Abstract:
The present invention provides methods for making plants resistant to parasitic weeds, the resistant transgenic plants, vectors and methods of integrated parasitic weed management.
Control of Parasitic Weeds

FIELD OF THE INVENTION

[0001] The field of the invention relates to the use of double-stranded RNA interference to control parasitic weeds in agronomic plants. In particular, this invention relates to methods of making corn or sorghum resistant to Striga spp. and to transgenic corn and sorghum that are resistant to striga. The invention also relates to methods of making Sunflower plants resistant to the parasitic weed Orobanche cutnana and transgenic plants produced from the method.

BACKGROUND OF THE INVENTION

[0002] Striga (witchweed) is a parasitic weed that attacks the major cereal crops (sorghum, millet, maize, rice). It penetrates the roots of its host and diverts essential nutrients, stunting its growth. Striga infests two-thirds of the 73 million hectares planted with these crops in Africa, and results in annual losses of 70% to 90%. The impact of striga is estimated at 4.1 million tons of cereal production lost, primarily to subsistence farmers, corresponding to an overall revenue loss of US$7 billion per year. Striga is the major obstacle to food production in Africa.

[0003] Each striga flower can produce thousands of tiny seeds, which are then spread very efficiently over large distances. These seeds can survive in the soil for up to 20 years and will germinate only in the vicinity of the proper host. None of the methods of control outlined below used against striga in the developed world can be practically managed in the third world.

[0004] Several technologies have been developed for Striga control. Injection of ethylene in the ground will trigger striga seeds germination even in the absence of a proper host. Because striga is an obligate parasite, the germinating seedlings will then die when they fail to attach to the roots of their host.

[0005] Striga is sensitive to several common herbicides used in developed countries but the cost of these chemicals is prohibitive in poor African countries. Recently a new approach developed by Dr. Gressel in collaboration with CIMMYT, based on coating the maize seeds with the herbicide Imazapyr, did show very encouraging results (Kanampiu et al, 2001), but this is limited to imidazolinone-resistant maize cultivars.
A bio-control fungus *Fusarium oxysporum* isolated from African soils free of *striga* infestations gave promising results in green house testing conditions but as most bio-control technologies, this approach appears to be limited to certain soils. Furthermore, preparation of the fungal spores ideally needs to be done by the farmer's family and this presents a practical challenge.

The selection of various cereal cultivars partially tolerant or resistant to *striga* has been reported but the performance of these cultivars in various field conditions as opposed to green house testing has been inconsistent. A recent pilot study, directed in Ethiopia by Dr. Gebisa Ejeta, which combines *striga* resistant sorghum cultivars with the use of inorganic fertilizers and water preservation measures, has shown very encouraging results. This, we believe, shows that successful *striga* control throughout Africa will most likely require multiple approaches. Therefore any new technology found to be efficient against this pest could become part of such Integrated Striga Management strategy.

The primary purpose of this invention is to control parasitic weeds such as *Striga* or *Orobanche*. This technology proves is easily engineered in other cereal crops such as sorghum which are critical staple food crops in sub-saharan Africa. One of the advantages of this method of *striga*-control would potentially be the reduction of *striga* seed banks since the *striga* seedlings would presumably be killed prior to their emerging above ground. Finally this invention is another tool in an Integrated Striga Management Strategy.

**SUMMARY OF THE INVENTION**

The present invention provides a method for increasing the resistance of a plant to a parasitic weed comprising the steps of:

1) introducing into a plant a DNA construct comprising a promoter operable in a plant operably linked to a DNA fragment at least 23 nucleotides encoding a sense RNA fragment of a transgene and a DNA fragment at least 23 nucleotides encoding an antisense RNA fragment of the transgene wherein the RNA fragments are capable of forming a double-stranded RNA molecule and a terminator; and

2) expressing said DNA construct in the plant;
[0013] The present invention also provides a method for increasing the resistance of a plant to a parasitic weed where the plant is a cereal such as, but not limited to, maize, sorgham, millet or rice, most preferably maize. In another embodiment the plants are legumes such as but not limited to, broad bean, clover, faba bean, lentil, chick pea, pea, common vetch, cowpea, groundnut, bambaranut, etc. In yet another embodiment the plants are tobacco, clover, solanaceous plants such as tomato, broom rape, Russian dandelion, or sunflowers. The present invention provides a method where the parasitic weed is a Striga spp., in particular, Striga asiatica or Striga hermonthica, or Striga gesnerioides or Orobanche spp., in particular, Orobanche cumana, crenate broomrape (Orobanche crenata Forsk.), nodding broomrape (Orobanche cernua Loeffl.), O. aegyptiaco and O. ramosa, and O. minor. 

[0014] The invention also provides methods for increasing the resistance of a plant to a parasitic weed wherein the transgene, or fragment thereof, used in the double-stranded construct is selected from the group consisting of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), alpha CTase (αCTase) which is part of a 4-protein ACCase (Acetyl-CoA carboxylase), enoyl-ACP reductase (ENR), VCL1 (the Arabidopsis VCL1 ortholog of S. cerevisiae Vpsl ó), or Adenylo-succinate synthase (AdSS). 

[0015] The present invention also relates to transgenic plants and plant cells that are resistant to parasitic weeds, preferably the parasitic weeds described above. 

[0016] The present invention further relates to methods of integrated parasitic weed management comprising growing seeds of the transgenic plants described above that are resistant to parasitic weeds described above.

[0017] The present invention also relates to the genetic constructs and plasmids described infra in the specification below.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

[0018] SEQ ID NO: 1 is the nucleotide sequence of plasmid pSTR6. 

[0019] SEQ ID NO: 2 is the nucleotide sequence of plasmid pSTR25. 

[0020] SEQ ID NO: 3 is the nucleotide sequence of plasmid pSTR26. 

[0021] SEQ ID NO: 4 is the nucleotide sequence of plasmid pSTR39. 

[0022] SEQ ID NO: 5 is the nucleotide sequence of plasmid pSTR40. 

[0023] SEQ ID NO: 6 is the nucleotide sequence of plasmid 11147. 

[0024] SEQ ID NO: 7 is the nucleotide sequence of plasmid 11148.
SEQ ID NO: 8 is the nucleotide sequence of plasmid 11057.
SEQ ID NO: 9 is the nucleotide sequence of plasmid 11058.
SEQ ID NO: 10 is the nucleotide sequence of plasmid 11238.
SEQ ID NO: 11 is the nucleotide sequence of plasmid 11239.
SEQ ID NO: 12 is the nucleotide sequence of plasmid 11240.
SEQ ID NO: 13 is the nucleotide sequence of plasmid 11241.
SEQ ID NO: 14 is the nucleotide sequence of plasmid 11146.

DETAILED DESCRIPTION OF THE INVENTION

Engineering corn for resistance to Striga

The present invention relates to a new strategy to engineer cereals for resistance to *striga* based on the novel RNA interference silencing technology, abbreviated as RNAi. First discovered in 1998 in *C. elegans* (Montgomery et al, 1998), it has been found to be a very general phenomenon in organisms as diverse as protozoa, animals, plants and fungi. RNAi has become one of the hottest topics in molecular biology in the last three years and even though it is not yet completely understood, a lot has been learned about its mechanism. In summary, double-stranded RNA molecules can induce the degradation of homologous RNAs, resulting in post-transcriptional gene silencing. It allows for efficient and specific gene silencing. Extremely potent and requiring only a few dsRNA molecules, it is reported to be 1000 times more efficient than antisense (Thakur. 2003).

Silencing of reporter genes as well as endogenous plant genes has been shown using RNAi (Waterhouse et al, 1998; Chuang and Meyerowitz, 2000; Levin et al, 2000). Furthermore and more importantly for our project, RNAi can be spread systemically within a plant via some yet uncharacterized signal, and can also be transmitted from a plant to a graft (Jorgensen et al, 1998; Vaucheret et al., 1998). It is believed that the systemic signal travels via the phloem.

No one has yet tested the possibility of using RNAi in plants to control parasitic weeds. In such an approach, a transgenic plant would express dsRNA molecules targeted against genes essential for the parasitic weed survival. As the parasite "feeds" on the host, it will take up dsRNA molecules (or the systemic signal molecule) which in turn will trigger silencing of its essential genes.
Various studies on *Striga* or *Orobanche* have shown that there was transfer of more than water between host and parasite, which would seem to indicate that there more than simple xylem connection between them.

The first example of this method is performed in maize to target the parasitic weed *striga*. The *striga* genes chosen as targets for our dsRNA constructs are known essential genes in plants, such as herbicide targets (EPSP synthase, target of glyphosate, a herbicide lethal to *striga*), as well as genes shown to be essential in plants, such as AdSS (adenylosuccinate synthetase, the first enzyme in AMP biosynthesis) or VCLI (Vacuolelessl), a gene required for vacuole formation and morphogenesis in *Arabidopsis*.

The following examples are illustrative and are not intended to be limiting.

**Examples**

**Experimental Design:** *Striga* genes chosen as RNAi targets and their source *Striga asiatica* leaves, roots and haustoria were collected from an infested corn field in Horry County, South Carolina, with the help of USDA-APHIS and an import permit from the North Carolina Department of Agriculture.

Total RNA was isolated from *striga* underground white tissue which contained roots and haustoria. The following essential genes or gene fragments were then cloned from total RNA using RT-PCR.

1- EPSPS (5-enoylpyruvylshikimate 3-phosphate synthase) is required for the synthesis of aromatic amino acids in plants and is the target of the herbicide glyphosate.

2- δCTase. δCTase is part of a 4-protein ACCase (Acetyl-CoA carboxylase) complex. This activity is needed for the initiation of fatty acid biosynthesis in the plastid. Maize does not have this particular gene, but uses a different type of ACCase for this activity.

3- ENR (enoyl-ACP reductase). ENR is also involved in fatty acid biosynthesis and was also shown to be essential in plants.

4- VCLI. The *Arabidopsis* VCLI (ortholog of *S. cerevisiae* Vps16) is an essential gene required for vacuole formation and morphogenesis. *Arabidopsis* VCLI is expressed throughout development, but especially in growing organs.

5- AdSS (Adenylo-succinate synthase). AdSS is a key step in AMP synthesis.

**Vectors and RNAi design**

The binary backbone vector is pNOV2 117
The intron used as the spacer fragment for the loop of the dsRNA is Adhl intron (Adh intron1 was acquired from Sogetol Corp. in plasmid pSOG14). The promoter driving the dsRNA is CMPS (aka prCMP-01 A 415 bp fragment - position 105-504 plus 16bp added during PCR to create BamHI flanking sites) from Cestrum Yellow Leaf Curling Virus Promoter. Contains Promoter region including TATA box and enhancer factors CmYLCV promoter Cestrium Yellow Leaf Curling Virus.

The plant selectable marker is PMI driven by ZmUbilnt.

In addition to the dsRNA constructs built using each of the target genes described above, we also made a chimeric construct which contains a fragment of each of the 5 target genes.

Transgenic maize lines

All constructs passed Quality Control (QC) at Syngenta Biotechnology Inc. and were transformed into Maize. Single-copy events were obtained and confirmed via genomic southern analysis. Four to five lines (events) per construct were chosen for testing on striga.

Listed below are the QC numbers of the different dsRNA constructs. There are two constructs for each target gene, one has the sense strand-spacer-antisense strand, the other antisense strand-spacer-sense strand. There are three constructs for EPSPS because various parts of the gene were chosen as targets, due to the high homology found between maize and striga EPSPS genes.

EPSPS constructs: pSTR6, pSTR25, pSTR26 (SEQ ID NOS: 1-3, respectively);

ACCase constructs: pSTR39, pSTR40 (SEQ ID NOS: 4 and 5, respectively);

ENR constructs: 11147, 11148 (SEQ ID NOS: 6 and 7, respectively);

VCL1 constructs: 11057, 11058 (SEQ ID NOS: 8 and 9, respectively);

AdSS constructs: 11238, 11239 (SEQ ID NOS: 10 and 11, respectively); and

Chimeric constructs: 11240, 11241 (SEQ ID NOS: 12 and 13, respectively).

There is also one more construct (QC number 11146) (SEQ ID NO: 14), which is a control construct to ensure functionality of the transcriptional fusion in which GUS was hooked up to the CMPS promoter.

The list of constructs and corresponding seeds ready for testing is shown in the Table 1. It shows a total of 13 constructs. Depending on the construct, between 2 to 10 independent events were selected after plant analysis and quality control. These selected events were then either selfed or when the primary event was not self-fertile, crossed back to wild-type maize. Therefore, the seeds available for testing represent a segregating...
Identification of the seeds carrying the transgenes can be done readily using immunostrips that detect the presence of the PMI plant selectable marker.

Table 1: Seeds for testing.

<table>
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<tr>
<th>construct</th>
<th>PlantID</th>
<th>envelope #</th>
<th># of seeds</th>
<th>Pollination type</th>
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</table>
Experimental Design for the Testing of the seeds

Extended Agar Gel Assay

The effect of the dsRNA transgenes in the transgenic plants can be tested using the Extended Agar Gel assay as essentially described in Mohamed et al. (Crop Sci. 43:1320-24 (2003) which is a modification of the agar gel assay by Hess et al. (Phytochemistry 31:493-97 (1992).

The EAGA can be used as an initial screening which would allow us to make multiple observations on particular parasitic events. This would be most useful if we find that the anticipated effects of the maize events armed with your constructs on *striga* occur within a week of infection.

Our "extended agar gel assay" involves the following steps:

1. Condition *striga* seed two weeks prior to infection.
2. Surface sterilize maize kernels and imbibe and germinate as described for the paper roll assay.
3. Test segregating populations with immunostrips for seedlings carrying the construct. Move positive seedlings into the containment facility.
4. Prepare sterile 0.7% agar and cool to 5OC.
5. Place a drop of conditioned *striga* seeds in the center of a 150mm Petri dish and pour 60 ml of liquid agar over these to distribute them evenly across the plate.
6. As agar begins to solidify, place germinated maize into agar such that its radicle points toward the center of the plate.
7. Incubate plates in darkness at 28C for 3-5 days.
8. Observe *striga* along the root of maize as it grows through the agar. Return to the incubator until subsequent observations in the following days.

Optimization of conditions is possible in order to get parasitic attachments on your material. Because the maize shoot is confined in the plates to the small space above the agar, it is hard to keep the things growing in this system for more than a week. This
system can be adapted to accommodate the large maize shoots (relative to sorghum) through the pilot studies. This or a similar cultural system allows one to screen many events in a smaller space and in less time required for the paper roll system. With sorghum, we do not get nearly the number of striga attachments in agar as we get in paper rolls. But the advantage of making nondestructive observations is an advantage to the agar assay.

[0074] The paper roll assay involves the following steps:

1. Condition striga seed beginning two weeks before infection of the maize host.

2. Surface sterilize maize kernels in 1% sodium hypochlorite and imbibe several hours in 5% Captan (fungicide) solution.

3. Germinate imbibed maize on filter papers.

4. Transplant germinated maize between sheets of germination paper, roll them into tubes and stand these in a beaker of water. Grow for 4-7 days.

5. Select healthy seedlings for presence of the transgene using immunostrips.

6. On the day before infection, spread conditioned striga seed on strips of glass fiber filter paper and pregerminate with ethylene.

7. Move selected seedlings into the containment facility for infection with striga.

8. Open the paper rolls in which the selected maize seedlings are growing to expose their roots. Lay the glass fiber containing germinated striga upon the exposed maize roots and reroll the germination papers.

9. Stand the rolls in glass jars to which moistened vermiculite covered with a layer of sand has been added. Place the jars in a growth chamber setting appropriate temperature, light and humidity conditions to favor maize growth. Irrigation is provided directly to the vermiculite layer by means of a tube placed in the jar with the paper rolls that penetrates through the sand to the vermiculite. The sand minimizes wicking of irrigation solution into the rolls themselves. Maize roots grow out from the bottom of the roots through the sand to the vermiculite. If the paper rolls are too wet, striga do not attach to their host.

10. After 5-8 days, remove rolls and open them to expose roots. Examine these under a dissecting microscope to observe striga attachments. As this is disruptive, several rolls containing one or a few infected seedlings should be grown so that multiple observations can be made over the course of the study.

10. Continue harvesting infected seedlings for observation every few days for up to 3 weeks.
Conditions can be optimized, such as timing of infection, growth chamber conditions, fertilization and irrigation frequency and timing of observations so that we get several attachments to examine during the study. The experiment will also include infecting and growing the appropriate control plants (plants transgenic for plasmid 11146; SEQ ID NO: 14) with which we can compare the parasitic interactions of plants containing the *striga* targeted constructs.

Each set of plants infected in the paper roll system will require up to 1 month to screen all events from germination of maize to final harvest.

A number of experimental parameters are measured, including but not limited to, counting the number of attachments and recording the growth stage and vigor of individual parasitic events, RNA extractions and histological observations. RT-PCR to compare message levels of targeted *striga* RNA is informative to molecularly characterize the effect of transgenic materials to attached *striga*. Also, fine dissection of endophytic tissues reveals the extent of vascular connections between host and parasite and any developmental differences between haustoria of *striga* attached to armed and control maize roots.

The most promising events from the paper roll assay will then be screened with *striga* in pots. Pot studies must also be conducted in our small containment facility in the growth chamber and take a few months for each event. Basically, this involves sowing the transgenic seeds in pots of soil inoculated with *striga* seeds then counting the number of striga plants that emerge in each pot.

Pot studies involve the following steps:

1. Place a coffee filter in the bottom of a small pot
2. Fill the pot with a clean sandy soil mix to about 2” to the top of the pot.
3. Sprinkle a fixed amount of *striga* seed across the surface of this soil.
4. Add more of the same soil mixture to fill the pots.
5. Wait to sow maize. Water thoroughly, but not excessively.
6. Water again after 4 days.
7. A week after filling pots, sow two or three maize kernels about 1/4” deep. This places them about an inch above the striga layer.
8. As maize emerges, test with immunostrips to check for presence of the transgene. Thin to one transgenic seedling per pot.
9. Continue to water regularly and fertilize only to the extent of maintaining good growth of maize in the growth chamber.
10. After approximately 6 weeks, when striga emerges, count the number of striga plants and note their vigor and internode length.

11. Striga can be removed and sampled for RNA, or left to monitor and include in counts taken in coming weeks.

Optimization of these conditions will optimize the informative value of these experiments.

The transgenic plant materials are also tested against *Striga hermonthica*. The later species is often the more restrictive to cereal production in Africa.

Molecular analysis also involves collection of RNA from attached striga in order to conduct studies using reverse transcription and PCR. Histological characterization of parasitic tissues is also performed.

References


What is claimed is:

1. A method for increasing the resistance of a plant to a parasitic weed comprising the steps of:

   1) introducing into a plant a DNA construct comprising a promoter operable in a plant operably linked to a DNA fragment at least 23 nucleotides encoding a sense RNA fragment of a transgene and a DNA fragment at least 23 nucleotides encoding an antisense RNA fragment of the transgene wherein the RNA fragments are capable of forming a double-stranded RNA molecule and a terminator; and

   2) expressing said DNA construct in the plant;

   wherein the expression of said DNA construct confers resistance to a parasitic weed.

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

3. The method of claim 2, wherein the cereal is maize, sorgham, millet or rice.

4. The method of claim 1, wherein the parasitic weed is a Striga spp.

5. The method of claim 4, wherein the Striga spp. is Striga asiatica or Striga hermonthica.

6. The method of claim 1 wherein the parasitic weed is an Orobanche spp.

7. The method of claim 6, wherein the Orobanche spp. is Orobanche cumana.

8. The method of claim 1, wherein the transgene, or fragment thereof, used in the double-stranded construct is selected from the group consisting of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), alpha CTase (αCTase) which is part of a 4-protein ACCase (Acetyl-CoA carboxylase), enoyl-ACP reductase (ENR), VCL1 (the Arabidopsis VCL1 ortholog of S. cerevisiae Vpsl6), or Adenylo-succinate synthase (AdSS).

9. A transgenic plant or plant cell resistant to a parasitic weed made using the method of claim 1.

10. A method of integrated parasitic weed management comprising growing seeds of the transgenic plant of claim 9.

11. A plasmid pSTR6, pSTR25, pSRT26, pSTR39, pSRT40, 11147, 11148, 11157, 11158, 11236, 11238, 11239, 11240, 11241 or 11246 having the SEQ IDNOs:l-14, respectively.