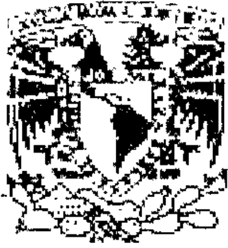

Universidad Nacional Untónoma de México

Instituto de Biotecnología

Tesis

Establecimiento de un sistema para clonar genes de maíz involucrados en la fotosíntesis y análisis genético de la biogénesis del cloroplasto en *Arabidopsis thaliana*

Que para obtener el grado de Doctora en Biotecnología
presenta:

María de la Luz Gutiérrez Nava

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Índice

Resumen i

Abstract *ii*

Introducción 1

Diferenciación del plastidio. 2

Control nuclear de la diferenciación temprana del plastidio 8

Señales que participan en la diferenciación del cloroplasto. 14

Organismos utilizados como modelos para el estudio del desarrollo del cloroplasto 16

El maíz como modelo de estudio. 18

Transposones como herramienta para el análisis de mutantes y la clonación de genes en maíz. 19

La familia de transposones *MuDR/Mu* 21

Objetivos del proyecto 24

Capítulo I Caracterizacion del elemento *MuDR-Zc* 25

Discusión 26

Evidencia de actividad Mutator en algunas líneas de Zapalote chico 26

En algunas líneas de Zapalote chico existe una alta frecuencia de mutación. 27

Caracteristicas únicas de las mutaciones inducidas en Zapalote chico. 27

Conclusiones 29

Caracterización *MuDR/Mu* en Zapalote chico. 29

Perspectivas 30

Capítulo II. Análisis genético de la biogénesis del cloroplasto en Arabidopsis thaliana. 31
Identificación de la inserción del T-DNA por TAIL-PCR en un gen predicho de una lipasa 39
Identificación de la inserción del T-DNA de la mutante 19,20 en un gen PPR 44
Discusión 48
Los genes ALBINO son requeridos para la diferenciación temprana del proplastidio y el desarrollo de diferentes tejidos de la hoja. 49
Los genes ALBINO codifican factores autónomos de la célula y factores no autónomos de la célula. 50
Las mutantes albinas son un pequeño grupo. 50
Conclusiones 52
Bibliografía 54

Resumen

Durante este trabajo utilizamos diferentes estrategias experimentales para estudiar la biogénesis temprana del cloroplasto. En primer lúgar tratamos de utilizar los transposones *MuDR/Mu* como herramienta para seleccionar mutantes y clonar los genes mutados. Para este fín llevamos a cabo la caracterización de este sistema de transposones en Zapalote chico y ello constituye una parte fundamental de este estudio.

En esta primera parte del trabajo, encontramos que en algunas líneas de Zapalote chico existe un elemento que es 94.6% idéntico al elemento autónomo *MuDR* y este es trancripcionalmente activo (a este elemento lo hemos llamado *MuDR-Zc*). En Zapalote chico la proteína predicha de 93 kD para el gen *mudrA* contiene 20 cambios de aminoácidos y 10 cambios para la proteína predicha de 23 kD del gen *mudrB*. Así mismo en esta raza de maíz los transcritos de *mudrA* son muy escasos, mientras que los transcritos de *mudrB* son tan abundantes como en líneas Mutator estándar con un bajo número de copias de *MuDR*. *MuDR-Zc* es capaz de trans-activar alelos reporteros en líneas Mutator inactivas. Una característica única de Zapalote chico es que las mutaciones causadas por *MuDR-Zc* son somaticamente estables. Este dato fue fundamental para el propósito de este trabajo y lo discutimos en el Capítulo I

En la segunda parte del proyecto realizamos un anális genético de la biogénesis del cloroplasto en Arabidopsis. En un tamizado de 1000 familias M3 mutagenizadas con EMS, 5000 familias mutagenizadas con T-DNA y todas las mutantes en pigmentos disponibles en el Centro de *Arabidopsis*, aislamos 99 mutantes con cambios en la pigmentación en las hojas. De estas identificamos 7 mutantes *albinas* que afectan biogénesis temprana del desarrollo del cloroplasto. Estas mutantes no expresan o expresan muy poco genes nucleares requeridos para el mantenimiento del proplastidio. En todas las mutantes excepto en una, los proplastidios no se diferencian lo suficientemente para formar la membrana tilacoidea. Además en este trabajo se muestra que existe una correlación entre la carencia del desarrollo del cloroplasto y desarrollo anormal de la hoja. Basado en nuestras observaciones de desarrollo del cloroplasto durante la embriogénesis, las mutantes *alb* que muestran una complementación parcial del desarrollo del

cloroplasto durante embriogénesis han sido clasificadas como mutaciones no autónomas de la célula mientras que mutaciones autónomas de la célula son aquellas que no presentan complementación por factores maternales durante la embriogénesis. El bajo porcentaje de mutaciones *albino* obtenido es nuestro tamizado de mutantes en pigmentos sugiere que un número de genes relativamente pequeño son requeridos para completar la biogénesis temprana del cloroplasto. La futura caracterización de estas mutantes *albino* aportaran datos importantes para el entendimiento de las vías que son requeridas para la diferenciación y desarrollo del cloroplasto.

Abstract

During this study we tried different experimental approaches to study early chloroplast biogenesis We first characterized the transposon *MuDR-Zc* with the intent to use this transposon as tool for cloning genes from corn. We found that some accessions of the maize land race Zapalote chico contain one to several copies of a full-length, transcriptionally active *MuDR*-like element (which we have called *MuDR-Zc*), as well as non-autonomous unmethylated elements. The sequence of the *MuDR-Zc* element is 94.6% identical to *MuDR*, with 20 amino acid changes in the 93 kD predicted protein of *mudrA* and ten amino acid changes in the 23 kD predicted protein of *mudrB*. In zapalote chico, *mudrA* transcripts are very rare, while *mudrB* transcripts are as abundant as in Mutator lines containing several copies of *MuDR*. *MuDR-Zc* can trans-activate reporter alleles in inactive Mutator backgrounds; they match the characteristic increased forward mutation frequency of standard Mutator lines, but only after outcrossing to another line. A unique characteristic of *MuDR-Zc* is that the new mutants recovered from Zapalote chico are somatically stable. This data was fundamental for the purposes of our work and we discuss this in Chapter One.

For the second part of this project we performed a genetic analysis of chloroplast biogenesis in *Arabidopsis*. We examined 1000 EMS M3 families, 5000 T-DNA families, and all chlorophyll pigmentation mutant lines available from the *Arabidopsis* stock center. In this screen we isolated 99 pigment mutants, and of these we have identified 7 *albino* mutants that affect early chloroplast biogenesis. These mutants have little or no expression of nuclear genes required for maintenance of the proplastid. In all but one mutant, proplastids do not differentiate enough to form any thylakoid membrane. In addition, we demonstrate a correlation between lack of chloroplast biogenesis and abnormal leaf development.

Based on our observations of chloroplast development during embryogenesis, *alb* mutants that display partial complementation of chloroplast development during embryogenesis have been classified as non-cell autonomous mutations, while cell autonomous mutants are those which are not complemented by maternal factors during embryogeneis. The low percent of *albino* mutants recovered in our screen for pigmentation mutants suggests that

a relatively small number of genes are required to complete early steps of chloroplast biogenesis. Further characterization of these *albino* mutants will begin to uncover the pathways that are required for differentiation and development of the chloroplast.

Introducción

La vida en la tierra, depende fundamentalmente de la energía derivada del sol. La fotosíntesis es el único proceso biológico capaz de absorber esta energía para sintetizar compuestos orgánicos y estos son utilizados como la única fuente de energía renovable de nuestro planeta.

Los organismos fotosínteticos absorben la luz del sol por medio de pigmentos. En plantas y algas este proceso se lleva a cabo en el cloroplasto. El cloroplasto es el resultado de una simbiosis ancestral entre una bacteria fotosintética aeróbica (parecida a las cianobacterias modernas) y una célula proto-eucarionte. Durante la evolución de la célula eucarionte, los organelos han desplazado a la mayoría de sus genes al núcleo, pero re-importan sus productos con la ayuda de péptidos de tránsito y una maquinaria de importación de proteinas, de tal forma que las proteínas son retenidas en el organelo. El resultado de esta endosimbiosis y transferencia de genes, es un sistema integrado en donde la expresión genética de los organelos es regulada por el núcleo (y no un organelo genéticamente semi-autónomo como se menciona frecuentemente en la literatura). La compartamentalización de la célula eucarionte involucra el desarrollo de vías que regulan la expresión de genes, la herencia coordinada de organelos y el flujo de productos entre los diferentes compartimentos genéticos (Herrmann R. G., 1997). De esta manera la diferenciación espacial y temporal del plastidio es regulada por la comunicación entre el organelo y el núcleo y es mantenida por numerosas actividades regulatorias que responden a estímulos y señales percibidas de otros compartimentos y está estrechamente coordinada con el estado de desarrollo de la planta.

Diferenciación del plastidio.

Los plastidios son organelos exclusivos de las células de plantas y algas. Como su nombre lo indica (del griego plastikos: "plásticos"), los plastidios poseen una extraordinaria capacidad de diferenciarse, desdiferenciarse y rediferenciarse. La abundancia de cada tipo de plastidio varia de acuerdo al tipo y al estado de la célula. En hojas, la vía principal de diferenciación del plastidio es la producción de cloroplastos; en raíz, de amiloplastos; en flores y frutos de cromoplastos y en plántulas que crecen en la obscuridad, de etioplastos. Los plastidios son responsables de una gran variedad de procesos tales como la fotosíntesis, la producción de almidón, de pigmentos, de ácidos grasos y de amino ácidos, así como de la síntesis de moléculas clave requeridas para la arquitectura y el funcionamiento básico de la célula (Kirk J. y R. Tilney-Bassett, 1967).

Los plastidios se reproducen por fisión binaria de los plastidios existentes, independientemente de la división celular. El proceso de fisión binaria del plastidio es morfológicamente similar al de la división de una célula bacteriana. Recientemente se demostró que genes homólogos a *ftsZ* de *Escherichia coli*, el cual participa en la formación del anillo de constricción que da origen a las dos células hijas, también están involucrados en la división del cloroplasto. (Osteryoung K. W. et al., 1998). Los estudios genéticos han demostrado que en angiospermas, los plastidios son heredados maternalmente en forma de proplastidios. Estos organelos son excluídos de las células espermáticas o degradados durante el desarrollo del gametofito masculino o durante la doble fertilización. En cambio en gimnospermas los pro-plastidios son heredados también a través del esperma (Warren G. y W. Wickner, 1996)

Los plastidios estan rodeados por una doble membrana y se originan de proplastidios que están presentes en células meristemáticas de tallos, raíz, embriones y endospermo, en

donde son mantenidos en un estado indiferenciado. La biología del proplastidio ha sido poco estudiada, quizás debido a que son muy difíciles de aislar de células meristemáticas. Sin embargo su estructura se conoce desde hace más de 30 años, por medio de microscopía electrónica.

En angiospermas existen aproximadamente 20 proplastidios por célula y su tamaño varía entre 0.2 a 1.0 µm. El sistema de membrana interna de este plastidio permanece pobremente desarrollado, consistiendo sólo de unas pocas invaginaciones y un número pequeño de sacos llamados lamela. Algunas de las invaginaciones de la membrana interna forman estructuras filamentosas y se ha propuesto que pueden ser túbulos. En el estroma (la matriz del plastidio) existen pocos ribosomas, escasas proteínas solubles y uno o dos nucleoides con finas fibras de DNA (Figura 1). La presencia de estas moléculas sugiere que existe un nivel basal de expresión de genes plastídicos y nucleares que permiten la perpetuación de la población de proplastidios en células del meristemo en división (Kirk J. y R. Tilney-Bassett, 1967). Los proplastidios se diferencian en concordancia al tipo de célula en la cual el plastidio reside; los mecanismos específicos que regulan esta diferenciación son aún desconocidos. Cuando en el primordio de la hoja, las células meristemáticas inician su diferenciación en células del mesófilo, el proplastidio inicia su diferenciación en cloroplasto. Los cambios que ocurren durante el desarrollo del plastidio se han visualizado por medio de estudios de microscopía electrónica. Durante este proceso, la invaginación de la membrana interna del plastidio continúa y se va formando una membrana interna a la que se le llama tilacoide. Al principio se observan sólo algunas membranas aisladas, pero conforme la diferenciación procede, el número de tilacoides se incrementa y se van apilando en dos, tres o más, a esta etapa del desarrollo se le llama plastidio pregranal debido a que los apilamientos

del tilacoide darán lugar al grana del cloroplasto maduro (Kirk J. y R. Tilney-Bassett. 1967) (Figura 1).

El tamaño y la composición del plastidio también cambian durante el desarrollo del cloroplasto. El volumen del plastidio puede incrementarse más de 100 veces en las células maduras del mesófilo, debido en gran parte a la acumulación de proteínas, lípidos y cofactores requeridos para la fotosíntesis. Estos componentes son derivados de númerosas vías biosínteticas que se activan durante la biogénesis del cloroplasto (Mullet J. E., 1988).

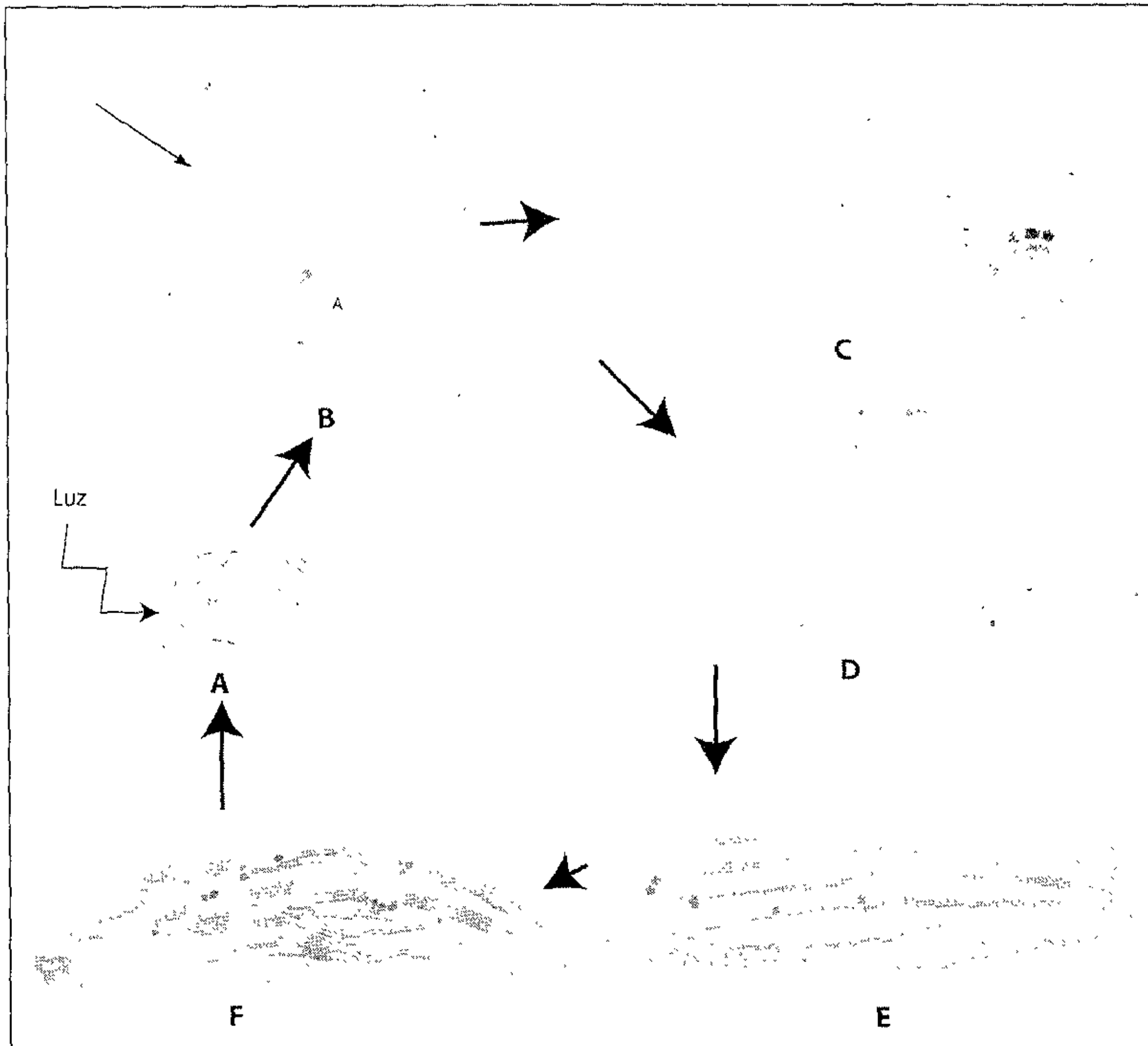


Figura 1. Imágenes de microscopia electrónica de diferentes estadíos de desarrollo del cloroplasto.

A). El proplastidio (Pp). B). Cuando los proplastidios son iluminados el volumen del proplastidio aumenta considerablemente y en el estroma se observa la membrana interior esparcida en todo el plastidio sin ningún tipo de organización, esporádicamente la membrana interna muestra invaginaciones(flecha pequeña), en la sección también se observa un pequeño granulo de almidón (A). C) La formación del tilacoide ha inciado pero existe sólo como pequeñas vesículas. D) El plastidio en un estadío intermedio de desarrollo con regiones muy cortas de fusión de tilacoides que forman el primer rudimento del grana. A este estadío se le llama estadío pregranal. E). Plastidio con formación extensiva del grana por apilamiento del tilacoide. F). Cloroplasto maduro con abundante membrana tilacoidea, las cuales estan

distintamente diferenciadas en grana y tilacoide estromal. B 77,000 X, C 94,300 X, D 60,000 X, E 75,750 X, F 43,500 X Modificado de Robertson D. y W. Laetsch, 1973.

En el cloroplasto maduro el tilacoide se encuentra completamente ensamblado con todos los complejos necesarios para la captura de la luz y el transporte de los electrones. Estos complejos son construidos por un gran número de subunidades polipeptídicas codificadas en el núcleo o en el genoma del plastidio. El tilacoide se organiza en distintas regiones, como ya se mencionó; las regiones en donde la membrana se apila da lugar al grana, las regiones que no están apiladas y están en contacto con el estroma, forman el tilacoide estromal. Estas regiones estan interconectadas y encierran un espacio interno conocido como lumen. Los cuatro complejos fotosínteticos de la membrana tilacoidea están distribuidos heterogéneamente, el fotosistema I (FSI)y el complejo de la síntetasa del ATP reside únicamente en el tilacoide estromal, es decir en las partes no apiladas de la membrana. Por otro lado el fotosistema II (FSII) se encuentra exclusivamente en el grana. A diferencia de los fotosistemas I y II, el complejo del citocromo b_6f está distribuído uniformemente en toda la membrana tilacoidea, a este fenómeno se le conoce como heterogeneidad lateral (Hoober J.K., 1984) (Figura 2).

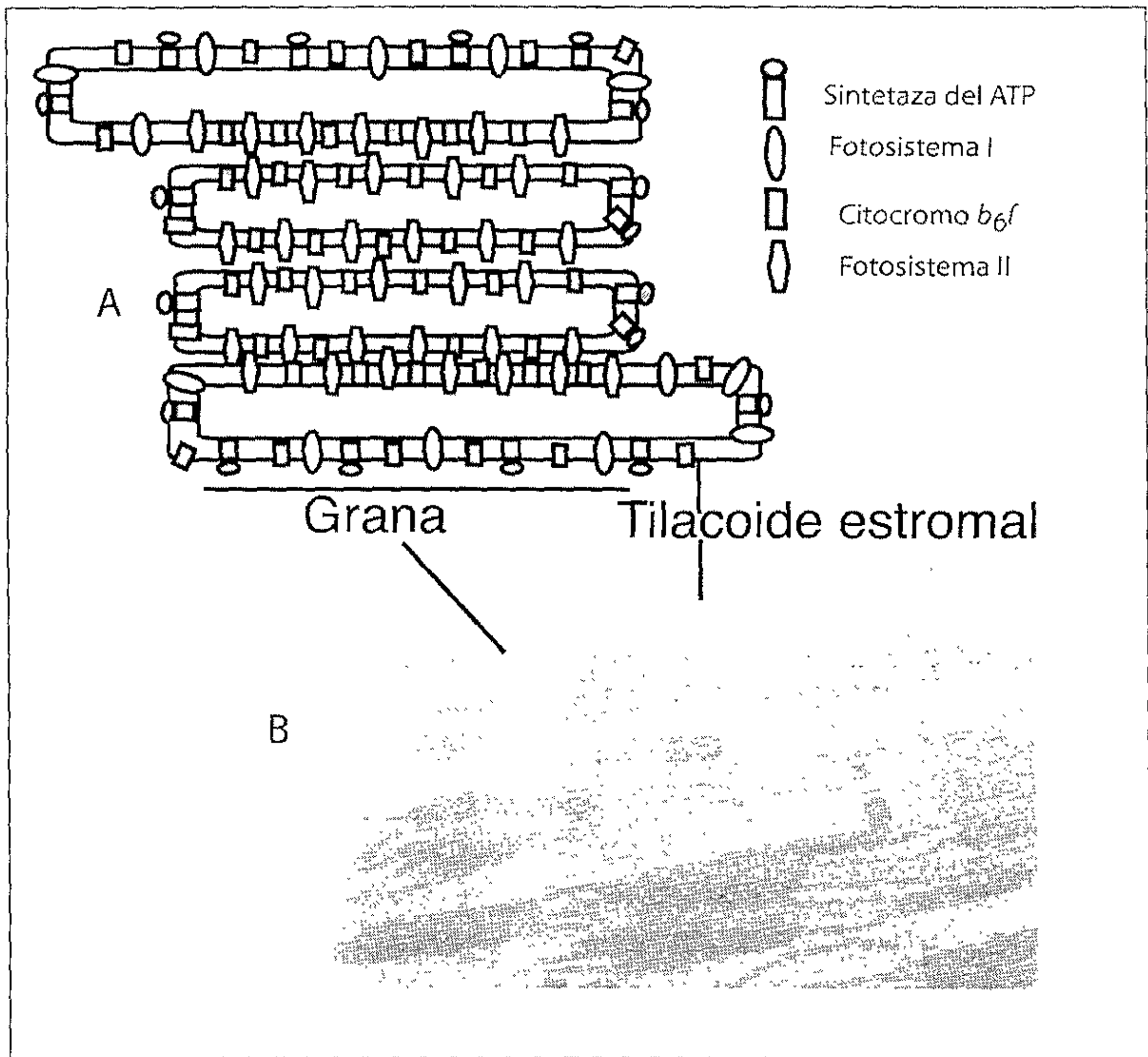


Figura 2. Heterogeneidad lateral de la membrana tilacoidea.

A). Diagrama en donde se muestra la compartamentalización del cloroplasto. B) Amplificación de una micrografía de la membrana tilacoidea en donde se observa el grana y el tilacoide estromal. El fotosistema II se encuentra en las regiones apiladas del tilacoide, así el Fotosistema I y la sintetasa del ATP se encuentran exclusivamente en el tilacoide estromal (regiones de la membrana no apiladas). El complejo del citocromo b_6f esta distribuído homogeneamente a través de todas las regiones de la membrana tilacoidea. El transporte de electrones entre el fotosistema I y el fotosistema II se lleva a cabo por medio de acarreadores de electrones móviles, tales como la plastocianina y la plastoquinona. Adaptado de Malkin R. y Niyogi K. 2000.

La fotosíntesis es uno de los procesos más importantes que ocurre en el cloroplasto. En las plantas, la fotosíntesis comprende una serie de reacciones que se llevan a cabo en diferentes partes del cloroplasto y que se dividen en dos fases: la fase luminosa que ocurre en la membrana tilacoidea en la cual se produce NADH y ATP y se libera oxígeno y el ciclo de Calvin, por otro lado, se lleva a cabo en el estroma del cloroplasto. En este ciclo se usan el NADPH y el ATP producidos en la fase luminosa para fijar el CO_2 atmosférico durante la producción de carbohidratos. (Malkin R. y K. Niyogi, 2000). Por muchos años este último ciclo ha sido llamado erróneamente fase obscura de la fotosíntesis, sin embargo actualmente está bien establecido que el ciclo de Calvin es regulado estrechamente por la luz. En el cloroplasto existen cuando menos dos mecanismos regulatorios dirigidos por la luz que modulan la actividad de varias enzimas fotosinteticas. Los cambios que ocurren en la concentración del Mg^{+2} y en el pH y después de la iluminación y que llevan a la activación de varias de estas enzimas es uno de ellos. Otro mecanismo de regulación del ciclo de Calvin es el sistema de señales iniciado por la luz llamado ferredoxina-thioredoxina. En este sistema, el flujo de electrones de la cadena fotosintética dispara una serie de reacciones redox que activan enzimas blanco a través de la reducción por tioredoxinas de los puentes disulfuro de estas proteínas. Las enzimas fotosintéticas reguladas por este sistema son activadas por reducción en la luz y desactivadas por oxidación en la obscuridad (Schürmann P. y J.-P. Jacquot, 2000).

Control nuclear de la diferenciación temprana del plastidio

El desarrollo del plastidio fotosíntetico a partir del proplastidio involucra la expresión coordinada de un gran número de genes nucleares y cloroplásticos. Los mecanismos que controlan este proceso están pobremente entendidos. Sin embargo, en los últimos años se ha

reconocido que la regulación de la transcripción de genes plastídicos por dos RNA polimerasas y varios factores sigma, tiene un papel clave en la diferenciación del plastidio en respuesta a cambios ambientales y señales internas del desarrollo de la planta. También se ha demostrado que estas señales son tejido y célula-específicas (Allison L. A. 2000).

La transcripción de genes del plastidio la llevan a cabo al menos dos RNA polimerasas, una de las cuales es parecida a la RNA polimerasa de eubacterias, tanto en su estructura como en el reconocimiento de los promotores. Esta enzima está compuesta de cuatro subunidades codificadas en el cloroplasto y se le ha llamado PEP por sus siglas en inglés (plastid-encoded plastid RNA polymerase). La otra enzima es codificada en el núcleo, consta de una sola subunidad y se le llama NEP (nuclear-encoded plastid RNA polymerase). Los genes que codifican esta última fueron recientemente clonados en Arabidopsis, maíz y trigo (Hedtke B. et al, 1997; Chang C. C. et al, 1999; Ikeda T. M. et al, 1999). PEP contiene un núcleo catalítico que se ensambla con factores sigma codificados en el núcleo para el reconocimiento selectivo de promotores del tipo σ^{70} (-10 /-35). Estos promotores no son reconocidos por NEP, los promotores de esta enzima se encuentran entre los promotores -35 y -10 y aún no se ha establecido una secuencia consenso (Hajdukiewicz P. T. J. et al, 1997; Hübschmann T. y T. Börner, 1998). Asimismo parece que PEP no reconoce los promotores de NEP. Existen distintas evidencias de que NEP transcribe principalmente genes de expresión constitutiva (genes *housekeeping*) y PEP transcribe principalmente genes fotosínteticos. La mayoría de los genes fotosínteticos poseen promotores que sólo son reconocidos por PEP (Hajdukiewicz P. T. J. et al, 1997). Los operones que contienen genes fotosínteticos, genes de expresión constitutiva y genes que codifican para otras funciones pueden ser transcritos por ambas RNA polimerasas. Los genes *rpoB* (que codifican la sub-unidad β de PEP), *rpl123 rpl 128* (genes que codifican

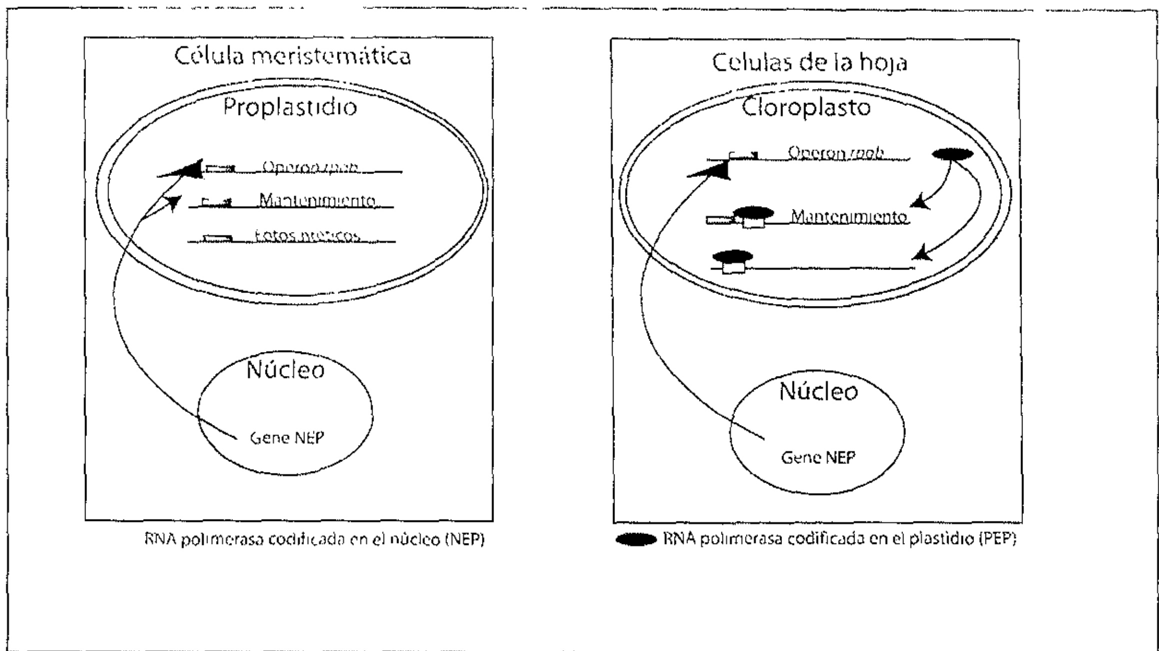


Figura 3. Control nuclear de la diferenciación temprana del cloroplasto.

El modelo propuesto en donde la expresión diferencial de genes durante el desarrollo del cloroplasto está basado en el reconocimiento de distintos promotores por NEP y PEP. En proplastidios, NEP transcribe genes de mantenimiento y los genes del operon *rpoB* que codifican las subunidades ß, ß', ß" de PEP. Así, en cloroplastos tanto genes fotosintéticos como de housekeeping son transcritos por PEP. Basado en Hajdukiewicz P. T. et al 1997.

En este escenario, NEP parece tener un papel primordial en el mantenimiento de los proplastidios y plastidios-no fotosinteticos; su principal función es la de transcribir los genes que son necesarios para la maquinaria de expresión de los genes involucrados en mantener al plastidio como un compartimento en donde se llevarán acabo diferentes vías metabólicas, tal como la biosíntesis de aminoácidos, de ácidos grasos y de ciertas fito-hormomas.

Otro factor importante en la regulación de la diferenciación del plastidio es la activación diferencial de factores sigma, los cuales a su vez modulan la actividad de transcripción de la PEP. En los últimos cuatro años ha habido un gran avance en el

entendimiento de cómo estos factores proveen al núcleo de un mecanismo para controlar la expresión de grupos de genes plastídicos en respuesta a señales internas del estado de desarrollo del plastidio o tipo de plastidio y a señales ambientales como la luz (Allison, L. A. 2000)

En Arabidopsis se han reportado seis factores sigma denominados AtSig A a la F respectivamente (Isono K. et al, 1997; Tanaka K. et al 1997; Yao J. et al, 1998) los cuales se expresan preferencialmente en tejidos de la hoja y muy poco en la raíz. La expresión de éstos en tejidos de la hoja se incrementa con la luz. Además fue demostrado que AtSig B se expresa 24 horas más temprano que AtSig A, sugiriendo que hay una cascada de expresión de estos factores lo cual es consistente con su papel en la inducción de la transcripción por luz en los cloroplastos maduros. En maíz se han descrito también cinco factores sigma plastídicos, ZmSig1*, ZmSig2*, ZmSig l, ZmSig 2 y ZmSig3 los cuales presentan un patrón de expresión muy específica (Tan S. y R.F Troxler, 1999; Lahiri S. D. y L. A. Allison, 2000) (Figura 4). Un estudio en donde se usaron anticuerpos que reconocen los factores ZmSig1* y ZmSig2* pero que no reconocen ninguno de los otros tres, mostró que ZmSig1* y ZmSig2* se acumulan preferencialmente en las hojas verdes, que no se acumulan en la raíz y que ZmSig2* se expresa también en etioplastos, lo que sugiere que éste puede estar involucrado en la transcripción de genes de mantenimiento ó *housekeeping* (Tan S. y R.F Troxler, 1999). Por otro lado se encontró que ZmSig3 se expresa en tejidos no fotosínteticos tal como raíz, hojas etioladas y en la porción basal de la hoja en donde se encuentran plastidios en proceso de diferenciación, mientras que ZmSig1 se expresa exclusivamente en tejidos que contienen cloroplastos maduros (Figura 4) (Lahiri S. D. y L. A. Allison, 2000). Así, parece que la expresión diferencial de factores sigma y por ende el reconocimiento de un promotor por dicho factor, responde a

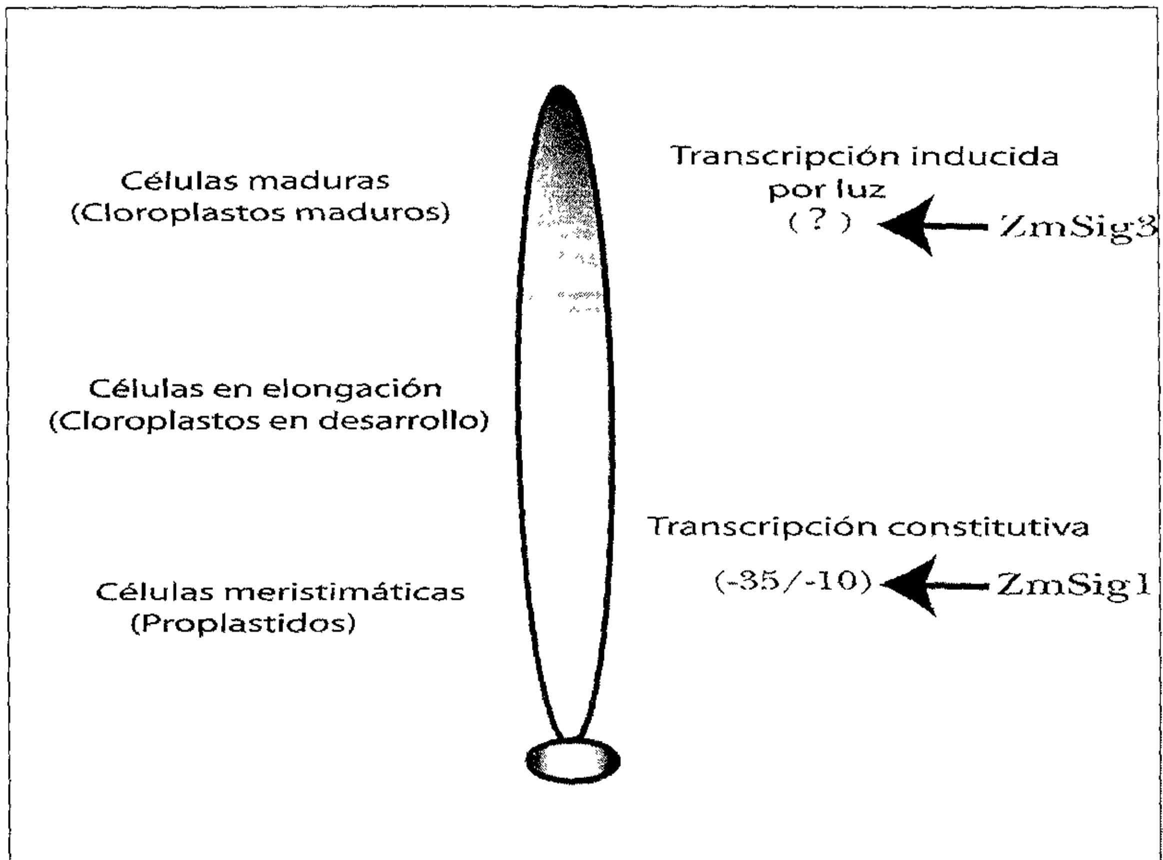


Figura 4. Modelo de la función de factores sigma durante el desarrollo del cloroplasto. En la hoja de maíz existe un gradiente de plastidios en diferentes estadíos de desarrollo, en la región meristemática en la base de la hoja existen solo proplastidios, la zona de elongación celular contiene cloroplastos en etapas tempranas de desarrollo y la parte superior de la hoja contiene células maduras con cloroplastos completamente desarrollados. ZmSig3 se expresa solo en la región meristemática de la hoja. ZmSig1 se expresa solo en tejidos en donde existen cloroplastos maduros. La expresión diferencial de factores sigma responde a señales como el estado de desarrollo de la planta, tipo de tejido y factores ambientales como luz. Estos factores reconocen un promotor específico dependiendo de la señal que se encuentre presente. Adaptado de Lahiri S. D. y A. Allison, 2000.

Señales que participan en la diferenciación del cloroplasto.

La única señal necesaria conocida hasta el momento para el desarrollo del cloroplasto es la luz . Las plantas durante todo su ciclo de vida tienen la capacidad de percibir la calidad, cantidad, dirección y duración de la luz y usar esta información como una señal para modular su crecimiento y desarrollo.

Las plantas regulan la expresión de sus genes espacial y temporalmente por la integración de señales celulares originadas por la luz, con programas intrínsecos de desarrollo a través de una maquinaria molecular que incluye: a) fotoreceptores, los cuales perciben la luz de diferentes regiones del espectro solar, b) segundos mensajeros que amplifican la señal y c) efectores celulares que traducen la señal al núcleo y disparan una cascada de cambios en la expresión de genes. Cuando una planta es iluminada y emerge del suelo, ocurre un proceso llamado fotomorfogénesis que se caracteriza por la inhibición del crecimiento del hipocótilo, la expansión de los cotiledones, la formación de pigmentos y tricomas así como la diferenciación de hojas y cloroplastos. La expresión de los genes cloroplásticos codificados en el núcleo como CAB y RBSC se incrementa más de 100 veces después de la iluminación (Chory J. et al, 1989). La cantidad y la calidad de luz también regulan la síntesis, la composición y la estequiometría de los pigmentos y las proteínas de los centros de reacción fotosínteticos FSI y FSII. Los cambios mediados por la luz en la biogénesis de los fotosistemas ocurre a muchos niveles, incluyendo: la regulación de la trancripción, la traducción y la estabilidad de los mRNA de los genes fotosínteticos; el apilamiento y la ultraestructura del tilacoide (revisado en Gruissem W. y J. C. Tonkyn, 1993). En contraste, cuando la plántula está creciendo bajo el suelo o en la

obscuridad, presenta hipocotilos elongados, cotiledones cerrados formando un gancho apical, los cotiledones no contienen cloroplastos sino etioplastos y no existe expresión de genes fotosínteticos. A este tipo de desarrollo se le llama etiolación ó escotomorfogénesis.

Las plantas superiores tienen al menos tres sistemas de fotoreceptores que median su respuesta a luz: los fitocromos responsables para la detección de luz roja/roja lejana del espectro visible (longitud de onda entre 600-800nm) aunque también tienen la capacidad de detectar la luz azul/luz ultra violeta-B. En Arabidopsis existen 5 tipos de fitocromos denominados phy A a la E. Los criptocromos son otros de los fotoreceptores de plantas los cuales detectan luz azul/luz ultravioleta-A. A la fecha han sido descritos 3 diferentes criptocromos, CRY1, CRY2 y NPH1 (non-phototropic hypocotyl). Los criptocromos, o receptores de luz ultravioleta-B, aún no han sido caracterizados molecularmente (Batschauer A.,1998).

Las aproximaciones genéticas en Arabidopsis han sido muy fructíferas para la identificación de genes nucleares que participan en la traducción de la señal luminosa y que afectan la biogénesis del cloroplasto y la morfogénesis en general. Estas mutantes pueden clasificarse en dos amplias categorías; a) mutantes en fotoreceptores, la mayoría de estas mutantes tienen fenotipos de plantas que crecen en la obscuridad, aún cuando están creciendo en presencia de luz monocromática que normalmente captura el fotoreceptor silvestre y b) las mutantes en transducción de la señal, estas mutantes tienen fenotipos similares a plántulas que crecen en luz cuando están creciendo en la obscuridad (Chory J. et al, 1989). A las mutantes del primer grupo se les ha llamado *hy*; varias de estas mutantes tienen defectos en fotoreceptores específicos, solo *hy5* no es una mutante en un receptor (Batschauer A., 1998). El gen HY5 ha sido clonado y se conoce que codifica un factor de transcripción que activa

genes que responden a luz (Oyama T et al 1997) Las mutantes del segundo grupo incluyen a: *cop* (constitutive photomorphogenesis) y *det* (de-etiolada) (para revisión ver Deng X. W., 1994; Chory J. et al, 1996). Los loci COP/DET son idénticos al previamente identificado FUS (fusca). Las mutantes *fus* se caracterizan por la gran acumulación de antocianinas aún en la obscuridad (Kwok S.F. et al, 1996). COP/DET/FUS actúan como represores de la fotomorfogénesis y son inactivados por señales de luz percibidas por los fotorreceptores.

Organismos utilizados como modelos para el estudio del desarrollo del cloroplasto

Debido a la importancia que tiene la fotosíntesis, la mayoría del esfuerzo de las investigaciones concernientes al desarrollo del cloroplasto se ha orientado a la comprensión del metabolismo asimilatorio y biosintético de este organelo, especialmente a la fijación del CO_2. El aparato fotosíntetico constituye el principal componente protéico del cloroplasto; de los cientos de proteínas que participan en la fotosíntesis, menos del uno por ciento son codificadas por el genoma del cloroplasto, la mayoría de las proteínas son codificadas en el núcleo. Las proteínas que son codificadas en el núcleo participan en muchos procesos del cloroplasto que incluyen transcripción, procesamiento de RNA mensajero, traducción, ensamble e inserción de proteínas, síntesis de lípidos y aminoácidos, entre otros. Debido a que en el genoma del cloroplasto hay pocos marcos de lectura abierta de función desconocida, lo mas probable es que la mayoría de factores que participan en procesos regulatorios en respuesta a señales ambientales y de desarrollo, sean productos de genes nucleares.

Se han utilizado diferentes estrategias experimentales a lo largo de los años para estudiar el desarrollo del cloroplasto. Históricamente, las aproximaciones bioquímicas resultaron ser muy útiles para identificar los genes estructurales de la maquinaria básica de la

fotosíntesis. También el uso de mutantes en el análisis de la fotosíntesis es una herramienta bien establecida. Los tres organismos más utilizados genéticamente para este propósito son: el alga unicelular *Chlamydomonas reinhardtii,* la dicotiledónea *Arabidopsis thaliana* y la monocotiledónea *Zea mays.* Chlamydomonas es única entre estos organismos, ya que tanto el genoma nuclear como el cloroplástico pueden ser transformados en individuos no fotosintéticos y producir progenie. En maíz y Arabidopsis, la mayoría de las mutantes fotosintéticas tienen que ser propagadas como heterócigas, por lo que es difícil identificar segundas mutaciones que supriman el defecto fotosintético. Por otro lado, las plantas presentan frecuentemente genes múltiples con funciones redundantes, lo cual dificulta determinar la función de los genes específicos (Grossman A. R., 2000).

Aunque *Chlamydomonas* es en muchos sentidos un organismo experimental ideal, no es útil para elucidar mecanismos regulatorios en plantas que involucran comunicación entre diferentes tejidos. Entre las plantas terrestres, el maíz ofrece propiedades únicas que facilitan el análisis de fenotipos mutantes y la clonación molecular de los genes mutados. La clonación de genes por la inserción de transposones ha sido una técnica muy exitosa. Recientemente, con el proyecto de la base de datos de maíz ZmDB (*Zea mays* Data Base), están siendo desarrolladas nuevas estrategias genómicas utilizando técnicas de PCR y rescate de plásmidos para identificar mutaciones en genes específicos mutados por un transposon (Gai X. et al, 2000). Otra ventaja es que los estudios bioquímicos se facilitan en esta planta por el gran tamaño de sus semillas, lo cual permite a la plántula crecer hasta que ha alcanzado estadios de tres o cuatro hojas, tiempo suficiente para colectar material de hojas o raíz. Por otro lado, en maíz existe un gradiente de proplastidios en maduración y cloroplastos maduros, separados

espacialmente a lo largo de la hoja, lo cual puede ser muy útil para estudios de desarrollo del cloroplasto.

Actualmente Arabidopsis es una de los modelos más utilizados, debido a que tiene un ciclo de vida corto, es una planta pequeña que permite hacer búsqueda de mutantes entre miles de plantas sin que se requiera de mucho espacio, su genoma ha sido totalmente secuenciado y se han generado una gran cantidad de marcadores moleculares que permiten el mapeo de mutaciones y la clonación de genes por posición en un tiempo relativamente corto.

El maíz como un modelo de estudio

El maíz es el principal cultivo extensivo en México y representa una fuente importante de alimentación. En México, el consumo anual per cápita de tortillas es de 180 kg/año (~0.5 kg al día) y se ha estimado que en áreas rurales las tortillas proveen cerca del 70% de las calorías adquiridas durante la alimentación (Salvador R., 1997). Pero el maíz es importante en México no solo por razones económicas, biológicamente tiene un gran valor ya que México es su centro de dispersión. Los datos antropológicos y genéticos sugieren que el maíz se originó a través de la domesticación del teocintle (*Zea mays* ssp. *parviglumis* o spp *mexicana*), un pasto relacionado al maíz actual (Goodman M. N. y W. I. Brown, 1988). La diversidad genética mas amplia del maíz se encuentra representada en las variedades criollas de nuestro país (Doebley J. y R. L Wang, 1997).

El maíz es la principal monocotiledónea utilizada como modelo en investigación básica para estudiar procesos fisiológicos fundamentales de cereales, los cuales contribuyen a nivel mundial con un 70% de las calorías de la dieta humana. La mayoría de los cereales son

tratados como un solo sistema genético, debido a que en su genoma el orden de los genes se ha conservado. Si los cromosomas individuales se disectan en segmentos, y estos segmentos se re-arreglan, los cromosomas pueden ser alineados en bloques que se relacionan entre sí (Moore et al., 1995). Así, las preguntas que se resuelvan utilizando al maíz como modelo, darán mucha luz sobre mecanismos semejantes que ocurren en otros cereales.

Los transposones como una herramienta para el análisis de mutantes y la clonación de genes en maíz.

Una de las ventajas más importantes que tiene el maíz como modelo de estudio es la presencia de varios sistemas de elementos móviles o transposones en su genoma, lo cual permite generar un gran número de mutantes y la clonación de sus genes por abanderamiento del transposón. El uso de transposones para la clonación de genes en maíz es en la actualidad el único método práctico disponible. Las familias de transposones *Ac/Ds* y *MuDR/Mu* han sido las más utilizadas en experimentos de mutagénesis. En líneas en donde los elementos regulatorios (llamados también elementos autónomos) *Ac* o *MuDR* están transcripcionalmente activos, los elementos no autónomos de las familias, los cuales tienen sitios de unión para la transposasa en las terminales invertidas repetidas (TIRs), son movilizados para crear una nueva inserción (Walbot V. 1992; S. L Dellaporta, 1996) . Durante un experimento de abanderamiento, la actividad del transposón es convenientemenete monitoreada con un alelo reportero que tiene un fenotipo que es visible después de la excisión de el transposón. En la Figura 5 se ilustra la progresión de eventos durante la movilización de un transposón, en este caso se ha usado como ejemplo el sistema *MuDR/Mu*, pero este mismo modelo podría

A

MuDR

Mu

Gene fotosintético
C

La expresión normal de *C* produce un pigmento fotosintético

B

MuDR activa la transposición de *Mu*

Mu puede transponer en *C*

Función del gene *c* interrumpida por la inserción de *Mu*

C

MuDR activa la transposición de *Mu* fuera del gen *c* durante el desarrollo de la hoja.

Gene *c* mutado

Reversión de la mutación de *c* a C

Gene *C* normal

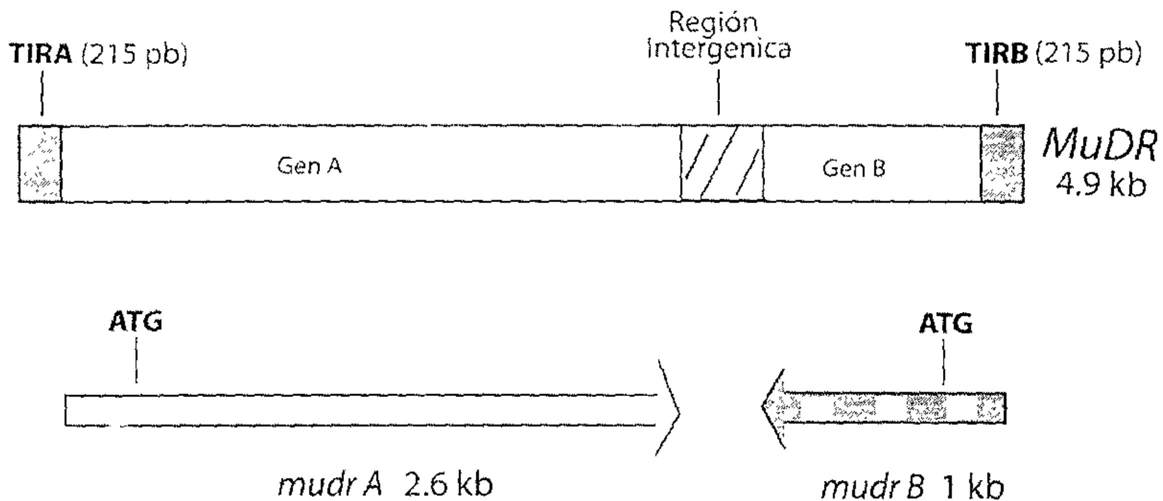
Sectores de reversión somática

Figura 5. Eventos que ocurren durante la movilización de un transposón.
A) El gen *C* codifica una enzima necesaria para la acumulación de clorofila en las hojas, cuando el gen C es activo las hojas son verdes. B) *MuDR* codifica una transposasa que activa la transposición de los elementos *Mu*. El gen *C* es inactivado cuando un elemento *Mu* se inserta dentro de el, lo cual origina un fenotipo mutado en donde las hojas no acumulan clorofila. C) Si durante el curso del desarrollo *Mu* transpone fuera del gen *c* la función del gen es restaurada y se observan sectores de reversión somática en donde hay acumulación de clorofila.

La familia de transposones *MuDR/Mu* tiene dos ventajas principales como sistema global de mutagénesis con respecto a *Ac/Ds*. a) *Mu* tiene un gran número de copias, por lo que su frecuencia de mutación es muy alta (10^{3} - 10^{5} por locus por generación). b) los elementos de esta familia se insertan preferencialmente en genes a través de todo el genoma. En cambio *Ac/Ds* tiene una baja frecuencia de mutación (10^{-6} por locus por generación) y estos elementos se insertan preferencialmente en sitios cercanos a la inserción original (Dellaporta S. L., 1996). Sin embargo la clonación de alelos mutados con *Mu* puede ser una tarea difícil, debido a que la mayoría de las líneas *Mu* contienen numerosos elementos y la identificación del gen mutado que está dando origen al fenotipo de interés, puede llevar varios años.

La familia de transposones *MuDR/Mu*

La familia *MuDR/Mu* también llamada Mutator es uno de los sistemas de transposones más diverso; está compuesta de cuando menos ocho elementos no autónomos *Mu1 - Mu8* cuya actividad es regulada por *MuDR*. Los diferentes elementos de esta familia comparten secuencias conservadas en las TIR de aproximadamente 200-215 pares de bases (pb). En las TIR se encuentra el sitio de unión de la transposasa (Benito M.-I. y V. Walbot, 1997). Las secuencias internas de estos elementos no están relacionadas a la secuencia interna de *MuDR*,



Intrones del gen A

Intrones del gen B

Figura 6. Estructura del elemento autónomo *MuDR*.

Con el objeto de estudiar la distribución de transposones Mutator en diferentes líneas de maíz actuales y tratar de averiguar el origen de estos transposones, el laboratorio de la doctora Virginia Walbot de la universidad de Stanford realizó una búsqueda en varias líneas de maíz de estos transpososones. Esta consistió en experimentos de hibridación tipo Southern utilizando como sonda al transposón *MuDR*. Se encontró que la única variedad natural que contenía un elemento relacionado a *MuDR*, pero con un menor número de copias, es la variedad mexicana Zapalote chico . Esta variedad es cultivada en el Istmo de Tehuantepec y se caracteriza por ser muy resistente a la sequía, las enfermedades y los vientos fuertes. Varias líneas (línea es usado como traducción de accessions) de Zapalote chico colectadas en el Istmo de Tehuantepec mostraron una elevada frecuencia de mutación siendo hasta 100 veces superior a la de otras veriedades de maíz aunque tres veces inferior a la de una línea *Mu*. Esto sugería fuertemente la presencia del elemento autónomo *MuDR* transcripcionalmente activo.

La presencia de este transposón en Zapalote chico representaba una ventaja para la selección de mutantes y la clonación de genes de interés en nuestro laboratorio por varias

razones: por un lado tiene un número de copias menor de elementos *Mu* y por otro lado Zapalote chico crece bien en México Ambas caracteristicas son muy importantes, ya que para la identificación del gen mutado se requiere de propagar la planta heteróciga cuando menos durante dos generaciones y las plantas *Mu* estándar crecen pobremente en algunas regiones de México en donde la intensidad de luz es muy alta.

La generación de mutantes fotosínteticas por la inserción de transposones es especialmente útil, debido a que el fenotipo es fácilmente visible y los sectores con reversión fenotípica por excisión del transposón son la prueba irrefutable de que la mutación es causada por la inserción de un transposón. En este trabajo exploramos la posibilidad de utilizarlos como herramienta para aislar mutantes en el desarrollo del cloroplasto

Objetivos del proyecto

El objetivo de este trabajo es identificar genes que participan en la biogénesis temprana del cloroplasto. Para ello se utilizaron como modelos maíz y Arabidopsis, debido a las diferentes ventajas que cada uno de ellos ofrece. Por un lado tratamos de establecer un sistema de clonación de genes en maíz utilizando como herramienta el transposón *MuDR* en Zapalote chico. Por lo que uno de los objetivos de esta parte del trabajo fue el análisis del transposón *MuDR* en Zapalote chico con el fín de poder establecer un sistema de clonación de genes en esta raza de maíz. En resumen, las dos preguntas que pretendíamos responder son: ¿existe en Zapalote chico el elemento *MuDR* activo?, ¿es posible desarrollar estrategias para clonar genes marcados con *Mu* en Zapalote chico? Si esto fuera posible, tendríamos una herramienta muy práctica para facilitar el desarrollo de la clonación de genes en una raza de maíz que crece bien en México.De ello se habla en el capítulo I de esta tesis. Por otro lado, en

Arabidopsis nos enfocamos a hacer un análisis genético de la biogénesis del cloroplasto a través de la caracterización de mutantes afectadas en el desarrollo temprano de este organelo; los resultados se presentan en el capítulo II.

Capítulo I

caracterización de *MuDR* en Zapalote chico

Existen varias características que definen a una línea Mutator estándar, entre ellas se encuentran; la presencia del elemento *MuDR* y elementos *Mu* no metilados. Ha sido demostrado que la metilación de estos elementos lleva a la pérdida de la actividad Mutator (Revisado en Walbot V. y G. N. Rudenko, 2001). La expresión abundante de los transcritos *mudrA* y *mudrB* es otra de tales características, los elementos autónomos pueden perder espontáneamente su actividad durante el desarrollo, un proceso que es acompañado por metilación llevando al silenciamiento transcripcional del elemento (Martienssen R., A. y A. Baron 1994). Una de las características que hacen de las líneas Mutator un sistema eficiente para la generación de mutantes y el abanderamiento de genes es su alta frecuencia de transposición (20 a 100 veces por arriba de las mutaciones espontáneas o de las mutaciones causadas por *Ac* y *Spm*) resultado del alto número de copias de elementos *Mu*. *MuDR/Mu* presentan transposición duplicativa en células pre-meióticas y células gametofíticas, las excisiones de elementos *Mu* ocurren sólo durante las últimas divisiones celulares de tejidos somáticos y se observan como pequeños sectores en donde se ha restaurado el fenotipo en anteras, aleurona y hojas. (Levy A. A. y V. Walbot, 1990). Nosotros examinamos cada una de estas características en Zapalote chico para determinar si estas se conservan y si es posible utilizar estos elementos para la selección de mutantes y el aislamiento de los genes mutados. A continuación se presentan los resultados.

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Transcriptionally Active *MuDR*, the Regulatory Element of the Mutator Transposable Element Family of *Zea mays*, Is Present in Some Accessions of the Mexican land race Zapalote chico

María de la Luz Gutiérrez-Nava,* Christine A. Warren,†,1 Patricia León* and Virginia Walbot†

**Instituto de Biotecnología, Universidad Nacional Autonoma de México, Apdo. 510-3, Cuernavaca, Morelos 62271, México and †Department of Biological Sciences, Stanford University, Stanford, California 94305-5020*

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ABSTRACT

To date, mobile *Mu* transposons and their autonomous regulator *MuDR* have been found only in the two known Mutator lines of maize and their immediate descendants. To gain insight into the origin, organization, and regulation of *Mutator* elements, we surveyed exotic maize and related species for cross-hybridization to *MuDR*. Some accessions of the mexican land race Zapalote chico contain one to several copies of full-length, unmethylated, and transcriptionally active *MuDR*-like elements plus non-autonomous *Mu* elements. The sequenced 5.0-kb *MuDR-Zc* element is 94.6% identical to *MuDR*, with only 20 amino acid changes in the 93-kD predicted protein encoded by *mudrA* and ten amino acid changes in the 23-kD predicted protein of *mudrB*. The terminal inverted repeat (TIR) A of *MuDR-Zc* is identical to standard *MuDR*; TIRB is 11.2% divergent from TIRA. In Zapalote chico, *mudrA* transcripts are very rare, while *mudrB* transcripts are as abundant as in Mutator lines with a few copies of *MuDR*. Zapalote chico lines with *MuDR*-like elements can *trans*-activate reporter alleles in inactive Mutator backgrounds; they match the characteristic increased forward mutation frequency of standard Mutator lines, but only after outcrossing to another line. Zapalote chico accessions that lack *MuDR*-like elements and the single copy *MuDR a1-mum2* line produce few mutations. New mutants recovered from Zapalote chico are somatically stable.

MOLECULAR, genetic, and anthropological data indicate that maize arose in what is now Mexico through domestication of teosinte, a grass closely related to present-day maize (Goodman and Brown 1988). Domestication is proposed to have occurred once, not more than 10,000 years ago (Doebley 1990). Because this is such a recent event on an evolutionary time scale, corn should have a very homogeneous genome. However, both the allelic diversity (Shattuck-Eidens *et al.* 1990) and the range of genome size (from 9.82 to 12.12 pg; Rayburn *et al.* 1985) are among the highest for any eukaryotic species. In the short time span since domestication, transposable elements and selection for growth in many habitats are proposed to have played important roles in first generating and then maintaining this diversity (Schwarz-Sommer *et al.* 1985; Kloeckener-Gruissem and Freeling 1995; Walbot 1996).

Characteristically, active transposable elements are found in a few populations within a species, and both the origin and maintenance of transposable elements remain enigmas (Capy *et al.* 1994). Within a lineage, transposons are transmitted in predictable, albeit non-Mendelian, patterns and often exhibit mechanisms to increase copy number. In addition, analysis of *P* and *Mariner* elements in *Drosophila* spp. and insertion sequences in bacteria implicate horizontal transmission as a possible explanation of the punctate distribution of active transposons, at least for some cases (Capy *et al.* 1994).

The Mutator transposons of maize are one of the most active transposable element families described in any organism. Standard Mutator activity is defined by a suite of characters: high forward mutation frequency (10^{-3} to 10^{-5} per gene per generation), frequent somatic excision late in development, and infrequent germinal excision ($<10^{-4}$ to 10^{-5} per gene per generation) (reviewed in Walbot 1992). These features have only been observed in plants derived from a single line of maize, first described as Mutator by Robertson (1978). These standard Mutator lines have multiple copies of a diverse family of transposable elements. *Mu* elements share ~210-bp Terminal Inverted Repeats (TIRs) and create 9-bp host sequence duplications at the site of insertion. The *Mu* transposon family is composed of at least six distinct subfamilies that are distinguished by unique internal sequences between the TIRs (reviewed in Bennetzen 1996). In standard Mutator lines, the 4942-bp *MuDR* regulatory elements are present in 5–50 copies (Hershberger *et al.* 1991), and non-autonomous *Mu* element copy numbers are even higher (Alleman and Freeling 1986; Taylor and Walbot 1987; reviewed in

Corresponding author: Virginia Walbot, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020.
E-mail: walbot@leland.stanford.edu

[1] *Present address:* Howard Hughes Medical Institute, Stanford University Medical School, Stanford, CA 94305-5428.

WALBOT 1991). All of the *Mu* elements exhibit non-Mendelian inheritance, with copy number maintained on outcrossing by replicative transposition late in the sporophytic or during the gametophytic phase of the life cycle (reviewed in WALBOT 1991; BENNETZEN 1996). Collectively, the multi copy *Mu* elements increase the mutation frequency 20-100-fold or more above the spontaneous level (reviewed in WALBOT 1992; BENNETZEN *et al.* 1993). In these standard Mutator lines, loss of Mutator activity is an epigenetic phenomenon rather than the result of segregation of *MuDR*; loss of activity is correlated with increased methylation of the regulatory *MuDR* and non-autonomous *Mu* elements (reviewed in CHANDLER and HARDEMAN 1992; BENNETZEN 1996).

A few exceptional Mutator lines contain only a single *MuDR* that segregates as a near-Mendelian factor; these lines were derived from standard, high copy number Robertson lines with the *a1-mum2* or *a1-mum3* reporter alleles (ROBERTSON and STINARD 1989, 1992; CHOMET *et al.* 1991; LISCH *et al.* 1995). In the most thoroughly analyzed example, the *MuDR* element is located on chromosome 2L (ROBERTSON and STINARD 1992; LISCH *et al.* 1995). At this location, it programs the standard pattern of high frequency somatic excision. However, few element insertions occur and both *MuDR* and *Mu* element copy numbers typically remain low (LISCH *et al.* 1995). Independently, SCHNABLE and PETERSON (1986, 1988, 1989a,b) described *Cy*/*r-cy*, a two-element transposable element system that has turned out to be a low activity Mutator line; *Cy* lines often contain a single, segregating regulatory element, now known to be a *MuDR* (HSIA and SCHNABLE 1996). The sequence of *MuDR* (HERSHBERGER *et al.* 1991) is identical to *Cy* (HSIA and SCHNABLE 1996) and, with the exception of a single, inconsequential base change, identical to the single *MuDR* in the *a1-mum2* lines (JAMES *et al.* 1993). Consequently, the differences between high- and low-activity Mutator lines cannot be explained by differences in the primary sequence of *MuDR*.

Based on Southern hybridization, all *Zea* species tested contain multiple, dispersed sequences homologous to segments of *Mu* elements; some of these widely shared sequences appear to be parts of genes that have become incorporated into a *Mu* element (TALBERT *et al.* 1989). By Southern hybridization, TIR-homologous sequences are not found beyond the genus Zea and the maize X Tripsacum hybrid species *Tripsacum andersonii* (TALBERT *et al.* 1990). However, genomic clones with sequences similar to *Mu* TIRs and limited regions of similarity to *MuDR* have been reported in rice (EISEN *et al.* 1994; ISHIKAWA *et al.* 1994). Within maize, *MuDR* is not widely distributed (HERSHBERGER *et al.* 1991), nor is Mutator activity. The largest survey to date tested maize lines for a *Cy* capable of activating somatic instability of *bz1-rcy*. SCHNABLE and PETERSON (1986) found that active *Cy* elements were nearly restricted to the original *Cy* line and Robertson's Mutator lines. Weak *Cy* activity was found sporadically in a few plants in 7 of 47 other lines surveyed, but this rare occurrence could represent activation of cryptic regulatory elements from the *Cy* parent; this possibility could not be tested as *MuDR* had not yet been cloned. Because standard Mutator activity creates so many mutations, it is not surprising that *MuDR* is apparently missing from most strains of corn.

We are interested in whether *MuDR* can be maintained in the maize genome. To address this question, we surveyed for *MuDR* in American inbreds, exotic maize lines, and *Zea* spp. by Southern blot hybridization. While nearly all lines had cross-hybridizing fragments, only the Mexican land race Zapalote chico had a multicopy cross-hybridizing band of approximately the correct size. Analysis of different accessions of Zapalote chico demonstrated that only a subset of the population contains *MuDR*-like elements; only the Zapalote chico lines with *MuDR*-like elements exhibited a high forward frequency.

The sequenced example of a *MuDR*-like element of Zapalote chico (*MuDR-Zc*) is highly similar to *MuDR* and encodes similar transcripts. Unlike standard Mutator lines, which generate new mutants during selfing and outcrossing, Zapalote chico exhibits hybrid dysgenesis. Self-pollinated lines produce few mutations, but outcrosses to non-Mutator lines activate a high forward mutation frequency. In addition, new mutants are somatically stable, at least in seedlings. Zapalote chico is cultivated by the Zapotecs, a Native American people of Oaxaca, Mexico. This line is their economic staple and by their oral history has been cultivated for more than 5000 years, tracing to their cultural origin in the highlands of Central Oaxaca. We discuss the possibility that selection for a high-yielding stable crop has resulted in the novel properties of the Mutator system in Zapalote chico.

MATERIALS AND METHODS

Mutator terminology: *MuDR* is the regulatory Mutator element, and this name replaces prior nomenclature: *MuA2* (QIN *et al.* 1991), *MuR1* (CHOMET *et al.* 1991), *Mu9* (HERSHBERGER *et al.* 1991), and *Cy* (HSIA and SCHNABLE 1996). *Mu* elements share ~200-bp TIRs with *MuDR* and require transcriptionally active *MuDR* to be mobilized.

Plant material: Active Mutator individuals with the *bz2-mu4::MuDR* allele (family M87) were used as the Mutator stock for DNA and RNA analysis; this is a multicopy, standard Mutator line with a *MuDR* transposon inserted in the second exon of the *bz2* gene (HERSHBERGER *et al.* 1991). The *bz2* tester line in inbred W23 was used as the non-Mutator stock for most molecular analyses and for the mutagenesis tests; an *a2* tester constructed in W23 by this lab, and an *a2* tester obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL) in a mixed nuclear background, were used for the Southern blot survey. For the original survey, all of the exotic lines of maize and the *Zea* spp. (listed in 1982 inventory as *Zea mexicana* luxurians, mexicana, nobogame, parviglumis, and peruviensis, *Z. perennis*, and *Z. diploperennis*) were obtained from the Stock Center. The *Zea* spp. collections now reside at the USDA Plant Introduction Station (Ames, IA). For subse-

quent experiments, existing Zapalote chico accessions were obtained from Pioneer Hi-Bred (Johnston, IA) and an overlapping set from CIMMYT (International Maize and Wheat Improvement Center, Texcoco, Mexico). Both RONALD PHILLIPS and RICHARD KOWLES provided several generations of crosses between Zapalote chico (cytogenetically many knobs) and Wilbur's Knobless Flint. Thirty-five new accessions were collected as individual ears in Juchitán, (Oaxaca, Mexico) (16.15N, 95.00W) directly from Zapotec farmers; two Tuxpeño X Zapalote chico F_1 hybrids and the F_2 backcross ears were donated by M. C. ARREDONDO, a retired Mexican corn breeder living near Juchitán. Tuxpeño is a widely adapted inbred line developed in Mexico and used as the foundation for breeding experiments.

Forward mutation test: Individuals were self-pollinated to assess the phenotypes of any pre-existing mutations and crossed as pollen to the *bz2* tester. The outcross seed were planted, and the F_1 plants were self-pollinated, yielding F_2 ears. Thirty progeny kernels of the F_2 and selfed parental ears were planted side-by-side in the summer field; mutants were counted in the F_2 only if they were clearly distinguishable from any segregating phenotype in the parent. All novel phenotypes recorded appeared to be recessive, present in ~one-quarter of the progeny. In ambiguous cases, *i.e.*, in which there was low germination or only one or a few mutant plants were present, a second sample of 30 kernels was planted and evaluated. Somatic mutability was scored by eye and by observation through a stereozoom microscope (×20).

Ten kernels of each Zapalote chico accession were grown in summer, 1993 (M designations), at Stanford. Seed were planted in late June to promote flowering, because maturation of neotropical maize is inhibited by long days in the temperate zone. In most accessions, only a few individuals reached maturity within 75–90 days and could be both self-pollinated and crossed as pollen parent to *bz2* tester. In the 1994 winter nursery in Molokai, Hawaii, Zapalote chico lines matured within 50–55 days, and additional representatives of some lines were self-pollinated and crossed to *bz2* tester. Of all the Zapalote chico samples examined, one line M59 = N234 was the most consistent in flowering at Stanford, and additional individuals of the original accession were tested for forward mutations during 1994–1995. To assess spontaneous mutation frequency in a non-Mutator line, the crossing scheme was used with the *bz2* tester. Four standard Mutator lines were used for comparison; two lines were selfed and crossed to *bz2* in 1993 (M88, *bz2-mu2::Mu1* reporter allele; M121, *bz2-mu1::Mu1* reporter allele), and two lines in 1994 (N190, *bz1-mu1::Mu1* early somatic excision line; N285, Mutator with *Bz1*-revertant alleles from the early excision of the *bz1-mu1::Mu1* reporter allele). Two *a1-mum2::Mu1*, single *MuDR* lines were obtained from D. ROBERTSON and evaluated in 1994 to compare the low *MuDR* copy number lines to Zapalote chico.

RNA blot analysis: Immature ears were collected from field-grown material of each line during the summer of 1994. Tissue was immediately frozen in liquid nitrogen and stored at −80° until RNA isolation. RNA was isolated by grinding the samples in liquid nitrogen, then extracting with Tri-Reagent (Molecular Research Center, Cincinnati, OH). Poly(A)$^+$ RNA was purified from total RNA using a Mini-oligo(dT) cellulose spin column kit (5 Prime→3 Prime, Boulder, CO).

For the RNA blots, 16–20 μg of poly(A)$^+$ RNA was electrophoresed through an agarose formaldehyde gel for 6 hr and transferred to Hybond-N (Amersham, Arlington Heights, IL) using standard techniques (SAMBROOK *et al.* 1989). Two probes were generated by PCR amplification from p*MuDR*. This plasmid was constructed and sequenced by R. J. HERSHBERGER; it contains a full-length *MuDR* element, recovered from the *bz2-mu4::MuDR* allele (HERSHBERGER *et al.* 1991), with a one base frameshift mutation in *mudrA* that allows maintenance in *Escherichia coli*. Probe PA contains 927 nucleotides of *mudrA* (positions 183–1100), and PB contains 978 nucleotides of *mudrB* (positions 3774–4752). A third probe, BX1.0 (HERSHBERGER *et al.* 1995), was recovered from a *Bam*HI (nucleotide position 2865) to *Xba*I (nucleotide position 3945) digest of p*MuDR*; this probe recognizes both *mudrA* and *mudrB* (Figure 1). Probes were labeled by the random primer method, using the DECAprime II Kit from Ambion, Inc. (Austin, TX) (FEINBERG and VOGELSTEIN 1983) and purified on push columns (Stratagene, La Jolla, CA). Prehybridization and hybridization were performed according to the protocol published for GeneScreen (Du Pont, Wilmington, DE) using 10% dextran sulfate. Filters were washed once in 2× SSPE, 1% SDS at room temperature for 10 min, once in 1× SSPE, 1% SDS at 65° for 15 min, and once in 0.1× SSPE, 0.1% SDS at 65° for 15 min. Autoradiography was performed for 12–72 hr at −80° using two intensifying screens.

DNA blot analysis: Maize genomic DNA was prepared from immature ears of selfed Zapalote chico accessions grown in 1994 (N designations in Tables) and purified as described by STAPLETON and WALBOT (1994). For Southern analysis, three μg of DNA were digested with restriction enzymes (BRL, Gaithersburg, MD) according to the manufacturer's instructions, electrophoresed through agarose gels, and blotted onto Hybond-N (Amersham). Probes were prepared as described above. The blots were prehybridized, hybridized, and washed as recommended by the membrane manufacturer. To quantify *MuDR* copy number, a plasmid containing *MuDR* was digested with *Sst*I or *Sst*I/*Dra*I, diluted to the proper concentration equivalent to a specific copy number in the maize genome, and electrophoresed next to restriction digests of maize genomic DNA. Blots were probed with BX1.0. In some cases, stripped blots were reprobed with a 380-bp fragment of *Adh1* as a loading control. To check for the presence of *Mu1* and the related *Mu2* elements, probe pA/B5 was used (TAYLOR and WALBOT 1987).

DNA amplification by polymerase chain reactions (PCR): DNA amplification reactions were performed in volumes of 25–100 μl overlaid with 50–100 μl paraffin oil. Each reaction contained 0.2 mM of each of the four deoxyribonucleotides, 100 ng of each oligonucleotide primer, a buffer (15 mM Tris pH 8.3, 50 mM KCl, 1% Gelatin, 1.8 mM $MgCl_2$), Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 50–100 ng of DNA. PCR reactions were carried out for 30 cycles of 1 min at 94°, followed by 1 min at 55°, and 1 min at 72°. The following DNA primers were used for *mudrA*: primer #183 5′-CGCCGTCTGGCAGGGCCTCTTGTCACCGTCTC-3′ with primer #1996 5′-GAATGTCATAGGTTGCATAG-3′ or primer #2017 5′-GATACGTTGGATACTGTAAG-3′ with primer #2282 5′-TATGGATGTAGAGACCTTAG-3′. For the *mudrA*/intergenic region primer #2281 5′-GATTCCAGAGATGTAGGTAT-3′ was used with primer #813 5′-CCAACCAAAGTAAGACCACA-3′. For the intergenic region to *mudrB* region, primer #2019 5′-GCCATTAGTTCTTACAACCT-3′ was used with primer #2109 5′-ACAATACGCGTTAACCAAACA-3′. To amplify *mudrB* primer #3773 5′-CTTGTACAGATCTTGTGACCAGTCGCA-3′ was used with primer #4752 5′-GTCCACAAATCGATGTTACGGTCGTT-3′. For TIRA primer #2466 5′-GCTGAGCCTCCTGCAGGGAGATAATTGCC-3′ was used with primer #2467 5′-CCATGGTACCAAAATCAGAG-3′. The resulting fragment contains all of TIRA, plus the region of the 5′ untranslated region that contains a transcriptional start site. To amplify TIRB, primer #2468 5′-TGAACGCCCTCCTGCAGGAGAGATAATTGC-3′ was used with primer #2470 5′-CAATCGGTACCCACAGGAGCAAGAG-3′. The resulting fragment contains TIRB plus the 5′ untranslated region of *mudrB*.

Plasmids: Seven plasmids were constructed by amplifying genomic DNA of Zapalote chico line N215 by PCR with the

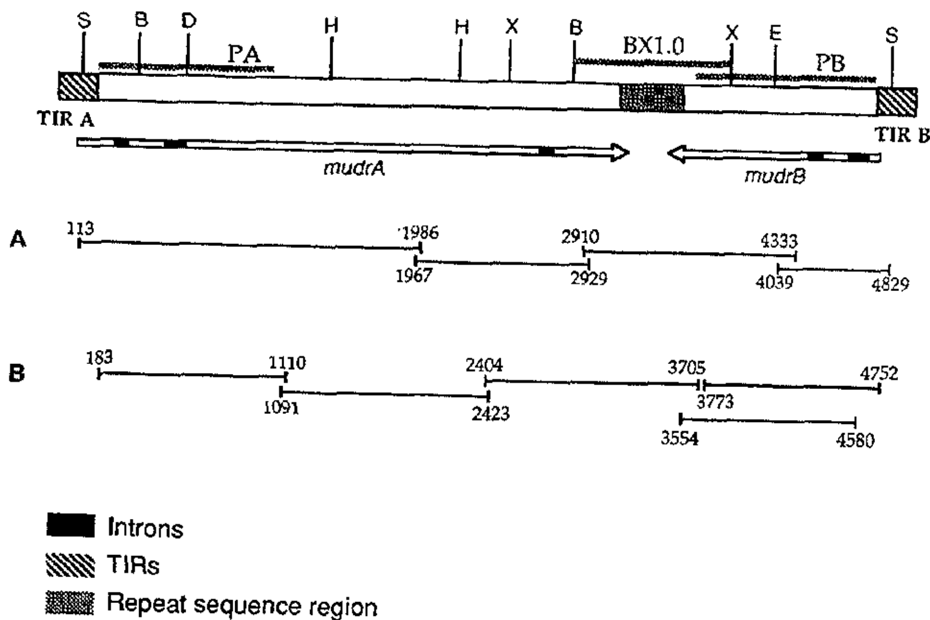


FIGURE 1.—Diagram of *MuDR*. DNA probes (PA, PB, BX1.0) used in Southern and Northern hybridizations are shown above the transposon structure. Below the transposon, the *mudrA* and *mudrB* convergent transcription units are illustrated; the first intron of each transcript is spliced ~100%, the second introns are spliced ~80%, as is the third intron of *mudrA*. A 120-bp in-frame intron of *mudrB* that is spliced in about 5% of transcripts of *MuDR* is not shown. (A) Regions of *MuDR* for which PCR amplification was attempted in various accessions of Zapalote chico. (B) PCR fragments generated in the cloning of the *MuDR*-like element. Nucleotide sequence numbering according to HERSHBERGER *et al.* (1991). B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; X, *Xba*I.

primers listed above. Amplified fragments were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The *MuDR-zc* element was cloned in overlapping fragments, because the full-length *MuDR* is toxic to *E. coli*. Bracketed numbers to the right of each plasmid correspond to the base pairs of the standard *MuDR* sequence (numbering according to HERSHBERGER *et al.* 1991). Plasmid pTIRAzc [1–455] has a 455-bp insert; pA1zch [183–1110] has a 927-bp insert; pA2zch [1091–2423] has a 1332-bp insert; pA3zch [2404–3705] has a 1301-bp insert; pA4zch [3554–4580] has a 1026-bp insert; pB1zch [3773–4752] has a 979-bp insert; and pTIRBzc [4476–4944] has a 468-bp insert.

DNA sequencing: *MuDR-Zc* regions were obtained as restriction fragments from the pAzch plasmid series and were subcloned into the M13mp19 vector for single-stranded sequencing (NORRANDER *et al.* 1983) with the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) or with the ABI 310 fluorometric automated sequencer. Both strands of all fragments were fully sequenced. To eliminate compression of bands that occurred when sequencing GC-rich regions, mixes containing deoxyinosine provided with the kit were used. Primers for sequencing were commercially available M13 primers; a few custom internal primers were used on long fragments. The *MuDR-Zc* sequence is registered in GenBank as accession number U75360.

RESULTS

Properties of *MuDR*: *MuDR* encodes two, convergently transcribed genes (Figure 1). The major transcription initiation sites are in the TIRs, and the most abundant transcripts are 2.8 kb (*mudrA*) and 1.0 kb (*mudrB*). Intron skipping, multiple polyadenylation sites, and a second transcription initiation site in the 5′UTR of *mudrA* result in four distinct transcript types for each gene (HERSHBERGER *et al.* 1995). Although the exact roles of the proteins encoded by *mudrA* and *mudrB* are unknown, *mudrA* encodes a polypeptide with homology, over an extended motif of ~150 amino acids, to a suite of bacterial transposons (EISEN *et al.* 1994). In addition, it has recently been demonstrated that *mudrA* encodes a DNA-binding protein which binds to specific sequences within the highly conserved *Mu* TIR, leading to the proposal that MURA is a transposase (BENITO and WALBOT 1997). Deletions within *mudrA* in lines with a single *MuDR* abolish somatic instability of the *a1-mum2* reporter allele; this evidence demonstrates that *mudrA* is essential for somatic excision (HSIA and SCHNABLE 1996; LISCH and FREELING 1994).

Distribution of *MuDR* elements: Previously, we reported that the 4.7-kb *Sst*I fragment characteristic of an intact, unmethylated *MuDR* element (Figure 1) was multicopy in Mutator lines and was not present in standard inbreds of maize (HERSHBERGER *et al.* 1991). Most non-Mutator lines did contain various sized fragments that hybridized to one or more internal *MuDR* probes, but there was no evidence for intact *MuDR* elements. To expand the analysis of distribution of *MuDR*-like elements, genomic

Southern blotting was used to screen additional inbreds, exotic lines, and *Zea* spp. for intact *MuDR* elements. Genomic DNA was digested with *Sst*I, which recognizes sites in unmethylated TIRs of *MuDR*, a Southern blot was prepared, and then hybridized with the BX1.0 fragment, which contains the 3′ portions of both *MudrA* and *MudrB* and the intergenic region (Figure 1).

Of the lines examined by this Southern blot survey, a Co-op accession of Zapalote chico had a fragment about the size expected for *MuDR* (~5.1 kb). This fragment was slightly larger than *MuDR* and was present in ~3–5 copies per genome (data not shown).

All of the other exotic lines examined, including Argentine popcorn, Tama flint, Strawberry popcorn, Papago flint, gourd seed, Northern flint, *Z. perennis*, *Z. diploperennis*, and five teosinte types (see MATERIALS AND METHODS), hybridized weakly to the central *MuDR* probe. Similar cross-hybridization has been found in some (W23, K55, and A188) standard maize inbred lines (data not shown).

Screening for *MuDR* in Zapalote chico lines by PCR and Southern analysis: The first Zapalote chico sample examined was collected in the 1950s from Oaxaca Mexico; it has been maintained by the Maize Genetics Stock Center, by periodically growing and selfing the line. Using eight sets of PCR primers that spanned most of *MuDR*, we determined that all regions of this putative regulatory element in Zapalote chico could be amplified from an immature ear DNA sample of one individual. Furthermore, seven of the fragments were the expected size, and each of these contained one or two restriction sites at the same positions as in *MuDR*. There were no polymorphisms for the 12 enzyme sites examined (data not shown). With the primer pair that spanned the intergenic region, however, several size variants were detected, ranging from 100 to 300 bp larger than the comparable region of *MuDR* (data not shown). As the intergenic region is composed of a complex set of repetitive elements (HERSHBERGER *et al.* 1995), we hypothesized that there had been an expansion of these motifs. Collectively, the results suggested that Zapalote chico contained elements that were very similar to *MuDR*.

To assess the distribution of *MuDR*-like elements in Zapalote chico populations, existing accessions were obtained from three other sources: seven examples of old accessions were obtained from CIMMYT, and a mostly overlapping set was obtained from Pioneer Hi-Bred. The CIMMYT materials were collected in the 1950s and 1960s, but then maintained under different growth conditions in central Mexico and in Iowa. RONALD PHILLIPS and RICHARD KOWLES contributed Zapalote chico X Wilbur's Knobless Flint hybrids, derived from a CIMMYT accession. Zapalote chico is classified as a land race, but it is also an economically important line. It is the only corn variety grown by the 300,000 Zapotecs living in southwestern Mexico. To obtain a current representation of Zapalote chico, 35 new accessions were collected in 1993 from farmers and from a corn-breeding program located in the main Zapotec population center, near Juchitán in the state of Oaxaca, Mexico. Most of these lines were successfully propagated at Stanford University in summer 1993. We conducted a more extensive investigation of the distribution of *MuDR* elements using Southern blot analysis and PCR experiments of genomic DNA samples from accessions that could be self-pollinated.

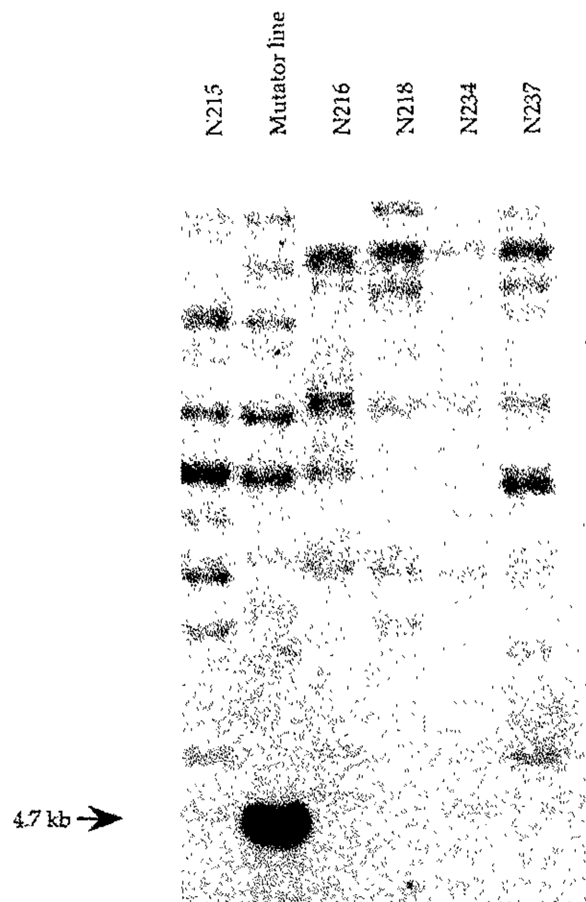


FIGURE 2.—Screening for *MuDR* in Zapalote chico lines by Southern analysis. 6–8 μg DNA samples were digested with *Sst*I and probed with BX1.0.

For Southern blot analysis, genomic DNA samples most likely to contain full-length elements (based on PCR screening; see Table 1 below) were digested with *Sst*I and probed with BX1.0. Figure 2 shows the ~4.7, kb *Sst*I fragment characteristic of an intact *MuDR* element was conserved in lines N215, N234, and N237, although the Zapalote chico hybridizing bands were always slightly larger (~50–100 bp) than *MuDR* from a standard Mutator line. Zapalote chico line N216 contained a fragment that is ~250 bp larger than *MuDR*; it may be similar to the larger *MuDR*-like element originally identified in the Maize Stock Center material.

TABLE 1

PCR survey for *MuDR* sequence in different accessions of *Zapalote chico*

Accessions	Primer sequence numbers			
	113–1986[a]	1967–2929	2910–4333	4039–4829
N200[c]	+[b]	–	–	+
Oax 50[c]	+	+	+	+
N201[c]	+	+	+	+
Oax 48[c]	+	+	–	+
N204[c]	–	–	–	+
N205[c]	–	–	–	–
N206[c]	–	–	–	–
Chis 224[c]	+	+	+	+
N207[c]	+	+	–	+
N211[d]	+	+	–	–
N213[d]	–	+	+	+
N214[d]	–	–	+	–
N215[d]	+	+	+	+
N216[d]	+	+	+	+
N217[d]	–	+	+	–
N219[d]	+	+	–	+
N220[d]	–	+	–	–
N221[d]	–	+	–	–
N222[d]	+	–	+	–
N226[d]	+	–	+	–
N230[d]	–	–	–	–
N234[d]	+	+	+	+
N236[e]	–	+	–	–
N237[e]	+	+	+	+
N240[f]	+	–	+	–
N241[g]	–	+	+	–
N249[g]	–	–	–	–
N252[h]	–	+	–	+
N255[d]	+	+	–	–
N257[d]	–	–	–	–
N264[c]	+	–	–	+
N267[c]	–	+	+	–
M4-1[c]	+	+	+	+

[a] Region amplified by PCR. Nucleotide numbering of *MuDR* according to HERSHBERGER *et al.* (1991).
[b] Plus symbol indicates amplification of this region by PCR.
[c] Accession obtained from CYMMIT and grown at Stanford.
[d] Accession collected from farmers in Oaxaca, Mexico.
[e] Accession was a cross of Tuxpeño with Zapalote chico.
[f] Accession obtained from Maize Stock Center.
[g] Accession obtained from R. PHILLIPS.
[h] Accession obtained from R. KOWLES; F_1 hybrid of Zapalote chico and Wilbur's knobless flint.

To extend our analysis to additional lines, PCR experiments were carried out on samples from individual selfed progeny, using four sets of primer pairs that span *MuDR* (Figure 1). As shown in Table 1, eight samples (24%) yielded PCR products of the expected size with all four primer pairs; these samples represent seven distinct accessions, with the Oaxaca 50 accession represented from two distinct sources (N201 and Oaxaca 50 directly from CIMMYT). The majority (6/8) of the accessions positive for *MuDR*-like elements represented the most recently collected material, indicating that *MuDR*-like elements exist in the current Zapotec crops. Twenty accessions (61%) yielded products from a subset of the primer pairs, and five accessions (15%) did not seem to contain any amplifiable fragments. In this analysis, the particular individual sampled from the Maize Stock Center lineage (N240) gave a positive PCR result with only two primer pairs, suggesting that this individual did not contain an intact *MuDR* element. Also, two of the CIMMYT lines, Oaxaca 48 and Chiapas 224, yielded different PCR products in the two versions sampled. We conclude that there is heterogeneity within some accessions, reflecting either heterogeneity in the original material or changes during propagation at stock centers.

For a more detailed analysis of these *MuDR*-like elements, the PCR fragments generated from lines N234

and N215 were digested with enzymes for restriction sites present in the transcribed region of authentic *MuDR* elements. All ten of these enzymes produced fragments of identical size in digests from active Mutator lines and both N234 and N215 Zapalote chico accessions (data not shown). These data suggest that the differences in length between Zapalote chico *MuDR*-like elements and *MuDR* will be found in the TIRs and/or in the intergenic region.

Inheritance of the *MuDR*-like element: To examine the propagation of *MuDR*-like elements through outcrosses with non-Mutator lines, Southern blots were perfomed in two lineages: (N234 = P56) and CIMMYT accession Oaxaca 2 (N264 and N265). The founder individual (M59) of the N234 lineage appears to have one copy of a *MuDR*-like sequence per haploid genome; it is 100 bp larger than *MuDR*. The M59 founder was crossed to *bz2* tester, a non-Mutator source, and the progeny (individuals of family P56) contain ~1 copy of *MuDR* (Figure 3A, and as discussed below a PCR survey of 28 P56 individuals were all positive for *MuDR*). All P56 individuals could contain a single copy of *MuDR* if the founder had been homozygous for one *MuDR* locus. When individuals of P56 were outcrossed a second time to *bz2* tester, the copy number of ~1 is maintained (compare parent P56-12 and outcross progeny, lanes 1 and 3; parent P56-17 and outcross progeny, lanes 2 and 4; Figure 3A). Figure 3B provides more evidence for transmission of the element through two outcrosses. M3-4 (lane 1) contains the *MuDR*-like element, and this element is maintained when outcrossed to *bz2* tester (MH3, lane 2) and when MH3 was selfed to produce line N265 (lane 3). Siblings of line N265 are shown to contain the element (O70, O70-1, and O70-4, lanes 4–6), which is again maintained on selfing (OH59, P61, lanes 7 and 9), as well as after a second outcross (O70-4 × *bz2*, lane 8).

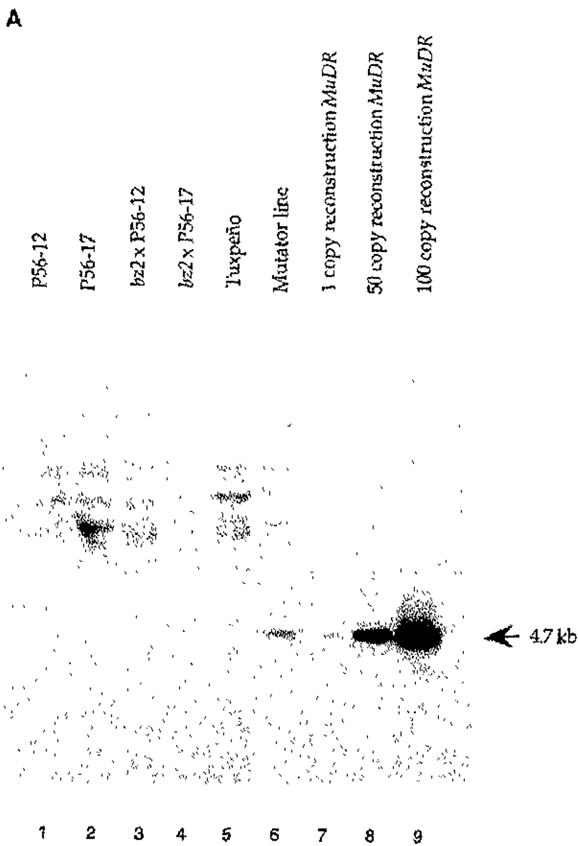
Figure 3C shows a lineage of progeny of M3-1 (a sibling of M3-4), which was outcrossed once, repeatedly selfed, and outcrossed once more. Selfed progeny of M3-1 do not show the *MuDR*-like element (lane 1). MH2 (lane 2), selfed F_1 progeny from an M3-1 outcross to *bz2* tester, do not show the element. Selfing of MH2 progeny produced line N264-1 (lane 3), which also lacks the *MuDR*-like element. However, after selfing of N264-1, bands of the correct *MuDR*-like element size appear in the siblings O69-5 and O59-9 (lanes 4 and 5) and persist in the F_1 of an outcross of O69-9 to *bz2* tester (lane 6). These results demonstrate that cryptic, presumably methylated copies of *MuDR*-like elements exist in Zapalote chico accessions, and that these elements can appear during a crossing program.

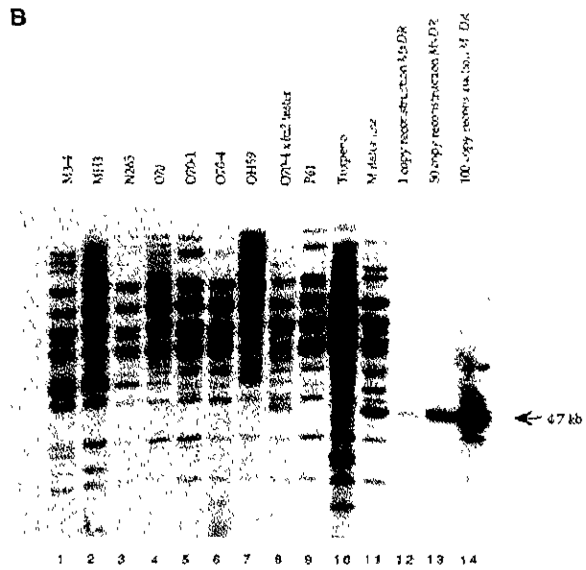
Larger hybridizing bands are present in all of the above Southerns, including Tuxpeño, a non-Mutator line. The relationship of these larger fragments to *MuDR* cannot be ascertained from the available data, although it is interesting that at least some part of *MuDR* is widely distributed in the genus. The fragments could represent disrupted copies of *MuDR*, sequence similarity to either *mudrA* or *mudrB*, or methylated intact *MuDR* element as we suggest for the M3 1 individual. *MuA*, for example, is a larger *MuDR*-like element recovered from a Mutator line; it is disrupted by several insertions (Qin and Ellingboe 1990). Internal deletions within *MuDR* that retain the TIRs produce *Sst*I fragments smaller than 4.7 kb (Hershberger *et al.* 1995), but deletions missing the *Sst*I site of one TIR could yield fragments larger than *MuDR*. As epigenetic loss of Mutator activity is correlated with DNA methylation, the larger fragments could also represent modified *MuDR*-like elements. The *Sst*I (=*Sac*I) sites (GAGCTC) in the TIRs are not followed by either G or NG, consequently, methylation of the "canonical" substrates CpG and CpNpG cannot explain the inability of these enzymes to digest methylated (epigenetic loss) *MuDR* elements (Martienssen and Baron 1994). Maize DNA can be methylated at other C residues (Wang *et al.* 1996), and it is possible that methylation at one or both of the internal C residues prevents digestion.

Distribution of *MuDR*-like elements in Zapalote chico families: Given the diversity between and within accessions of Zapalote chico, we wished to determine the inheritance of *MuDR*-like elements in individual lines in which a founding individual was demonstrated to contain one or a few copies of the *MuDR*-like element. Our strategy was to PCR amplify the *MuDR*-like element in two halves (positions 113–2423, yielding a 2310-bp fragment, and from positions 2404–4829, yielding a 2425-bp fragment) that cover nearly the entire element. PCR analysis of 28 individuals of line P56 (progeny of *bz2* × M59) indicated that all were positive for both halves of the *MuDR*-like element (data not shown); these data indicate either homozygosity of the M59 parent (although it was estimated to contain only a single *MuDR*-like element by Southern blotting) or copy number maintenance. Line P57 are progeny of the original Zapalote chico accession M62 (Tuxpeño X Zapalote chico) crossed to *bz2* tester. In the 28 second outcross progeny examined, all tested positive for both halves of *MuDR* (data not shown). As the original individual had only 1–2 copies of *MuDR*, it seems likely that copy number is maintained in the stock either by transposition or by recruitment of formerly cryptic elements.

We conclude from the combination of PCR analysis and Southern blot hybridization tests that the parental Zapalote chico lines, which had only 1–2 copies of the *MuDR*-like element, transmitted the element to all progeny examined. This is circumstantial evidence that replicative transposition of the *MuDR*-like element occurs in Zapalote chico as is proposed for all *Mu* elements in standard lines (Bennetzen 1996). The analysis is compromised, however, by possible recruitment of cryptic *MuDR*-like elements during the crossing scheme.

DNA sequence analysis: Because *MuDR* and the gene, cDNA, and exon3 of *mudrA* are unstable in *E. coli*, the





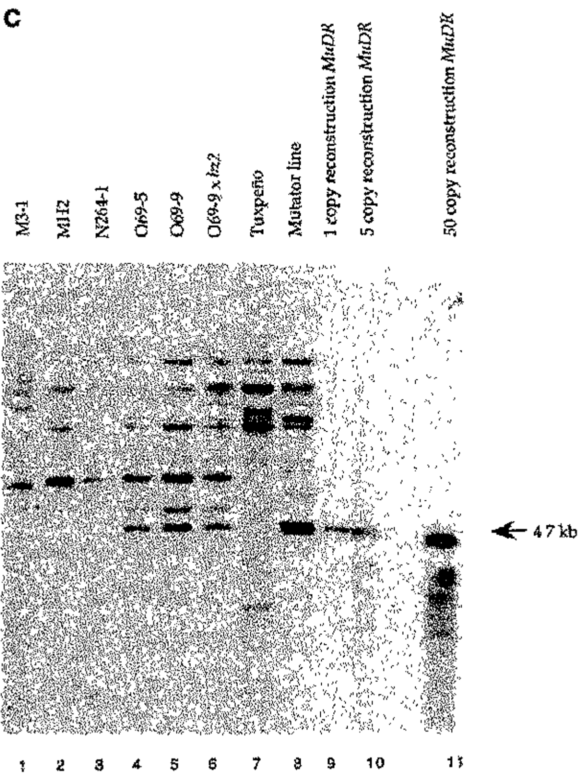


FIGURE 3.—Inheritance of the *MuDR*-like element on outcrossing of *MuDR*-like lines to *bz2* tester. Three μg samples were digested with *Sst*I and probed with BX1.0. (A) Line P56, the F_1 cross of M59 × *bz2*, and its progeny after a second outcross to *bz2* tester. (B) CIMMYT accession Oaxaca 2, line M3-4 and its derivatives. (C) Line M3-1, a sibling of M3-4 and its derivatives. All Southern blots contain the non-Mutator line Tuxpeño, a Mutator line, and a copy reconstruction of *MuDR* plasmid. See text for details of each lineage.

element and large subclones of it cannot be stably maintained on bacterial plasmids. Stable derivatives inevitably contain frameshift and deletion mutations that destroy the large open reading frame within exon3 (HERSHBERGER *et al.* 1991, 1995). As we wished to obtain the sequence of the *MuDR*-like element of Zapalote chico without selecting for mutations during cloning, segments of the *MuDR*-like element(s) of line N215 were cloned in five overlapping fragments. The cloning strategy is shown in Figure 1. Given that Southern analysis indicated only that ~3 copies of a *MuDR*-like element are present in N215 (Figure 2) and that the 10-enzyme-restriction survey demonstrated that the reading frames had the expected enzyme sites, we reasoned that an element sequence assembled from these pieces would represent a single, full-length element. It is possible, however, that the individual pieces sequenced are from different, but very closely related, elements.

The complete sequence for the deduced element assembled from the overlapping clones is shown in Figure 4, and the sequence comparisions to *MuDR* are summarized in Table 2A. The *SstI*/*SstI* internal fragments of *MuDR* and the cloned *MuDR*-like element from line N215 share 94.6% DNA sequence identity. In comparison to the known sequence of *MuDR*, it is possible to identify two putative coding regions in the *MuDR*-like element which correspond to the *MudrA* and *MudrB* genes of *MuDR*. The greatest divergence between the two elements is found in the intergenic region and in the sequence of the first intron of the *MudrA* gene. The intergenic region of the *MuDR*-like element contains a number of nucleotide insertions, including an additional copy of a direct repeat sequence; these insertions likely account for the slightly higher apparent molecular weight of *MuDR*-like *SstI* framents on genomic Southern blots (Figure 2). However, the putative coding regions and intron locations are highly conserved. As shown in Table 2B, there are only 10 nonsynonymous codon changes in MURB, and 20 nonsynonymous changes in the much larger MURA. Based on the high degree of conservation of the MURA and MURB proteins, we will designate the *MuDR*-like element of N215 Zapalote chico as *MuDR-Zc*.

Comparison of the TIR sequences of *MuDR* and N215 showed that TIRA of *MuDR-Zc* is 100% identical to TIRA of *MuDR*, whereas TIRB of *MuDR-Zc* is 91% identical to the TIRB of *MuDR*. The two TIRs of *MuDR-Zc* are only 88.1% identical to each other. Although the left and right TIRs of other *Mu* elements are rarely identical, the extent of divergence between the TIRs of *MuDR-Zc* is much higher than in *Mu1*–*Mu8* (WALBOT 1991). In *MuDR*, there are only two base changes in the first 180 bp of the TIRs, and overall the 215-bp TIRs are 96% identical (HERSHBERGER *et al.* 1991). These nearly identical TIRs of *MuDR* contain the promoter regions and major transcription start sites for the two genes (BENITO and WALBOT 1994; HERSHBERGER *et al.* 1995).

Transcription of *MuDR-Zc*: The biological significance of *MuDR-Zc* is best addressed by determining whether *MuDR-Zc* is an active element. Active and inactive Mutator lines can be distinguished by the presence or absence, respectively, of *MuDR*-hybridizing transcripts. The expression of the *MuDR-Zc* was examined by Northern blot hybridization. Figure 5 shows the analysis of a standard Mutator line and several Zapalote chico lines that yielded PCR (N201), or both PCR and Southern hybridization results (N215, N234, and N237), consistent with full-length *MuDR*-like elements. In poly(A)$^+$ RNA samples, the active Mutator plants of standard lines have abundant transcripts for both *mudrA* (Figure 5A, lane 5) and *mudrB* (Figure 5B, lane 6). The *MuDR* transcripts are relatively abundant as they are readily observed in total RNA (data not shown; see HERSHBERGER *et al.* 1995). In the Zapalote chico samples, however, it was technically difficult to visualize *mudrA* transcripts using total RNA. With poly(A)$^+$ RNA, very low levels of *mudrA* could be detected as a faint band of ~2.8 kb (Figure 5A, lanes 1–3). These transcripts are similar in size to those produced by the standard Mutator plants. The Zapalote chico *mudrB* transcripts were easily detected with poly(A)$^+$ RNA (Figure 5B, lanes 1–4). Surprisingly, the *mudrB* probe identified two different sized transcripts, one slightly larger (1.05 kb) and one slightly smaller (0.95 kb) than the 1.0-kb transcript from the standard Mutator line.

Unexpectedly, we also observed novel-sized RNAs in the non-Mutator *bz2* tester line that hybridized with the *MuDR* probe (Figure 5A, lane 4, and Figure 5B, lane 5). Similar size transcripts are also present at very low, comparable levels in the standard Mutator sample and in some of the Zapalote chico samples. The *mudrA* and *mudrB* gene probes may fortuitously recognize ubiquitous maize transcripts. In standard Mutator lines, transcripts as long as the entire element (4.9 kb) and truncated transcripts from internally deleted *MuDR* elements have been reported (HERSHBERGER *et al.* 1995). However, the cross-hybridizing material in the poly(A)+ sample from *bz2* tester is the first report of any cross-hybridization with a non-Mutator line.

Non-autonomous *Mu* elements in Zapalote chico accessions: One hallmark of active Mutator lines shared by both standard and low copy number lines is the presence of unmethylated *Mu* elements. For the *Mu1* and related *Mu2* elements, methylation status is conveniently assessed by Southern blot analysis after digestion with *HinfI*. There is a recognition site for this enzyme near the end of each TIR of the 1.4-kb *Mu1* and 1.7-kb *Mu2* elements. As shown in Figure 6, derivatives of accessions N201, N215, N234, and N237, four accessions with full-length *MuDR*-like elements, yield both the 1.3- and 1.6-kb expected fragments that hybridize to a probe that can detect both *Mu1* and *Mu2*. Considering the *Mu1* and *Mu2* elements together, it appears that the Zapalote chico accessions examined contain ~3–10 cop-

GAGATAATTGCCATTATAGACGAAGAGCGGAAGGGATTCGACGAAATGGAGGCCATGGCGTTGGCTTCTATGATCTGGAG 80
ACGCAGAGGACAGCCAATCGCCAAAACAGAAAGGTGACAGCGCTTGGAGCTCCTTAAACAGGTATTACTCTCCTGTCGGC 160
*GTTTACCGTTCGCCCGCGCACACGCCGTCTGGCATACTCCTCTTGTCACCG*TCTCTCCTCTAAATGCTCTCTGGTTCGGC 240
CTGCTCGCGGCAGCTGGCGTACTCCTCTCCTCGCCGAATTGGACTGCTCTCGGGAGCTGGCGTCTTCCTACTGCGGCTGC 320
TTCCGGTTTCCTGTTCGTGAGTTCTCCTGCTATCCTCTGTCTCCCATGGCTATCTTATGTGAACCATGGCTATCGTGTTC 400
CCCTCACCGAACCCGGTTGTGAACTTAGGTTTTCTCTGATTTTGGATCC**ATG**GACTTCACGCCCAGTTGCAATTCGCCAG 480
ACTCCAACCACATTCCCAACTCCCCCGATGTAGATCCGGCATTGGGCGAAACAGGTGGCAGTGAGGTGAGTTCAATTTAG 560
ATACATTGTCTTCAATTTCTAAAATAGTGTTGGATGGTCTCGTCCTGCATGGTCTTCAATTTTTAAAACAGTCTCGCTTA 640
TGCTTGGAGGCTCCTACATTGTCTGTAATATAGGGACTTCAGAAGATTGATGGGGAATCACAACTGGACTGGGATTCGAT 720
TATAGTTTCAGATGTATTGGATGATGAAGGCAGAGTACAAGTACCATCCGAAAATGAGATATATTTTAATCTTGGACTCA 800
ATAAAGAGGATGAGGCTGCCAATAATAGGTTTTCTGGCAGTGGTACAAATTGTCATGCACAAGGAAGTTTGGATACGGAC 880
AACGAAGACCACCATGCTGATCAGCCTTGTCAAGACTACATTCCAGACGAAAAGAGGGTGGTGTATAATAGGATGAATCC 960
TTCTATGCAGCCAGGTTGTTTGTTTCCTAACATGAAAGAATTTAGGATTGCTATGCGACAGTATGCAATAAAACATGAGT 1040
TCGAGCTTGGAATTGAAGTTACTTCGACAACAAGATACGTTGGATACTGTAAGGGTGGTGATTGCCCTTGGAGGATATAT 1120
GCACGTGAAGAGAAGAAAGGATTGCCTACTATTGTGGTAGCTGTACTAGATGATGTTCACACTTGCACATCTAGTGGAAG 1200
GAGGGGGACTACTAGGCCAACTTGTGGTTGGGTCGCATTCCACGCTAAACCCTTGCTCATGAAGAAACCACAAATGGGTG 1280
GTAAAGAGTTACAACAAACACTACAGACAACTCATAACGTCACTATTGGGTATGATACAGTTTGGAAGGGGAAAGAGAAG 1360
GCTTTGAGAGAGTTGTATGGATCTTGGGAGGAAAGCTTCCAGCTCTTGTACTCTTGGAAGGAGGCTGTAATTGCAGTGAT 1440
GCCCGATAGTGTGATTGAGATTGATGTTATTTTGGAAGATGGAAGTACTATTTTAGTCGATTCTTTTGTGCCTTTGGTC 1520
CATGCATATCTGGGTTCCGAGATGGGTGCAGACCTTATCTTAGTGTGGACTCGACAGCATTGAACGGTAGATGGAACGAA 1600
CATCTTGCATCTGCTACTGGTGTAGATGGCCACAATTGGATGTACCCAGTATGTTTTGGCTTTTTCCAAGCTGAGACGGT 1680
TGACAATTGGATTTGGTTCATGAAACAGCTGAAAAAGGTTGTGGGTGATATGACACTTCTAGCTATATGTTCAGATGCAC 1760
AAAAAGGTCTGATGCATGCTGTAAATGAGGTATTTCCTTATGCTGAGAGAAGAGAATGCTTCAGACACTTAATGGGAAAC 1840
TATGTGAAACACCATGCTGGTTCAGAGCACATGTATCCAGCAGCAAGGGCCTATAGGAGAGATGTATTTGAACACCATGT 1920
TACCAAGGTCAGAAATGTTCACAAGATTGCTGAGTACTTAGACCAACACCATAAATTCCTTTGGTACAGGAGTGGTTTCA 2000
ACAAAGATATCAAATGTGATTACATCACAAATAACATGGCTGAGGTTTATAATAACTGGGTTAAAGACCACAAAGACCTT 2080
CCTGTGTGTGATTTGGCTGAGAAAATTAGGGAGATGACAATGGGACTGTTTCATCGTAGGCGAAGGATTGGTCATAAGCT 2160
TCATGGTATTATTTTGCCATCTGCTTTAGCGATACTAAAGGCTCGCACTAGAGGGTTGGGCCACTTGTCCATTGTAAAAT 2240
GTGACAACTACATGGCAGAGGTACGAGACAGCACTAATTGTATGACTAAACATGTCGTGAATGCAGAACTGAAACAGTGT 2320
TCTTGTGAGGAATGGCAACACACTGGGAAACCGTGTCAACATGGTCTAGCCCTAATAATAGCCCAAGATTCCAGAGATGT 2400
AGGTATGGAAAATTTTGTTGACGATTATTACTCTACTGAAAGATTCAAGATAGCATATTCTAGAAGGGTGGAACCAATTG 2480
GTGATCGTTCGTTTTGGCCATCCGTTGATTTCGCCAGTGGAGTGTTTGCACCAATAGCTAGAAGAGGTCTTGGAAGACAA 2560
CGAAAAAATAGAATTAAAAGCTGTCTCGAGGGTAGGAGTGCTAGAAAAAAAAGTACCAACGAAAATGAGAAAACGAAAAA 2640
GCGACTCAAAAGGCAATACACTTGTCCTAATTGTGGTGAATTGGGACACCGCCAATCTAGCTACAAGTGCCCTTTGAATG 2760
GGACAAAAAAAAGGCTAGTTCTCAACTTACTTCTATATGTTCAATTTATATTAGTACTCGTGAACTAAATGTTTGAGTAT 2800
TTTTTTGAGTAGGAAAAGGAAACCACGGATGAACACCACAAAAAATTGGATCCCTAAAGAGCTTCGGACTTCTTCACAGA 2880
ATGTACCAGAACAGCCAGACGTAGCAGAGGAAATCACTGAACAAGAGCTAGAAGATCCACAGCCAGAGACAGAACAATTG 2960
GGTCTTGCACTCTTCCAGCCGCTGGGTGCACAAATCACTGAACAAGAGGCCGATGAACCAGCCCAACAAGCTCCACCTGC 3040
TTCTCCACCACCGACAAGGAAATGGCTAGTGAAGAAAATCACCCCCAAGAAAAGACTGAGGATTAGTGCTCAGAAGAAGC 3120
AGTAT**TAA**CTGCTAAGAACAACCACCGTGCTCAGAAGAAACACAGTATTTGTTGTAAGACAACCACTGTATTTGTTGTAA 3200
GACTGTTATGTAAGACTGCTACGAACAAACACTATGTAACCTCCACCTGTATTGGTTGTAAGACTGCTAAGAACAAGCCC 3280
AGTGTATTTGTTGTAAGACTGTTCAGTTTTAGTTGCCAGTTCGGTGCTTCCCAGGTTCAGTTTTATTTCAGTTCCCAGTT 3360
CGGTGCTTCCCAGTTTTCCCAGTTCGGTCTTGCAATTGTTGCTGCTTGCAATTGACGCCCAGTTCGGTCACTTGCAATTG 3440
GGTCTTGCAATTGTTGCTGCTTGCCCAGTTTCAGAGAAACAGATTGCTTGCAATTGTCGCCCAGTTCAGAGAAACAGATT 3520
GCTTGCCCAGTTTCAGAGAAATAGAGAGCAGAAAACAGATAAAATATTACACAAAACAGATGACATATGACACACATGAA 3600
TAACAGTGAGCCATTAGTTCTTACAACCTCATCTCCACAACACAGAAAACAGACAACACTAATGTTCTTACAAACGCCAT 3680
TCATCAGGCCTTAACACGACAACAAACACTAGGGTTCTTACATCAGATAATAGGTCATACAACAAATATCAGTTGCGTCC 3760
TTCCAAAATATATCCCAGACAGACCAAAATGACACCAGAATGAAACCAACCCACCAAAGGCAACCTCAAGTCCACAA**CTA** 3840
CATGTTACGGTCGTTTATCTCTTCGAACCTGTAGTTTATGACACGATAGTGCTCTTCGAATGAGACATTAGCTTTAATCT 3920
CTTCCCACTTGAAGTCTTCAAAAACCATTTCCCATAGCTCTGGATCTTCTGTACTGTACCCATCACCAAGTTCATCATCA 4000
TCTAGAGGATGTTCATCATCTACGGAAGGGTTGTCGTAAAGATCCCAAGTTGGATTCTTTGTTTCTAAATCTTCTTCACA 4080
GACAATTGAAGTTTTGTCTAGCTCCTTGCACACATGTTCCAGATGCCCGAGAACCTTACCTATCTTGCCACCTTGTACCT 4160
CTGGAATAGTCGAACACAATTTTTGAACCGAATGCAACAGTTTAGTGTGCCTCCTCATTTCTTGAATAGCATATCTCAAC 4240
GAATTCTTTATGTTCACATGATACTTGAAGTCGACAAGATCCTGTGCAACAACCAGATGACAATCCATTTCTATCAACAA 4320
AAAAAATGAGTGTATGAGTTTGATAATCCATACCACATTCGATGAGGCCTTAACTGCTTCAACCTTGTCCACGGCAATGG 4400
CGGCGAACTGAACCGGAGCCTTGGCCTCCTTCTCAGCAACAAATACCGCGCATCTCGCCTCCGAGGCCGCAACGGCAGCA 4480
GCACGAGCAGCTTCCACAGCATCTGCAACCACTTTGCTCGGTGGACAATC**CAT**CCGCACAGGAGCAGAGAAATTACGGCT 4560
AGGGTTTCTGGATTCAGGATGGGGGCGGCATGAACAAGGAACGACGGCTAGGGTTCGGCAACGCGTTAACCATACAAGGC 4640
AGAGATGGGAATCGGTGCAGCATTGTCGGAGCAGGAGAACTCACCGCTGAGAATAGGAGACCGCAAGCAGCCGCAAGAGG 4720
AAGAAGAGAAGCAGGCGGCGATGTCGACCCCGAGAGGAGTCCAATTCGGCGACGGAAGATATGCGAAT*GGTCACAAGAGG* 4800
AGTACAAGGGACGCCGTCTGCGCAGGCGAACGGTAAACGGGGACAGCAGAGTAATACCTGTTTACGCAGCTCCAAGCGCT 4880
GTCACCTTTCCAATTTGGCGATTGGTTGTCGTCTGCGTCTCCAGAACAGAGAAACCAACGCCATGGCCTCCATTTCGTCG 4960
AATCCCGTCCGCTCTTCGTCTACAATGGCAATTATCTC 4998

FIGURE 4.—Complete nucleotide sequence of the 4998-bp *MuDR-zc* element of accession N215. The TIRs are in italics. Bold bases are the ATG of *mudrA* (position 450), stop codon of *mudrA* (position 3126), start codon of *mudrB* (position 4531, in antisense orientation), and the stop codon of *mudrB* (position 3838; in antisense orientation).

TABLE 2

Summary of differences between *MuDR* and *MuDR Zc* sequences

Region		% Identity
A. Comparison of DNA sequences		
TIRA: *MuDR* to *MuDR-Zc*		100
mudrA: *MuDR* to *MuDR-Zc*		97.6
mudrB: *MuDR* to *MuDR-Zc*		95.2
TIRB: *MuDR* to *MuDR-Zc*		91.0
TIRA to TIRB of *MuDR-Zc*		88.1
Type of Change	Number in MURA	Number in MURB
B. Comparison of MURA and MURB predicted proteins[a]		
Synonymous codons	26	1
Conservative changes	9	7
Charged to neutral	3	1
Neutral to charged	8	0

[a] Based on the fully spliced MURA of 823 amino acids and the 207 amino acid MURB with intron 3 retained.

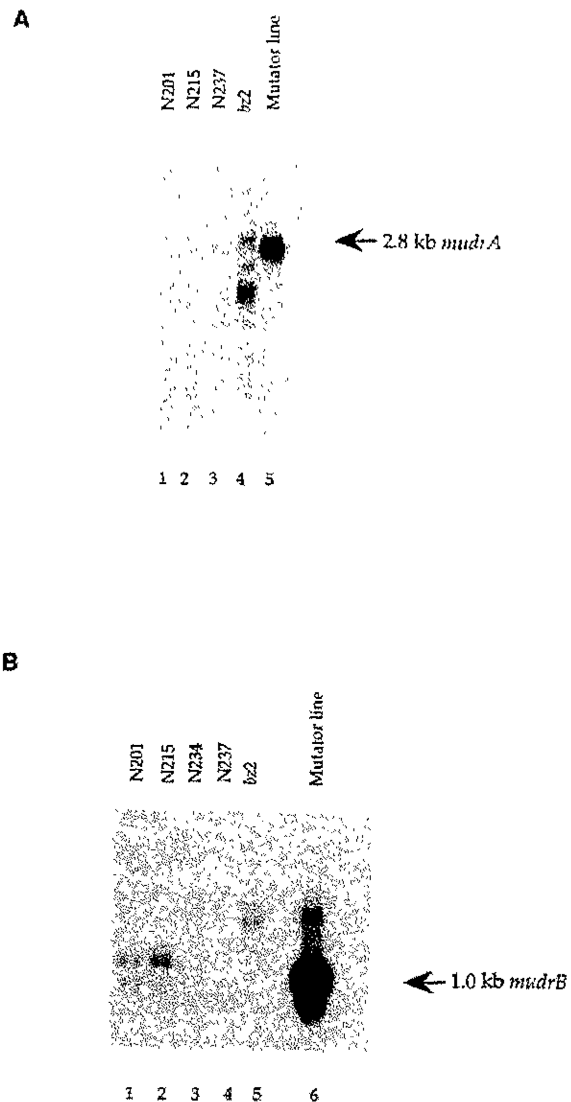


FIGURE 5.—Northern hybridization analysis of Mutator lines and Zapalote chico. Each sample contains 16–20 μg of poly (A)+ RNA. (A) The probe is PA corresponding to internal *mudrA* sequence. (B) The probe is PB corresponding to internal *mudrB* sequence.

ies of these non-autonomous elements in an unmethylated form. Although *Mu1* elements typically predominate in standard Mutator individuals (TAYLOR and WALBOT 1987), the *Mu2* elements are more abundant in the Zapalote chico accessions examined. In addition, the probe detects additional size classes that may represent one of the common deleted forms of these *Mu* elements (reviewed in WALBOT 1991), novel types of *Mu1*-derivatives or methylated copies of *Mu1* or *Mu2*. Other known or novel *Mu* elements may also be present.

Non-Mutator lines contain from zero to three *Mu1* and *Mu2* elements (BENNETZEN 1984; CHANDLER *et al.* 1986; CHANDLER and WALBOT, 1986). These elements are completely stable in position and copy number, and they remain methylated in a non-Mutator line. On crossing with a standard, active Mutator line, the *Hin*fI sites in the termini of the *Mu1* element in inbred line B37 lost methylation and could be digested with methylation-sensitive enzymes, such as *Hin*fI (CHANDLER *et al.* 1988). Thus, the moderate copy number and presence of unmethylated non-autonomous *Mu* elements suggest that these accessions of Zapalote chico are active Mutator lines.

Elevated forward mutation frequency in some Zapalote chico accessions: The multiple copies of unmethylated *Mu* regulatory and non-autonomous elements in some accessions of Zapalote chico are similar to what is found in standard, active Mutator lines. On the other hand, the low abundance of *MuDR*-related transcripts is more similar to the single copy *MuDR a1-mum2* lines (QIN and ELLINGBOE 1990) in which *MuDR* transcripts are only reliably detected with poly(A)+ RNA. The standard and single-copy *MuDR* lines both program the same pattern of high frequency somatic excision of *Mu* elements from reporter alleles, but the lines differ in the frequency of new mutants recovered (ROBERTSON and STINARD 1992). Given that the Zapalote chico lines share specific properties with each of the two characterized types of Mutator lines, we were interested in defining the forward mutation frequency.

We used the test devised by ROBERTSON (1978). Each individual is self-pollinated to score pre-existing recessive mutants; each individual is also crossed to a non-Mutator line, and multiple F_1 plants are grown and self-pollinated to score for new mutants generated in the gametes of the presumptive Mutator parent. As *Mu* insertions occur late in development, new mutants are

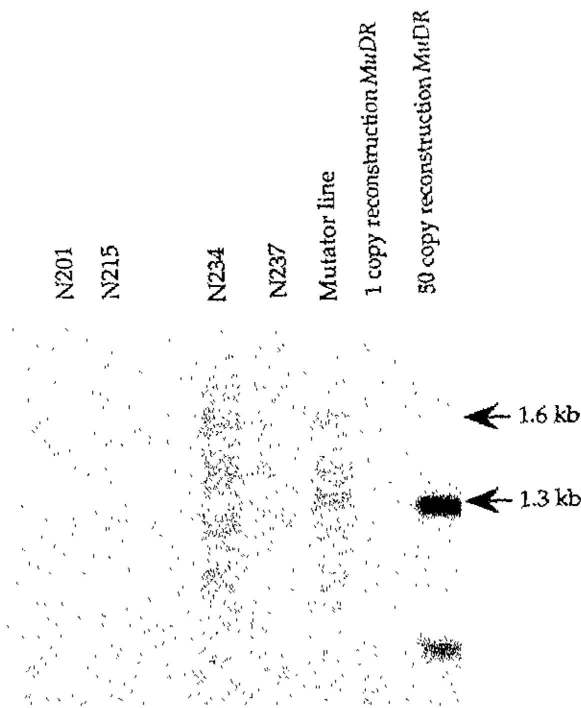


FIGURE 6.—Southern blot analysis of non-autonomous *Mu* elements in four accessions with full-length *MuDR-Zc* elements. DNA was digested with *Hin*f I and probed with pA/B5, which hybridizes to both *Mu1* and *Mu2*.

almost always recovered in only a single gamete (ROBERTSON 1981; reviewed in WALBOT 1991). One or a few individuals in most accessions of Zapalote chico were self-pollinated and crossed as pollen to *bz2* tester. Mutants recovered in the parental selfed ear and the F_2 selfed ear were scored as visible seedling traits 10 and 28 days after germination in the summer field. For comparison, we also assessed forward mutation frequency with several standard Mutator lines, two single-copy *MuDR* lines with the *a1-mum2* reporter allele, and the *bz2* tester in the W23 inbred line.

As expected, the four standard Mutator lines generated many mutants (Table 3D). On selfing, nearly half (28/62) of the parental plants segregated 3:1 wild-type:mutant for a pre-existing, visible seedling mutation. Common recessive phenotypes included albinos, zebra-striped leaves, pale green, pale yellow, and developmental mutants with twisted, shredded or midrib-only leaves. In the outcross to *bz2* tester, followed by selfing, 240 new mutants were observed in 835 families (29% mutation frequency) for the four standard Mutator lines. In the control for spontaneous mutation, no seedling mutants were observed in the selfed *bz2* tester (Table 3F). We can estimate spontaneous mutation frequency in the *bz2* tester if we also consider *defective kernel* (*dek*) mutations; one new *dek* mutation was recovered from the *bz2* tester (1/120 = 0.8%), a value similar to the spontaneous mutation frequency found in other non-Mutator lines examined in this test (ROBERTSON 1981). The *dek* phenotype is among the most common recessive class in standard Mutator lines, representing failure of the embryo, endosperm or both (reviewed in WALBOT 1991). There were 131 new *dek* mutations (131/835 = 16%) in the standard Mutator sample, ~20-fold more than in *bz2*.

In contrast to standard Mutator lines, the single *MuDR a1-mum2* line had a low forward mutation frequency (one mutant/344 families, Table 3E). Thus, the forward mutation frequency characteristic of standard Mutator may require multiple copies of *MuDR* and, most likely, a large population of non-autonomous elements.

The forward mutation test was completed before we classified the Zapalote chico accessions for *MuDR*-like elements and was therefore unbiased in selecting individuals for analysis. For simplicity, however, Table 3 groups lines on the basis of their *MuDR* phenotype. The four Zapalote chico accessions shown by PCR to contain all four segments of a *MuDR*-like element (Table 1) generated many new mutants after outcrossing as pollen parent to *bz2*; we observed 79 new mutations in 187 families (42%). The frequency of new mutations is equal to the most active standard Mutator line (M121), which gave 106 new mutants in 255 families (42%). In contrast, for the 11 Zapalote chico accessions in which PCR failed to detect all four segments of *MuDR*-like elements, the forward mutation frequency was low (five mutants/392 families = 1.2%, Table 3B); this is within the range of the spontaneous mutation frequency in other non-Mutator lines analyzed by this test (ROBERTSON 1978), and similar to our results with *bz2* and with the two single-copy *MuDR* lines. A third group of 10 Zapalote chico accessions was analyzed for mutation frequency but not tested by PCR (Table 3C). These lines yield an intermediate value, with 31 new mutants in 217 families (14.2%). This group is clearly heterogeneous, with some accessions generating multiple mutants and some none.

Because both plastids and mitochondria are maternally transmitted in maize, reciprocal crosses between disparate lines often lead to defective kernels as a result of nuclear-cytoplasmic incompatibility. We observed many *dek* mutants in the self-pollinated F_2 ears of the Zapalote chico outcrosses, particularly among accessions that also gave rise to seedling mutants (data not shown). This class was excluded from analysis, however, because it is unknown whether mutations or incompatibility are responsible for the small or defective kernel phenotype (ALLEN *et al.* 1989).

Unusual features of mutant induction in Zapalote chico: It seems likely that both Mutator activity and *MuDR*-like elements are unevenly distributed in Zapalote chico populations. Only a subset of the Zapalote chico accessions qualify as Mutator lines by the forward mutation assay. This assay does not pinpoint what types

TABLE 3

Forward mutation frequency

Line	Self-pollinated parent		Outcrossed to *bz2*		N
	No. of Individuals	No. of Mutants	No. of F_1 Families	No. of F_2 Families	
A. Zapalote chico accessions with *MuDR*-like elements					
M2	2	0	33	11	N201
M25	1	0	6	3	N215
M26	1	0	19	11	N216
M59	11	1	83	47	N234
M62	4	1	41	13	N237
Total	19	2	182	85	
B. Zapalote chico accessions without intact elements					
M1	1	0	14	0	N200
M5	4	1	41	1	N204
M6	1	0	11	1	N205
M7	2	1	40	0	N206
M8	1	0	75	0	N207
M24	2	1	44	0	N214
M29	3	2	42	0	N219
M30	3	1	47	2	N220
M32	2	0	25	1	N222
M36	2	0	32	0	N226
M41	2	0	21	0	N230
M58	1	1	16	1	N233
Total	24	7	408	6	
C. Zapalote chico accessions not classified by PCR					
M3	2	1	28	2	N202
M4	1	1	16	4	N203
M17	1	0	17	3	N208
M18	1	0	16	4	N209
M33	2	0	26	0	N223
M34	2	1	37	0	N224
M35	3	1	29	4	N225
M37	1	0	17	3	N227
M38	1	0	9	0	N228
M40	2	0	22	11	N229
Total	16	4	217	31	
D. Standard Mutator lines					
M88	2	1	32	6	n.a.
M121	20	11	255	106	n.a.
N285	20	9	309	57	n.a.
N190	20	7	239	71	n.a.
Total	62	28	835	240	
E. Single *MuDR a1-mum2* line					
N55	10	0	197	0	n.a.
N56	10	0	147	1	n.a.
Total	20	0	344	1	
F. *bz2* tester					
M72	10	0	120	0	

of elements cause mutations. It is possible that some Zapalote chico lines contain several types of transposable elements.

One curious feature of the analysis is that Zapalote chico lines yielded few mutants on selfing. In the subset of lines with *MuDR*-like elements, we identified only two visible seedling mutations among the 19 parents (Table 3A). In subsequent years, continuous selfing of these lines, and tests with more individuals from the original accessions, have produced few new mutants (data not

TABLE 4

Reactivation test with cryptic *bronze2* reporter alleles

Reporter allele[b]	No. tested	Number of ears with spotted kernels[a] after the indicated cross			
		(X)	to *bz2*	Zapalote chico[c]	Standard Mutator
bz2::MuDR	25	0	0	12	8
bz2::Mu1-mu1	17	0	0	0	1
bz2::Mu1-mu2	13	0	0	3	11

[a] Ears were scored as positive if at least 5% of the progeny kernels exhibited the frequent, fine spotting phenotype.

[b] The *bz2::MuDR* allele (formerly called *bz2-mu4*) has a full-length *MuDR* element inserted in the second exon (Hershberger *et al.* 1991); the other alleles have *Mu1* insertions in the first (*-mu2*) and second (*-mu1*) exons of *Bz2*. For the test, unspotted kernels were chosen from lines that were fully inactivated (*bz2::Mu1-mu1*), scored as no somatic mutability over several generations or from lines that were just inactivating; in the latter lines, selfed ears had just a few very lightly spotted kernels while progeny ears on *bz2* tester had no somatically unstable kernels.

[c] Results are pooled for the N237 and N264-derived *bz2* Zapalote chico lines.

shown). The number of visible mutants was similar to what we found in the "no *MuDR* group" (seven visible mutations in 24 parents) and the unclassified group (four mutants in 16 parents). The Zapalote chico accessions contain more "mutants" than *bz2*, but one plausible explanation is that temperature-sensitive alleles were recognized as mutant at Stanford that have no mutant phenotype in the much warmer conditions of Oaxaca.

The low incidence of visible mutants in Zapalote chico lines containing *MuDR* (2/19) is particularly striking considering the incidence of such pre-existing mutants in standard Mutator lines (28/62). In contrast, the F_2 ears from the outcross part of the forward mutation test exhibit similar frequencies of newly induced mutants. In its native habitat, only selfing or crosses within Zapalote chico germplasm occur, because Zapotec farmers grow only this type of corn. The activation of a high forward mutation frequency on crossing with a heterologous line suggests that hybrid dysgenesis occurs. We completed too few exact reciprocal crosses between Zapalote chico and *bz2* to determine whether the elevated mutation frequency results when an active Zapalote chico individual is the female parent as well as the pollen donor.

A second curious feature of the many new seedling mutants produced by the various Zapalote chico accessions is that none displayed somatic variegation. Typically, small wild-type sectors indicative of late somatic excision are visible in at least half of all new mutants produced by a standard Mutator line (Robertson 1981; reviewed in Walbot 1991). In the collection of mutants produced for this study, we also found that about half of the new albino, pale green and yellow mutants recovered from standard Mutator lines had visible dots of green on the first leaf (data not shown). The absence of somatic reversion is a novel property of new mutants produced in Zapalote chico.

Zapalote chico lines with *MuDR-Zc* can restore somatic mutability to cryptic *bz2* mutable reporter alleles: The lack of somatic instability of newly induced mutations in unknown genes made it difficult to analyze whether *Mu* elements were involved. To gain more direct evidence that *MuDR-Zc* elements were genetically active, we used a *trans*-activation test for Mutator activity. Lines derived from N237 and N264 with full-length *MuDR-Zc* (based on Southern blot hybridization) were crossed twice with *bz2*, in effect creating *bz2* tester lines after selection for individuals without the dominant *C-I* allele. This allele prevents anthocyanin accumulation and was present in most Zapalote chico accessions. For the activation test, inactive Mutator lines homozygous for one of three well-characterized *bronze2* alleles with precisely mapped *Mu* element insertions were selected from our collection; these lines contain multiple, methylated copies of *MuDR* and somatically stable *Mu* elements. As shown in Table 4, each inactive individual was self-pollinated and crossed to *bz2* tester to score spontaneous reactivation of somatic mutability at the cryptic reporter allele; no instance of spontaneous reactivation was observed in the 55 individuals tested. On crossing to Zapalote chico or standard Mutator *bz2* lines, fine purple spotting indicative of late, frequent somatic excision was restored in from zero to 85% of the test crosses. Such wide variation in reactivation is typical of Mutator reactivation tests (Walbot 1986).

DISCUSSION

A high forward mutation frequency is a defining characteristic of standard Mutator lines; mutation frequency is elevated 20–100-fold above spontaneous or above what is observed in active *Ac* or *Spm* lines (reviewed in Walbot 1992). Mutations in Mutator lines are caused by a diverse family of *Mu* elements, which share ~200-bp TIRs. Germinal insertion and somatic excision activities are controlled by the regulatory element *MuDR*. To date, *MuDR* has been found only in standard Mutator

lines, in their immediate derivatives, and in the *Cy* germplasm (BENNETZEN 1996). In the standard U.S. germ plasm, land races, and *Zea* spp. we have examined, we find evidence for unmethylated *MuDR*-like elements and Mutator activity in Zapalote chico. Even within this land race, only a subset of accessions appear to contain full-length elements.

For a neotropical maize, Zapalote chico is relatively tolerant of long daylength. It can be grown to maturity in the temperate zone and crossed with U.S. germplasm. Because it is so adaptable and contains many traits of potential agronomic importance, Zapalote chico has been used in breeding for disease, insect, and wind-damage resistance (MUÑOZ *et al.* 1992). Zapalote chico contains large numbers of prominent heterochromatic knobs, and this line has been used in maize cytogenetic research (GOODMAN and BROWN 1988).

Several lines of evidence indicate that some accessions of Zapalote chico qualify as Mutator lines. First, they exhibit a high forward mutation frequency, similar to standard Mutator lines. Second, they contain multiple, unmethylated copies of non-autonomous *Mu* elements. *Mu* elements are methylated in inactive or non-Mutator lines (CHANDLER *et al.* 1988). Third, they contain multicopy unmethylated and transcriptionally active *MuDR*-like elements, which to date have been found only in standard Mutator lines (BENNETZEN 1996). Fourth, *MuDR*-like element copy number is maintained through several outcrosses to non-Mutator lines. Approximately one-fourth of the Zapalote chico accessions examined appear to have Mutator activity by one or more of these criteria.

Molecular analysis of *Mu* elements in Zapalote chico accessions: The three sequenced examples of *MuDR* are nearly identical, and it was expected that a search for additional Mutator sources would identify only this element. We have cloned the *MuDR-Zc* element in several fragments from one accession of Zapalote chico (N215) that contains several copies of the putative regulatory transposon. The *MuDR-Zc* sequence assembled from the fragments is highly similar, but clearly diverged, from the *MuDR* present in standard and the derived low-copy *MuDR* Mutator lines. *MuDR-Zc* is 4998 bp, 56 bp larger than the 4942-bp *MuDR*. Overall, *MuDR* and *MuDR-Zc* exhibit 94.6% DNA sequence identity. Identity is highest in TIRA and in the coding regions, with the intergenic region being the most divergent part of the element. At the amino acid level, the *mudrA*-like gene (*mudrAzc*) is more similar to that of *MuDR*, 97%, than the *mudrB*-like gene (*mudrBzc*), 95.2%. A portion of *MuDR-Zc* was also cloned and sequenced from N234; in the region 4398–4524, this sequence is identical to *MuDR-Zc* of line N215.

Southern blot analysis clearly demonstrates the presence of intact ~5.0-kb *MuDR-Zc* elements in N215. Because *MuDR-Zc* was cloned in fragments by PCR amplification, however, we do not have proof that all of the polymorphisms exist in the same element. It is also possible that a few of the nucleotide polymorphisms are from PCR mutation. Because *MuDR* is toxic to *E. coli*, point mutations are common during attempts to clone the intact element; for this reason we cloned *MuDR-Zc* in pieces that appear to be tolerated in *E. coli*. However, we were also able to amplify the fragment in two, large overlapping PCR fragments (position 113–2423 yielded a 2310-bp fragment; positions 2404–4829 yielded a 2425-bp fragment). Future recovery of overlapping genomic clones of *MuDR-Zc* and cDNA clones will confirm the distribution of sequence differences within individual *MuDR-Zc* elements in line N215. To gain a better understanding of the diversity of *MuDR*-like elements, full sequencing of elements from additional Zapalote chico accessions could be informative as well.

Evidence for Mutator activity in some Zapalote chico accessions: Several approaches were taken to demonstrate that some Zapalote chico lines not only carry intact *MuDR*-like elements but may also have an actively transposing population of *Mu* elements. The first measure of Mutator activity was by Northern analysis, because it has been shown that only active Mutator lines express *MuDR* transcripts (HERSHBERGER *et al.* 1995). Second, we examined the methylation status and copy number of *Mu* elements and the transmission of *MuDR*-like elements to progeny. The third measure was a forward mutation test to determine if any Zapalote chico accessions had an elevated mutation frequency, and whether mutation frequency correlated with *MuDR*-like elements. Fourth, we examined the ability of Zapalote chico to activate somatic instability in inactive Mutator lines.

Northern analysis demonstrated that *MuDR-Zc* is actively transcribed; however, the levels and patterns of expression are different from standard Mutator lines (HERSHBERGER *et al.* 1995). *mudrA* and *mudrB* transcripts are easily detected in total RNA of standard Mutator lines and are approximately equally abundant, although in immature (prefertilization) ears there is an ~1:4 ratio of *mudrA:mudrB* transcripts (HERSHBERGER *et al.* 1995). Low transcript abundance is characteristic of the single-copy *MuDR* lines, but these lines have approximately equal amounts of transcript from genes A and B (QIN *et al.* 1991; JAMES *et al.* 1993). In Zapalote chico, however, *mudrAzc* transcript levels are extremely low, while those of *mudrBzc* are relatively more abundant. Both transcripts are only readily detected from poly(A)$^+$ RNA. As the *mudrAzc* and *mudrBzc* transcripts are approximately the size of standard Mutator transcripts, we infer that the TIRs also act as the promoter elements in Zapalote chico, as well as constituting part of the 5′ UTR of each transcript type. In the sequenced example of *MuDR-Zc*, TIRA is identical to TIRA of *MuDR* but TIRB is only 91% identical. The differences in TIRB may allow a higher level of *mudrBzc* transcription or increased transcript stability. It is possible that *mudrA*

and *mudrB* differ in transcript abundance because there is Zapalote chico-specific host regulation or new forms of autoregulation by the *MuDR*-like elements.

It is not clear why there are two *mudrBzc* transcripts in the Zapalote chico accessions examined. It is possible that these transcripts are produced by two different, but related, *MuDR-Zc* elements. It is also possible that they are produced by alternative transcription start sites, differential splicing or different polyadenylation events from a single transcription unit. As mentioned earlier, both alternative splicing and multiple polyadenylation sites exist in *mudrB* transcripts in standard Mutator lines (HERSHBERGER *et al.* 1995), and such post-transcriptional events may explain the two transcripts found in Zapalote chico. We also observed novel-sized RNAs in the *bz2* tester line that hybridized with the *MuDR* probe. These cross-hybridizing RNAs may result from fortuitous similarity or regions of similarity to *MuDR* in this non-Mutator line.

Methylation has previously been shown to be correlated with the loss of activity of *MuDR* (MARTIENSSEN and BARON 1994). In inactive Mutator lines with methylated *Mu* elements, *Mu* copy number decreases by approximately half with each successive outcross to a non-Mutator line (WALBOT and WARREN 1988). Neither *MuDR-Zc* elements nor *Mu1* and *Mu2* are methylated at the enzyme sites examined. Furthermore, the *MuDR-Zc* copy number is maintained on outcrossing: parents with just 1–3 elements transmit them to all progeny and through at least two outcrosses. Maintenance of *Mu*-element copy number is a key property of active Mutator lines (ALLEMAN and FREELING 1986; WALBOT and WARREN 1988), although we are uncertain whether transposition or demethylation of cryptic elements is responsible for copy number maintenance in Zapalote chico.

In the test for forward mutation frequency, we established that standard Mutator lines have a high forward mutation frequency (29% of families contain a new visible seedling mutation) compared to the low frequency of a standard inbred line of maize (*bz2* tester in W23 background) or a single *MuDR* line (*a1-mum2*). In Zapalote chico, an elevated mutation frequency correlates with the presence of *MuDR*-like elements, but transposable elements of additional families and *Mu* elements of several types may contribute to the observed mutation frequency. The accessions for which there is molecular evidence of regulatory elements had a 42% forward mutation frequency, matching the level of the most active standard Mutator line. All of the new mutations recovered appear to be recessive, based on segregation data (data not shown).

Several properties of Mutator activity in Zapalote chico are distinct from both standard and single-copy *MuDR* Mutator lines. In contrast to standard Mutator lines, we found few mutations segregating in the original Zapalote chico parents. New mutants occur after outcrossing Zapalote chico as pollen donor onto a non-Mutator line. Consequently, new mutations occur as a result of hybrid dysgenesis and must be induced during or after fertilization. In standard Mutator lines, many new mutations are recovered as single-kernel events, indicative of *Mu* insertions that affect single gametophytes (reviewed in WALBOT 1991; CHANDLER and HARDEMAN 1992). When an active Mutator plant is used as a pollen donor, nonconcordant embryo and endosperm mutations occur in ~20% of the new mutants selected at *Y1* (ROBERTSON and STINARD 1993). The lack of correspondence between the embryo and endosperm genotypes indicates that *Mu* insertions can occur after the mitosis that separates the two sperm in each pollen grain (ROBERTSON and STINARD 1993). Our data provide evidence that mutations in Zapalote chico sperm can be induced even later, after fertilization, provided the sperm interact with a non-Mutator egg.

A second unusual feature of new mutations induced in Zapalote chico is that they are not somatically mutable. Frequent late somatic excision is characteristic of both standard and low-copy Mutator lines (reviewed in WALBOT 1991; LISCH *et al.* 1995). If the Zapalote chico mutations are caused by *Mu* insertions, then the lack of somatic instability suggests that there is novel developmental regulation of element excision behavior. In Mutator lines losing activity, often assessed by a loss of somatic excision at a reporter allele, the levels of *MuDR* transcripts decline precipitously (JOANIN *et al.* 1996). The low abundance of *MuDR-Zc* transcripts may similarly be below the threshold required to program somatic excision.

A mutation screen is currently in progress to isolate mutations in anthocyanin reporter genes, using Zapalote chico accessions with *MuDR-Zc* elements. The isolation of a *Mu* element inserted into a known gene will provide the opportunity to analyze the type of insert and its excision behavior more precisely. It is possible that mutations in Zapalote chico lineages result from more than one type of transposon.

Implications of hybrid dysgenesis: The seed accessions used in this study were gathered from different sources, and at different times. Only a subset of the Zapalote chico lines contains *MuDR-Zc* and exhibits an elevated mutation frequency. In the past, Zapalote chico has been included in a variety of corn-breeding programs. In crosses with other lines, however, hybrids are often abandoned because of high sterility (MUÑOZ *et al.* 1992) or poor vigor (W. TRACY, personal communication). Yet this land race is a commercial crop when grown and maintained by inbreeding by the Zapotec farmers in Oaxaca, México. Zapalote chico is the staple of the human and animal diet of the Zapotec people. Zapotecs prize this variety of corn for preparation of totopos, a baked corn cracker that is the main starchy food in their diet. We hypothesize that the Zapotec farmers have selected for the alterations in Mutator activity that

we observe as a low abundance of transcripts and unremarkable mutation frequency in inbred Zapalote chico.

The apparent restriction of a high forward mutation frequency to outcrosses involving Zapalote chico may be the explanation for the stability of this line in crop fields. Our molecular and genetic observations confirm a Zapotec myth that their corn will kill other lines of maize if interbred. This myth is one reason Zapotecs grow only Zapalote chico to ensure a reasonable yield. The basis of this myth may be hybrid dysgenesis. This phenomenon was first described by analysis of the repression and activation of *P* elements in *Drosophila melanogaster* in crosses that involved wild-caught and laboratory flies (reviewed by ENGLES 1989). With the appropriate combination of breeding scheme and *P*-element types, this transposable element family is quiescent, effectively tamed.

Similarly, we found a low-mutation frequency after selfing Zapalote chico lines with transcriptionally active *MuDR*-like elements. This contrasted with the high-mutation frequency observed in the progeny of these same plants crossed as pollen donor to inbred W23 and in the derivatives of the Tuxpeño X Zapalote chico lines crossed to W23. The difference in mutation frequencies suggests that Zapalote chico germplasm could contain a novel factor that suppresses Mutator activities or has lost a host factor required for activation. When Zapalote chico is crossed as pollen donor to other lines, the "repressor" of Mutator activity is missing or ineffective. The somatic stability of new mutants in the dysgenic crosses is also striking, and again suggests that the *MuDR-Zc* or the Zapalote chico background confers novel and stabilizing properties on the Mutator transposons. Further genetic and molecular analysis will be required to identify the proposed repressor, if it exists, and to probe the interactions of standard *MuDR* and *MuDR-Zc*.

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Discusión

Con el propósito de poder utilizar la familia de transposones *MuDR/Mu* en Zapalote chico como una herramienta para la generación de mutantes y la clonación de genes, llevamos a cabo la caracterización de estos transposones en Zapalote chico por medio de diferentes estrategias experimentales. Los resultados obtenidos se discuten en seguida.

Evidencia de actividad Mutator en algunas líneas de Zapalote chico.

En este trabajo mostramos que la secuencia completa de *MuDR-Zc* es 94.6% similar a *MuDR*. Por experimentos de hibridización RNA-DNA tipo*Northern blot*, encontramos que los elementos *MuDR-Zc* son transcripcionalmente activos, sin embargo los patrones de expresión de *mudrA-zc* y *mudrB-zc* son diferentes de las líneas Mutator estándar. En Zapalote chico los niveles de *mudrA-zc* y *mudrB-zc* son extremadamente bajos y aunque *mudrB* es un poco más abundante ambos transcritos fueron posibles de detectar sólo cuando se utilizó RNA poli $(A)^+$. Por otro lado observamos que en Zapalote chico existen dos transcritos de *mudrB-zc*, uno de aproximadamente 1.05 kb y otro un poco mas pequeño (0.95 kb) que el observado en líneas Mutator estándar. Es posible que estos transcritos sean el producto de diferentes sitios de iniciación de la transcripción, procesamiento diferencial del mensajero o que existan sitios de poliadenilación múltiples de una misma unidad transcripcional. Otra posibilidad es que estos transcritos se originen de dos distintos *MuDR-Zc*. Un dato interesante de estos experimentos es que en líneas *bz* tester (líneas no Mutator) se observaron varios transcritos de diferentes tamaños que hibridan con las sondas de *MuDR* , en líneas Mutator estándar también se observaron transcritos de similar tamaño pero los niveles de expresión fueron mas bajos. En

líneas Mutator estándar se han reportado transcritos tan grandes como el elemento entero o transcritos más pequeños que provienen de elementos *MuDR* truncados (Hershberger R. J. et al 1995). Sin embargo es la primera vez que se reporta hibridación de los transcritos de *MuDR* en líneas no Mutator como *bz2*. Estos resultados representan una parte importante de este trabajo pues ellos constituyerón el primer precedente de futuras investigaciones de *MuDR* y sus derivados, actualmente se investiga si estos elementos contribuyen a la regulación de la actividad de Mutator durante el desarrollo de la planta (Rudenko G. N. y V. Walbot 2001).

En algunas líneas de Zapalote chico existe una alta frecuencia de mutación.

Probablemente una de las características más sobresalientes de líneas Mutator es su alta frecuencia de transposición. En este trabajo encontramos que en Zapalote chico una frecuencia alta de transposición se correlaciona con la presencia del elemento *MuDR-Zc* y en algunas de las líneas la frecuencia de transposición fue tan alta como en líneas Mutator estándar. Sin embargo, una característica distinta en Zapalote chico es que las mutaciones nuevas ocurren sólo después de que el polen de Zapalote chico es usado en cruzas con líneas no Mutator, las nuevas mutaciones deben de ser inducidas durante o después de la fertilización causando una especie de disgénesis hibrida. Esto sugiere que en Zapalote chico podría existir un factor que suprime la actividad Mutator o que ha perdido un factor necesario para su activación.

Características únicas de las mutaciones inducidas en Zapalote chico.

Como ya se mencionó, una de las características más importantes de seleccionar mutantes inducidas por transposones es que estas mutaciones son somáticamente inestables y los sectores de reversión son una prueba de que la mutación es causada por la inserción de un

transposón Un rasgo distintivo de Zapalote chico y determinante para los objetivos de este trabajo es que las mutaciones generadas por *MuDR-Zc* no son somáticamente mutables. Esta es una propiedad nueva de Zapalote chico, lo que indica que en esta raza de maíz existe una regulación de la excisión del transposon diferente a la presente en líneas Mutator estándar. Ha sido demostrado que el producto de *mudr*A es suficiente para programar la excisión somática, quizás la baja expresión de este gen en Zapalote chico no sea suficiente para permitir la excisión de este transposón. Actualmente se estan realizando experimentos para aislar mutaciones en un gen reportero de la ruta de las antocianinas usando líneas de Zapalote chico con elementos *MuDR-Zc* para poder analizar el tipo de inserción y tratar de averiguar el por qué no existen excisiones de estos transposones en esta raza de maíz. Después de analizar estos resultados, concluimos que para los propósitos de este trabajo no era posible usar Zapalote chico para generar mutantes y la posterior clonación de los genes, pues el hecho de que no existan excisiones somáticas de este transposón deja la duda de si la mutación de interés está siendo causada por un transposón *Mu*, por otro transposón o la causa de la mutación es otra. Sin embargo, Zapalote chico sigue siendo interesante para estudiar la biológía de Mutator y sería bastante interesante analizar si en Zapalote chico existen factores endógenos de su genoma que llevan a una regulación distinta de estos transposones que en líneas Mutator estándar. Por otro lado uno de los problemas con la que algunos grupos de trabajo se han enfrentado, es que despúes de ciertas generaciones la mutación de interés en líneas estandár Mutator se puede perder debido a la excisión del transposón; así, en Zapalote chico existe la posibilidad de estudiar mutaciones de genes ya conocidos sin el riesgo de que se pierda la mutación pues las mutaciones producidas por elementos *Mu-zc* son estables. Sin embargo, para

poder establecer un sistema de abanderamiento de genes en Zapalote chico es necesario futuros estudios del comportamiento de estos transposones.

Conclusiones

Caracterización *MuDR/Mu* en Zapalote chico.

Después del análisis de varias líneas de Zapalote chico encontramos que en algunas de estas líneas existe este elemento activo que conserva varias características de líneas Mutator estándar, como son: una frecuencia de mutación alta, contienen copias multiples de elementos *Mu* no-autónomos y el elemento *MuDR* es activo transcripcionalmente. Estos elementos se conservan después de varias cruzas con líneas no mutator, lo que sugiere que hay amplificación del elemento por transposición replicativa. A este elemento lo hemos llamado *MuDR-Zc*.

El sistema *MuDR-Zc/Mu-zc* tiene caracteristicas distintivas del de líneas estándar; en Zapalote chico se observan mutaciones nuevas sólo después de que polen de Zapalote chico es usado en cruzas con líneas no Mutator. Otra característica inusual en Zapalote chico es que las mutaciones no son somáticamente inestables, esto último fue determinante para los objetivos de este trabajo y concluimos que no es posible utilizar esta raza de maíz para la seleccíon de mutantes y la posterior caracterización de sus genes. Sin embargo puede ser útil para entender más de los mecanismos de la nueva actividad de elementos Mu en Zapalote chico.

Perspectivas

El hecho de que en Zapalote chico se encuentren los elementos *MuDR/Mu* activos y además se conserven varias de las características de líneas Mutator estándar pero con algunos rasgos distintivos, ofrece la ventaja de poder conocer más del comportamiento de estos elementos y los posibles factores que regulan su actividad en Zapalote chico. Análisis genéticos y moleculares podrían identificar dichos factores y estudiar si estos interactúan también con *MuDR* de líneas estándar. Por otro lado, una vez mejor caracterizado este sistema, podría ser útil para identificar mutaciones en genes ya conocidos sin el temor de perder la mutación en cruzas futuras ya que las mutaciones en Zapalote chico son estables.

Capítulo II

Análisis genético de la biogénesis del cloroplasto en *Arabidopsis thaliana*.

En el embrión de una planta existen diferentes identidades celulares formando órganos y cada célula contiene proplastidios. Estos proplastidios se diferencian durante el desarrollo de la planta de acuerdo al programa de la célula en la cual ellos se encuentran, lo que refleja su plasticidad estructural y metabólica.

El metabolismo de los diferentes tipos de plastidios está en estrecha relación con la función del tejido en el cual se encuentran, por ejemplo, su función principal en hojas iluminadas es la asimilación de CO_2 y captación de luz por el cloroplasto. Mientras que los plastidios en la raíz estan principalmente involucrados en la asimilación de nitrógeno inorgánico. Los amiloplastos que acumulan grandes cantidades de almidón, se comportan como reservorios en raíz y tubérculos. Los cromoplastos presentes en pétalos, frutas y raíces sintetizan grandes cantidades de carotenoides. La interconversión entre los diferentes tipos de plastidios está acompañada por cambios dramáticos en la estructura del plastidio, incluyendo el desarrollo o el desensamble del sistema de membrana interna y la adquisición de enzimas específicas de la vía biosíntetica en la cual participan (Kirk J. T. O. y R. A. E. Tilney-Bassett, 1978).

La biogénesis del plastidio es genéticamente compleja e involucra la participación de cientos de genes distribuídos en el núcleo y en el mismo organelo. Se sabe poco de cómo la

célula coordina la expresión de genes en ambos compartimentos en respuesta a diferentes estímulos.

El plastidio mejor estudiado es el cloroplasto, por un lado debido al importante papel que juega en la fijación del CO_2 atmosférico en compuestos orgánicos y por otro lado, a que son relativamente fáciles de aislar de células del mesófilo en comparación con otro tipo de plastidios. El control nuclear de la diferenciación del cloroplasto ha sido documentado por numerosos estudios bioquímicos de la síntesis del aparato fotosintético, así como por una buena cantidad de mutantes nucleares que bloquean la función de este organelo (para revisión ver Somerville C., 1986). Sin embargo, pocas mutaciones de genes nucleares que regulan el desarrollo del cloroplasto han sido sujetos a análisis molecular (León P. 1998). En la tabla I se incluyen gran parte de ellas. La mayoría de estas mutantes fueron aisladas por su fenotipo verde pálido o amarillo, algunas han sido clasificadas como albinas, como es el caso de *alb*3, aún cuando presenta un fenotipo amarillo. Como se observa en la tabla I, estas mutantes tienen defectos en una gran variedad de las funciones del cloroplasto que incluyen, el importe de proteínas a través de la maquinaria general de importación, como es el caso de *ppi1* (Jarvis P. et al 1998) y *ppi2* (Bauer J. et al 2000). Estos genes codifican las proteínas Toc33 y Toc159 respectivamente, las cuales forman parte del aparato de translocación de proteínas de la membrana externa e interna del cloroplasto, *alb3* (Long D. et al 1993;
Sundberg E. et al 1997), *hcf106* (Voelker R. y A. Barkan. et al 1995; Settles A. M. et al 1997) *tha1* (Voelker R. y A. Barkan et al 1995; Voelker R. et al 1997) y *tha4* (Walker M. B. et al 1999.) son ejemplos de mutaciones que afectan la maquinaria de inserción de proteínas a la membrana tilacoidea.

Durante mucho tiempo se ha propuesto que el estado de desarrollo del cloroplasto regula la expresión de genes nucleares cuyas proteínas son destinadas a este organelo (Taylor W.C. 1989). Por ejemplo, en plantas en donde el desarrollo del cloroplasto ha sido detenido por un inhibidor como norflurazona, el cual interrumpe la síntesis de carotenoides, no se lleva a cabo la expresión de genes nucleares como CAB y RBCS que codifican proteínas del cloroplasto con función fotosintética. Así se ha hipotetizado que una señal cloroplástica (llamada factor plastídico) provee al núcleo con la infomación acerca del estado de desarrollo del organelo y esta señal regula la expresión de genes nucleares. Se ha especulado que la señal plastídica puede ser un metabolito, un segundo mensajero o una macromolécula (Somanchi A. y S. P. Mayfield, 1999). Susek y colaboradores diseñaron diferentes estrategias experimentales para identificar señales plastídicas, tanto positivas como negativas, que regularan la expresión de genes nucleares (Susek R. E. et al 1993; Li H.-m. et al 1995). Como resultado de este trabajo aislaron una serie de mutantes entre ellas *cue1* y *gun5*; recientemente fueron clonados los genes de ambas mutantes (Mochizuki N. et al 2000; Streatfield S. J. et al 1999); *cue* codifica para el translocador de fosfoenolpiruvato/fosfato y *gun5* la subunidad ChlH de la Mg-quelatasa, la que participa en la biosíntesis de la clorofila (tabla 1). En el caso de *gun5* se ha propuesto que la acumulación de precursores de clorofila puede interactuar con dicho factor plastídico y modular negativamente la expresión de un juego de genes nucleares.

Por otro lado, el grupo de Mache R.. ha demostrado que genes nucleares que codifican componentes del aparato de traducción del plastídio se expresan muy temprano durante la biogénesis del cloroplasto y anteceden la expresión de cualquier otro gen plastídico (Mache R. et al 1997) y que los genes plastídicos que se expresan en las primeras horas después de la imbibición de la semilla, son transcritos exclusivamente por NEP, lo que sugiere que la

biogénesis temprana del plastidio está bajo el estricto control del núcleo (Harrak H 1995, Mache R. et al 1997).

Como se observa en los datos de la tabla I la mayoría de las mutantes reportadas hasta ahora inhiben el desarrollo del cloroplasto afectando estadíos específicos del ensamble de la membrana del tilacoide y la producción del aparato fotosintético. Los cloroplastos en estas mutantes son generalmente pequeños y contienen tilacoides semidesarrollados. Las mutantes que bloquean completamente el desarrollo del cloroplasto dan lugar a plastidios que se asemejan proplastidios, este tipo de mutantes son relativamente raras, quizás deb letales. Sin embargo, cuando este tipo de mutaciones son somáticamente i sucede en aquellas causadas por transposones, la inestabilidad puede [illegible] sectores revertantes silvestres que permiten la viabilidad de la planta c[illegible] mutante *dag* de *Antirrhinum* (Chatterjee M. et al 1996) de *dcl* del jito[illegible] 1996), de *vdl* (Wang Y. et al 2000) del tabaco y *iojap* del maíz. [illegible] pocas reportadas que afectan el desarrollo temprano del clorop[illegible] clonados; hasta la fecha se conoce sólo la función de *Vdl* ya que el [illegible] *Dag* y *Iojap* no tienen similitud con ningún otro gen reportado en las bases de datos. Arabidopsis es otra mutante de la biogénesis del cloroplasto y la función del gen fue conocida hasta hace poco (Mandel A. A. et al 1996; Estévez J. et al 2000) (tabla I y artículo) En conclusión hasta el momento se conoce poco a nivel molecular de las fases tempranas del desarrollo del cloroplasto.

El objetivo de esta parte del trabajo, es realizar un análisis sistemático de los pasos tempranos de la biogénesis del cloroplasto a través de la selección de mutantes albinas. Para ello se seleccionó como modelo a la planta de *Arabidopsis thaliana* por todas las ventajas que

ella ofrece y que ya han sido mencionadas. A continuación se presenta el artículo que contiene la primera parte de los resultados de este trabajo. En una segunda parte se presentan los resultados de la clonación de la secuencia genómica adyacentes al T-DNA de dos de las mutantes aisladas durante este trabajo

Tabla 1

Mutante	Organismo	Función	Referencia
alb3 (*albino3*)	*Arabidopsis thaliana*	ALB3 interviene en la integración de proteínas en la membrana tilacoidea a través de la vía SRP	Long, D. et al 1993. Sundberg E. et al 1997.
cla1-1 (*cloroplastos alterados*)	*Arabidopsis thaliana*	1-desoxi-D-xilulosa 5 fosfato sintasa	Mandel A. A. et al 1996. Estévez J. et al 2000
cue1 *chlorophyll a/b binding protein(CAB) gene underexpressed*	*Arabidopsis thaliana*	translocador de fosfoenolpiruvato/fosfato	Li H-m. et al 1995. Streatfield S. J. at al 1999.
dcl-m (*defective chloroplasts and leaves-mutable*)	*Lycopersicon esculentum*	Desconocida	Keddie J. S. et al 1996.
dga (*differentiation and greening*)	*Antirrhinum majus*	Desconocida	Chatterjee M. et al 1996
gun5 (*genomes uncoupled*)	*Arabidopsis thaliana*	GUN5 codifica la sub-unidad ChlH de la enzima Mg-quelatasa	Susek R. E. et al 1993 Mochizuki N. et al 2001.
hcf106 (*high-chlorophyll fluorescent*)	*Zea mays*	HCF106 es una proteína de la membrana tilacoidea, participa en la translocación de proteínas por la vía ΔpH	Miles C. D. y D. J. Daniel 1974. Voelker R. y A. Barkan. 1995. Settles A. M. et al 1997.

ij (*iojap*)	*Zea mays*	Desconocida	Walbot V. y E. H. Coe 1979 Han H. D. et al 1992
im *immutants*	*Arabidopsis thaliana*	IM actúa como una oxidasa terminal en la desaturación de carotenos. IM se encuentra en la membrana tilacoidea.	Wetzel C. M. et al 1994. Carol P. et al 1999.
lpe1-m1 (*leaf permease1-mutable1*)	*Zea mays*	*Lpe1* codifica una proteína con similitud a una familia de proteínas de bacterias que transportan purinas y pirimidinas	Schultes N. P. et al 1996.
oli (*Olive*)	*Antirrhinum majus*	OLI tiene homología con el producto de dos genes de bacterias necesarios para la quelación de metales de tetrapirroles.	Hudson A. et al 1993.
pac (pale cress)	*Arabidopsis thaliana*	Desconocida	Reiter R. S. et al 1994.
ppi1 (*plastid protein import*)	*Arabidopsis thaliana*	PPI1 (Toc 33) tiene 51% de similitud con Toc34. Es un componente del aparato de importación de proteínas de la membrana externa del cloroplasto.	Jarvis P. et al 1998
ppi2 (*plastid protein import*)	*Arabidopsis thaliana*	PPI2 (Toc 159) es el receptor de la proteína de tránsito del aparato de importación de proteínas de la membrana externa.	Bauer J. et al 2000
tha1 (*thylakoid assembly*)	*Zea mays*	THA1 es homólogo de SecA de bacterias. Participa en la translocacion de un juego de proteínas hacia el lumen del tilacoide.	Voelker R. y A. Barkan 1995. Voelker R. et al 1997.

tha4 (*thylakoid assembly*)	*Zea mays*	THA4 es requerido para la translocación de proteínas a través de la vía ΔpH	Walker M. B. et al 1999.
vdl (*variegated and distorted leaf*)	*Nicotiana tabacum*	DVL codifica una proteína putativa con actividad de RNA helicasa	Wang Y. et al 2000.

Six novel *albino* loci define cell autonomous and non-cell autonomous factors required for early chloroplast biogenesis and leaf development in *Arabidopsis thaliana*

Maria de la Luz Gutiérrez-Nava[1]*, C. Stewart Gillmor[2]*, Luis F. Jiménez[3], and Patricia León[1]

* These authors contributed equally to this work.

[1]Dept. of Plan Molecular Biology, IBT, UNAM

[2]Carnegie Institution, Stanford Iniversity

[3]Electron Microscopy Laboratory, UNAM

ABSTRACT

In a screen of 99 pigment afected lines of Arabidopsis we have identified 9 mutations that affect early stages of chloroplast biogenesis. These mutations fall into 7 complementation groups and include a novel allele of *cla1*. *albino* mutants all have less than 2% of Wt levels of chlorophyll, little or no expression of nuclear genes required for chloroplast maintenance, and some of the mutants even do not have expression of genes required for maintenance of the proplastid. In all mutants but *alb8*, proplastids do not differentiate enough to form even early thylakoid membranes. These *alb* mutations also have moderate to severe effects on leaf development, and the strongest *alb* mutations lack most internal layers of the leaf. In addition, analysis of *alb* mutants during embryogenesis allows us to differentiate between the loss of non-cell autonomous factors, where partial complementation of chloroplast development is observed during embryogenesis, and cell autonomous factors, which are not complemented by maternal factors during embryogenesis. The low percentage of pigment lines we recovered that result in true albino phenotypes suggests that a relatively small number of genes are required to complete early steps of chloroplast biogenesis.

INTRODUCTION

Chloroplasts are the biosynthetic and assimilatory powerhouses of plant cells, responsible for the fixation of CO_2, manufacture of starch, fatty acids and pigments and the synthesis of amino acids from inorganic nitrogen. The capacity of a plastid to house several biosynthetic pathways or perform photosynthesis is strictly linked to its state of differentation. In higher plants, chloroplast develop from proplastids which are present primarily in meristematic cells and in young postmitotic cells. As meristematic cells begin to differentiate into the mesophyll and palisade cells proplastids begin to differentiate into chloroplasts. Proplastids are small organelles (0.2-0.5 μm in diameter), that lack thylakoid membranes but do contain low amounts of DNA, RNA, ribosomes and soluble proteins. During development, plastid volume increases dramatically: the increase in plastid volume can be greater than 100 fold. When the proplastid reaches an average size of 1.0 μm the inner layer of the membrane begins to invaginate into the stroma (Mühlethaler and Frey-Wyssling, 1959). This process continues until there are many flat sacs or thylakoids laying in the stroma, and as differentiation proceeds, the number of thylakoids increases. Also these thylakoids are now seen to occur almost invariably in stacks of two, three or more. Further, the number of thylakoids in each stack increases until eventually the typical grana of the mature chloroplast are produced (Kirk and Tilney-Bassett, 1978).

It has been suggested that progressive extension of the thylakoid system results from fusion of vesicles to its edges that in turn are derived from small invaginations of the inner plastid envolope (Whatley et al., 1982). The thylakoid membranes have a lipid composition similar to that of the chloroplast inner membranes (Carde et al., 1982; Douce et al., 1984; Morré et al., 1991). However no connection between these membranes can be found in later

states of maturation, and thylakoids seem to be maintained by a flux of inner membrane vesicles (Carde et al., 1982; Douce et al., 1984). Recently, several proteins have been reported that seem to be involved in formation of vesicles. Pftf, which shares a high degree of amino acid sequence similarity with NSF of yeast and animals and FtsH of bacteria, has been isolated from hot pepper and implicated in the vesicle fusion (Hugueney et al., 1995). DL1, a dynamin-like protein, is also thought to be involved in the formation of vesicles (Park et al., 1998). VIPP1 is a protein that is involved in the budding of vesicles from the inner envelope, and which in turn is required for the maintenance of a structurally and functionally intact thylakoid membrane. Deletion of the *VIPP1* gene is deleterious to thylakoid membrane formation (Kroll et al., 2001).

The conversion of proplastids into chloroplasts is accompanied by high transcription levels of plastid genes encoding the transcription/translation apparatus during early chloroplast biogenesis followed by a decrease once mature chloroplasts are established. Chloroplast photosynthetic genes are highly expressed only later in development (Bisanz-Seyer et al., 1989). This suggests that one central regulatory point of chloroplast differentiation is the activation of chloroplast transcription. It has been shown that differential transcription of genes encoding the transcription/translation apparatus occurs early in chloroplast development of barley (Baumgartner et al., 1993). In spinach, nuclear genes encoding plastid ribosomal proteins are expressed very early during the first hours following seed imbibition and in advance of the expression of the plastid encoded genes, the expression of these genes is dependent on activation of specific promoter elements and precedes the expression by two days of the two ribosomal RNA operons and other photosynthetic genes encoded in the chloroplast or photosynthetic genes encoded in the nucleus. These results suggest that the early signals for

chloroplast development originate from the nucleus (Mullet et al., 1993; Harrak et al 995, Mache et al., 1997).

Despite the importance of plastids little is understood about the regulation of their differentiation, their role in plant development and the molecular basis of plastid nuclear communication. Recently, genetic approaches have begun to yield insights into the mechanisms of chloroplast biogenesis. Mutants in several different species have been identified in which plastid development is severely compromised leading to a pale or albino phenotype that is often seedling lethal (Somerville C. R. 1986). From such mutants a variety of genes have been cloned and characterized that appear to have a role in controlling plastid differentiation and development.

Mutants that completely block chloroplast development should give rise to plastids that are very small, lack internal membranes and resemble proplastids. Few true albino mutants of this type have been characterized. To date the earliest-acting mutants reported are *pac, dcl, dag, iojap, cla1*, and *vdl* (Reiter et al., 1994; Keddie et al., 1996; Chatterjee et al., 1996; Shumway and Weier, 1967; Mandel et al., 1996; Wang et al., 2000). All these mutants have chloroplast structures characteristic of proplastids, while plastids from *pac, dcl* and *cla1* display some single thylakoid membranes and accumulate chlorophyll and carotenoids at low levels. In *dag, iojap* and *vdl* no thylakoid differentiation occurs and they show typical small vesicles and invaginations of the inner membrane seen in proplastids. In addition, cells lacking DAG function do not express RBCS or CAB, whose expression is thought to be required for transcriptional and/or translational activity of the chloroplast and for nuclear gene expression (Susek and Chory, 1992). This suggests that DAG acts very early in chloroplast biogenesis. DAG has no similarity to proteins of known function. *iojap* plastids

have a programmed loss of the ribosomes (Walbot and Coe, 1979). The nucleotide sequence of *iojap* shows no significant sequence similarity with proteins listed in the database (Han et al, 1992) however it is associated with the 50S plastid ribosomal subunit, suggesting a role for Ij in protein translation (Han and Martienssen, 1994). In *pac* mutant plants distinct chloroplast encoded transcripts are affected, as the mutation mainly changes the maturation pattern of chloroplast mRNAs. It has been suggested that PAC has a specific role in chloroplast mRNA maturation (Meurer et al ., 1998). *CLA1* encodes 1-deoxy-D-xylulose-5-phosphate synthase, which is involved in biosynthesis of isoprenoids. Inactiviation of this gene affects normal development of the chloroplast and etioplast, but does not affect amyloplast structure (Estévez et al, 2000). The predicted DCL sequence shows no significant homology to any protein. *dcl-m* plants appear affected only in the development of the chloroplast and no other plastid type (Keddie et al 1996). The *vdl* mutant of tobacco contains undifferentiated plastids and shows variegated leaves and abnormal roots and flowers. *VDL* encodes a plastid DEAD box RNA helicase which suggests that plastid RNA helicases are involved in early plastid differentiation and plant morphogenesis (Wang et al., 2000).

In addition to affecting chloroplast development, the mutants *pac* and *cla1* in *Arabidopsis, dag* in *Antirrhinum, dcl* in tomato, and *vdl* in tobacco show loss of palisade mesophyll cell differentiation within the leaf. In these mutants the palisade layer is missing and is replaced with smaller spherical-shaped cells similar to those observed in spongy meshophyll. In addition, air spaces are present more often in mutant leaves that in wt leaves. Analysis of these mutants has led to the hypothesis that a signal derived from the normal functional chloroplast interacts with a dorsoventralizing signal to cause periclinal elongation of palisade and mesophyll cells.

To gain a better understanding of the genes which are necessary for early steps of chloroplast biogenesis, we conducted a genetic screen for pigmentation lines in *Arabidopsis*. Of the 99 lines we obtained from our screen, 9 lines showed an extreme albino phenotype and contain less than 2% of Wt levels of chlorophyll. These 9 *albino* (*alb*) lines define 7 loci, and include a novel allele of the previously described *cla1*. In this work we examine the affect of these *albino* mutations on chloroplast and nuclear photosynthetic gene expression, chloroplast development, and leaf development. In addition, we differentiate between cell autonomous and non-cell autonomous factors which are required for early chloroplast development. The low number of true *albino* mutations recovered in our screen suggests that relatively few genes are required for the earliest steps of chloroplast biogenesis.

MATERIALS AND METHODS

Plant material and growth conditions: Arabidopsis thaliana (L) Heyhn. ecotypes Landsberg *erecta* (L*er*), Columbia (Col), Wassilewskija (WS), C24, L*er*/Lim/Dij, and Dijon were used in this study as is indicated. For experiments involving plants grown under sterile conditions, seeds were surface-sterilized and plated on germination medium (GM) containing 1X Murashige and Skoog basal salts (Gibco BRL, Grand Island, NY), 2% (w/v) sucrose, 1X B5 vitamin solution (Gamborg's, Sigma, Inc., St Louis, MO), 0.05 % (w/v) MES [2-(N-morpholino) ethanesulfonic acid], solidified with 0.8% (w/v) phytoagar. Adult plants were grown in Metro-Mix 200 (Grace Sierra, Milpitas, CA) soil under 16 hrs light 8 hrs dark at 24 ^{o}C . Seeds were incubated at 4 ^{o}C to break dormancy prior to germination.

All of the mutants isolated as part of this study were recovered from different sources: T-DNA insertion and Fast neutron mutagenesis were provided by Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). Ethyl mathanesulfonate mutants (EMS) and some T-DNA lines from Chris Somerville (Carnegie Institute of Whasington Stanford, CA), some all the albinos lines from the Arabidopsis Biological Resource Center (ABRC).

Complementation analysis were done by crossing heterozygous mutants plant to one another in all combination possible and the resulting F1 embryos scored for the presence or absence of the mutant phenotype.

Determination of genetic map positions Genetic mapping was done according to Lukowitz et al., 2000. Mapping populations were generated by manually crossing heterozygous mutants plants to Ler or Col ecotypes background . F1 plants were allowed to self pollinate and set seed. F2 plants tissue homozygous or heterozygous were collected and

bulked, DNA pools were used to assign a rough position on the genetic map by identifying linked genetic markers. To find flanking markers DNA was prepared from individual F2 plant samples and examined with markers from the region. About 140 chromosomes were analysed.

Northern blot analysis Seedlings were grown on GM medio +2 % sucrose plates for 16 days 16 hrs light 8 dark 24 °C. Total RNA was was prepared by extration with Triazol Reagent (Sigma, Inc., St Louis, MO). 15 µg of total RNA per lane from seedlings was fractionated by electrophoresis in 1.2% (w/v) agarose gel and transferred onto Gene Screen nylon membrane (New Life Science Products., Boston, MA) and fixed in a drier at 80°C for 2 hrs. Hybridization was done with PSE buffer. Hybridization and washes were done a high stringency conditions according to standard procedures.

Chlorophyll determination Determination of total carotenoids and chlorophylls was conducted following the protocol reported by Lichtenthaler and Wellburn (1983).

Confocal microscopy Late stage embryos were dissected from heterozygous plants and mounted in a solution of 300mM Mannitol on large coverslips, and were imaged on a Nikon inverted fluorescence microscope equipped with a 20x Nikon objective and a Bio-Rad MRC 1024 confocal head. Confocal reconstructions were made from ~1µm optical sections using NIH Image and Adobe Photoshop.

Light and electron microscopy For transmission electron microscopy, tissues were fixed with 6% (w/v) glutaraldehyde in phosphate-buffered saline (pH 7.2) for 10 h and post fixed in 1% (w/v) osmium tetroxide in the same buffer for several hours. After dehydration in a graded series of ethanol and propylene oxide, samples were embedded in Epoxy resin. For electron microscopy, 60 nm thin sections were obtained and mounted on formvar-coated copper grids (Electron Microscopy Sciences, Fort Washington, PA). For contrast, 3% (w/v)

uranyl acetate and .3% (w/v) lead citrate were used. Grids were observed with transmission electron microscope (EM-10, Carl Zeiss, Jena, Germany) operating at 80 KV. For light microscopy, samples were treated as described above and 0.5 - μm semi-thin sections were obtained. The section were stained with 1% (w/v) toluidine blue and observed in brightfield with a light microscope (Standard, Carl Zeiss).

RESULTS

Identification of *albino* mutants: Historically, much effort in plant biology has been concentrated in the biochemistry and structure of different plastid types. In recent year a clear molecular picture of chloroplast development is emerging, the core components of the chloroplast protein import are probably been identified and much of the biogenesis and assambly of photosynthetic proteins in thylakoid membranes is know (for review Bauer et al., 2001; Wollman et al., 1999). By contrast, little is known about genes required for early steps of chloroplast differentiation. Mutants that are disrupted in early stages of chloroplast biogenesis might be expected to resemble proplastids. Chloroplasts arrested at this stage would be expected to have little or no photosynthetic activity and also lack photosynthetic pigments. Thus, seedling mutants with chloroplasts arrested at very early stages of differentiation should display a white, true albino phenotype. Of the mutants previously described in *Arabidopsis,* only *cla1* (Mandel et al., 1996) and *pac* (Reiter et al., 1994) fit this criteria.

To identify mutants which most likely affect early stages of chloroplast differentiation, lines available from the Arabidopsis Biological Resource Center (ABRC) classified as 'albino' were examined. 33 lines were obtained from the ABRC, all of which were homozygous lethal and were be propagated as heterozygotes. Lines were grown in tissue culture media supplemented with sucrose, and examined visually. Mutant lines representative of the observed phenotypic spectrum are shown in Figure 1. The majority of mutants lines were not true albinos, but in fact had considerable amount of green (Figure 1B) or yellow (Figure 1C) pigments, and which are referred to here as pale green or yellow phenotypes. However, two lines, *alb2* and CS213 (Figure 1D), did fit our phenotypic criteria for albino mutants, as they

were completely lacking in photosynthetic pigments when viewed with a dissecting microscope. The fact that only two out of 33 ABRC lines classified as albinos fit our strict phenotypic criteria for a true albino phenotype suggested that the number of *albino* loci might be small enough to be tractable for analysis. In view of this, a genetic screen to isolate more *albino* mutants was performed.

A genetic screen to isolate lethal mutations must be done by examining families so that heterozygous siblings can be used to propagate the mutation of interest. Since Wt embryos develop chloroplasts during embryogenesis (Mansfield and Briarty, 1991), late stage embryos which lack chlorophyll can be easily scored, and plants which are heterozygous for a pigment mutation segregate approximately 1/4 white embryos, as shown in Figure 2A. Thus, M3 families can be effectively screened before seed dessication while the seed are still developing in siliques of the M2 mother plant, saving the step of harvesting individual families for screening by germinating seedlings on plates. In this manner, 1000 EMS M2 and 5,000 T-DNA mutagenized lines were examined for siliques containing approximately 1/4 pale or white embryos. 69 lines segregating morphologically normal pale or white embryos were recovered and used for further analysis.

In total, 99 pigment lines were obtained from the survey of ABRC 'albino' lines and our genetic screen. Based on seedling pigmentation, these 99 lines were classified into three categories: albino, pale green, and yellow. Lines in which no green or yellow pigment could be observed under a dissecting scope were classified as albino. Pale green lines contained less chlorophyll than Wt seedlings, while yellow lines primarily lacked chlorophyll, but retained yellow (carotenoid) pigments. Of the 99 mutant lines, 61 were scored as pale green, 29 as yellow and only 9 as albino (Figure 1 and Table 1).

Complementation crosses using all pairwise combinations of the nine *albino* mutants identifed demonstrated that these 9 lines comprise 7 complementation groups (see Table 2). ABRC stock center line CS213 was found to be allelic to the previously described *cla*1-1 mutation, and was thus designated *cla*1-2. *alb*2-1, which was also obtained from the ABRC, was not found to be allelic to any of the other *albino* mutants. Of the remaining 7 mutants, three were found to be allelic and were designated *alb*7-1, *alb*7-2, and *alb*7-3. The remaining four mutant lines were non-allelic and were designated *alb*4-1, *alb*5-1, *alb*6-1 and *alb*8-1. All mutants segregate as monogenic, nuclear recessive mutations with normal transmission (data not shown).

To support the visual observation that our *albino* mutants were indeed serverely lacking in chlorophyll and carotenoid pigments, pigment levels in all 6 albino mutants were quantified (Table 3). Pigments from 18 day old seedlings were extracted in acetone and quantified by their absorption spectra by the method of Linchtenhaler and Wellburn (1983). All albino mutants contained 2% or less of Wt chlorophyll per weight compared with Wt seedlings. *alb2, alb*4, *alb*5 and *alb*7 had approximately 3% of Wt level of carotenoid, while *alb*6 and *alb*8 had 12.9 and 9.6 % of Wt carotenoids, respectively. This data support the visual classification of these mutants as true *albino* mutants that are severely lacking chlorophyll and carotenoid pigments.

Map positions of *albino* mutants:

We crossed our *alb* mutants to the Columbia ecotype and selfed the F1 heterozygous plants to generate F2 mapping populations. Rough map positions for each *alb* locus were generated by pooling 50 mutant seedlings and using pcr-based SSLP markers to check for linkage to a set of SSLP markers spaced at regular intervals throughout the genome (Lukowitz

et al., 2000). For more fine scale mapping, we obtained recombination frequencies with flanking SSLP markers for all *alb* mutations, using populations of approximately 70 F2 plants. *alb* mutations map to chromosomes III, IV and V and map positions, along with recombination frequencies with closely linked markers, are shown in Figure 3.

albino seedling morphology: Although all *albino* mutants are morphologically normal during embryo development (see Figure 2 and Figure 8 below), dramatic differences are observed during seedling growth. *alb* mutants grow much more slowly than Wt seedlings, have serious defects in leaf morphogenesis, and are seedling lethal. *alb* seedling phenotypes after 21 days of growth on MS sucrose plates are shown in Figure 4.

The mutant with the most severe seedling morphology is *alb7* (Figure 4E). This mutant develops small, bumpy cotyledons which curve over so that the adaxial surface touches the hypocotyl. *alb7* cotyledons lack petioles, and cells of cotyledons are very swollen, especially on the adaxial side of the cotyledon, which presumably causes the cotyledons to curl downward. The first leaves of *alb7* are round, finger-like projections, and lack any differentiation between petiole and blade. The other five *alb* mutants have somewhat more normal leaf morphology, with a clear differentiation between petiole and blade, though the leaves have a gnarled, bumpy appearance. This is presumeably due to lack of internal tissue layers of the leaf and abnormal development of epidermal cells (see Figure 7 below). The leaf blades are small and distorted, and leaves of *alb2* (Figure 4A), *alb4* (Figure 4B) and *alb5* (Figure 4C) also curl under at the ends, presumably due to increased cell expansion on the adaxial side. *alb6* (Figure 4D) and *alb8* (Figure 4F) have the most normal morphology: cotyledons and leaves have a more normal shape, and do not fold over. All mutants except *alb7-1* produce trichomes.

Leaves of *alb*2-1 mutants are opaque, in contrast to the other 5 *alb* mutants, which have a crystalline appearance such that they are almost transparent. *alb*4, *alb*5, *alb*7, and *alb*8 accumulate significant amounts of anthocyanin during seedling development, presumably due to physiological stress. By contrast, *alb*2 and *alb*6 do not accumulate anthocyanins, and *alb*6 leaves have a subtle yellow color, most likely due to the increased amount of carotenoids in this mutant relative to the other *alb* mutants (see Table 3). When grown in tissue culture on MS sucrose medium, *alb*2 can bolt but will not produce any seeds. All of the other mutants will not bolt, even after 5 weeks in tissue culture, and after 4-5 weeks the mutant seedlings have the appearance of callus (data not shown).

When mutants were grown in absence of light, seedlings displayed the characteristic wild type skotomorphogenic pattern: long hypocotyls and unexpanded cotyledons (data not shown). Also, all mutants respond normally to blue and red light (data not shown). This suggests that signal transduction in response to light is functional in the *albino* mutants.

When the mutants are grow in hormone Indole acetic acid, Benzylaminopurine, Naphthalene acetic acid and Isopentil adenine the phenotype of them do not change wich seems to indicate that this mutants do not have a deffect in the production of any of this hormone.

Plastid structure in *albino* mutants: To assess the effect of *alb* mutations on chloroplast development at the ultrastructural level, plastids of the first leaf of 3-week old seedlings were examined by electron microscopy. Representative chloroplasts and plastids from Wt and *albino* mutants are shown in Figure 5. Compared with the Wt chloroplast shown in Figure 5A, the plastids of albino mutants are all arrested at an early stage of differentiation, and generally resemble proplastids. Plastids of *alb4*(Figure 5C), *alb*5 (Figure 5D), and *alb*7 (Figure 5F) lack

appressed internal membranes, although all three have large vesicle-like structures with unknown contents. By contrast, *alb*2 (Figure 5B) and *alb*8 (Figure 5G) contain short, linear appressed membranes, while *alb*6 contains long appressed membranes. Based on plastid morphology, chloroplast development is arrested earliest in *alb*4, 5 and 7, at a slightly later stage in *alb* 2 and 8, and yet later in *alb*6.

Analysis of nuclear and chloroplast gene expression in *albino* mutants: EM analysis of plastids in *albino* mutants suggested that chloroplast differentiation is arrested at a very early stage in these mutants. To further characterize the differentiation state of plastids in the *albino* mutants, the expression of nuclear and chloroplast-encoded genes which are known to be expressed at different stages of chloroplast development was determined.

It have been show that the nucler gene *rpl*21 encoding plastid ribosomal proteins are expressed very early after seed imbibition, preceding the expression of the chloroplast-encoded genes photosynthetic and nonphotosynthetic, *rpl*21 mRNA is present in root and seeds in much lower amount that in leaves. (Lagrange et al., 1993; Harrak et al., 1995). Thus,*rpl*21 consitutes a very early marker of plastid differentiation. As shown in Figure 6, *rpl*21 is expressed at a level similar to Wt in *alb*2, *alb*4, *alb*5, and *alb*6, but is not expressed in *alb*4 and *alb*7. This suggests that plastids of *alb*4 and *alb*7 are not translationally competent. To check expression of the earliest transcribed chloroplast genes, we used *rrn16S* and *accD* genes. The 16S rRNA gene is the most highly expressed plastid gene in proplastids (krupinska and Falk, 1994) therefore, it represents a good tool to measure plastid transcriptional activities during the early phases of chloroplast development. *accD* is the chloroplast-encoded subunit of acetyl-CoA carboxylase, which is involved in lipid biosynthesis in the plastid. *accD* is

transcribed exclusively by a nuclear RNA polymerase. it have been proposed that this polymerase plays an important role in non-green plastids and it is required for maintaining the proplastid (Hajdukiewicz et al., 1997). Similar to the expression pattern of *rpl21*, *accD* is not expressed in *alb4* and *alb7*, but is expressed at levels similar to Wt in the other 4 *albino* mutants. The *rrn16S* gene is expressed very low in *alb4* but is not expressed in *alb7* suggesting that this mutant is the most early affected during chloroplast biogenesis.

Nuclear and a chloroplast-encoded genes which are required for photosynthesis are late molecular markers for chloroplast biosynthesis and function. The expression of nuclear-encoded *RBCS*, the essential enzyme of the calvin cycle and chloroplast encoded *psbA*, which encodes the D1 protein, a protein of the reaction center of photosystem II (Bruick and Mayfield, 1999) were determined in *albino* mutants. *alb4* and *alb7* show no expression of either gene, *alb2* and *alb5* show intermediate (but low compared to Wt) expression, and *alb6* and *alb8* show relatively high levels of expression, but still less than Wt. Thus, northern analysis of nuclear and chloroplast gene expression suggests that *alb4* and *alb7* are arrested most early in chloroplast development, *alb2* and *alb5* can progress farther, since they express *RBCS* and *psbA* at low levels, and *alb6* and *alb8* are the least severe, perhaps even having some photosynthetic capacity. The developmental state of the plastids in albino mutants based analysis of gene expression is consistent with our analysis of plastid structure by electron microscopy. Plastids of *alb4* and *alb7* are arrested at the earliest stages, while *alb2* and *alb5* develop enough to express photosynthetic genes at low levels, and *alb6* and *alb8* express significant amounts of photosynthetic genes.

Leaf structure of albino mutants To determine the tissue structure of the leaf of albino mutants, Wt and albino mutant leaves were embedded and sectioned at the midpoint of the leaf blade, as best as could be determined due to the severely altered structure of the albino leaves. As shown below, the failure of the palisade and mesophyll cells to divide and expand normally, or the total absence of these tissues, explains the altered morphology of albino leaves.

alb7 has the most severe effect on leaf development. As previously shown in Figure 4, *alb7* produces round finger like leaves that lack apparent dorso-ventrality. Figure 7F shows a cross-section through the first leaf of *alb7*. The leaf consists of an inner cylinder of vascular tissue, surrounded in most places by a single layer of epidermal cells which are drastically expanded compared to the Wt epidermal cells shown in Fig. 7A. *alb7* totally lacks mesophyll tissue and instead has a large empty space between the epidermis and the vascular tissue. Similar to *alb7*, but less severe, *alb5* (Fig. 7D) and *alb8* (Fig. 7G) lack mesophyll cells in many parts of the leaf and consist mostly of vascular cylinders surrounded by epidermal cells. The shape of the cells of the epidermis is variable and *alb8* has many round cells. *alb2* (Fig. 7B) and *alb4* (Fig. 7C)have internal cell layers in addition to the epidermis and vacular tissue, but the shapes and sizes of these cells are variable. In addition, no differentiation between palisade parenchyma and spongy mesophyll tissue is visible. In fact, inner cells of the leaves of *alb2* and *alb4* elongate parallel to the surface of the leaf, unlike Wt palisade cells which elongate perpendicular to the surface of the leaf. Leaves of *alb6* show the most normal morphology. The thickness of the leaf is even, and cell morphology is relatively normal, as elongated palisade and spongy mesophyll cells are distinguishable.

These severe leaf defects suggest that either early steps in plastid development are necessary for proper leaf structure (such as in the *dcl* mutant of tomato (Keddie et al., 1996) or the *dag* mutant of *Antirrhinum* (Chatterjee et al., 1996), or that the severe chloroplast phenotypes we have selected are the result of loss of a factor important in multiple aspects of plant development.

ALBINO genes encode cell autonomous and non-cell autonomous factors required for chloroplast biogenesis: *Arabidopsis* embryos form morphologically normal chloroplasts during embryogenesis that are evident to the eye in late stage embryos, which become very green (Mansfield and Briarty, 1991). Since the embryo develops within several cell layers which comprise the embryo sac, which are in turn inside the silique, it is unlikely that the levels of light and carbon dioxide normally required for sufficient fixation of carbon into sugars can penetrate the silique. Thus, the embryo must derive its carbon, water and mineral needs from the mother plant. This flow of nutrients from the mother plant to the embryo could also conceiveably carry other small molecules and metabolites from the mother plant to the embryo.

Figure 8A-D show confocal reconstructions of Wt, *cla1-1*, *alb6* and *alb8* embryos at the early bent cotyledon stage of development. Embryo morphology is visualized using a GFP fusion protein which is targeted to the plasma membrane, and chlorophyll autofluorescence is shown in red. The Wt embryo in Figure 8A shows high red fluorescence indicative of the chlorophyll in chloroplasts present throughout the embryo. Although all of the albino mutants presented in this work have been shown to have practically no chlorophyll during seedling development (see Table 3), during embryogenesis *cla1-1*, *alb6* and *alb8* mutant embryos accumulate significant amounts of chlorophyll (Figure 8B-D). *cla1-1* has recently been shown

to be a non-cell autonomous mutation, as cla1-1 seedlings can be complementated by growth on medium containing 1-Deoxyxylulose 5-Phosphate (DX), the product of the CLA1 enzyme (Estévez et al., 2000). Externally supplied DX is thus able to diffuse from the growth medium into the plant and substitute for the lack of DXS activity in the cla1-1 mutant. Diffusion of DX from the mother plant to cla1 embryos is also almost certainly responsible for the partial complementation of *cla*1 embryos. It is interesting to note that, unlike in Wt embryos which have greater amounts of chlorophyll in the cotyledons (Figure 8A), the non-cell autonomous *alb* mutants have relatively more chlorophyll autofluorescence in the hypocotyl than in the cotyledons. This may reflect the proximity of the hypocotyl to the micropylar end of the ovule, the presumed entry site of water and metabolites from the mother plant to the embryo. Since *alb*6 and *alb*8 also accumulate chlorophyll during embryogenesis, it is likely that this is also due to diffusion of the factor that is normally produced by the ALB6 and ALB8 gene products from the mother plant to the albino embryo, and which allows partial complementation of the mutant phenotype during embryogenesis.

As shown in Figure 8E-H, *alb*2, *alb*4, *alb*5 and *alb*7 do not produce any chlorophyll during embryogenesis, as no chlorophyll autofluorescence was detected in mutant embryos. It is likely that these genes code for the production of cell autonomous factors which must be synthesized within each cell for their normal function, or which cannot travel from the mother plant to the embryo.

DISCUSSION

Our analysis of the six *albino* mutants presented in this paper shows that these six *ALBINO* genes are required for very early steps of proplastid differentiation into chloroplasts. *albino* mutants have plastids which resemble proplastids, and have low or no expression of genes required for early chloroplast function and photosynthesis. *ALBINO* gene products are also directly or indirectly required for correct development of the mesophyll layers of the leaf, and for leaf morphogenesis. As demonstrated by observation during embryogenesis, the *ALBINO* genes can be classified as affecting either cell autonomous or non-cell autonomous processes. This work is the first systematic genetic study of genes required for early chloroplast differentiation.

ALBINO genes are required for early steps of proplastid differentiation Differentiation of the proplastid to a mature chloroplast occurs in several steps. As represented in simplified from in Figure 9, proplastids first increase in size, form single thylakoid membranes, and then eventually develop the combination of single and stacked membranes characteristic of stroma and grana thylakoids. This process is accompanied by the expression of certain nuclear and chloroplast encoded genes. Early expressed genes include those required for transcription and translation of genes required for chloroplast function, such as *rpl21* and *rrn16S*. At later steps in differentiation, *psb*A and *RBCS* genes which encode proteins involved in the light capture and carbon fixation steps of photosynthesis are expressed.

By considering both plastid morphology and the pattern of gene expression in each individual *albino* mutant, an ordered pathway for *ALBINO* gene expression required for plastid differentiation can be proposed (see Figure 9). Two lines of evidence support *ALB7* as the

earliest required gene. *alb7* plastids lack any kind thylakoid-like internal membranes, and display only large vesiculated structures. Second, *alb7* mutants do not express any of the probes characterized in this study, even the early probes *rrn16S*, *rpl21* and *accD* which are normally expressed during the early growth phase of the proplastid. The plastid and gene expression phenotype of *alb4* is very similar to *alb7*, except that *alb4* mutants express the *rrn16S* gene at low levels, suggesting that *ALB4* is required slightly after *ALB7*. Our data suggests that *ALB2* and *ALB5* function after *ALB7* and *ALB4*. Like *alb7* and *alb4*, *alb2* and *alb5* plastids lack thylakoid membranes, but both mutants express the early markers *rrn16S*, *rpl21* and *accD* at levels comparable to Wt, and express the late markers *RBCS* and *psbA* at low levels. ALB8 appears to be the next gene required. Plastids of *alb8* mutants have small thylakoid membranes, express all three early markers at levels similar to Wt and the late markers *RBCS* and *psbA* at relatively high levels. *alb6* mutants have the least severe phenotype, and thus *ALB6* is the last acting *ALBINO* gene. *alb6* plastids have significant, single thylakoid membranes, and express all three early markers at high levels, and *RBCS* and *psbA* at a similar level to *alb8*. Thus, our collection of albino mutants represents a series of genes which are required at discrete stages during a narrow window of early chloroplast differentiation.

ALBINO genes are required for formation and differentiation of mesophyll cells, and leaf morphogenesis The *albino* mutants described here all display abnormal leaf development, including absent or undifferentiated mesophyll tissue, irregularly shaped and enlarged cells, and altered overall morphology. A similar link between chloroplast biogenesis and leaf development has previously been observed in the *dag* mutant of *Antirrhinum*, the *dcl* mutant of tomato, the *pac* and *cla1* mutants of *Arabidopsis*, and *iojap* in corn. *dag* and *vdl* are both

albino mutants with chloroplasts arrested at an early stage of development, as plastids of both the mutants lack thylakoid membranes, and mutant sectors are lacking or severly reduced in development of mesophyll tissue. DAG, VDL and IOJAP appear to be required for chloroplast differentiation at an earlier stage than PAC, DCL and CLA1 since these latter mutants have some thylakoid membranes and also express RBCS genes, indicative of some chloroplast function. These mutants suggest that a signal derived from a normal functional chloroplast causes periclinal elongation of palisade cells to form the normal layer of palisade cell. It has also been shown that palisade cell periclinal expansion is affected by light quantity levels and hormone metabolism, as mutants which affect these processes show differences in leaf development (Pike K. and Lopez-Juez R. 1999)

It is interesting to observe that there is a good correlation between the severity of plastid differentiation and leaf development phenotypes in the *albino* mutants presented here. *alb7* is the most extreme with the earliest plastid arrest and the most deformed leaves, while the least severe mutant *alb6* has plastids which contain some thylakoid membranes, and leaves with semi-normal morphology and tissue differentiation. The other four *albino* mutants have plastid and leaf morphology intermediate between those of *alb7* and *alb6*. This correlation of plastid and leaf phenotypes suggests either that a functioning chloroplast is essential for leaf development, as has previously been proposed by (Reiter et al., 1994; Chatterjee et al., 1996; Keddie et al., 1996 and Wang et al., 2001) or that the *ALBINO* genes are independantly required for plastid differentiation and leaf development. The lower density of mesophyll cells in albino mutants reflects a premature cessation of cell division. It has been well documented that partitioning of organelles in eucaryotes is tightly coupled with the cell cycle (for review Warren and Wickner 1996). In yeast, several mutants in mitochondrial and vacuolar

inheritance have delayed cytokinesis and accumulate multiply budded cells (McConnell and Yaffe 1990; Xu and Wickner 1996). It have been show that mutations affecting chloroplast function can have strong effects on cell number and size within the leaf. The *Inhibitor of striate* gene in maize, encodes aprotein that is similar to the bacterial phosphatases that regulate carbon metabolism, and is thought to encode a chloroplast protein selectively supress cell proliferation within chlorotic stripes, indicating that this protein may play an important role in modulating cell proliferation within the leaf (Park et al., 2000).The lack of normal elongation in mesophyll cells of albino mutants could also be a direct effect of the physical lack of chloroplasts in developing mesophyll cells which then perturbs cell shape control during mesophyll cell expansion. A compensatory mechanism appears to function between the number of the chloroplast within a cell and their size, since a very tight correlation exists between the total amount of chloroplast area within the cell, measured as the product of chloroplast number and chloroplast size and cell size. In mature cells the chloroplast population can occupy up to 70% of the surface area of the cell and ~20% of total cell volume (Pyke, 1997).

ALBINO genes encode cell autonomous and non-cell autonomous factors During embryogenesis developing embryos rely on delivery of water, minerals and fixed carbon from the mother plant to the ovule. It is also conceivable that small metabolites, which are not normally required for development of Wt embryos, might also be delivered to the embryo. The *cla1* mutant has already been demonstrated to be a non-cell autonomous mutation (Estévez et al., 2000), as *cla1* seedlings can be rescued by supplementation of the missing factor in the growth medium. We demonstrate here that *cla1* embryos are partially complemented during embryogenesis, as shown by the formation of chloroplasts during development of *cla1* embryos. This complementation occurs with greater intensity in the hypocotyl of the embryo,

which is situated at the micropylar end of the ovule, at the entry point of maternal transport from the micropyle. Using this embryo complementation as a criteria, we demonstrate that *alb*6 and *alb*8 are also non-cell autonomous mutations, as they exhibit partial complementation during embryogenesis, while *alb*2, *alb*4, *alb*5 and *alb*7 are cell autonomous mutations which are not complemented during embryogenesis. It is interesting to note that *alb*6 and *alb*8 (as well as *cla*1), which exhibit complementation during embryogenesis, and also have the least severe seedling phenotypes in terms of chloroplast differentiation and leaf development. The weaker seedling phenotypes (compared to cell autonomous mutants) might be due to the partial rescue during embryogenesis. Seedling phenotypes might then be less severe either because having a number of functional chloroplasts during embryogenesis allows the production of all kinds of important compounds which are normally produced by the chloroplast or simply because a small amount of the missing factor supplied to the embryo remains during seedling growth and allows partial complementation during this stage also.

To our knowledge, this is the first time that complementation of a mutant by diffusion of maternal factors has been observed during embryogenesis. Conceivably, this could occur with any mutant which is lacking a molecule with properties which allow it to diffuse through the plant, including most plant hormones. If this is a general phenomenon, it could have important implications for the interpretation of mutants which appear not to express a mutant phenotype during embryogenesis.

albino mutants are a small class of pigment mutations

In our collection of pigment mutants, we found that only 9 mutant lines, or 9% of the total, have an albino phenotype where seedlings are almost totally lacking in green and yellow pigments. The percentage of *albino* mutants out of total pigment mutations has previously

been estimated to be much higher (Jürgens personal communication), but differences between growth conditions may explain the discrepancy in numbers. Jürgens et al grew seedlings on water-agar plates, without sucrose, vitamins or MS salts, which may exasperate the phenotype of mutants which we would classify as pale green or pale yellow. Also, these mutant lines were classified after only 5 days of growth. If anything, our estimation of the number of seedling pigment mutants which are albinos may be low, due to the fact that our mutant screen was conducted during embryogenesis, and thus more subtle pigment mutants, such as virescent mutants, would not be recovered by our screening technique.

Although the size of our mutant screen was relatively small, it is possible to make an estimate of the number of loci in Arabidopsis which mutate to an albino seedling phenotype. Previous screens with the M2 pools used for our albino embryo screen have shown that 2,000 M2 plants is approximately equivalent to a one-genome mutagenesis (W. Lukowitz, S. Gillmor, A. Roeder and C. Somerville, unpublished). This means that on average, each gene which mutates with average frequency will be represented by one mutant allele for every 2,000 M2 plants examined. Our screen comprised 1,000 EMS M2, 5,000 T-DNA lines and 30 pigment lines from the ABRC, and thus a one-half genome sampling is a reasonable estimate for the size of our screen. Since we found albino mutant alleles at 7 loci (including the *cla*1-2 allele we recovered), a conservative upper limit for the number of albino loci which would be recovered in a saturating screen might be 30, while our data suggests that the actual number might be closer to 20. Since all of our albino mutants have plastids which are arrested at very early steps of chloroplast differentiation, we propose that the number of genes required for early chloroplast differentiation is between 20 and 30.

This work demonstrates that genes required for proplastid growth and early differentiation, including the formation of the first thylakoid membranes of the chloroplast, can be recovered by screening for seedling mutants with an *albino* phenotype. Our characterization of albino mutants provides a conceptual framework for a more large scale genetic analysis of chloroplast biogenesis in Arabidopsis. The cloning of the *ALBINO* genes presented here will undoubtedly provide some of the first insights into the molecular basis of early plastid differentiation.

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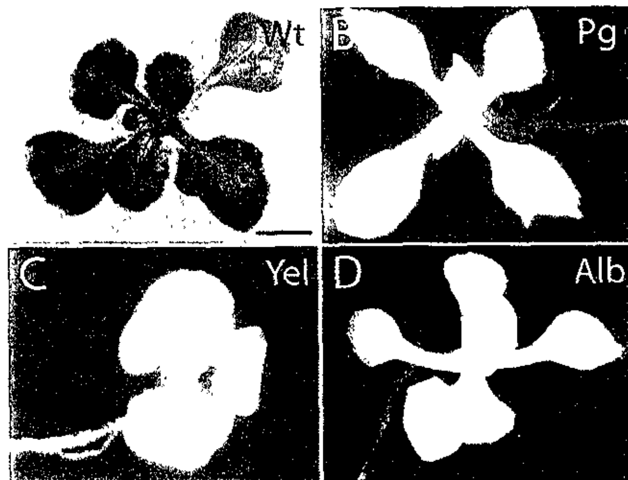


Figure 1

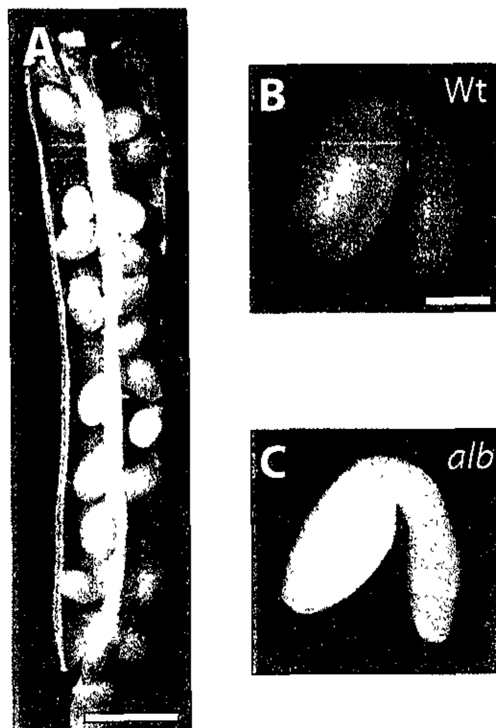


Figure 2

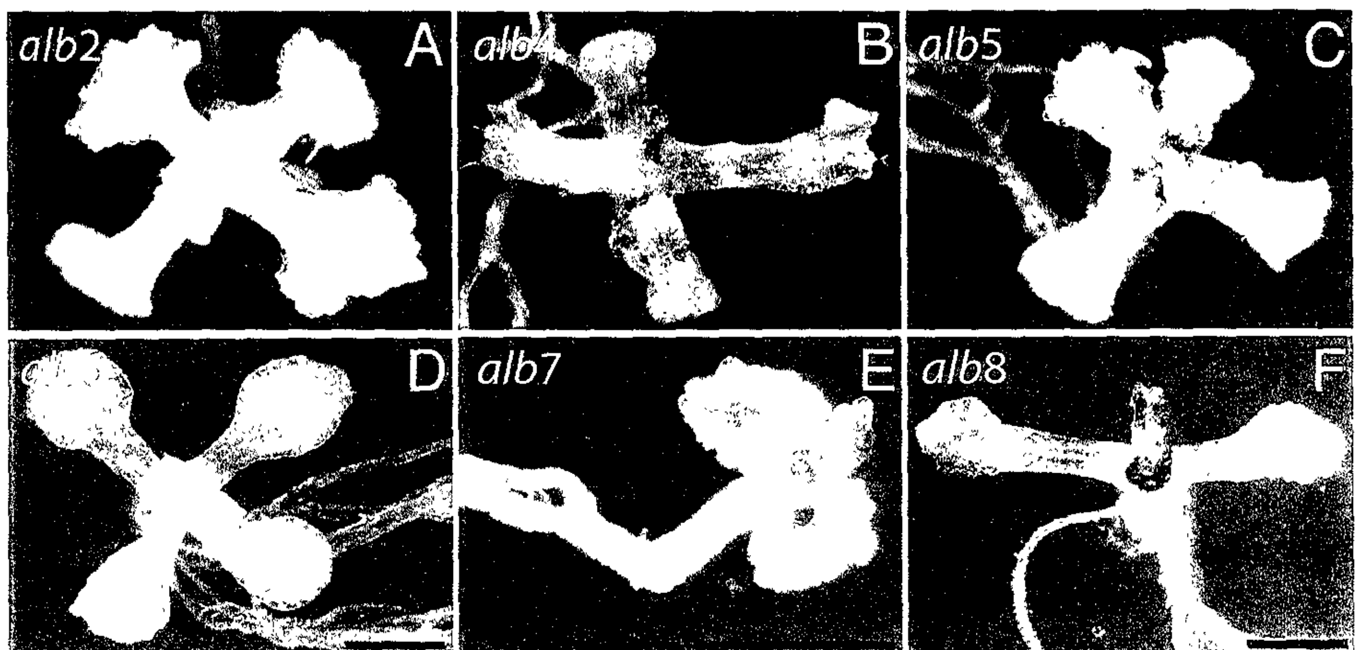
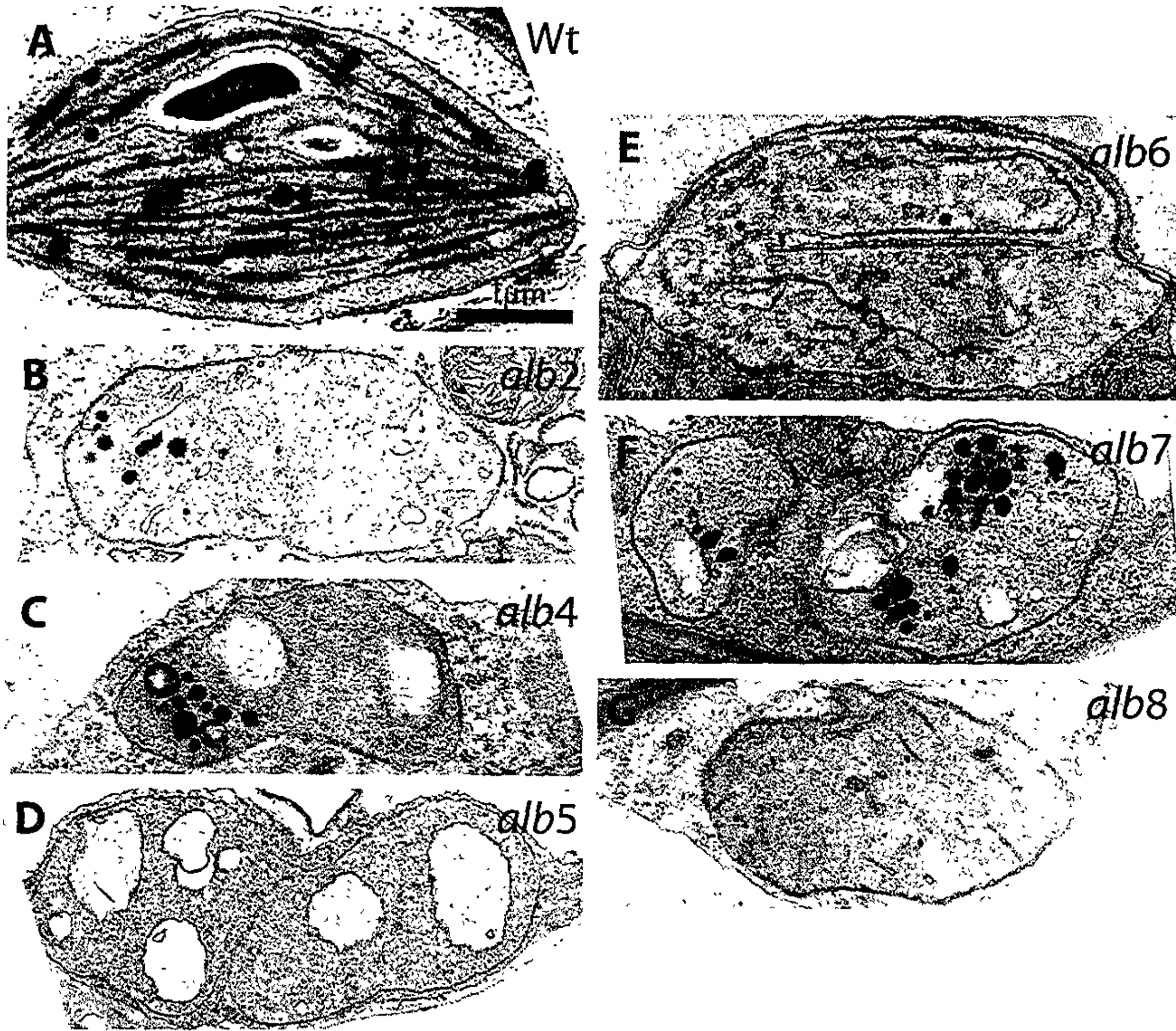


Figure 4



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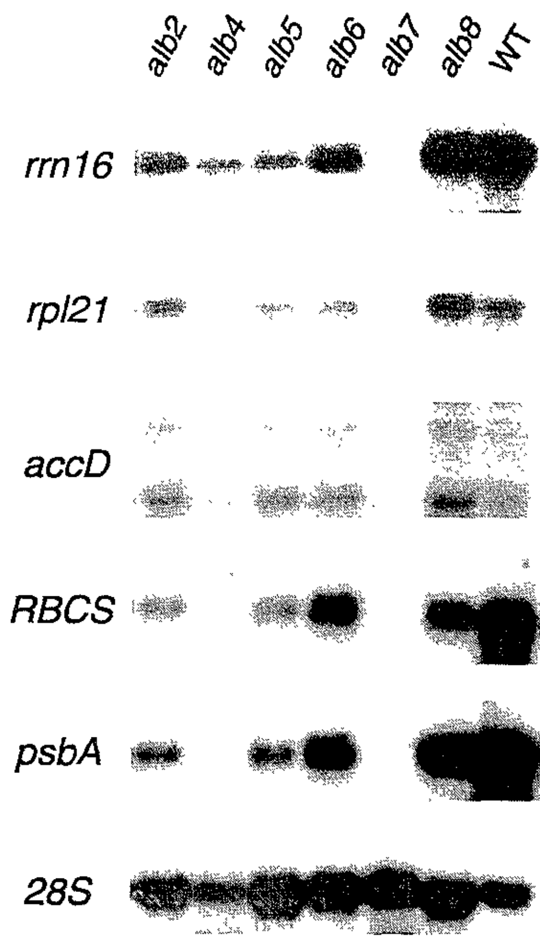

Figure 6

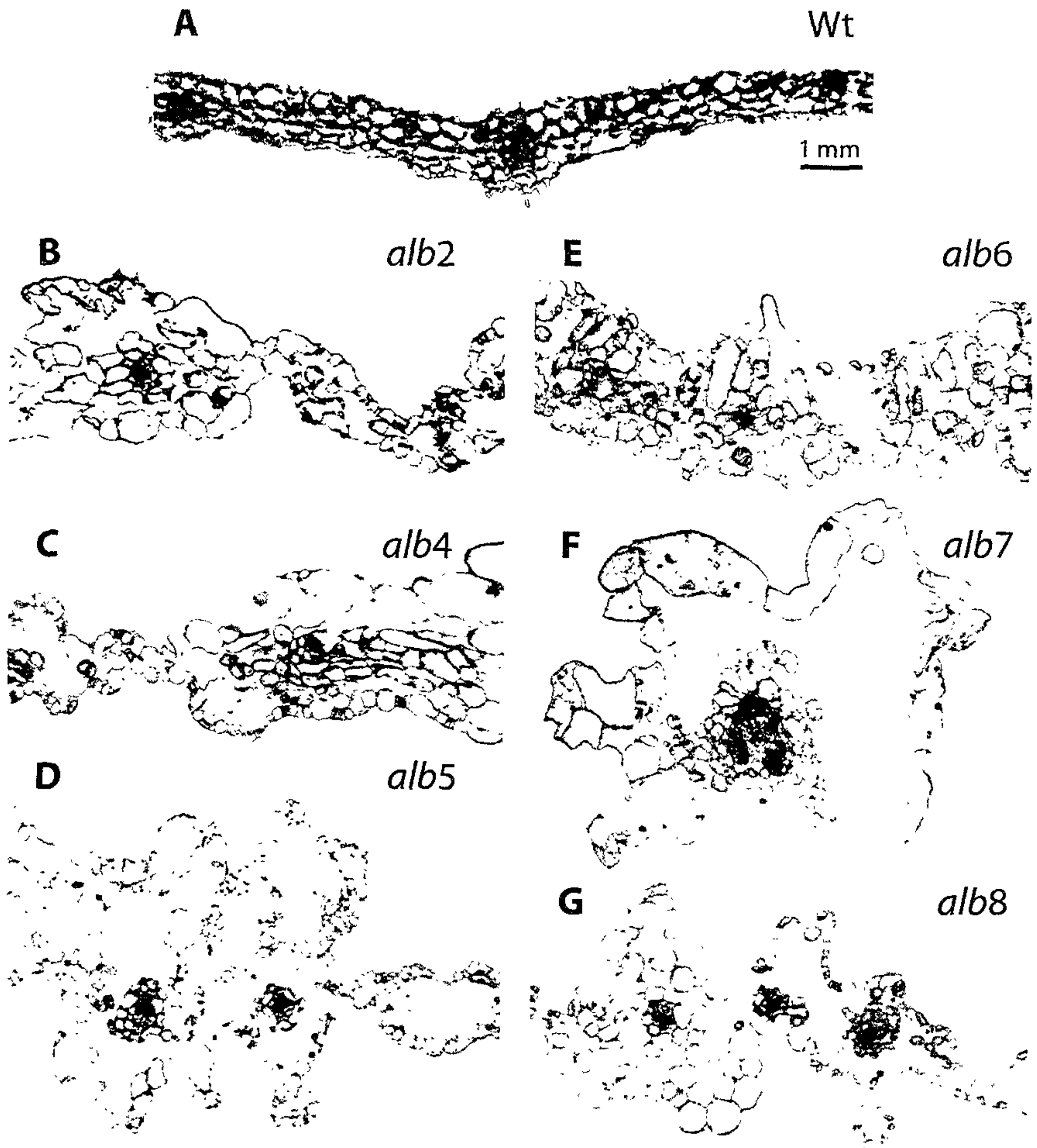

Figure 7

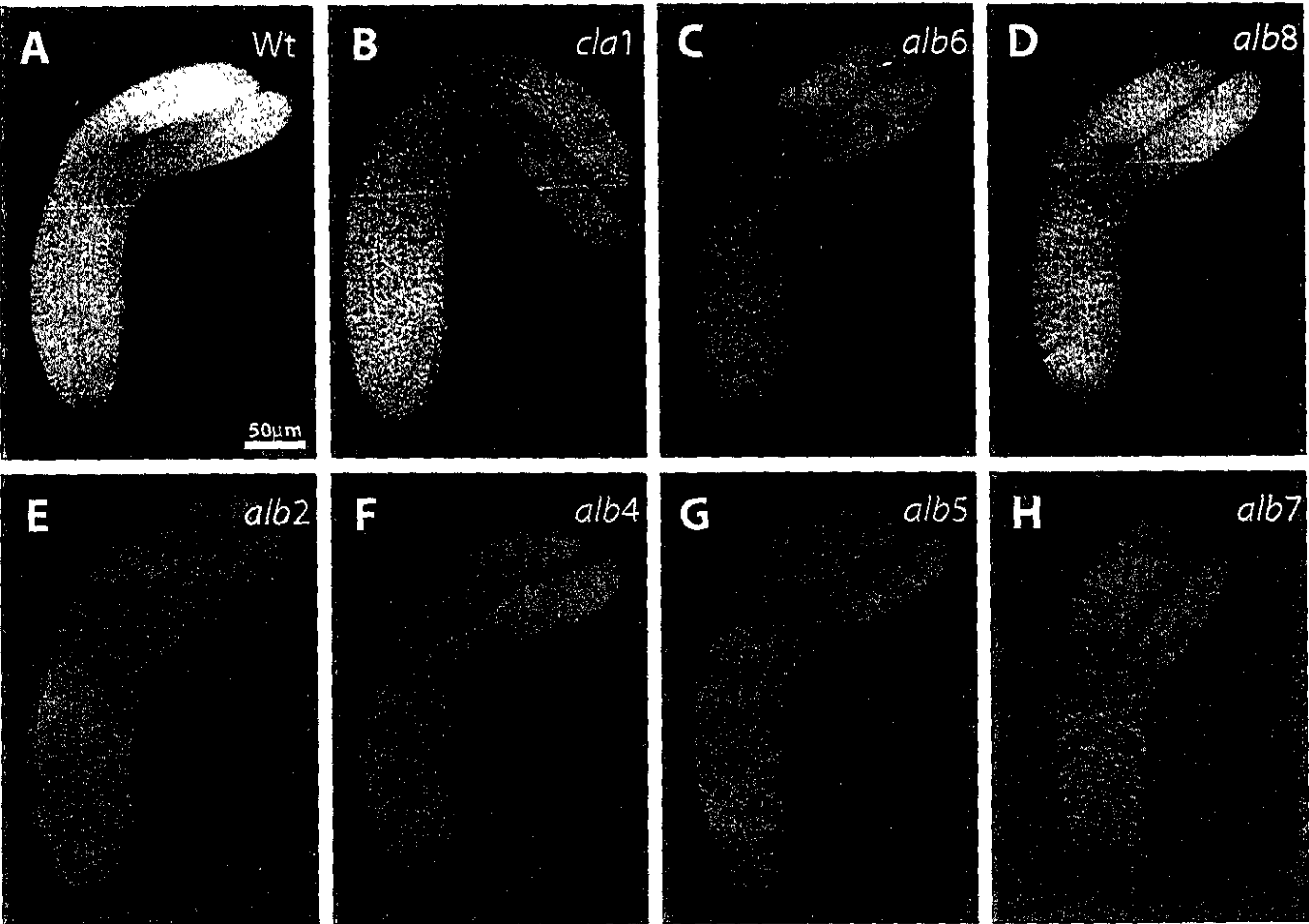

Figure 8

Figure 1. Phenotypic spectrum of pigment mutants. Representative lines for each phenotype are shown. Plants were grown on MS + 2% sucrose for 21 days. A wild type (Wt). B. Pale green (Pg). C yellow (Yel) and D albino (Alb).

Figure 2. A.silique from heterozygous plant for a pigment mutation. Aproximately 1/4 white embryos segregate. B Wild type embryon. C. *alb* embryon.

Figure 3. Map positions of *albino* mutants.

Figure 4. *albino* seedling morphology. Plants were grown on MS + 2% sucrose for 21 days.

Figure 5. Transmission electron microscopic (TEM) examination of plastids of wild-type and homozygous *albino* mutants. Plants were grown on MS + 2% sucrose for 21 days and the second leaf of a representative plant for each phenotype was fix for TEM analysis.

Figure 6.Northern analysis of plastid- and nuclear-encoded genes. Total RNA from *albino* and wild-type plants grown under standard conditions was fractionated on agarose gels, transferred to nylon membrane and hybridised with labelled probes for *rrn16*, *rpl21*, *accD*, *RBCS*, *psbA* and *28S*.

Figure 7. Light micrographs of cross sections of seedling leaves of *albino* mutants and wild-type plants. Plants were grown on MS + 2% sucrose for 21 days and the second leaf of a representative plant for each phenotype was fix for light microscopic analysis.

Figure 8. Confocal reconstruction of wild-type and *albino* embryos at the early bent cotyledon stage of development.

TABLE 1

Classification of pigment lines used in this study

Phenotype	Number
Albino	9
Pale green	61
Yellow	29
Total	99

TABLE 2

***albino* mutants described in this study**

allele	mutagen	ecotype	source
*cla*1-2	E	Dijon	ABRC
*alb*2-1	NMS	L*er*/L/D	ABRC
*alb*4-1	EMS	L*er*	T.S.
*alb*5-1	EMS	L*er*	T.S.
*alb*6-1	EMS	L*er*	T.S.
*alb*7-1	EMS	L*er*	T.S.
*alb*7-2	FN	L*er*	T.S.
*alb*7-3	T-DNA	C24	T.S.
*alb*8-1	EMS	L*er*	T.S.

Abbreviations used: E, Ethylenimine; NMS, Nitrosomethyl urea; EMS, Ethyl Methanesulfonate; FN, Fast neutron; L*er*, Landsberg *erecta;* D, Dijon; L, Limoges; ABRC, *Arabidopsis* Biological Resource Center; T.S., This study

TABLE 3

Relative amounts of chlorophyll A, chlorophyll B and carotenoids in albino mutants compared to Wt

	Chlorophyll A	Chlorophyll B	Carotenoids
*alb*2-1	0.5 ± 0.02 %	1.1 ± 0.02 %	3.5 ± 0.06 %
*alb*4-1	0.4 ± 0.01 %	0.6 ± 0.04 %	3.6 ± 0.13 %
*alb*5-1	1.0 ± 0.18%	1.3 ± 0.10 %	3.9 ± 0.35 %
*alb*6-1	1.5 ± 0.06 %	1.2 ± 0.003 %	12.9 ± 0.50 %
*alb*7-1	0.15 ± 0.02 %	0.3 ± 0.06 %	3.5 ± 0.12 %
*alb*8-1	1.5 ± 0.15 %	2.2 ± 0.50 %	9.6 ± 1.35 %

Resultados: segunda parte

Identificación de la inserción del T-DNA por TAIL-PCR en un gen predicho de una lipasa

De las mutantes albinas que se aislaron en este trabajo, el alelo *alb7-3* fué generado por la inserción de T-DNA. El análisis de segregación de la resistencia al antibiótico y los experimentos de hibridación DNA:DNA tipo *Southern blot* (figura 8) indicarón que existe una sola inserción del T-DNA la cual podría estar generando el fenotipo albino, por lo que se decidió clonar la secuencia genómica adyacente al T-DNA.

La secuencia genómica que flanqueaba el T-DNA fue aislada por el método Thermal Asymmetric Interlaced-PCR (TAIL-PCR) descrito por Liu Y.-G. et al 1995. En la figura 7 se muestra un mapa del T-DNA (Goddijn O. J. et al 1993) con que fué generada la mutante *alb7-3* así como la posición de los oligos utilizados para ubicar la zona de inserción. Los oligos degenerados que se utilizaron en este trabajo fueron AD2 y AD3 así mismo las condiciones de PCR fueron idénticas a las que se mencionan en Liu Y.-G. et al 1995.

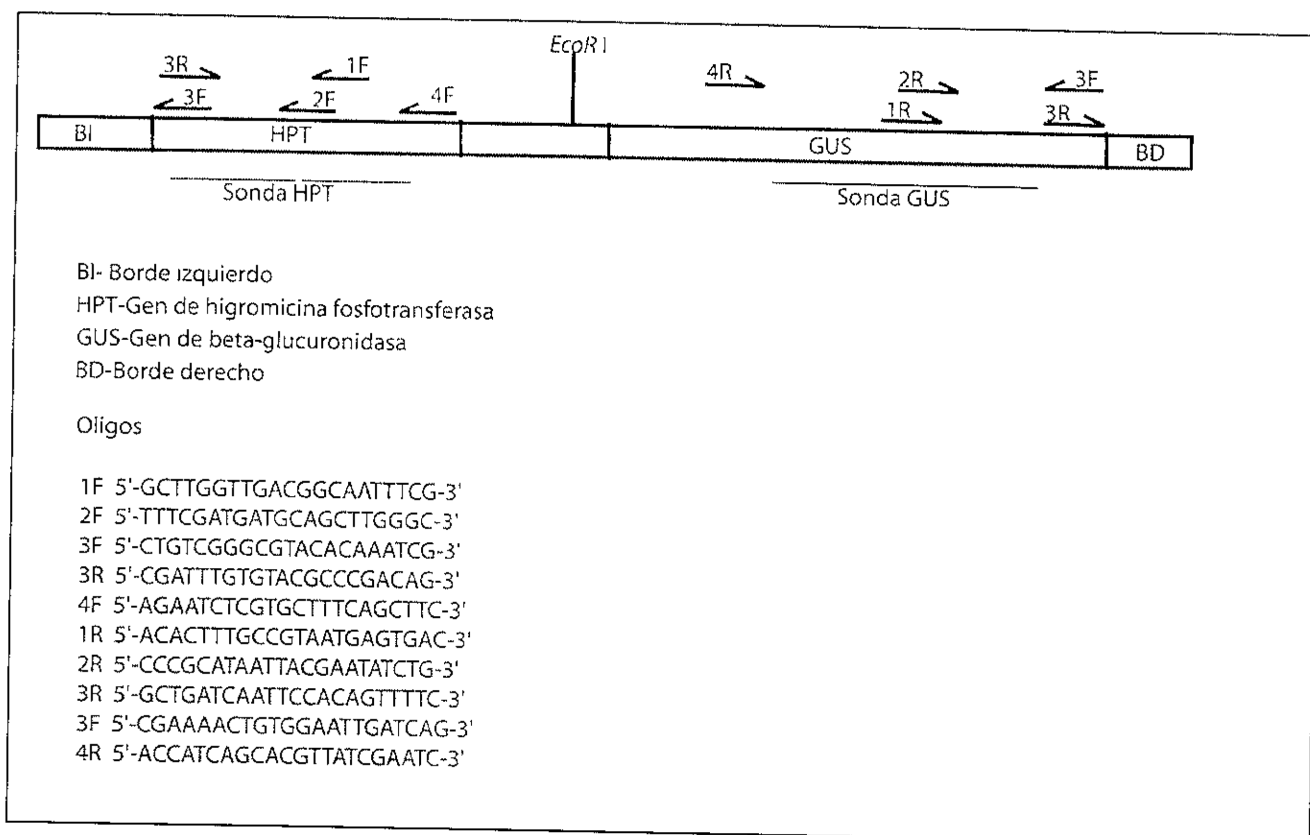

Figura 7. Mapa del T-DNA (pMOG55).

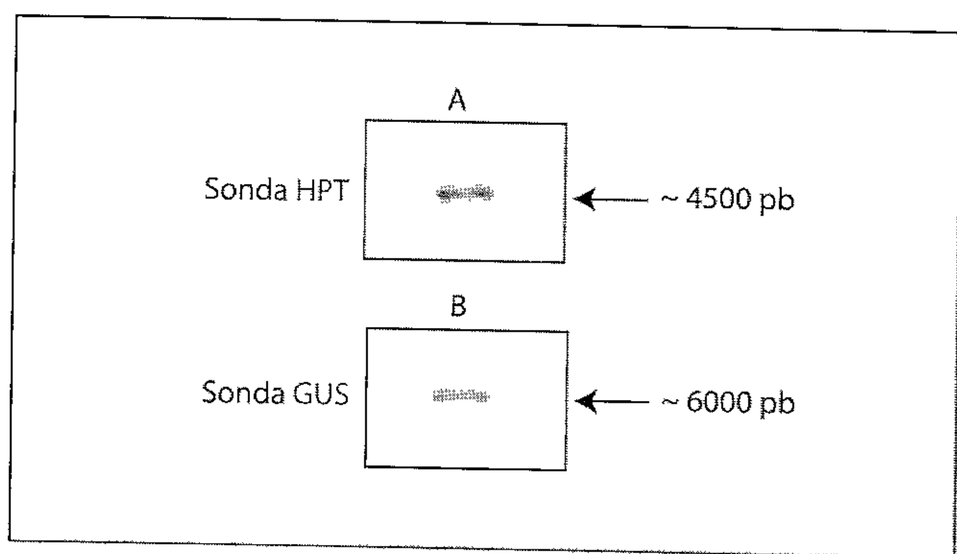

Figura 8. Análisis por *Southern* de la inserción del T-DNA.

Southern blot de 4 µg de DNA genómico de la mutante heteróciga *alb7-1* digerido con la enzima *Eco*R1. En el panel A se muestra la hibridación con la sonda HTP y en el panel B con la sonda GUS.

Cuando se compararon los resultados de la secuencia genómica aislada con la base de datos nos dimos cuanta que está se encuentra en el cromosoma 5, mientras que la mutante *alb7-1* se habia ubicado en el cromosoma III durante el mapeo de las mutantes con SSLPs (ver artículo), por lo que concluímos que la inserción del T-DNA no es la causa del fenotipo albino. Sin embargo, los resultados obtenidos son bastante interesantes. La inserción del T-DNA interrumpe la función de un gen que tiene similitud con el gen de una lipasa y se han reportado cuando menos 12 ESTs para este gen, lo que indica que éste se expresa abundantemente. En la figura 9 se muestra la secuencia del gene (no procesada y procesada) el cual hemos llamado 7815 así también se señala el sitio de la inserción del T-DNA. El análisis de la proteína predicha a partir de la secuencia nucleotídica, sugiere que está se localiza en el cloroplasto y en ella se identificó la secuencia consenso conservada para la familia de las lipasas: [LIV]-X-[LIVAFY]-[LIAMVST]-G-[HYWV]-S-X-G-[GSTAC] se piensa que la serina es el residuo activo (Hong, Y. W. et al 2000). En la figura 10 se muestra la secuencia de la proteína predicha y el motivo se señala con un rectángulo.

En las plantas los lípidos tienen una gran diversidad de funciones, son componentes estructurales de membranas (galactolípidos y fosfolípidos) así mismo protegen las membranas contra daños de radicales libres(tocoferoles), son compuestos activos en las reacciones de transferencia de electrónes durante la fotosíntesis (clorofila y otros pigementos), participan en la señalización celular (ácido abscísico, giberelinas y brasinosteroides y oxilipinas) entre otras

funciones. Considerando la importancia de los lípidos en plantas sería interesante caracterizar la mutante 7815 y tratar de establecer el papel de este gen en la vía metabólica de los lípidos.

```
1    [illegible]                                                                        7815 Genomic
1    [illegible]                                                                        7815 spliced

81   CCCAAATCGGTCAACT[illegible]TATTC[illegible]TCAAAAAATCCAG[illegible]TGATTCGAAC[illegible]AAA[illegible]CCGATGC[illegible] 7815 Genomic
81   CCCAAATCGGTCAACTTTGTTATTCTC[illegible]TTCAAAAAATCCAGAC[illegible]TGATTCGAACTGAAA[illegible]CCGATGCTTA[illegible] 7815 spliced

161  TTGTTGGTGAGAATCATGACTTGG[illegible]CCAGAAGAGGAGAA[illegible]CAACGTCAAAGG[illegible]TTTGATTCCTGGTTTGGCT 7815 Genomic
161  TTGTTGGTGAGAATGATGACTTGG[illegible]CCAGAAGAGGAGAATCAACGTCAAAGG[illegible]TTTGATTCCTCCTTTGCC[illegible] 7815 spliced

241  GATCAATCAAATGCTGAAATTGCTCCTCCAATCAGTCATTCTCAC[illegible]GAGTGGAAGCCCAAGCTTAGAGTACATTATGA 7815 Genomic
241  GATGAATCAAATGGTCAAATTGCTGCTCCAATCAGTCATTCTCACTGGAGTGGAAGCCCAAGCTTAGAGTACATTATGA 7815 spliced

321  GAAAGCCGGTTGTGACAATCTCCATGCTCCTCGGTGTTGTTTCTTCCTGGCTTTGGCGTTGGTTCATTTCACTATGAGA 7815 Genomic
321  GAAAGCCGGTTGTGACAATCTCGATGCTCCTCGGTGTTGTTTCTTCCTGGCTTTGGCGTTGGTTCATTTCACTATGAGA 7815 spliced

401  AGCACCTTACCCATTTGGGAAGCCATTATCCACTATGGCCTATTGATTTTCTTGGACAGGGTTTATCTCTCCCTACTGAA 7815 Genomic
401  AGCACCTTACCGATTTCGCAAGGGATTATCCAGTATGGCCTATTGATTTTCTTGGACAGGGTTTATCTCTCCCTACTGAA 7815 spliced

481  GATCCTACTACCATGACTGAAGAAACCAGTTCCTCGGAACATAAGGAACCATTTTGGGGATTTGGTGACAAAACTGAACC 7815 Genomic
481  GATCCTACTACCATGACTGAAGAAACCAGTTCCTCGGAAGATAAGGAACCATTTTGGGGATTTGGTGACAAAACTGAACC 7815 spliced

561  GTGGGCTGATCAACTTGTATTCTCTCTGGATCTCTGGAGGGATCAAGTTCAGTATTTTGTAGAAGAGGTTAGCAATATCT 7815 Genomic
561  GTGGGCTGATCAACTTGTATTCTCTCTGGATCTCTGGAGGGATCAAGTTCAGTATTTTGTACAAGAGGTTA--------- 7815 spliced

641  GAGTTCAGTTGTTCTATTACTCTTTAAGCTGTTGATTTTGTTCTTTTAGTCGTAATTTCAAAAGCAGTGTGTTAGCTTTT 7815 Genomic
632  -------------------------------------------------------------------------------- 7815 spliced

721  TTTTTTTAGTGTACATTGTTCCTATGTGTCCTATACTCATCGATTTCAATAGTTTTCAGCTCAGAACCTGAAAGTGTGCG 7815 Genomic
632  -------------------------------------------------------------------------------- 7815 spliced

801  TATGGAGTCTTCTGGTGTTGCTTTGGGAATCGGAAAGTTTAGGATGCACTAGATATTAGAATTCATCTCTGTTCTTTGAT 7815 Genomic
632  -------------------------------------------------------------------------------- 7815 spliced

881  TCCCTTTGTGTTCTAGTTTGAGATCACGATCTTTTTCATCTGAAATAAGCTGCTGAGTCGGTAAGAGTATGTGGTATTTG 7815 Genomic
632  -------------------------------------------------------------------------------- 7815 spliced

961  TGTTGTACAGGTTATCGGTGAGCCTGTGTACATTGCAGGGAACTCACTTGGAGGGTATGTAGCTCTCTACTTTGCAGCAA 7815 Genomic
632  ----------------TCGGTGAGCCTGTGTACATTGCAGGGAACTCACTTGGAGGGTATGTAGCTCTCTACTTTGCAGCAA 7815 spliced

1041 CCCATCCTCACCTGGTTAAGCGTCTTACCTTGCTTAATGCAACACCTTTCTGGGCTTTCTTCCCTAATCCAGTAAGATCC 7815 Genomic
698  CCCATCCTCACCTGGTTAAGGGTGTTACCTTGCTTAATGCAACACCTTTCTGGGGTTTCTTCCCTAATCCAGTAAGATCC 7815 spliced

1121 CCAAAGCTAGCACGTCTCTTTCCATGGCCCGGAGCATTCCCTCTGCCGGAAAGAGTGAAAAAAATCACAGAATTGGTGTA 7815 Genomic
778  CCAAAGCTAGCACGTCTCTTTCCATGGCCCGCAGCATTCCCTCTGCCGGAAAGAGTGAAAAAAATCACAGAATTGGTGT- 7815 spliced

1201 AGGCAGACTTTATTAACACATCTCCTCACCTCAAAACTATCATTTTCTATTTCTATCTCTATCAGTCTAATATCTTTACT 7815 Genomic
857  -------------------------------------------------------------------------------- 7815 spliced

1281 TCCCAATGAAACTGCAGGTGGCAAAAGATAAGTGATCCTGAAAGCATAGCTGAGATACTTAAACAGGTCTACACAGACCA 7815 Genomic
857  ------------------GGCAAAAGATAAGTGATCCTGAAAGCATAGCTGAGATACTTAAACAGGTCTACACAGACCA 7815 spliced

1361 TTCTATCAATGTGGATAAAGTATTCTCACGTATTGTGGAGGTCACACAGCATCCGGCTGCTGCAGCATCGTTTGCTTCAA 7815 Genomic
918  TTCTATCAATGTGGATAAAGTATTCTCACGTATTGTGGAGGTCACACAGCATCCGGCTGCTGCAGCATCGTTTGCTTCAA 7815 spliced

1441 TCATGCTTGCTCCTGGTGGAGAGCTATCTTTCTCCGAAGCTTTATCTAGGTATAAGACAGTGTGGTTTCTTGTTTTTATA 7815 Genomic
998  TCATGCTTGCTCCTGGTGGAGAGCTATCTTTCTCCGAAGCTTTATCTAGGT----------------------------- 7815 spliced

1521 TGTTTTTTACTCTGCGTGATTGGTGTTATTGCTTATGAGGAATGCTGCTATTTGATGGTTTATGCTAGGTGTAAGGAAAA 7815 Genomic
1049 ----------------------------------------------------------------------GTAAGGAAAA 7815 spliced

1601 CAATGTTCAGATATGTCTCATGTATGGAAGAGAAGATCCATGGGTGAGACCGTTATGGGGAAAGAAGATAAAGAAGGAAA 7815 Genomic
1059 CAATGTTCAGATATGTCTCATGTATGGAAGAGAAGATCCATGGGTGAGACCGTTATGGGGAAAGAAGATAAAGAAGGAAA 7815 spliced

1681 TCCCCAACGCTCCATACTACGAGATCAGCCCAGCGGGTCACTGCCCACACGATGAAGTCCCTGAGGTATAATGAACTAGT 7815 Genomic
1139 TCCCCAACGCTCCATACTACGAGATCAGCCCAGCGGGTCACTGCCCACACGATGAAGTCCCTGAGGT------------- 7815 spliced

1761 CAATAAATTCTTCAAACTCTGCAATTAGGTATAATGAACCCACCATTTGGGATATGAAAACGAGCAGGTGGTGAACTATC 7815 Genomic
1206 ----------------------▼ Inserción del T-DNA ----------------------GGTGAACTATC 7815 spliced

1841 TGATGCGCGGGTGGATCAAGCACCTGGAGTCTGGTGGTTTTGAAGCGCTCCCGCTTTTGGAGGACACTGAAGAAGATTGG 7815 Genomic
1217 TGATGCGCGGGTGGATCAAGCACCTGGAGTCTGGTGGTTTTGAAGCGCTCCCGCTTTTGGAGCACACTGAAGAAGATTCG 7815 spliced

1921 GAGGAGTCCAGGATTGGTAGAGAAATTGAGTTCCCGAGAGATGGTTGGAAAAAAGCAGTGAATCTGTGGTTATATGGGTC 7815 Genomic
1297 GAGGAGTCCAGGATTGGTAGAGAAATTGAGTTCCCGAGAGATGGTTGGAAAAAAGCAGTGAATCTGTGGTTATATGGGTC 7815 spliced

2001 AAACTATACGTACTGGAGAGGAGTTAGAGAATCTTTCAGATCCAGTTTTATAAGGGTGTTTGGAGGGAAGTCTGCATAGA 7815 Genomic
1377 AAACTATACGTACTGGAGAGGAGTTAGAGAATCTTTCAGATCCAGTTTTATAAGGGTGTTTGGAGGGAAGTCTGCATAG  7815 spliced

2081 AGAAGCATGGAACAGTCGTCTAGTGTAAATTAATTGTAAT                                         7815 Genomic
1455                                                                                   7815 spliced
```

Figura 9. Secuencia no procesada y procesada del gen 7815

MEHSLNVVPQCSVVTWSSKLATKRLVPNRSSLLFSGVKKSRLVIRSGNSDGYVVGENDDLG
RIARRGESTSKVLIPGLPDESNGEIAARISHSHCEWKPKLRVHYEKAGCDNLDAPAVLFLPGF
GVGSFHYEKQLTDLGRDYRVWAIDFLGQGLSLPTEDPTTMTEETSSSEDKEPFWGFGDKTEP
WADQLVFSLDLWRDQVQYFVEEVIGEP[VYIAGNSLGG]YVALYFAATHPHLVKGVTLLNAT
PFWGFFPNPVRSPKLARLFPWPGAFPLPERVKKITELVWQKISDPESIAEILKQVYTDHSINVD
KVFSRIVEVTQHPAAAASFASIMLAPGGELSFSEALSRCKENNVQICLMYGREDPWVRPLWG
KKIKKEIPNAPYYEISPAGHCPHDEVPEVVNYLMRGWIKHLESGGFEALPLLEDTEEDWEES
RIGREIEFPRDGWKKAVNLWLYGSNYTYWRGVRESFRSSFIRVFGGKSA

Figura 10. Secuencia de la proteína predicha para el gen 7815.

En el rectángulo se señala la secuencia consenso de los 10 aminoácidos característicos de lipasas de animales, bacterias y hongos.

Identificación de la inserción del T-DNA de la mutante 19,20 en un gen PPR

Al inicio de este proyecto en la etapa de la selección de las mutantes observamos que una de ellas, la cual llamamos 19,20 presentaba un fenotipo bastante interesante. Cuando esta mutante es crecida en medio GM suplementada con 2% de sacarosa, la plántula tiene un fenotipo amarillo que se torna verde pálido después de aproximadamente 10 días. Esto sugiere que el gen mutado puede estar regulado durante el desarrollo. Esta mutante fue generada por la inserción de T-DNA y el análisis de la segregación del fenotipo con la resistencia al antibiótico higromicina indica que existe una sola inserción del T-DNA. Sin embargo en el *Southern blot* con las sondas del borde derecho e izquierdo se observan varias bandas, esto se puede deber o bien a que ocurrió un rearreglo del T-DNA durante la transformación, como a que existe más de una inserción del T-DNA. Para descartar esta última posibilidad actualmente se esta realizando el análisis de cosegregación del T-DNA con el fenotipo en la progenie de 100 plantas proveniente de un solo individuo inicial.

Para clonar la secuencia genómica adyacente al T-DNA se construyó un sub-banco genómico de la mutante. Se digirieron 4 μg de DNA con la enzima *Hind* III, se corrió en un gel por electroforésis y se cortó el fragmento de agarosa del tamaño en donde hibrida el borde izquierdo, previamente visto en los análisis por *Southern blot*. El DNA se eluyo por electroforesis y se clonó en el vector Lambda ZAP Express® (Stratagene). Una de las clonas que se aisló contiene un fragmento genómico de 140 pb adyacente al borde izquierdo. Esta secuencia fue comparada con el banco de datos. En la figura 11 se muestra la secuencia del gen con el cual se encontró 100 % de similitud en el genoma de Arabidopsis, asi mismo se señala el sitio de inserción del T-DNA (este gen no contiene intrones). En la figura 12 se

muestra la proteına predicha para dicho gen, la cual contiene tres motivos PPR (pentatricopeptide repeat)

PPR es una secuencia degenerada de 34 aminoácidos identificada en una amplia variedad de proteínas presente en tandem de 3-16 motivos los cuales están involucrados en interaciones proteína-proteína. En Arabidopsis existe una familia de aproximadamente 200 genes que contienen tándems de este mismo motivo. Dos tercios de estas proteínas estan destinadas al cloroplasto o mitocondria, pero hasta la fecha la función de ninguna de ellas ha sido caracterizada. En otros organismos solo se han caracterizado tres genes que contienen este motivo, en maíz el gene *crp*1 (Barkan, A. et al 1994) que es un miembro de esta misma familia. Una secuencia similar es encontrada en PET309 de *Saccharomyces cerevisiae* (Manthey, G.M. et al 1995)y *cya*-5 de *Neurospora crassa* (Coffin, J.W. et al 1997). Interesantemente estos 3 genes codifican para proteínas involucradas en el procesamiento o traducción de RNAs mensajeros de cloroplasto o mitocondria. El hecho que *crp*1 tiene un fenotipo verde pálido, sugiere que los miembros de la familia PPR no son redundantes y que estas proteínas se unen específicamente a ciertas secuencias. (Small. I. D. y Peeters, N. 2000).

Sería interesante análizar si el gen 19,20 tiene una función parecida al de los genes ya descritos; más experimentos son necesarios para especificar el papel fisiológico de este gen y corroborar finalmente que el fenotipo es debido a la mutación de dicho gen.

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ATGGGTCTCCTTCCCGTCGTCGGAATTACTTCGCCGGCGCTCATCACCCACAAAAATCACGCCAATCCAAAGATAC
AGAGACATAATCAATCTACCTCCGAGACCACTGTTTCATGGACTTCTCGCATCAATCTCCTCACGCGCAATGGTCG
ATTAGCGGAGGCAGCGAAGGAATTCTCCGATATGACACTCGCCGGCGTAGAGCCTAACCACATCACTTTCATAGCT
CTACTCTCCGGGTGTGGTGATTTTACCTCCGGAAGTGAAGCCTTGGGCGATTTGCTTCATGGGTATGCTTGTAAACT
TGGTCTTGATAGAAACCATGTCATGGTTGGCACCGCAATTATCGGCATGTACTCCAAACGCGGCCGTTTTAAGAAG
GCTAGATTGGTTTTTGATTACATGGAAGATAAAAATTCGGTTACTTGGAATACAATGATCGATGGGTACATGAGAA
GTGGTCAAGTCGATAACGCTGCTAAGATGTTCGACAAAATGCCTGAACGAGACTTGATTTCTTGGACGGCTATGAT
AAATGGCTTTGTTAAGAAAGGTTATCAAGAGG ▽ AAGCTTTGTTATGGTTCCGTGAAATGCAGATTTCTGGAGTAA
AACCAGATTACGTTGCTATTAGCTGCTCTTAACGCTTGCACAAACCTTGGTGCTCTCTCATTTGGATTATGGGTACA
TCGTTATGTTTTGAGTCAGGATTTCAAGAACAATGTGAGGGTGAGTAATTCACTGATCGATTTGTATTGTCGATGCG
GGTGTGTGGAGTTTGCTCGACAAGTTTTTTACAACATGGAGAAACGAACCGTAGTTTCGTGGAATTCAGTCATTGTT
GGTTTTGCTGCTAATGGAAATGCACATGAATCATTAGTCTACTTCAGGAAAATGCAAGAGAAAGGCTTTAAACCTG
ACGCAGTCACTTTCACTGGGGCGCTTACCGCGTGTAGCCATGTAGGTCTAGTTGAAGAAGGTCTTCGATATTTTCA
AATTATGAAATGTGATTACAGAATCTCGCCTCGAATTGAGCATTATGGATGCTTAGTGGATTTGTATAGTCGGGCT
GGGAGATTGGAAGACGCTTTGAAGTTGGTGCAGAGCATGCCAATGAAGCCAAATGAAGTTGTGATTGGATCGTTG
CTTGCAGCTTGCAGCAATCATGGGAACAATATCGTCTTAGCAGAGAGGCTGATGAAGCATCTTACCGACCTGAATG
TGAAAAGCCATTCAAATTATGTGATTCTCTCGAATATGTATGCTGCTGATGGAAAATGGGAAGGAGCAAGTAAGAT
GAGAAGGAAGATGAAGGGTCTCGGTCTAAAAAAGCAGCCTGGGTTTAGTTCGATAGAGATTGATGATTGCATGCA
TGTGTTCATGGCCGGTGACAATGCCCATGTTGAGACCACTTATATCCGCGAGGTCCTGGAGCTTATTTCTTCTGATT
TGCGATTACAGGGCTGTGTAGTTGAAACCCTTGCTGGTGATCTCCTCAATGCTT

Figura 11. Secuencia del gen 19,20, ▽ señala el sitio de la inserción del T-DNA

AKEFSDMTLAGVEPNHITFIALLSGCGDFTSGSEALGDLLHGYACKLGLDRNHV
MVGTAIIGMYSKRGRFKKARLVFDYMEDKNS
ISWTAMING [illegible] KKGYQE [illegible] LWFREMQISGVKPDYVAIIAALNA
CTNLGALSFGLWVHRYVLSQDFKNNVRVSNSLIDLYCRCGCVEFARQVFYNME
KRTVVSWNSVIVGFAANGNAIESLVYFRKMQEKGFKPDAVTFTGALTACSHV
GLVEEGLRYFQIMKCDYRISPRIEHYGCLVDLYSRAGRLEDALKLVQSMPMKPN
EVVIGSLLAACSNHGNNIVLAERLMKHLTDLNVKSHSNYVILSNMYAADGKWE
GASKMRRKMKGLGLKKQPGFSSIEIDDCMHVFMAGDNAHVETTYIREVLELIS
SDLRLQGCVVETLAGDLLNA

Consenso PPR .TYNALINAYAK.G..EEA..LY..M...G..PN.

Figura 12 Proteína predicha para el gen 19,20.
Los motivos PPR estan señalados con diferentes colores, al final se muestra la secuencia consenso para el motivo PPR.

Discusión.

El desarrollo del cloroplasto se ha estudiado desde diferentes puntos de vista. El trabajo clásico sobre la biogénesis del cloroplasto durante los años 1960 a 1970 fue en su mayoría descriptivo, principalmente basado en los cambios que ocurren en la ultraestructura del plastidio durante las diferentes fases del desarrollo del organelo (Robertson D y McMillan Laetsch 1974). Fue en estos años que se estableció que en las hojas de plantas monocotiledoneas había un gradiente de diferenciación del plastidio a lo largo de la hoja. Entre los años de 1960 a 1980 por estudios bioquímicos se obtuvo un avanze substancial en el conocimiento a cerca del proceso de la fotosíntesis y de las proteínas componentes de la membrana fotosintética. También en este periodo algunos de los genes que codifican para proteínas involucradas en la fotosíntesis fueron identificados por la secuenciación parcial de aminoácidos (Haley, J. y L. Bogorad, 1989).

Uno de los trabajos pioneros en estudiar genes nucleares involucrados en el desarrollo del cloroplasto en plantas superiores fue iniciado por William Taylor, Alice Barkan y Donald Miles. El grupo de A. Barkan es uno de los únicos grupos que ha hecho un estudio sistemático del desarrollo del cloroplasto. Este grupo ha utilizado mutantes de maíz generadas por la inserción de transposones *Mu* para identificar mutantes afectadas en el transporte de electrones fotosintéticos. Estas mutantes presentan una fluorescencia alta de la clorofila por lo que se les ha llamado *hcf* (*h*igh chlorophyll *f*luorescence) (Barkan, A. et al., 1986; Barkan, A. et al., 1995).

En los últimos años se ha reportado un número pequeño de mutantes que afectan estadios tempranos de la biogénesis del plastidio. La clonación de algunos de estos genes ha iniciado a dar luz de los diferentes factores requeridos para la biogénesis de este organelo. Por ejemplo, el aislamiento de CLA1 (Estévez, J. M. et al., 2000) ha mostrado que la vía de los isoprenoides es importante para el desarrollo del cloroplasto. Sin embargo, no ha habido un estudio sistemático de mutantes que estén afectadas en la biogénesis temprana del cloroplasto.

Los genes ALBINO son requeridos para la diferenciación temprana del proplastidio y el desarrollo de diferentes tejidos de la hoja

Este trabajo representa un avance significativo en el conocimiento de la biogénesis del plastidio por diferentes razones. Utilizando como criterio la ausencia de pigmentos en las mutantes (observadas en el microscopio de disección) hemos seleccionado un grupo de aproximadamente 100 mutantes. Los fenotipos de estas mutantes se han clasificado de acuerdo a la presencia de pigmentos en verde pálido, amarillas y albinas. Interesantemente de estas mutantes sólo un pequeño grupo son albinas (aproximadamente el 10%). Dado que estamos interesados en estudiar biogénesis temprana del clorolasto nos hemos enfocado a estudiar el grupo de las 6 mutantes albinas (las cuales hemos llamado *alb*). Para estas mutantes llevamos a cabo una cuidadosa caracterización fenotípica, los datos de este análisis (principalmente la expresión de genes y las imágenes de microscopia electrónica de los plastidios) mostraron que existe un gradiente de severidad entre ellas. Las mutantes *alb* tienen un fenotipo más severo que cualquiera de las mutantes albinas publicadas anteriormente sin que sean embriones letales y representan el grupo de albinas más grandes hasta la fecha reportado

Un aspecto importante de este trabajo es que provee evidencia de que existe un vínculo entre la diferenciación del cloroplasto y el desarrollo de la hoja ya que en todas las mutantes albinas que se analizarón, se observa la ausencia de células del mésofilo.

Los genes ALBINO codifican factores autónomos y no autónomos de la célula.

Este estudio muestra elegantemente que las mutantes albinas analizadas estan afectadas en factores que pueden ser autónomos o no autónomos de la célula; lo que permite diferenciar (especular o conocer) que clase de genes están afectados en las diferentes mutantes. Por ejemplo si uno está interesado en la biosíntesis de hormonas o en metabolitos hechos en el cloroplasto uno puede enfocarse en las mutantes no autónomas de la célula. Por el contrario, si se tiene interés en estudiar regulación de la transcripción del plastidio uno puede enfocarse a estudiar las mutantes autónomas de la célula. Por otro lado, nadie ha mostrado que una mutación que cause defectos en el embrión pueda ser parcialmente complementada por productos maternales durante la embriogénesis. El hecho de que el embrión en Arabidopsis desarrolle cloroplastos durante la embriogénesis es una característica única que permite visualizar esto facilmente. Pero tal vez esto pueda ser un principio general para mutaciones que afectan embriogénesis si ellas están afectadas en factores capaces de difundirse; en este tipo de mutantes podría haber un rescate parcial de la mutación durante la embriogénesis que hace que el fenotipo sea menos severo de lo que realmente es. Así muchas mutantes que presentan un fenotipo anormal durante las etapas tempranas de la plántula, pero no durante embriogénesis, podría deberse a que existió una rescate maternal de la mutación

Las mutantes *albinas* son un pequeño grupo.

Aún cuando el tamizado que se realizó en este trabajo es relativamente pequeño, éste se hizo con un banco equilibrado de semillas mutagenizadas. En un tamizado previo de 12000 plantas M2 (provenientes del mismo grupo de semillas usadas para nuestro estudio), Lukowitz W. y Gillmor S. aislaron un promedio de 6 alelos para 5 genes diferentes, esto muestra que 2000 plantas M2 equivalen aproximadamente a un genoma mutagenizado; por lo que podemos predecir que hemos tamizado la mitad de un genoma. Dado que encontramos sólo 7 mutantes albinas (incluyendo *cla1*) esto sugiere que el número de genes que mutan y dan un fenotipo albino es alrededor de 20. Éste es un número de genes razonables con el que se puede empezar a entender las bases moleculares de la biogénesis del cloroplasto.

Con respecto a la clonación del gen de la mutante 19,20 todavía se requiere una corroboración estrícita de que el fonotipo de la mutante es causado por el que se aisló. Sin embargo la naturaleza del gen aislado nos permite hacer ciertas especulaciones ya que este gen contiene secuencias con homología del gen *crpl* de maíz. En maíz CRP1 participan en la traducción y procesamiento de ciertos RNA cloroplásticos. El hecho de que la mutante *crp1* de maíz tenga un fenotipo similar al de la mutante 19,20 y la proteína predicha para tal gen tenga motivos PPR conservados, como es el caso del gen de la mutante 19,20, apunta que el gen afectado podría estar involucrado en funciones similares que *crp1*.

Conclusiones

En este trabajo se reportan seis mutantes *albinas*, cuyos genes son requeridos para la diferenciación temprana del proplastido en cloroplasto. Estas mutantes tienen plastidios que semejan proplastidios, la expresión de genes requeridos para la función temprana del cloroplasto o genes fotosintéticos es muy baja o en algunos casos nula. Los productos de los genes *ALBINO* son directa o indirectamente requeridos para el desarrollo normal de las células del mesófilo y la morfogénesis de la hoja. Los genes *ALBINO* afectan diferentes procesos de una manera autónoma y no-autónoma de la célula. Esta es la primera vez que ha sido observado durante embriogénesis la complementación de una mutante por difusión de un factor maternal. Probablemente esto puede pasar con cualquier mutante que carezca de una molécula capaz de difundir de la planta madre hacia el embrión, esto incluye a la mayoría de las hormonas. Si esto es un fenómeno general, podría tener implicaciones importantes para la interpretación de aquellas mutantes que parecen no expresar un fenotipo mutante durante la embriogénesis.

Este trabajo muestra que los genes requeridos para la diferenciación del proplastidio incluyendo la formación de la membrana tilacoidea del cloroplasto, pueden aislarse por tamizados de plántulas con fenotipo *albino.*

La caracterización de mutantes albinos que aquí se presenta provee un marco conceptual para un análisis genetico a mayor escala de la biogenésis del cloroplasto en Arabidopsis. Este trabajo es el primer estudio genético sistemático de los genes requeridos para la diferenciación temprana del cloroplasto. La clonación de los genes *ALBINO* cuyos resultados

se presentan aquí contribuyen con los primeros conocimientos de las bases moleculares de la diferenciación temprana del cloroplasto.

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