleic acid;

generating one or more progeny plants from said cells; and

identifying at least one of said progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

26. A method for producing a *Brassica* plant, said method comprising:

providing a plant comprising a mutation in a fad2 and/or fad6 nucleic acid;

identifying at least one of said plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

27. A method for producing a *Brassica* plant, said method comprising:

introducing a nucleic acid construct into *Brassica* cells, wherein said nucleic acid construct comprises a regulatory element operably linked to an omega 6 fatty acid desaturase (fad6) nucleic acid molecule;

generating one or more progeny plants from said cells; and

identifying at least one of said progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans* relative to a corresponding control plant.

28. A method for producing a *Brassica* plant, said method comprising:

increasing the ratio of oleic acid to linoleic acid in *Brassica* cells;

generating one or more progeny plants from said cells; and identifying at least one of said progeny plants that exhibits enhanced resistance to *Sclerotinia sclerotiorum* and/or *Leptosphaeria maculans*.

29. The method of claim 28, wherein said oleic acid is cytosolic or chloroplastic.

30. The method of claim 28, wherein said linoleic acid is cytosolic or chloroplastic.

31. The method of claim 28, wherein said increase in said ratio of oleic acid to linoleic acid is due to a decrease in expression of fad2 and/or fad6.

32. A method of producing a crop, comprising:

growing plants of a variety that has been identified as having enhanced resistance to *S. sclerotiorum* and/or *L. maculans* relative to a corresponding control variety; and

harvesting seeds produced on such plants.

33. The method of claim 32, wherein said variety is an F_1 hybrid canola variety.

34. A transgenic plant comprising a nucleic acid construct, said construct comprising a regulatory element operably linked to an omega 6 fatty acid desaturase nucleic acid, said regulatory element conferring expression in at least one vegetative tissue of said plant, wherein said plant has enhanced resistance to *S. sclerotiorum* and/or *L. maculans* in said at least one tissue, relative to the corresponding resistance in the corresponding tissue of a corresponding control plant.

35. The plant of claim 34, wherein said plant has enhanced resistance to *S. sclerotiorum* and said regulatory element confers expression in leaf tissue.

36. The plant of claim 34, wherein said plant is a *Brassica* plant.

37. The plant of claim 36, wherein said plant is a *Brassica napus* plant.

38. The plant of claim 36, wherein said plant has enhanced resistance to *L. maculans* and said regulatory element confers expression in stem tissue.

39. A transgenic plant comprising a nucleic acid construct, said construct comprising a regulatory element operably linked to a delta-12 fatty acid desaturase (fad2) nucleic acid, said regulatory element conferring expression in at least one vegetative tissue of said plant, wherein said plant has enhanced resistance to *S. sclerotiorum* and/or *L. maculans* in said at least one tissue, relative to the corresponding resistance in the corresponding tissue of a corresponding control plant.

40. The plant of claim 39, wherein said plant has enhanced resistance to *S. sclerotiorum* and said regulatory element confers expression in leaf tissue.

41. The plant of claim 39, wherein said plant is a *Brassica* plant.

42. The plant of claim 41, wherein said plant is a *Brassica napus* plant.

43. The plant of claim 41, wherein said plant has enhanced resistance to *L. maculans* and said regulatory element confers expression in stem tissue.

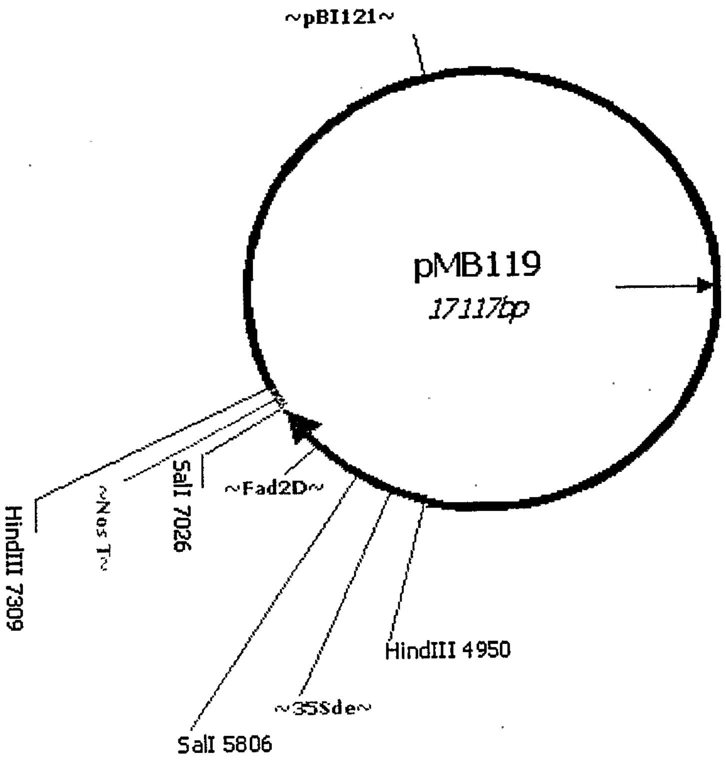
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Figure 1