

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 6
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

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Asunto: Radicación: 9-33753- -6
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 3797
 Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2007/081527				
Fecha de Presentación Internacional	16/10/2007				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 68 a 125	01/Abril/2009
Secuencias:	Folios 126 a 129	01/Abril/2009
Reivindicaciones:	Folios 130 a 136	01/Abril/2009
	Folios 181 a 186	26/Diciembre/2012
Dibujos:	Folios 138 a 144	01/Abril/2009
Prioridad:	US 60/852.308	16/Octubre/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones (Art 34 D 486 y Art 19, 22 y 34 PCT)

Se aceptan las modificaciones a las reivindicaciones en los folios 181 a 186 ya que no amplían la materia inicialmente presentada, y sobre dicho capítulo se realiza el análisis de patentabilidad.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En la presente solicitud se estudiarán las 39 reivindicaciones de los folios 181 a 186, ya que el solicitante canceló la tasa de presentación y de modificación.

Título de la solicitud: “Métodos y composiciones para mejorar el estado sanitario de las plantas”

El problema planteado en esta solicitud consiste en suministrar métodos para el mejorar el rendimiento de cultivos tratados con dicamba. Para resolver este problema técnico la solicitud en estudio proporciona métodos para mejorar el estado sanitario de las plantas, los cuales consisten en la aplicación de dicamba u otro sustrato de defensa lo cual tiene un efecto ventajoso contra el estrés biótico o abiótico tal como el estrés oxidativo y a enfermedades de la planta.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N15/82

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 28 y 39 que se refieren a métodos para mejorar la salud o la productividad de una planta, población de plantas o semillas no son claras, puesto que dan a entender que el compuesto 3,6 DCSA es obtenido como un producto del metabolismo mediado por DMO. Sin embargo según se entiende de las reivindicaciones 15 y 31 dicho compuesto es aplicado sobre las plantas o semillas, y no es obtenido por medio del metabolismo de la planta modificada con DMO. Se sugiere al solicitante modificar la reacción de dicha reivindicación, de manera que sea claro que 3,6 DCSA es aplicado externamente y que no es obtenido mediante el metabolismo de la planta.

Las reivindicaciones 16 a 19 y 32 a 35 no tienen sustento en la descripción para todas las plantas reclamadas, en la descripción se divulga que los métodos reclamados fueron aplicados solamente a plantas de soja y algodón.

6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 16/Octubre/2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO2004057957	Method for increasing the efficacy of agricultural chemicals	15/Julio/2004
D2	Science 25. Vol. 316 no. 5828 pp. 1185-1188	Dicamba Resistance: Enlarging and Preserving Biotechnology-Based Weed Management Strategies	Mayo/2007:

El documento D1¹ divulga métodos para aumentar la eficiencia de químicos para la agricultura. Estos métodos incluyen la aplicación de un reactivo químico a una planta o semilla de planta bajo condiciones efectivas para para realizar su función prevista (Pág. 1 resumen).

¹URL: http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20040715&CC=WO&NR=2004057957A2&KC=A2

El documento D2² divulga tratamientos que involucran el uso de un gen DMO bacteriano introducido mediante ingeniería genética, el cual es capaz de inactivar las moléculas de dicamba cuando se expresa a partir de ya sea el genoma nuclear o del genoma del cloroplasto de las plantas transgénicas transformadas (Pág. 1185, columna 2). Enseña que el primer paso en la mineralización completa de dicamba por la bacteria *Pseudomonas maltophilia* en el suelo es convertir dicamba a 3,6-DSCA, un compuesto que carece de actividad herbicida apreciable (Pág. 1185, columna 3 párrafo 1).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste “*Un método para mejorar la salud de una planta o población de plantas que comprende: I. Proporcionar a la planta ácido 3,6-dicloro salicílico (3,6 DCSA), un producto del metabolismo mediado por DMO (dicambamonooxigenasa), o un análogo del mismo en una cantidad que mejora la salud de la planta en comparación con una planta del mismo genotipo no provista con 3,6 DCSA; ó II. Proporcionar dicamba a la planta, o producto del metabolismo mediado por DMO, o análogo del mismo donde la planta a la que se le proporcionó dicamba es una planta transgénica que comprende un transgen que codifica DMO,*
Para aumentar el rendimiento de la planta con respecto a una planta del mismo genotipo cultivada en las mismas condiciones pero sin entrar en contacto con el dicamba o un producto del metabolismo del dicamba mediado por DMO.”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Método para potencia el rendimiento de una planta	Método para mejorar la eficacia de productos químicos agrícolas
Proporcionar a la planta ácido 3,6-dicloro salicílico (3,6 DCSA), un producto del metabolismo mediado por DMO (dicambamonooxigenasa), o un análogo del mismo en una cantidad que mejora la salud de la planta en comparación con una planta del mismo genotipo no provista con 3,6 DCSA Ó:	Comprende la aplicación de uno o más productos químicos agrícolas a plantas transgénicas o semillas transgénicas transformadas con una molécula de ácido nucleico que codifica una proteína que induce una respuesta hipersensible bajo condiciones efectivas para el producto químico agrícola para cumplir con su función, pero con una mayor eficacia (Pág. 4 línea 21 a 26)
Proporcionar dicamba a la planta, o producto del metabolismo mediado por DMO, o análogo del mismo donde la planta a la que se le proporcionó dicamba es una planta transgénica que comprende un transgen que codifica DMO	El método incluye la aplicación de dicamba a plantas transformadas con péptidos que proporcionan resistencia a dicho herbicida (Pág. 46 Reivindicación 28).

Las características esenciales de la composición de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual da a conocer un método para mejorar la eficacia de productos químicos agrícolas, el cual comprende la aplicación de uno o más productos químicos agrícolas a plantas transgénicas o semillas transgénicas transformadas con una molécula de ácido nucleico que codifica una proteína que induce una respuesta hipersensible bajo condiciones efectivas para el producto químico agrícola para cumplir con su función, pero con una mayor eficacia (Pág. 4 línea 21 a 26). El método incluye la aplicación de dicamba a plantas transformadas con péptidos que proporcionan resistencia a dicho herbicida (Pág. 46 Reivindicación 28).

En consecuencia, la reivindicación 1 literal II no es nueva, según lo divulgado en el documento D1.

Por su parte, las reivindicaciones 28 y 39, y dependientes, no mencionan alguna característica que haga nueva a la reivindicación 1, por lo tanto, tampoco cumplen con lo establecido en el mencionado artículo 16 de la Decisión 486.

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “*Un método para mejorar la salud de una planta o población*

2URL:<http://www.sciencemag.org/content/316/5828/1185.short>

de plantas que comprende: I. Proporcionar a la planta ácido 3,6-dicloro salicílico (3,6 DCSA), un producto del metabolismo mediado por DMO (dicambamonooxigenasa), o un análogo del mismo en una cantidad que mejora la salud de la planta en comparación con una planta del mismo genotipo no provista con 3,6 DCSA; ó II. Proporcionar dicamba a la planta, o producto del metabolismo mediado por DMO, o análogo del mismo donde la planta a la que se le proporcionó dicamba es una planta transgénica que comprende un transgen que codifica DMO,

Para aumentar el rendimiento de la planta con respecto a una planta del mismo genotipo cultivada en las mismas condiciones pero sin entrar en contacto con el dicamba o un producto del metabolismo del dicamba mediado por DMO.”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D5
Método para potencia el rendimiento de una planta	Método para mejorar la eficacia de productos químicos agrícolas	Método de control de malezas
Proporcionar a la planta ácido 3,6-dicloro salicílico (3,6 DCSA), un producto del metabolismo mediado por DMO (dicambamonooxigenasa), o un análogo del mismo en una cantidad que mejora la salud de la planta en comparación con una planta del mismo genotipo no provista con 3,6 DCSA O:	Comprende la aplicación de uno o más productos químicos agrícolas a plantas transgénicas o semillas transgénicas transformadas con una molécula de ácido nucleico que codifica una proteína que induce una respuesta hipersensible bajo condiciones efectivas para el producto químico agrícola para cumplir con su función, pero con una mayor eficacia (Pág. 4 línea 21 a 26)	Estudia los efectos de la aplicación de dicamba a cultivos transformado con DMO, explica que 3, 6 DCSA es un producto del metabolismo de dicha enzima.
Proporcionar dicamba a la planta, o producto del metabolismo mediado por DMO, o análogo del mismo donde la planta a la que se le proporcionó dicamba es una planta transgénica que comprende un transgen que codifica DMO	El método incluye la aplicación de dicamba a plantas transformadas con péptidos que proporcionan resistencia a dicho herbicida (Pág. 46 Reivindicación 28).	

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D1 da a conocer un método para mejorar la eficacia de productos químicos agrícolas, el cual comprende la aplicación de uno o más productos químicos agrícolas a plantas transgénicas o semillas transgénicas transformadas con una molécula de ácido nucleico que codifica una proteína que induce una respuesta hipersensible bajo condiciones efectivas para el producto químico agrícola para cumplir con su función, pero con una mayor eficacia (Pág. 4 línea 21 a 26). El método incluye la aplicación de dicamba a plantas transformadas con péptidos que proporcionan resistencia a dicho herbicida (Pág. 46 Reivindicación 28).

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de D1 consiste en que el documento no hace referencia al uso de 2,6 DCSA.

El efecto que logra el incluir el uso de 2,6 DCSA es que mejorar la salud de las plantas sometidas a estreses bióticos o abióticos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar el procedimiento conocido en el documento D1 para mejorar la salud de las plantas sometidas a estreses bióticos o abióticos”.

Sin embargo, el documento D2 divulga que las aplicaciones pre-emergentes de dicamba en o cerca del cultivo transgénico se podrían hacer sin daños a los cultivos o pérdida significativa de rendimiento. También enseña que el dicamba se combina con el glifosato para mejorar el grado de control de malas hierbas y permitir un control de las malas hierbas tolerantes a los herbicidas sin daños a los cultivos. El documento divulga los efectos de la aplicación de dicamba a cultivos transformado con DMO, y explica que 3,6 DCSA es un producto del metabolismo de dicha enzima, dichos métodos demostraron que permitían la aplicación del herbicida sin afectar el cultivo, eliminando las malezas. Por lo tanto, habría

sido evidente para el experto en la materia, que la aplicación de dicamba o un producto del metabolismo mediado DMO como 3,6 DCSA , que por fuerza se encuentra en la planta después de la aplicación de dicamba, podrían mejorar la salud de la planta que tiene el estrés biótico o abiótico.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría producto del metabolismo mediado DMO en el procedimiento divulgado en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Por su parte, las reivindicaciones 28 y 39, y dependientes, que corresponden a el mismo método de la reivindicación 1, pero con un objetivo diferente (mejorar el rendimiento) no mencionan alguna característica esencial que provea nivel inventivo a la reivindicación 1, por lo tanto, tampoco cumplen con lo establecido en el mencionado artículo 18 de la Decisión 486.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2004057957	1 a 39	Novedad Nivel inventivo
D2	Science 25. Vol. 316 no. 5828 pp. 1185- 1188	1 a 39	Nivel inventivo

9. Respuesta a los argumentos del señor solicitante

Argumentos con respecto a D1

En referencia al argumento de la solicitante, según el cual el documento D1 no divulga o sugiere el uso de dicamba o un producto del metabolismo mediado por DMO en un método para mejorar la salud de la planta, y que por el contrario enseña que el dicamba es un herbicida que puede dañar muchos cultivos comerciales y que la expresión de DMO en plantas tratadas simplemente hace que las plantas sean tolerantes a dicamba. Se aclara al solicitante si bien el documento D1 no hace referencia específica a que la aplicación de dicamba a plantas modificadas con DMO mejora la salud de la planta, el método descrito por ellos no se diferencia en ninguna característica esencial del método presentado por el solicitante en la reivindicaciones 1, 28 y 39, tal como se aclaró en el ítem examen de patentabilidad, además en el método descrito en el documento D1 se infiere la presencia del metabolito 3,6 DCSA ya que es producto de la función metabólica de la planta modificada con DMO en presencia de dicamba, por tanto es claro para un persona normalmente versada en la materia que dicho metabolito se encontraría presente en el método descrito por el documento D1 y por tanto tendría el efecto descrito por el solicitante.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-02-26

Elaboró: Sandra Carolina Crespo
Revisó: Consuelo Leguizamon Leguizamon



Dicamba Resistance: Enlarging and Preserving Biotechnology-Based Weed Management Strategies

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Dicamba Resistance: Enlarging and Preserving Biotechnology-Based Weed Management Strategies

Mark R. Behrens,^{1*} Nedim Mutlu,^{1*} Sarbani Chakraborty,¹ Razvan Dumitru,¹ Wen Zhi Jiang,¹ Bradley J. LaVallee,^{2†} Patricia L. Herman,^{1‡} Thomas E. Clemente,^{2,3,4} Donald P. Weeks^{1,4§}

The advent of biotechnology-derived, herbicide-resistant crops has revolutionized farming practices in many countries. Facile, highly effective, environmentally sound, and profitable weed control methods have been rapidly adopted by crop producers who value the benefits associated with biotechnology-derived weed management traits. But a rapid rise in the populations of several troublesome weeds that are tolerant or resistant to herbicides currently used in conjunction with herbicide-resistant crops may signify that the useful lifetime of these economically important weed management traits will be cut short. We describe the development of soybean and other broadleaf plant species resistant to dicamba, a widely used, inexpensive, and environmentally safe herbicide. The dicamba resistance technology will augment current herbicide resistance technologies and extend their effective lifetime. Attributes of both nuclear- and chloroplast-encoded dicamba resistance genes that affect the potency and expected durability of the herbicide resistance trait are examined.

In the past decade, the availability of biotechnology-derived herbicide-resistant and insect-resistant traits has led to striking advancements in agricultural crop management systems throughout the world. These “input traits” have contributed to greater productivity per hectare, decreased production costs, greater flexibility and efficiencies in production regimes, reduced pesticide use, and improved farmer health (1–3). In 2006, more than 100 million hectares worldwide were planted with crops having biotechnology-derived traits (4). In the United States, for example, Roundup (glyphosate)-resistant crops were planted on almost 90% of the soybean acreage and 60% of the cotton acreage in 2005, along with about 18% of the corn crop (3). The recent emergence of weeds resistant to the herbicides used year after year for weed control in fields of herbicide-resistant crops has prompted serious concerns regarding the long-term availability of the facile and economically important weed control provided by current herbicide-resistant crop plants. Also at risk is the greatly expanded use of no-till or reduced-till planting procedures that are made possible by “burndown” of weeds before planting of herbicide-resistant crops. These integrated practices minimize soil loss due to water and wind erosion resulting from traditional methods of soil tillage (3).

Among the glyphosate-tolerant weed species currently posing the greatest danger to agricultur-

al productivity are several broadleaf plants such as giant ragweed (*Ambrosia trifida*), horseweed (*Conyza canadensis*), waterhemp (*Amaranthus rudis*), Palmer amaranth (*Amaranthus palmeri*), and common ragweed (*Ambrosia artemisiifolia*) (3, 5). To combat these pernicious weeds and to address the potential emergence of other herbicide-resistant broadleaf weeds, we have targeted the development of crop plants resistant to treatment with dicamba. Dicamba is a widely used, low-cost, environmentally friendly herbicide that does not persist in soils and shows little or no toxicity to wildlife and humans (6–10). Use of the dicamba resistance trait alone or in combination with other herbicide resistance traits will allow rotation of herbicides or use of mixtures of herbicides that will greatly suppress several present or future herbicide-resistant weeds. Here, we describe the use of a genetically engineered bacterial gene, *DMO* (dicamba monooxygenase), that encodes a Rieske non-heme monooxygenase capable of inactivating dicamba when expressed from either the nuclear genome or chloroplast genome of transgenic plants. The *DMO* enzyme acts to destroy the herbicidal activity of dicamba before the herbicide

can build to toxic levels in dicamba-treated transgenic plants, as shown below.

As the first step in the complete mineralization of dicamba, the soil bacterium *Pseudomonas maltophilia* (strain DI-6) converts dicamba to 3,6-dichlorosalicylic acid (DCSA) (11, 12) (Fig. 1A), a compound that lacks appreciable herbicidal activity. The enzyme system responsible for this conversion in the bacterium is the three-component enzyme dicamba *O*-demethylase. This enzyme system serves as an electron transfer chain in which electrons from NADH (the reduced form of nicotinamide adenine dinucleotide) are shuttled through a reductase to a ferredoxin and finally to the terminal component DMO (13–15). The ferredoxin component of dicamba *O*-demethylase closely resembles the ferredoxin found in plant chloroplasts. Thus, to potentially take advantage of a source of reduced ferredoxin in chloroplasts of transgenic plants to supply electrons for the DMO reaction (and to eliminate the need for the bacterial reductase and ferredoxin genes), we included a chloroplast transit peptide-coding region upstream of the *DMO* gene to allow targeting of DMO to the chloroplast. The *DMO* expression cassette (Fig. 1B) contained the strong peanut chlorotic streak virus gene promoter *FLt36* (16) and a terminator region from the pea Rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase) small subunit gene. The goal then was to determine whether expression of DMO from this expression vector in transgenic broadleaf plants could provide protection against the normally lethal effects of dicamba.

Because of ease of transformation and regeneration, *Arabidopsis thaliana*, tomato, and tobacco were used as model systems to test whether expression of the *DMO* gene alone (i.e., without the ferredoxin and reductase components of dicamba *O*-demethylase) could impart herbicide resistance after application of dicamba. *Agrobacterium*-mediated gene transfer was used to introduce the *DMO* expression cassette into the nuclear genome of the respective plant species. In regard to tobacco, we used DNA, RNA, and protein blot analyses to test several independently derived T₁-generation plants for the presence and expression of the *DMO* gene (fig.

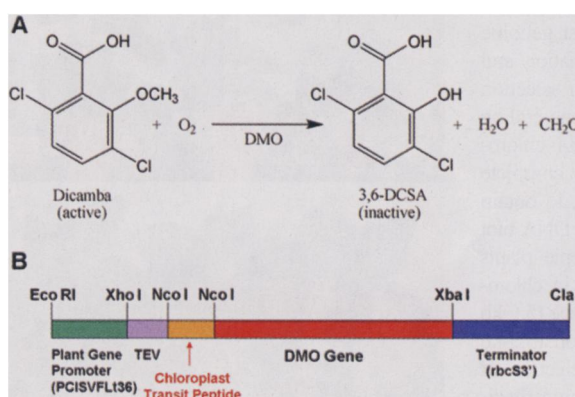


Fig. 1. Dicamba inactivation. (A) Conversion of dicamba to DCSA by DMO. **(B)** Genetically engineered version of the *DMO* gene for expression in higher plants, using the *FLt36* promoter from peanut chlorotic streak virus, a translational enhancer from the tobacco etch virus (TEV), a chloroplast transit peptide-coding region from the pea Rubisco small subunit gene for chloroplast localization of DMO, and a terminator region from the pea Rubisco small subunit gene (*rbcS3'*).

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S2). RNA blots demonstrated highly variable levels of *DMO* mRNA in individual transformants that, in general, did not correlate closely with the amount of DMO enzyme produced. We noted (most easily in lanes 2 and 6, fig. S2) that although most of the precursor DMO molecule containing the chloroplast transit peptide was cleaved to the mature form, not all of the precursor was processed.

Most dicotyledonous plants, such as tobacco, are quite sensitive to treatment with dicamba, an auxin-type herbicide. Figure 2A illustrates this point by showing nontransgenic tobacco plants not treated (leftmost plant) and treated with increasing amounts of dicamba. Herbicide damage symptoms are pronounced after spraying dicamba even at the low level of 0.017 kg/ha. Symptoms are quite severe at 0.28 kg/ha and 0.56 kg/ha, the levels normally used for weed control in agricultural applications.

Treatment of transgenic tobacco plants containing the *DMO* gene with 5.6 kg/ha (10 to 20 times the recommended application rate) caused few if any symptoms, whereas a nontransgenic plant suffered severe damage (Fig. 2B). Damage to the lower leaves of the transgenic plants could be duplicated by spraying plants with the surfactant-containing solvent solution used as the vehicle for dicamba application. Leaves produced after treatment of the transgenic plants with dicamba exhibited no visible signs of damage (Fig. 2C). Transgenic tomato plants carrying the genetically engineered *DMO* gene, likewise, showed no damage to newly emerged leaves (fig. S2B) after spraying with dicamba at concentrations as high as 5.6 kg/ha. *Arabidopsis* expressing the *DMO* gene also displayed strong resistance to treatment with dicamba at 1.12 kg/ha, the highest level tested (fig. S3). Over a range of dicamba concentrations tested, an unexpected finding was the observation that tobacco plants transformed with a *DMO* expression cassette lacking a transit peptide-coding region were resistant to treatments with dicamba at levels on average only slightly below that of plants containing *DMO* genes bearing transit peptide-coding regions (fig. S4; see below).

To determine whether DMO could function exclusively inside chloroplasts, we created the pDMO1 vector bearing the *DMO* gene coding region (fig. S5). This vector allows integration of the *DMO* gene into the chloroplast genome of tobacco by homologous recombination and the isolation of transformants through selection for antibiotic resistance. The *DMO* gene coding region was driven by the strong *psbA* chloroplast gene promoter, containing the complete *psbA* 5'-untranslated region sequence, to obtain high levels of DMO expression. Initial DNA blot analyses of antibiotic-resistant transgenic plants (fig. S6A) demonstrated the presence in chloroplast genomes of both the *DMO* transgene (5.6-kb band) and the native *psbA* gene region (3.3-kb band). Repeated regeneration and selection of transgenic plants on antibiotic-containing medi-

um resulted in apparently homoplasmidic chloroplasts bearing the *DMO* gene fragment but not the endogenous native gene region (fig. S6B). Only chloroplast transformants expressing the DMO enzyme were resistant to treatment with dicamba (fig. S7). T₁, T₂, and T₃ generations of progeny from two independently derived chloroplast transformants were tested for resistance to treatment with dicamba at various doses. All exhibited high levels of resistance. Indeed, chloroplast genome transformants displayed no apparent damage (other than "solvent-only damage" to lower leaves) when sprayed with dicamba at a rate of 28 kg/ha (fig. S8). Only transitory damage was observed when plants were treated with extremely high dicamba applications of 112 and 224 kg/ha. At these extremely high levels, initial damage was caused primarily by surfactants and other components of the solvent in which dicamba was delivered. New apex tissues and leaves growing from the damaged plants displayed nearly normal to normal phenotypes, showed no decrease in growth rates, and retained the ability to produce usual numbers and quality of seeds.

The above results were consistent with the hypothesis that reduced ferredoxin in tobacco chloroplasts could be the donor to DMO of electrons needed for oxidation of dicamba to DCSA. As a direct test of this hypothesis, we examined the ability of purified spinach ferredoxin to support the conversion of dicamba to DCSA in

the presence and absence of DMO purified from *P. maltophilia* (strain DI-6) or overproduced and purified from *Escherichia coli* (table S1). Results of these experiments demonstrated that reduced ferredoxin from spinach or *Clostridium pasteurianum* was fully capable of donating electrons to DMO in vitro, as measured either by dicamba degradation or by DCSA appearance.

The exceptionally high levels of resistance to dicamba displayed by tobacco plants carrying the *DMO* gene in the chloroplast genome, relative to plants bearing the *DMO* gene as a nuclear gene, suggested the possibility that chloroplast-encoded DMO was produced in greater abundance. Comparison of the amounts of oxygenase as percentage of total soluble protein, fraction of fresh weight, or fraction of dry weight (table S2) showed that chloroplast transformants produced about 20 times as much DMO as did nuclear transformants synthesizing DMO with a chloroplast transit peptide, and about 40 times as much DMO as did nuclear transformants synthesizing DMO without the peptide. The ability to achieve high levels of herbicide resistance and the ability to block gene dissemination through "pollen flow" are attractive features of incorporating the *DMO* gene into the chloroplast genomes of important crop plants as soon as the techniques for such approaches prove practical (17, 18).

Genetic studies of the inheritance of the *DMO* gene in chloroplast transformants revealed that inheritance was maternal, as expected, and was

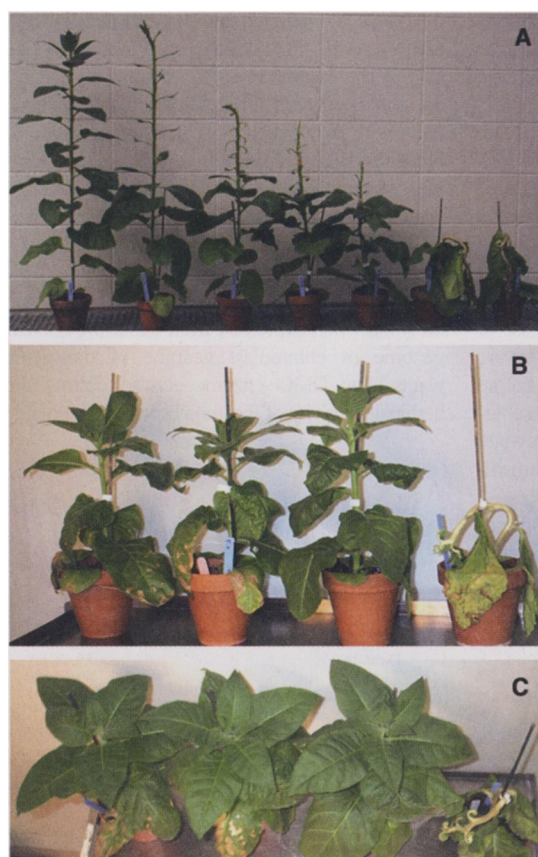


Fig. 2. Effects of dicamba treatment on nontransgenic tobacco plants and plants transformed with a genetically engineered *DMO* gene. (A) Demonstration of the sensitivity of nontransgenic tobacco plants to treatment with increasing doses of dicamba (left to right: 0, 0.017, 0.034, 0.07, 0.14, 0.28, and 0.56 kg/ha). (B) Three independently derived T₁-generation tobacco plants carrying the dicamba resistance gene (three plants at left) and a nontransgenic plant (right) treated with dicamba at a level of 5.6 kg/ha. (C) Top view of plants in (B).

mostly Mendelian in the case of plants carrying DMO as a nuclear gene (table S3). Most plants examined by DNA blot analysis contained a single DMO gene insert. Moreover, T₃ and T₄ progeny maintained the original levels of expression in regard to herbicide resistance whether they contained single or multiple copies of the DMO gene.

The prime value of the dicamba resistance technology is related to its use in major field crops in which management of broadleaf weeds is essential to maximize production. Because soybean is one such crop, we transformed the soybean varieties Thorne (Ohio State University) and NE3001 (University of Nebraska) with the same DMO expression cassette (Fig. 1B) used to transform tobacco, tomato, and *Arabidopsis*. As a means to derive marker-free soybean transformants, a two-T-DNA binary plasmid was assembled. In this plasmid, the marker-gene T-DNA element carried a bar gene cassette under the control of the *Agrobacterium tumefaciens* nopaline synthase promoter (nos), and the second, separate, T-DNA element carried the DMO expression cassette. More than 50 transgenic soybean events were produced, and seeds from the T₁, T₂, and T₃ generations were collected. Among the population of primary transformants generated, one marker-free event was identified that harbored only the DMO cassette. Most transgenic soybean events showed resistance to treatment with dicamba at 2.8 kg/ha and 5.6 kg/ha under greenhouse conditions (fig. S9) and complete resistance to dicamba at 2.8 kg/ha (the highest level tested in field trials) (Fig. 3). Initial field studies with five independent soybean events on University of Nebraska farms over the past 3 years revealed no compromise in agronomic performance—including yield, date to flowering, height, and lodging—in the transgenic plots treated with dicamba application (1.5 kg/ha) at preplant, V3 stage, or dual preplant spray treatment coupled with post-emergence treatments at the V3 stage of plant development when compared with non-herbicide-treated, weed-free plots of the parental soybean variety Thorne.



Fig. 3. Effects of dicamba treatments on non-transgenic soybean plants and transgenic plants containing the genetically engineered DMO gene. Nontransgenic (1) and transgenic soybean plants (2) treated under field conditions with dicamba at a level of 2.8 kg/ha are pictured 8 days after spraying.

Dicamba resistance in all of the plants tested did not require cotransformation with either ferredoxin or reductase genes from *P. maltophilia* (strain DI-6). These results showed that the plants contained one or more molecules that could transfer the requisite electrons to DMO to allow conversion of dicamba to DCSA. The initial targeting of DMO to the chloroplasts by means of a transit peptide sequence was aimed at using reduced ferredoxin abundantly available in the chloroplasts. However, transformation of tobacco plants with a DMO gene construct lacking a chloroplast transit peptide-coding sequence unexpectedly resulted in plants that were highly resistant to treatment with dicamba. Results from our limited trials with a small number of T₁-generation plants indicated that the level of resistance obtained with these transgenic plants was only slightly lower on average than that obtained with tobacco plants producing DMO containing a transit peptide.

These observations raise important questions in regard to the molecules in transgenic plants that can productively donate electrons to DMO. The fact that homoplasmidic chloroplasts producing DMO internally from a DMO gene integrated into the chloroplast genome show resistance to extremely high levels of dicamba (fig. S8) and the fact that purified DMO can function in vitro with reduced spinach chloroplast ferredoxin (table S2) both suggest that chloroplast ferredoxin can productively interact with DMO to allow electron transfer. However, the source of electrons for DMO produced from nuclear genes lacking a chloroplast transit peptide-coding sequence remains unknown. Presuming that ferredoxins do not reside outside of the plant chloroplasts, one must consider the possibility that an unknown cytoplasmic protein can provide DMO with a steady supply of electrons. Alternatively, DMO itself might contain a gratuitous chloroplast transit peptide that allows sufficient DMO to enter the chloroplasts to provide protection from dicamba moving into the cell after dicamba treatment. Further studies, such as microscopic localizations in situ of DMO with and without a chloroplast transit peptide and/or isolation and identification of cytoplasmic proteins that can interact “indiscriminately” with DMO to supply electrons, will be needed to resolve the questions emanating from the present observations.

It is illuminating to consider that dicot plants like tobacco display distinct injury symptoms even at levels of dicamba treatment as low as 0.001 to 0.01 kg/ha (Fig. 2A). Many transgenic tobacco, tomato, *Arabidopsis*, and soybean plants containing a nuclear-encoded DMO gene were fully resistant to treatments with dicamba at or above 5.8 kg/ha. This demonstrates that the DMO gene, present even in a single copy and expressed at relatively moderate rates (table S2), is capable of decreasing the sensitivity of dicot plants to applications of dicamba by at least a factor of 5000.

Dicamba is an “auxin”-type herbicide that mimics the effects of excess quantities of the natural plant hormone indole-3-acetic acid (IAA) when applied to dicotyledonous plants. It has been used for more than 40 years to efficiently control most broadleaf weeds. Yet despite its widespread use, no new noxious and economically important dicamba-resistant weed species have appeared (5). One possible reason for such a situation may be that dicamba may act on some, if not all, of the IAA receptors that are essential in controlling normal growth and development of all plants. If so, the appearance of new dicamba-resistant weeds may not happen rapidly. This is especially true if the dicamba resistance gene is “stacked,” for example, with the widely used glyphosate resistance gene to allow farmers to alternate herbicide applications between dicamba and glyphosate or to use mixtures of the two herbicides together. In either case, appearance of weeds resistant to either dicamba or glyphosate will be greatly suppressed. Moreover, the ability to use either or both herbicides before planting or at a variety of points during crop development will allow producers excellent weed control with greater flexibility in their crop management practices. This may be particularly important in the control of existing glyphosate-resistant weeds, such as horseweed, in which application of dicamba before planting can control emerged or emerging glyphosate-resistant weeds. Thus, dicamba-resistant crops can be a valuable asset in strategies to control currently existing herbicide-resistant weeds and to suppress the appearance of additional herbicide-resistant weeds that ultimately could threaten the long-term use and value of current herbicides and herbicide-resistant crops. Likewise, dicamba-resistant crops should further encourage the use of conservation tillage practices that greatly decrease soil erosion and foster more sustainable and environmentally friendly farming.

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royalties from the marketing of dicamba-resistant technology if crop seeds containing the resistance gene are marketed by Monsanto, the licensee of this technology from the University of Nebraska.

Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5828/1185/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 to S3

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A Manganese(IV)/Iron(III) Cofactor in *Chlamydia trachomatis* Ribonucleotide Reductase

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In a conventional class I ribonucleotide reductase (RNR), a diiron(II/II) cofactor in the R2 subunit reacts with oxygen to produce a diiron(III/IV) intermediate, which generates a stable tyrosyl radical (Y•). The Y• reversibly oxidizes a cysteine residue in the R1 subunit to a cysteinyl radical (C•), which abstracts the 3'-hydrogen of the substrate to initiate its reduction. The RNR from *Chlamydia trachomatis* lacks the Y•, and it had been proposed that the diiron(III/IV) complex in R2 directly generates the C• in R1. By enzyme activity measurements and spectroscopic methods, we show that this RNR actually uses a previously unknown stable manganese(IV)/iron(III) cofactor for radical initiation.

Ribonucleotide reductases (RNRs) provide all organisms with 2' deoxyribonucleotides for DNA synthesis (1, 2). All known RNRs are thought to initiate ribonucleotide reduction by using a cysteine thiyl radical to abstract the hydrogen atom from the 3'-carbon (3, 4). Three distinct strategies to generate the initiating cysteinyl radical (C•) have been described and are, in part, the basis for division of the RNRs into three classes. Class II and III RNRs use strategies involving the 5'-deoxyadenosyl radical, generated either by homolysis of the Co-C bond of 5'-deoxyadenosylcob(III)alamin (class II) or by reductive cleavage of the 5'-C-S bond of S-adenosyl-L-methionine by a separate activase protein (class III), as the ultimate oxidant for cysteine activation. The 5'-deoxyadenosyl radical either generates the C• directly (class II) or generates a stable glycy radical (G•) that reversibly oxidizes the cysteine (class III) (3, 5, 6). In a conventional class I RNR (e.g., from *Homo*

sapiens, *Saccharomyces cerevisiae*, or aerobic *Escherichia coli*), a binuclear iron center in its cofactor subunit, R2, reacts with oxygen to oxidize a tyrosine residue by one electron to a stable tyrosyl radical (Y•). The Y• in R2 generates the C• in the catalytic subunit, R1, where nucleotide reduction occurs (7).

An unexpected adaptation of the class I functional architecture was revealed by the recent characterization of the RNR from the bacterium *Chlamydia trachomatis* (8), an obligate intracellular parasite and important human pathogen. The presence of a phenylalanine in place of the tyrosine residue in R2 that normally harbors the essential initiating Y• was revealed first by sequence comparisons (8) and subsequently by x-ray crystallography (9). Consistent with these findings, no evidence for a Y• was found in biochemical studies (9–11). Sequences of R2 genes from other organisms revealed that the absence of the Y• is not specific to the chlamydial RNRs (9). Notably, the presence of genes encoding such R2 proteins in the genomes of other pathogens (e.g., *Mycobacterium tuberculosis*) suggested that the novel RNRs might have arisen as an adaptation to the host's immune response (9) and might present specific targets for design of new antibacterial drugs.

To explain how the *C. trachomatis* RNR can function without the essential Y•, Nordlund, Gräslund, and co-workers suggested that an Fe₂^{III/IV} cofactor might be produced in R2 by re-

action of the Fe₂^{III/II} cluster with O₂ and directly oxidize the cysteine in R1 (9). Biochemical studies by Gräslund and co-workers supported this hypothesis by demonstrating both stabilization of the reactive Fe₂^{III/IV} complex in the presence of the other reaction components (R1, substrate, and allosteric effector) (10) and induction of the Fe₂^{III/IV} state from Fe₂^{III/III}-R2 under these turnover conditions (11).

Although the reported induction and stabilization of the Fe₂^{III/IV}-R2 complex were consistent with the earlier Nordlund/Gräslund hypothesis, the fact that this form was never enriched to greater than ~30% of the total protein (10) left open the possibility that a different form was responsible for the modest activity observed. After preparing His₆-affinity-tagged forms of *C. trachomatis* R2 and the N-terminally truncated Δ(1 to 248)-R1 (12) reported by McClarty and co-workers to be more stable than full-length R1 (8), we noted a distinct lack of correlation between the catalytic activities and iron contents of different preparations of R2 (13).

By reductive chelation of iron from the purified protein and subsequent dialysis against ethylenedinitrilotetraacetate (EDTA) (12), R2 was isolated with less than 0.02 equivalents (equiv) iron (14) and very low catalytic activity [velocity (v)/[R2] ≤ 0.01 s⁻¹] (15). The metal-depleted R2 so obtained was not detectably activated by addition of 2 equiv of Fe^{II}. By contrast, addition of both Fe^{II} and Mn^{II} was found to activate the metal-depleted R2 by a factor of more than 50 (Fig. 1). A Mn^{II}:Fe^{II} ratio of unity gave maximal activation (Fig. 1A), and a total of two divalent metal ions per monomer was sufficient for ~85% of maximal activation (Fig. 1B).

The results in Fig. 1, in particular the 1:1 Mn:Fe ratio giving maximal activity, suggest the use of a Mn/Fe cofactor rather than a Fe₂ cofactor by *C. trachomatis* RNR. Because the raison d'être of the R2 subunit and its metal cofactor is to transiently oxidize the cysteine residue in R1 by one electron (7), the fully reduced complex formed upon addition of the divalent metal ions to the protein (Mn^{II}/Fe^{II}-R2) cannot be active but, by analogy with other class I RNRs, might react with O₂ to produce an oxidized state that functions in catalysis. Indeed, we observed no turnover after addition of an O₂-free solution containing R2, Mn^{II}, and Fe^{II} (0.75 equiv of

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