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(54) Title: METHODS FOR THE IDENTIFICATION OF INHIBITORS OF FLAVANONE 3-HYDROXYLASE EXPRESSION OR ACTIVITY IN PLANTS

(57) Abstract: The present inventors have discovered that Flavanone 3-hydroxylase ("FHT") is essential for plant growth. Specifically, the inhibition of FHT gene expression in plant seedling results in small and chlorotic seedlings. Thus, FHT can be used as a target for the identification of herbicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit FHT expression or activity. The present invention can be used to identify compounds having herbicide activity by contacting a compound with FHT and detecting the presence and/or absence of binding between the compound and the FHT, or alternatively, detecting a decrease in FHT expression or activity.

METHODS FOR THE IDENTIFICATION OF INHIBITORS OF FLAVANONE 3-HYDROXYLASE EXPRESSION OR ACTIVITY IN PLANTS

FIELD OF THE INVENTION

The invention relates generally to plant molecular biology. In particular, the invention relates to methods for the identification of herbicides.

BACKGROUND OF THE INVENTION

Flavanone 3-hydroxylase is an enzyme acting in the central part of the flavonoid biosynthesis pathway. Flavonoids are plant phenolic compounds involved in leguminous plant-microbe interactions. Flavanone 3-hydroxylase is generally encoded by a single gene and seems to have a key position for the regulation in this pathway. Charrier has reported promoter-GUS studies of the Medicago sativa (alfalfa) flavanone 3-hydroxylase gene in Nicotiana benthamiana. Charrier et al., 30 PLANT MOL. BIOL. 1153-68 (1996) (PMID: 8704126).

Flavanone 3-hydroxylase (EC 1.14.11.9) ("FHT") is commonly referred to as the following: naringenin 3-dioxygenase; flavanone 3-hydroxylase; flavanone 3-dioxygenase; flavanone 3b-hydroxylase; flavanone synthase I; (2S)-flavanone 3-hydroxylase. FHT is know to catalyze the reaction naringenin + 2-oxoglutarate + O_2 <=> 3-dihydrokaempferol + succinate + CO_2.

cDNA clones for this gene have been isolated from a number of plants and the enzyme has been purified from plants. A genomic clone encoding flavanone 3-hydroxylase was isolated from Arabidopsis thaliana. Pelletier et al., 111 PLANT PHYSIOL. 339-45 (1996) (PMID: 8685272)).

Noting in the literature to date describes the lethal effects of over-expression, antisense expression, or knock-out of this gene in plants. Thus, the prior art has not suggested that FHT is essential for plant growth and development. It would be desirable utilize this enzyme for evaluating plant growth regulators, especially herbicide compounds.

SUMMARY OF THE INVENTION

The present inventors have discovered that antisense expression of a FHT cDNA in *Arabidopsis* causes developmental abnormalities, and small and chlorotic plant seedlings. Thus, the present inventors have discovered that FHT is essential for normal seed development and growth, and can be used as a target for the identification of herbicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit FHT expression or activity, comprising: contacting a candidate compound with a FHT and detecting the presence or absence of binding between the compound and the FHT, or detecting a decrease in FHT expression or activity. The methods of the invention are useful for the identification of herbicides.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the Flavanone 3-hydroxylase reaction.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "binding" refers to a noncovalent interaction that holds two molecules together. For example, two such molecules could be an enzyme and an inhibitor of that enzyme. Noncovalent interactions include hydrogen bonding, ionic interactions among

charged groups, van der Waals interactions and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

As used herein, the term "Flavanone 3-hydroxylase (EC 1.14.11.9)" is synonymous with "FHT" and refers to an enzyme that catalyses the conversion of naringenin, 2-oxoglutarate, and O_2 to 3-dihydrokaempferol, succinate, and CO_2, as shown in Fig. 1.

As used herein "NAD+"/"NADH" refer to the coenzyme "nicotinamide adenine dinucleotide," a participant in many redox reactions in biological systems. The nicotinamide ring of NAD+ accepts a hydrogen atom plus an electron (a hydride ion, H-) forming NADH. The ring is no longer stable, and therefore the added hydride ion is easily transferred to other molecules.

The term "herbicide," as used herein, refers to a compound that may be used to kill or suppress the growth of at least one plant, plant cell, plant tissue or seed.

The term "inhibitor," as used herein, refers to a chemical substance that inactivates the enzymatic activity of FHT. The inhibitor may function by interacting directly with the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

A polynucleotide may be "introduced" into a plant cell by any means, including transfection, transformation or transduction, electroporation, particle bombardment, agroinfection and the like. The introduced polynucleotide may be maintained in the cell stably if it is incorporated into a non-chromosomal autonomous replicon or integrated into the plant chromosome. Alternatively, the introduced polynucleotide may be present on an extra-chromosomal non-replicating vector and be transiently expressed or transiently active.

The "percent (%) sequence identity" between two polynucleotide or two polypeptide sequences is determined according to the either the BLAST program (Basic Local Alignment Search Tool: Altschul & Gish, 266 METH. ENZYMOL. 460-480 (1996); Altschul, 215 J. MOL. BIOL. 403-10 (1990)) in the Wisconsin Genetics Software Package (Devererreux et al., 12 NUCL. ACID RES. 387 (1984)), Genetics Computer Group, Madison, Wisconsin (NCBI, Version 2.0.11, default settings) or using Smith Waterman

Alignment (Smith & Waterman, 2 ADV. APPL. MATH 482 (1981)) as incorporated into GENEMATCHER PLUS (Paracel, Inc., Internet-accessible interface using the default settings and the version current at the time of filing). It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

"Plant" refers to whole plants, plant organs and tissues (e.g., stems, roots, ovules, stamens, leaves, embryos, meristematic regions, callus tissue, gametophytes, sporophytes, pollen, microspores and the like) seeds, plant cells and the progeny thereof.

By "polypeptide" is meant a chain of at least four amino acids joined by peptide bonds. The chain may be linear, branched, circular or combinations thereof. The polypeptides may contain amino acid analogs and other modifications, including, but not limited to glycosylated or phosphorylated residues.

The term "specific binding" refers to an interaction between FHT and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence or the conformation of FHT.

Embodiments of the Invention

The present inventors have discovered that inhibition of FHT gene expression strongly inhibits the growth and development of plant seedlings. Thus, the inventors are the first to demonstrate that FHT is a target for herbicides.

Accordingly, the invention provides methods for identifying compounds that inhibit FHT gene expression or activity. Such methods include ligand binding assays, assays for enzyme activity and assays for FHT gene expression. Any compound that is a ligand for FHT, other than its substrates, naringenin, 2-oxoglutarate, and O_2, may have herbicidal activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as herbicides.

Thus, in one embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a FHT with the compound; and

b) detecting the presence and/or absence of binding between the compound and the FHT, wherein binding indicates that the compound is a candidate for a herbicide.

By "FHT" is meant any enzyme that catalyzes the interconversion of naringenin, 2-oxoglutarate, and O_2 with 3-dihydrokaempferol, succinate, and CO_2. The FHT may have the amino acid sequence of a naturally occurring FHT found in a plant, animal or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the FHT is a plant FHT. The cDNA (SEQ ID NO:1) encoding the FHT protein or polypeptide (SEQ ID NO:2) can be found herein as well as in the TIGR database at locus F24M12_280.

By "plant FHT" is meant an enzyme that can be found in at least one plant, and which catalyzes the interconversion of naringenin, 2-oxoglutarate, and O_2 with 3-dihydrokaempferol, succinate, and CO_2. The FHT may be from any plant, including both monocots and dicots.

In one embodiment, the FHT is an *Arabidopsis* FHT. *Arabidopsis* species include, but are not limited to, *Arabidopsis arenosa, Arabidopsis bursifolia, Arabidopsis cebennensis, Arabidopsis croatica, Arabidopsis griffithiana, Arabidopsis halleri, Arabidopsis himalaica, Arabidopsis korshinskyi, Arabidopsis lyrata, Arabidopsis neglecta, Arabidopsis pumila, Arabidopsis suecica, Arabidopsis thaliana* and *Arabidopsis wallichii*. Preferably, the *Arabidopsis* FHT is from *Arabidopsis thaliana*.

In various embodiments, the FHT can be from barnyard grass (*Echinochloa crus-galli*), crabgrass (*Digitaria sanguinalis*), green foxtail (*Setana viridis*), perennial ryegrass (*Lolium perenne*), hairy beggarticks (*Bidens pilosa*), nightshade (*Solanum nigrum*), smartweed (*Polygonum lapathifolium*), velvetleaf (*Abutilon theophrasti*), common lambsquarters (*Chenopodium album L.*), *Brachiara plantaginea, Cassia occidentalis, Ipomoea aristolochiaefolia, Ipomoea purpurea, Euphorbia heterophylla, Setaria spp, Amaranthus retroflexus, Sida spinosa, Xanthium strumarium* and the like.

Fragments of a FHT polypeptide may be used in the methods of the invention. The fragments comprise at least 10 consecutive amino acids of a FHT. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 or at least 100 consecutive amino acids residues of a FHT. In one embodiment, the fragment is from an *Arabidopsis* FHT. Preferably, the fragment contains an amino acid sequence conserved

among plant Flavanone 3-hydroxylases. Such conserved fragments are identified in Grima-Pettenuti et al., 21 PLANT MOL. BIOL. 1085-1095 (1993). Those skilled in the art could identify additional conserved fragments using sequence comparison software.

Polypeptides having at least 80% sequence identity with a plant FHT are also useful in the methods of the invention. Preferably, the sequence identity is at least 85%, more preferably the identity is at least 90%, most preferably the sequence identity is at least 95% or 99%.

In addition, it is preferred that the polypeptide has at least 50% of the activity of a plant FHT. More preferably, the polypeptide has at least 60%, at least 70%, at least 80% or at least 90% of the activity of a plant FHT. Most preferably, the polypeptide has at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the activity of the *A. thaliana* FHT protein.

Thus, in another embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting the compound with at least one polypeptide selected from the group consisting of: a plant FHT, a polypeptide comprising at least ten consecutive amino acids of a plant FHT, a polypeptide having at least 85% sequence identity with a plant FHT, and a polypeptide having at least 80% sequence identity with a plant FHT and at least 50% of the activity thereof; and

b) detecting the presence and/or absence of binding between the compound and the polypeptide, wherein binding indicates that the compound is a candidate for a herbicide.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a FHT protein or a fragment or variant thereof, the unbound protein is removed and the bound FHT is detected. In a preferred embodiment, bound FHT is detected using a labeled binding partner, such as a labeled

antibody. In a variation of this assay, FHT is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for a herbicide, it can be tested for the ability to inhibit FHT enzyme activity. The compounds can be tested using either *in vitro* or cell based enzyme assays. Alternatively, a compound can be tested by applying it directly to a plant or plant cell, or expressing it therein, and monitoring the plant or plant cell for changes or decreases in growth, development, viability or alterations in gene expression.

Thus, in one embodiment, the invention provides a method for determining whether a compound identified as a herbicide candidate by an above method has herbicidal activity, comprising: contacting a plant or plant cells with the herbicide candidate and detecting the presence or absence of a decrease in the growth or viability of the plant or plant cells.

By decrease in growth, is meant that the herbicide candidate causes at least a 10% decrease in the growth of the plant or plant cells, as compared to the growth of the plants or plant cells in the absence of the herbicide candidate. By a decrease in viability is meant that at least 20% of the plants cells, or portion of the plant contacted with the herbicide candidate are nonviable. Preferably, the growth or viability will be at decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring plant growth and cell viability are known to those skilled in the art. It is possible that a candidate compound may have herbicidal activity only for certain plants or certain plant species.

The ability of a compound to inhibit FHT activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. FHT catalyzes the irreversible or reversible reaction of naringenin, 2-oxoglutarate, and O_2 to 3-dihydrokaempferol, succinate, and CO_2. Methods for detection of naringenin, 2-oxoglutarate, and O_2, and/or 3-dihydrokaempferol, succinate, and CO_2, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a naringenin, 2-oxoglutarate, and O_2 with FHT;

b) contacting the naringenin, 2-oxoglutarate, and O_2 with FHT and the candidate compound; and

c) determining the concentration of 3-dihydrokaempferol, succinate, and/or CO_2 after the contacting of steps (a) and (b).

If a candidate compound inhibits FHT activity, a higher concentration of the substrates (naringenin, 2-oxoglutarate, and O_2) and a lower level of the products (3-dihydrokaempferol, succinate, and CO_2) will be detected in the presence of the candidate compound (step b) than in the absence of the compound (step a).

Preferably the FHT is a plant FHT. Enzymatically active fragments of a plant FHT are also useful in the methods of the invention. For example, a polypeptide comprising at least 100 consecutive amino acid residues of a plant FHT may be used in the methods of the invention. In addition, a polypeptide having at least 80%, 85%, 90%, 95%, 98% or at least 99% sequence identity with a plant FHT may be used in the methods of the invention. Preferably, the polypeptide has at least 80% sequence identity with a plant FHT and at least 50%, 75%, 90% or at least 95% of the activity thereof.

Thus, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting naringenin, 2-oxoglutarate, and O_2 with a polypeptide selected from the group consisting of: a polypeptide having at least 85% sequence identity with a plant FHT, a polypeptide having at least 80% sequence identity with a plant FHT and at least 50% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a plant FHT;

b) contacting the naringenin, 2-oxoglutarate, and O_2 with the polypeptide and the compound; and

c) determining the concentration of 3-dihydrokaempferol, succinate, and/or CO_2 after the contacting of steps (a) and (b).

Again, if a candidate compound inhibits FHT activity, a higher concentration of the substrates (naringenin, 2-oxoglutarate, and O_2) and a lower level of the products (3-dihydrokaempferol, succinate, and CO_2) will be detected in the presence of the candidate compound (step b) than in the absence of the compound (step a).

For the *in vitro* enzymatic assays, FHT protein and derivatives thereof may be purified from a plant or may be recombinantly produced in and purified from a plant, bacteria, or eukaryotic cell culture. Preferably these proteins are produced using a baculovirus or *E. coli* expression system. Methods for the purification of flavanone 3-hydroxylase may be described in Purification of recombinant flavanone 3beta-hydroxylase from petunia hybrida and assignment of the primary site of proteolytic degradation. Lukacin et al., 375 ARCH BIOCHEM. BIOPHYS. 364-70 (2000) (PMID: 10700394). Other methods for the purification of FHT proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides plant and plant cell based assays. In one embodiment, the invention provides a method for identifying a compound as a candidate for a herbicide, comprising:

a) measuring the expression of FHT in a plant or plant cell in the absence of the compound;

b) contacting a plant or plant cell with the compound and measuring the expression of FHT in the plant or plant cell;

c) comparing the expression of FHT in steps (a) and (b).

A reduction in FHT expression indicates that the compound is a herbicide candidate. In one embodiment, the plant or plant cell is an *Arabidopsis thaliana* plant or plant cell.

Expression of FHT can be measured by detecting FHT primary transcript or mRNA, FHT polypeptide or FHT enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. *See e.g., Current Protocols in Molecular Biology,* (Ausubel et al., eds., Greene Publishing and Wiley-Interscience) (1995). The method of detection is not critical to the invention. Methods for detecting FHT RNA include, but are not limited to amplification assays such as

quantitative PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using a FHT promoter fused to a reporter gene, bDNA assays and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, His Tag and ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy and enzymatic assays. Also, any reporter gene system may be used to detect FHT protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with FHT, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art. Examples of reporter genes include, but are not limited to: chloramphenicol acetyltransferase (Gorman et al., 2 MOL. CELL BIOL. 1104 (1982); Prost et al., 45 GENE 107-111 (1986)); β-galactosidase (Nolan et al., 85 PROC. NAT. ACAD. SCI. USA 2603-2607 (1988)); alkaline phosphatase (Berger et al., 66 GENE 10 (1988)); luciferase (De Wet et al., 7 MOL. CELL BIOL. 725-737 (1987)); β-glucuronidase ("GUS"); fluorescent proteins; chromogenic proteins, and the like. Methods for detecting FHT activity are described above.

Chemicals, compounds, or compositions identified by the above methods as modulators of FHT expression or activity can then be used to control plant growth. For example, compounds that inhibit plant growth can be applied to a plant or expressed in a plant, in order to prevent plant growth. Thus, the invention provides a method for inhibiting plant growth, comprising contacting a plant with a compound identified by the methods of the invention as having herbicidal activity.

Herbicides and herbicide candidates identified by the methods of the invention can be used to control the growth of undesired plants, including both monocots and dicots. Examples of undesired plants include, but are not limited to barnyard grass (*Echinochloa crus-galli*), crabgrass (*Digitaria sanguinalis*), green foxtail (*Setana viridis*), perennial ryegrass (*Lolium perenne*), hairy beggarticks (*Bidens pilosa*), nightshade (*Solanum nigrum*), smartweed (*Polygonum lapathifolium*), velvetleaf (*Abutilon theophrasti*), common lambsquarters (*Chenopodium album L.*), *Brachiara plantaginea, Cassia occidentalis, Ipomoea aristolochiaefolia, Ipomoea purpurea, Euphorbia*

heterophylla, Setaria spp, Amaranthus retroflexus, Sida spinosa, Xanthium strumarium and the like.

EXPERIMENTAL

Plant Growth Conditions

Unless, otherwise indicated, all plants are grown Scotts Metro-Mix™ soil (the Scotts Company) or a similar soil mixture in an environmental growth room at 22°C, 65% humidity, 65% humidity and a light intensity of ~100 μ-E m^{-2} s^{-1} supplied over 16 hour day period.

Seed Sterilization

All seeds are surface sterilized before sowing onto phytagel plates using the following protocol.

1. Place approximately 20-30 seeds into a labeled 1.5 ml conical screw cap tube. Perform all remaining steps in a sterile hood using sterile technique.
2. Fill each tube with 1ml 70% ethanol and place on rotisserie for 5 minutes.
3. Carefully remove ethanol from each tube using a sterile plastic dropper; avoid removing any seeds.
4. Fill each tube with 1ml of 30% Clorox and 0.5% SDS solution and place on rotisserie for 10 minutes.
5. Carefully remove bleach/SDS solution.
6. Fill each tube with 1ml sterile dI H_2O; seeds should be stirred up by pipetting of water into tube. Carefully remove water. Repeat 3 to 5 times to ensure removal of Clorox/SDS solution.
7. Fill each tube with enough sterile dI H_2O for seed plating (~200-400 μl). Cap tube until ready to begin seed plating.

Plate Growth Assays

Surface sterilized seeds are sown onto plate containing 40 ml half strength sterile MS (Murashige and Skoog, no sucrose) medium and 1% Phytagel using the following protocol:

1. Using a pipette with 200 µl tip, carefully fill tip with seed solution. Place 10 seeds across the top of the plate, about ¼ in down from the top edge of the plate.
2. Place plate lid ¾ of the way over the plate and allow to dry for 10 minutes.
3. Using sterile micropore tape, seal the edge of the plate where the top and bottom meet.
4. Place plates stored in a vertical rack in the dark at 4°C for three days.
5. Three days after sowing, the plates transferred into a growth chamber with a day and night temperature of 22 and 20°C, respectively, 65% humidity and a light intensity of ~100 µ-E m^{-2} s^{-1} supplied over 16 hour day period.
6. Beginning on day 3, daily measurements are carried out to track the seedlings development until day 14. Seedlings are harvested on day 14 (or when root length reaches 6 cm) for root and rosette analysis.

Example 1
Construction of a Transgenic Plant expressing the Driver

The "Driver" is an artificial transcription factor comprising a chimera of the DNA-binding domain of the yeast GAL4 protein (amino acid residues 147) fused to two tandem activation domains of herpes simplex virus protein VP16 (amino acid residues 413-490). Schwechheimer *et al.* (1998) *Plant Mol Biol 36*:195-204. This chimeric driver is a transcriptional activator specific for promoters having GAL4 binding sites. Expression of the driver is controlled by two tandem copies of the constitutive CaMV 35S promoter.

The driver expression cassette was introduced into *Arabidopsis thaliana* by agroinfection. Transgenic plants that stably expressed the driver transcription factor were obtained.

Example 2
Construction of Antisense Expression Cassettes in a Binary Vector

A fragment, fragment or variant of an *Arabidopsis thaliana* cDNA corresponding to SEQ ID NO:1 was ligated into the PacI/AscI sites of an *E.coli/Agrobacterium* binary vector in the antisense orientation. This placed transcription of the antisense RNA under the control of an artificial promoter that is active only in the presence of the driver transcription factor described above. The artificial promoter contains four contiguous binding sites for the GAL4 transcriptional activator upstream of a minimal promoter comprising a TATA box.

The ligated DNA was transformed into *E.coli*. Kanamycin resistant clones were selected and purified. DNA was isolated from each clone and characterized by PCR and sequence analysis. The DNA was inserted in a vector that expresses the *A. thaliana* antisense RNA, which is complementary to a portion of the DNA of SEQ ID NO:1. This antisense RNA is complementary to the cDNA sequence found in the TIGR database at locus F24M12_280. The coding sequence for this locus is shown as SEQ ID NO:1. The protein encoded by these mRNAs is shown as SEQ ID NO:2.

The antisense expression cassette and a constitutive chemical resistance expression cassette are located between right and left T-DNA borders. Thus, the antisense expression cassettes can be transferred into a recipient plant cell by agroinfection.

Example 3
Transformation of *Agrobacterium* with the Antisense Expression Cassette

The vector was transformed into *Agrobacterium tumefaciens* by electroporation. Transformed *Agrobacterium* colonies were isolated using chemical selection. DNA was prepared from purified resistant colonies and the inserts were amplified by PCR and sequenced to confirm sequence and orientation.

Example 4
Construction of an *Arabidopsis* Antisense Target Plants

The antisense expression cassette was introduced into *Arabidopsis thaliana* wild-type plants by the following method. Five days prior to agroinfection, the primary inflorescence of *Arabidopsis* thaliana plants grown in 2.5 inch pots were clipped in order enhance the emergence of secondary bolts.

At two days prior to agroinfection, 5 ml LB broth (10 g/L Peptone, 5 g/L Yeast extract, 5 g/L NaCl, pH 7.0 plus 25 mg/L kanamycin added prior to use) was inoculated with a clonal glycerol stock of Agrobacterium carrying the desired DNA. The cultures were incubated overnight at 28°C at 250 rpm until the cells reached stationary phase. The following morning, 200 ml LB in a 500 ml flask was inoculated with 500 µl of the overnight culture and the cells were grown to stationary phase by overnight incubation at 28°C at 250 rpm. The cells were pelleted by centrifugation at 8000 rpm for 5 minutes. The supernatant was removed and excess media was removed by setting the centrifuge bottles upside down on a paper towel for several minutes. The cells were then resuspended in 500 ml infiltration medium (autoclaved 5% sucrose) and 250 µl/L SILWET L-77 (84% polyalkyleneoxide modified heptamethyltrisiloxane and 16% allyloxypolyethyleneglycol methyl ether), and transferred to a one liter beaker.

The previously clipped *Arabidopsis* plants were dipped into the Agrobacterium suspension so that all above ground parts were immersed and agitated gently for 10 seconds. The dipped plants were then covered with a tall clear plastic dome in order to maintain the humidity, and returned to the growth room. The following day, the dome was removed and the plants were grown under normal light conditions until mature seeds were produced. Mature seeds were collected and stored desiccated at 4 °C.

Transgenic *Arabidopsis* T1 seedlings were selected. Approximately 70 mg seeds from an agrotransformed plant were mixed approximately 4:1 with sand and placed in a 2 ml screw cap cryo vial.

One vial of seeds was then sown in a cell of an 8 cell flat. The flat was covered with a dome, stored at 4°C for 3 days, and then transferred to a growth room. The domes were removed when the seedlings first emerged. After the emergence of the first primary leaves, the flat was sprayed uniformly with a herbicide corresponding to the chemical

resistance marker plus 0.005% SILWET (50 µl/L) until the leaves were completely wetted. The spraying was repeated for the following two days.

Ten days after the first spraying resistant plants were transplanted to 2.5 inch round pots containing moistened sterile potting soil. The transplants were then sprayed with herbicide and returned to the growth room. These herbicide resistant plants represented stably transformed T1 plants.

Example 5
Effect of Antisense Expression in *Arabidopsis* Seedlings

The T1 antisense target plants from the transformed plant lines obtained in Example 4 were crossed with the *Arabidopsis* transgenic driver line described above. The resulting F1 seeds were then subjected to a PGI plate assay to observe seedling growth over a 2-week period. Seedlings were inspected for growth and development. The transgenic plant line containing the antisense construct exhibited significant developmental abnormalities during early development.

A clear 1:1 segregation ratio was observed in the antisense line, which demonstrates that the antisense expression of this gene resulted in significantly impaired growth and that this gene represents an essential gene for normal plant growth and development. The transgenic line containing the antisense construct for Flavanone 3-hydroxylase exhibited significant seedling abnormalities. Seedlings were small and chlorotic.

Example 6.
Cloning and Expression Strategies/Extraction and Purfication of the FHT protein.

The following protocol may be employed to obtain the purified FHT protein.

Cloning and expression strategies:
FHT gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags. Evaluate the expression of recombinant protein by SDS-PAGE and Western blot analysis.

Extraction:
Extract recombinant protein from 250 ml cell pellet in 3 mL of extraction buffer By sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000 x g for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:
Purify recombinant protein by Ni-NTA affinity chromatography (Quiagen). Purification protocol: perform all steps at 4oC:

- Use 3 ml Ni-beads (Quiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 7
Assays for Testing Inhibitors or Candidates for Inhibition of FHT Activity

The enzymatic activity of FHT may be determined in the presence and absence of candidate inhibitors in a suitable reaction mixture, such as described by any of the following known assay protocols:

Radiochemical assay:
This assay is based on the conversion of 2-oxo[1-^{14}C]glutarate in the enzyme assay to dihydrokaempferol and $^{14}CO_2$, which are formed in a molar ratio of 1:1, as described by Britsch. Britsch & Grisebach ,156 EUR. J. BIOCHEM 569-77 (1986).

NADH assay:
The product of the forward reaction, succinate, is itself a substrate for the enzyme Succinate Semialdehyde Dehydrogenase (E.C. 1.2.1.24). This enzyme is capable of converting succinate to Succinic semialdehyde in an NADH dependant manner. Using a coupled enzyme system, the loss of NADH can be monitored by either absorbance at 340nm or flourescence at ex.340/em.460 to assay for enzymatic activity.

Although the invention has been described with respect to certain embodiments thereof, it will be also understood that it is not to be so limited in that changes and modifications can be made therein which fall within the full intended scope of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a FHT with a compound; and

b) detecting the presence and/or absence of binding between the compound and the FHT, wherein binding indicates that the compound is a candidate for a herbicide.

2. The method of claim 1, wherein the FHT is a plant FHT.

3. The method of claim 2, wherein the plant FHT is an *Arabidopsis* FHT.

4. The method of claim 3, wherein the FHT is SEQ ID. NO. 2.

5. A method for determining whether a compound identified as a herbicide candidate by the method of claim 1 has herbicidal activity, comprising: contacting a plant or plant cells with the herbicide candidate and detecting the presence or absence of a decrease in growth or viability of the plant or plant cells.

6. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a compound with at least one polypeptide selected from the group consisting of: an amino acid sequence comprising at least ten consecutive amino acids of a plant FHT, an amino acid sequence having at least 85% sequence identity with a plant FHT, and an amino acid sequence having at least 80% sequence identity with a plant FHT and at least 50% of the activity thereof; and

b) detecting the presence and/or absence of binding between the compound and the polypeptide, wherein binding indicates that the compound is a candidate for a herbicide.

7. A method for determining whether a compound identified as a herbicide candidate by the method of claim 6 has herbicidal activity, comprising: contacting a plant or plant cells with the herbicide candidate and detecting the presence or absence of a decrease in growth or viability of the plant or plant cells.

8. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting a naringenin, 2-oxoglutarate, and O_2 with a FHT;

b) contacting the naringenin, 2-oxoglutarate, and O_2 with the FHT and a candidate compound; and

c) determining the concentration of at least one of naringenin, 2-oxoglutarate, and O_2, and/or 3-dihydrokaempferol, succinate, and CO_2 after the contacting of steps (a) and (b).

9. The method of claim 8, wherein the FHT is a plant FHT.

10. The method of claim 9, wherein the plant FHT is an *Arabidopsis* FHT.

11. The method of claim 10, wherein the FHT is SEQ ID. NO. 2.

12. A method for identifying a compound as a candidate for a herbicide, comprising:

a) contacting naringenin, 2-oxoglutarate, and O_2 with a polypeptide selected from the group consisting of: a polypeptide having at least 85% sequence identity with a plant FHT, a polypeptide having at least 80% sequence identity with a plant FHT and at least 50% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a plant FHT;

b) contacting the naringenin, 2-oxoglutarate, and O_2 with the polypeptide and a compound; and

c) determining the concentration of at least one of naringenin, 2-oxoglutarate, and O_2, and/or 3-dihydrokaempferol, succinate, and CO_2 after the contacting of steps (a) and (b).

13. A method for identifying a compound as a candidate for a herbicide, comprising:

a) measuring the expression of a FHT in a plant or plant cell in the absence of a compound;

b) contacting a plant or plant cell with the compound and measuring the expression of the FHT in the plant or plant cell; and

c) comparing the expression of FHT in steps (a) and (b).

14. The method of claim 13 wherein the plant or plant cell is an *Arabidopsis* plant or plant cell.

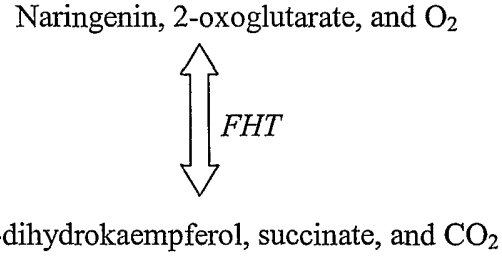
15. The method of claim 14, wherein the FHT is SEQ ID NO 2.

16. The method of claim 13, wherein the expression of FHT is measured by detecting FHT mRNA.

17. The method of claim 13, wherein the expression of FHT is measured by detecting FHT polypeptide.

Figure 1. The Flavanone 3-hydroxylase (FHT) reaction.

Pathway:



Substrates:

Naringenin, 2-oxoglutarate, and O_2

Products:

3-dihydrokaempferol, succinate, and CO_2

Function:

The FHT enzyme is involved in the conversion of naringenin, 2-oxoglutarate, and O_2 to 3-dihydrokaempferol, succinate, and CO_2 in plants.

SEQUENCE LISTING

<110> Davis, Keith

Zayed, Adel

Ascenzi, Robert

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Hoffman, Neil

Gorlach, Jorn

Woessner, Jeff

Hamilton, Carol

Phillips, Kenneth

<120> METHODS FOR THE IDENTIFICATION OF INHIBITORS OF FLAVANONE 3-HYDROXYLASE EXPRESSION OR ACTIVITY IN PLANTS

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