COLEGIO DE POSTGRADUADOS

INSTITUCIÓN DE ENSEÑANZA E INVESTIGACIÓN EN CIENCIAS AGRÍCOLAS

CAMPUS MONTECILLO

POSTGRADO EN BOTANICA

EXPRESION DIFERENCIAL DE PROTEÍNAS NUCLEARES Y CLOROPLASTÍDICAS DE CÉLULAS CLOROFÍLICAS DE *Bouteloua gracilis* EN ESTRÉS OSMÓTICO

BLANCA MORENO GÓMEZ

T E S I S

PRESENTADA COMO REQUISITO PARCIAL
PARA OBTENER EL GRADO DE:

DOCTORA EN CIENCIAS

MONTECILLO, TEXCOCO, EDO. DE MÉXICO

2015

La presente tesis titulada: **Expresión diferencial de proteínas nucleares y cloroplastídicas de células clorofílicas y mutantes de *Bouteloua gracilis* en estrés osmótico** realizada por el alumno: **BLANCA MORENO GOMEZ** bajo la dirección del Consejo Particular indicado, ha sido aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS

BOTÁNICA

CONSEJO PARTICULAR

CONSEJERO

DR. F. VICTOR CONDE MARTINEZ

DIRECTOR

DR. G. ARMANDO AGUADO SANTACRUZ

ASESOR

DR. EDMUNDO GARCIA MOYA

ASESORA

DRA. ALEJANDRINA ROBLEDO PAZ

ASESORA

DRA. ADRIANA DELGADO ALVARADO

Montecillo, Texcoco, Estado de México,

Expresión diferencial de proteínas nucleares y cloroplastídicas de células clorofílicas y mutantes de *Bouteloua gracilis* en condiciones de estrés osmótico

Blanca Moreno Gómez, Dra.

Colegio de Postgraduados, 2015

RESUMEN

Las suspensiones celulares clorofílicas y mutantes de *Bouteloua gracilis* son un modelo único en el mundo dentro de la familia de las gramíneas que es ideal para el estudio de las respuestas celulares a estreses abióticos, como estrés hídrico o salino. Debido a su elevada resistencia al estrés osmótico, a la presencia de cloroplastos desarrollados, a la acumulación de altos niveles de clorofila, a la sintenia y semejanza de su genoma con el de algunos cereales de importancia agrícola y al confinamiento en los cloroplastos de ciertas enzimas que son fundamentales para la respuesta vegetal a los factores ambientales, las suspensiones celulares clorofílicas de *B. gracilis* constituyen un modelo único para el estudio y el análisis de la respuesta de las plantas al estrés hídrico a nivel celular. Actualmente, la participación de los cloroplastos en la respuesta de las plantas al déficit hídrico ha sido poco estudiada. Sin embargo, el empleo de herramientas bioquímicas y moleculares, como la proteómica, nos permite estudiar las proteínas nucleares y cloroplastídicas de respuesta a estrés osmótico, conocimiento necesario para poder dilucidar algunas de las funciones enzimáticas codificadas en los cloroplastos que están relacionadas con la respuesta de las plantas al estrés hídrico y osmótico. En el presente estudio se lograron identificar proteínas diferenciales entre una línea celular clorofílica y una línea silvestre mutante carente de clorofila, así como algunas proteínas de respuesta a estrés osmótico en las células silvestres de *Bouteloua gracilis*. Estos resultados sientan las bases para el diseño de estrategias biotecnológicas que permitan reducir el impacto del estrés hídrico y mejorar la productividad vegetal.

Palabras clave: *Bouteloua gracilis*, estrés hídrico, cloroplastos, proteómica,

Differential expression of nuclear and chloroplast proteins of chlorophyllic and mutants cells from *Bouteloua gracilis* in conditions of osmotic stress.

Blanca Moreno Gómez, Dra.

Colegio de Postgraduados, 2015

ABSTRACT

Chlorophyllic and mutants cell suspensions of *Bouteloua gracilis* are a unique model in the world within the grass family, which is ideal for studying cellular responses to abiotic stresses such as water or saline stress. Due to its high resistance to osmotic stress, to the presence of well-developed chloroplasts, to the accumulation of high levels of chlorophyll, to the genetic similarity and synteny to the the genomes of some agriculturally important cereals and to the confinement of some enzymes, which are fundamental for the plant response to the environmental factors, the chlorophyllic cells suspensions of *B. gracilis* are a unique model for the study and analysis of the plant response to water stress at the cellular level. Currently, the participation of chloroplasts in the plant response to water deficit has been partially studied. However, the use of biochemical and molecular tools, such as proteomics, allow to study nuclear and chloroplast proteins which are responsive to osmotic stress, knowledge that is necessary for elucidating some of the enzymatic functions encoded in chloroplasts which are related to the plant response to water and osmotic stress. In the present study, we were able to identify diferential proteins between a wild type-chlorophyllic green cell line and a yellow mutant cell line that is devoid of chlorophyll, as well as some responsive proteins to water stress in the *B. gracilis* wild type cells. These results provide the basis for designing of biotechnological strategies aimed to reduce the impact of water stress and improve the productivity of plants.

Keywords: *Bouteloua gracilis*, water stress, chloroplast, proteomic

AGRADECIMIENTOS

Al **Consejo Nacional de Ciencia y Tecnología (CONACyT)** por la beca otorgada para la realización de este trabajo

Al **Colegio de Postgraduados**, en especial al **Posgrado en Botánica** por la oportunidad y las facilidades brindadas para continuar con mi formación académica

A los integrantes de mi consejo particular **Dr. Gerardo Armando Aguado Santacruz, Dr. F. Victor Conde Martínez, Dr. Edmundo García Moya, Dra. Alejandrina Robledo Paz** y **Dra. Adriana Delgado Alvarado**, mi más sincero agradecimiento por su tiempo, paciencia, sabiduría y disposición que cada uno de ustedes aportó para que este trabajo se llevara a cabo.

Al **Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP)-**Campo experimental Bajío-Celaya, Gto, por el apoyo otorgado y las facilidades proporcionadas para desarrollar la parte experimental de este trabajo.

Un agradecimiento especial a mis compañeras y amigas **Betzaida Jiménez Francisco** y **Ma. Guadalupe Valadez Bustos** por la disposición que siempre tuvieron para apoyarme en la tramitología durante todo el periodo de mis estudios.

CONTENIDO

Página

LISTA DE FIGURAS

Página

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE CUADROS

Página

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

CAPÍTULO I

INTRODUCCIÓN GENERAL

1.1. Respuesta de las plantas al estrés hídrico

La sequía constituye el principal obstáculo para la producción de alimentos a nivel mundial (Boyer, 1982; Edmeades et al., 1989). En México, al igual que en muchas regiones del mundo, la errática y escasa precipitación (comúnmente menos de 600 mm anuales) limita la actividad agrícola en áreas de temporal e influencía de manera determinante la dinámica y productividad de los cultivos y comunidades vegetales naturales (Aguado-Santacruz y García-Moya, 1998). Debido a su importancia en la producción agrícola mundial, grandes esfuerzos en diferentes áreas del conocimiento han sido enfocados a conocer el comportamiento de las plantas bajo condiciones de sequía desde la perspectiva morfológica, fisiológica ecológica, y hasta la molecular. Desde una perspectiva molecular, la comprensión integral de la percepción al estrés con la resultante cascada de señales que finalmente desembocan en la generación de una respuesta o grupo de respuestas tendientes a reducir el impacto del estrés en la planta es complicado, dada la gran variedad de elementos que participan en estos procesos: ABA, citocininas, auxinas, giberelinas y etileno como mensajeros primarios en los eventos de transducción de señales, calcio e IP_3 como segundos mensajeros en las respuestas al estrés, y solutos compatibles, HSPs (heat shock proteins o chaperonas), LEA's (Late Embryogenesis Abundant Proteins), osmotina, dehidrinas y rehidrinas, compuestos antioxidantes y acuaporinas, como elementos de respuesta para contrarrestar los efectos del déficit hídrico (Aguado-Santacruz, 2006). Por otro lado, se ha encontrado que la mayoría de los genes que son inducidos en respuesta a estrés osmótico o sequía son regulados por ABA (Skriver y Mundy, 1990).

1.2. Suspensiones celulares de *Bouteloua gracilis* como modelo de estudio

El pasto forrajero conocido como navajita azul, *Bouteloua gracilis* [H.B.K.] Lag. ex

Steud., es la gramínea más importante de los pastizales semiáridos de México (Vázquez, 1988; Orozco, 1993) y también del pastizal corto (shortgrass prairie) de los Estados Unidos (Sims et al., 1973). Se considera a México como un centro probable de diversificación del género (Rzedowski, 1975). Una de las características más relevantes de esta gramínea y que explica su amplia distribución a través del pastizal semiárido de Norteamérica, desde el sur de Canadá hasta las mesetas centrales de México, es su elevada tolerancia al pastoreo, a condiciones de agobio hídrico (Hoover et al., 1948; Havard-Duclos, 1979) y a la ocurrencia de fuego (Osborne, 2008). Como referencia se pueden mencionar los potenciales hídricos de hoja que resultan en disminución del crecimiento de maíz y *B. gracilis*. En maíz -1.0 MPa resultan en la detención del crecimiento foliar (Westgate y Boyer, 1985), mientras que en *B. gracilis* valores de -4.5 MPa no afectan significativamente su productividad o capacidad reproductiva (Alcocer-Ruthling et al., 1989). Estas características hacen de *B. gracilis* un modelo ideal para el análisis de las respuestas de las plantas al estrés hídrico y la búsqueda de nuevos mecanismos de tolerancia a la sequía.

En el año 2001, se obtuvieron los primeros cultivos celulares que poseen cloroplastos desarrollados dentro de las gramíneas (Aguado-Santacruz et al., 2001), a partir de *Bouteloua gracilis*. Con base en estos cultivos celulares clorofílicos se ha desarrollado una línea de investigación que ha permitido dilucidar algunos aspectos importantes de la participación de los cloroplastos en la respuesta de las plantas al estrés osmótico a nivel celular. Uno de ellos es el aumento de clorofila en función de la tensión osmótica aplicada, inducida ya sea por polietilenglicol (García-Valenzuela et al., 2005) o sal (Moreno-Gómez, 2006; 2009), fenómeno que parece estar ligado a la vía de señalización mediada por ácido abscísico.

Asimismo, se generaron dos bibliotecas substractivas de cDNA de respuesta a estrés osmótico, a partir de las células clorofílicas de *B. gracilis* (Delgado-Sánchez, 2007; Martínez-Castillo, 2007). En esta investigación se lograron clonar 288 EST´s de respuesta a estrés osmótico, los cuales se agruparon en 101 grupos funcionales. De un análisis funcional de los genes identificados, se encontró que los genes más representados se relacionaron con procesos como el metabolismo de azúcares y la

fotosíntesis. Destacan un 4% de las secuencias obtenidas que no se relacionan con genes ya referidos en las bases de datos disponibles. Las mayores homologías de estos EST´s se relacionaron con genes de otras gramíneas como maíz, cebada y arroz. Asimismo, se presentaron homologías con otros EST´s encontrados en las bases de datos y que fueron obtenidos a partir de otras plantas en condiciones de estrés salino, frío o sequía. Algunos de estos EST´s correspondieron a proteínas codificadas en cloroplasto, o bien a proteínas codificadas en núcleo pero que posteriormente se transportan a estos organelos (Fructuosa bifosfato aldolasa, aspartato aminotransferasa, proteínas del lumen de los tilacoides). Los resultados de la secuenciación de los EST´s de respuesta a estrés osmótico están disponibles en la base de datos mazorca del CINVESTAV (http:10.0.0.100/cgi-in/maiz/subindex.pl?proyecto=bouteloua).

Es importante mencionar que algunos de los resultados expuestos anteriormente han sido confirmados por análisis diferencial de la expresión de proteínas en las células clorofílicas de *Bouteloua gracilis* crecidas bajo condiciones de choque osmótico (Ruiz-Espinoza, 2004). Por ejemplo un transcrito, identificado por nuestro grupo en la biblioteca de EST's de respuesta a estrés osmótico, correspondió a la enzima gliceraldehido 3-fosfato deshidrogenasa. Esta proteína la cual se ha relacionado también por otros autores con la respuesta al estrés en plantas (Russell y Schas, 1989; Grant et al., 1999), fue encontrada en el trabajo de proteómica realizado por Ruiz-Espinoza (2004) utilizando las células clorofílicas de *B. gracilis* en choque osmótico. En este mismo estudio de proteómica se logró el aislamiento de 11 polipéptidos diferenciales que fueron expresados de manera diferencial en las células clorofílicas sometidas a choque osmótico.

Más recientemente hemos obtenido una línea celular mutante espontánea ('TADH'-UC) de las células clorofílicas que se caracteriza por no presentar cloroplastos desarrollados y que probablemente muestre alguna alteración en la ruta de biosíntesis de la clorofila. Dicha proposición está basada en lo reportado en investigaciones de mutantes de clorofila (Newell y Rienits, 1975; Maluf et al., 1997). Al no presentar cloroplastos desarrollados, esta línea mutante constituye un modelo ideal y complementario al de células clorofílicas para el análisis de la participación de

los cloroplastos en la respuesta al estrés osmótico de las plantas.

1.3. Importancia de los cloroplastos en la respuesta de las plantas al estrés

Los cloroplastos juegan un papel primordial en la bioquímica y fisiología de las plantas. Además de su bien conocida participación en la fotosíntesis, los cloroplastos intervienen en procesos fundamentales como la síntesis de hormonas, ácidos grasos y lípidos, aminoácidos, vitaminas (B1, K1 y E), nucleótidos, metabolitos secundarios como alcaloides e isoprenoides y son requeridos para la asimilación de nitrógeno y azufre.

El conjunto de eventos que conllevan a la adaptación o tolerancia a la deficiencia de agua involucra una estrecha interacción de genes nucleares y cloroplastídicos a través de mecanismos pobremente descritos. De entre lo poco que se conoce se puede mencionar, por ejemplo, que el núcleo modula la expresión genética del cloroplasto y que los productos genéticos codificados en el núcleo son necesarios para todos los procesos que ocurren en el cloroplasto (Ellis, 1984; Schuster et al., 1999). Por otro lado, se desconoce por qué las células de algunas plantas incrementan sus contenidos de clorofila bajo condiciones de estrés osmótico (García-Valenzuela, et al., 2005) o salino (Locy et al., 1996; Chang et al., 1997), el tipo y cantidad de señales que el núcleo envía al cloroplasto en respuesta a condiciones de estrés hídrico, o de modo más amplio, el tipo de redes de comunicación que se establecen entre estos dos organelos bajo condiciones subóptimas de crecimiento.

La definición de las funciones cloroplastídicas que operan bajo condiciones de estrés hídrico es fundamental en temas centrales, y actualmente en fuerte controversia, dentro del campo de la fisiología vegetal como el esclarecimiento de qué tanto de la reducción en la fijación de CO_2 observada en plantas en sequía, se debe a efectos directos (metabólicos) del déficit de agua en el aparato fotosintético y qué tanto a efectos estomáticos (Griffiths y Parry, 2002). En esta controversia se ha encontrado, por ejemplo, que el aparato fotosintético es notoriamente tolerante a la deshidratación. La reducción en la tasa fotosintética, por debajo de un 70% en el

contenido relativo de agua (CRA), es debida principalmente, a efectos de tipo estomático (Kaiser, 1987; Tourneux y Peltier, 1995), mientras que niveles más severos de estrés (entre 70 y 30% en el CRA) causan una inhibición directa, pero reversible de la actividad fotosintética (Chaves, 1991; Kaiser, 1987). Se desconoce, a la fecha, sí las proteínas de respuesta a sequía participan directamente en la protección del aparato fotosintético. Se ha mencionado la producción de proteínas específicas de cloroplasto.

La elucidación de estas incógnitas trasciende aspectos científicos básicos, siendo de primordial importancia en el diseño de estregias para la manipulación genética de los cloroplastos (Ellis, 1984) que puedan ser enfocadas, por ejemplo, a incrementar la eficiencia de expresión y disminuir los riesgos de dispersión de transgenes (Daniell, 2002), o aumentar la productividad de las plantas mediante el control de la síntesis de componentes fotosintéticos (Nieuwhof y van de Dijk, 1988; Peters et al., 1992).

Por otro lado, se considera que los cloroplastos son un blanco importante dentro de la biotecnología del estrés hídrico (Hayashi et al., 1997), debido a que diferentes proteínas relacionadas con la respuesta de las plantas a la deficiencia de agua son producidas en estos organelos. Dentro de estas proteínas se encuentran aquellas que se relacionan con la producción de solutos compatibles, o enzimas involucradas en su biosíntesis, y que son compartamentalizadas en estos organelos. Por ejemplo, el osmoregulador glicina betaína se localiza principalmente en cloroplastos (Robinson y Jones, 1986) donde se supone que estabiliza el aparato fotosintético y, por ende, la tasa fotosintética, durante condiciones de estrés hídrico (Rhodes y Hanson, 1993). Las proteínas BADH (Betaína Aldehido Deshidrogenasa) y colina monooxigenasa son enzimas involucradas en la biosíntesis de glicina betaína que se encuentran localizadas casi exclusivamente en el estroma de los cloroplastos (Weigel et al., 1986; Brouquisse et al., 1989). Los cloroplastos son un importante centro de producción de especies reactivas de oxígeno durante el estrés hídrico (Noctor y Foyer, 1998), por lo que cuentan con sistemas de detoxificación para la inactivación de radicales oxígeno y H_2O_2 que se generan en situaciones de deficiencia de agua (Bohnert y Shen, 1999) y de otras restricciones ambientales.

Adicionalmente en estos plástidos se encuentra codificada la información para la producción de la enzima (zeaxantina epoxidasa) que lleva a cabo la primer reacción de la biosíntesis de ABA mediante una epoxidación de la zeaxantina en anteraxantina y violaxantina.

A pesar de la importancia de comprender las funciones cloroplastídicas que intervienen en los procesos relacionados con la tolerancia al estrés hídrico en las plantas, al momento se cuenta con información más bien fragmentaria que permite conocer la estructura del cloroplasto y su genoma, mientras que, por otro lado, es escaso el conocimiento sobre la participación de los genes cloroplastídicos y su interacción con genes nucleares, durante situaciones de déficit de agua. Se espera que esfuerzos futuros en la ingeniería genética del estrés hídrico consideren el estudio de enzimas relacionadas con la síntesis de osmolitos, y otras proteínas protectoras de los cloroplastos a fin de aumentar la eficiencia de esta tecnología (Sakamoto y Murata, 2000).

1.3.1. Proteómica del cloroplasto

El proteoma del cloroplasto es el producto de genes nucleares y cloroplastídicos (Goldschimdt-Clermont, 1998). Aunque proteínas de cloroplasto codificadas en el núcleo son traducidas en ribosomas 80S e importadas después hacia los cloroplastos, las proteínas que son el producto del genoma del cloroplasto son traducidas en ribosomas 70S, usualmente en su forma madura. Debido a que el ADN de los plástidos en plantas superiores codifica para menos de 100 proteínas, el genoma nuclear es responsable de más del 95% de las proteínas que conforman el proteoma del cloroplasto (Martin y Herrmann, 1998). Las proteínas cloroplastídicas codificadas en núcleo son generalmente sintetizadas como proteínas precursoras con péptidos de tránsito de cloroplasto escindibles. Estos péptidos son de entre 30 y 120 aminoácidos de longitud, son bajos en residuos acídicos, pero altos en residuos hidroxilados y tienen poca estructura, posiblemente con una alfa hélice corta amfifílica (Von Heijne et al., 1989; Emanuelsson y Von Heijne, 2001). Debido a problemas técnicos y a que algunos de las proteínas del cloroplasto no

necesariamente poseen cTP´s que permitan identificarlas mediante programas de análisis bioinformática, es difícil estimar de manera confiable el número de proteínas que son compartamentalizadas en cloroplasto. Considerando estos factores de error, se estima que las proteínas confinadas en el cloroplasto probablemente sean más de 2,000 (van Wjik, 2004).

1.3.2. Proteómica comparada del cloroplasto: un reto para la definición de nuevas funciones cloroplastídicas orientadas a contrarrestar el efecto del estrés osmótico

La proteómica ha sido raramente enfocada hacia el funcionamiento del cloroplasto bajo condiciones de estrés (van Wijk, 2004). Esto puede ser atribuido a dificultades experimentales para obtener suficiente resolución dinámica del proteoma del estroma, la complejidad del proteoma de las membranas hidrofóbicas y la novedad de algunas de las herramientas de proteómica comparada. Se ha comprobado, por ejemplo, la dificultad para analizar el estroma soluble del cloroplasto debido a la presencia de una gran cantidad de proteínas involucradas en la fijación y el metabolismo de carbono (van Wijk, 2004). Las subunidades grande y pequeña de la Rubisco por si solas representan del 50-60% del total de la proteína del estroma del cloroplasto y, por otro lado, se supone que solamente de 10 a 15 especies de proteínas componen cerca del 90% de la biomasa proteómica del cloroplasto.

A pesar de estas dificultades, se han logrados avances en el análisis del proteoma del cloroplasto. Kubis et al. (2003), estudiando una planta mutante de *Arabidopsis thaliana* con una interrupción en Toc33, una proteína importante del aparato de importación de cloroplasto localizada en la membrana externa, encontraron que en las plantas mutantes dos subunidades solubles abundantes del complejo de ruptura del agua (OEC23 y OEC33) fueron menos abundantes, mientras que otras proteínas de tipo chaperón (Hsp70, Hsp90 y Cpn70) se sobreexpresaron. Hippler et al. (2001) analizaron membranas fotosintéticas de tilacoides de *Chlamydomonas reinhardtii* las cuales fueron aisladas a partir de un mutante deficiente en PSI (en el cual el gen ycf4 está interrumpido) y un mutante crd1 que bajo condiciones de deficiencia de cobre es condicionalmente reducido en PSI y

LHCI. Estos autores demostraron que la pérdida de LHCI y PSI puede ser visualizada en geles bidimensionales. Por su parte Lonosky et al. (2004) mediante análisis proteómico estudiaron la biogénesis de cloroplastos en hojas verdes de maíz y propusieron una metodología estadística para el análisis de los resultados de los estudios de proteómica en los que la repetibilidad sigue siendo un problema.

La composición del proteoma del cloroplasto y los niveles relativos de expresión de las proteínas del cloroplasto no son estáticos sino que varían en función del estado de desarrollo, así como de las condiciones ambientales. Las plantas han desarrollado diferentes mecanismos para mantener el funcionamiento de la maquinaria fotosintética durante situaciones de estrés ambiental, como la protección de los centros de reacción mediante la reducción del tamaño de las antenas, activación de sistemas de disipación de energía mediante la activación de cambios en la composición de los carotenoides (ciclo de las xantofilas), la producción de desactivadores de radicales de oxígeno y la activación de mecanismos de reparación o de nuevas vías de síntesis de los fotosistemas (Phee et al., 2004).

En este contexto, se ha demostrado, por ejemplo, que los genes que codifican proteínas de unión de clorofila a/b de los complejos cosechadores de luz son subregulados por la exposición a una alta intensidad de luz (Oelmuller, 1989; Taylor, 1989), mientras que otros genes de respuesta a estrés como aquellos relacionados con el sistema antioxidativo son sobreregulados, es decir, incrementan en situaciones de estrés (Rossel et al., 2002). Sin embargo, muchas de las proteínas responsables de los sistemas de defensa ante el estrés son aún desconocidas. Estos antecedentes anticipan que los estudios de proteómica de cloroplasto, deben proveer importantes nociones sobre la respuesta de estos organelos en plantas sujetas a sequía.

OBJETIVOS

General

Identificar, por espectrometría de masas, las proteínas nucleares y cloroplastídicas de células clorofílicas en suspensión de *Bouteloua gracilis,* que presenten cambios en los niveles de expresión como respuesta a un estrés osmótico y comparar los proteomas de la línea celular silvestre y la línea celular mutante carente de clorofila.

Específicos

Optimizar el método de aislamiento y purificación de cloroplastos

Optimizar una metodología de extracción y separación de proteínas del cloroplasto por electroforesis bidimensional

Identificar las proteínas nucleares y del cloroplasto, de respuesta al estrés osmótico en ambas líneas celulares

Identificar las proteínas diferenciales entre la línea celular silvestre y la línea celular mutante carante de clorofila de *Bouteloua gracilis*.

HIPOTESIS

El estrés osmótico induce cambios en la expresión de proteínas nucleares y cloroplastídicas de *Bouteloua gracilis,* especialmente en proteínas relacionadas con el metabolismo, fotosíntesis, almacenamiento, estructura celular y defensa. Mientras que, la mutación en las células amarillas carentes de clorofila, es causada por los cambios en la expresión de las proteínas que participan en el aparato fotosintético de las células clorofílicas de *Bouteloua gracilis.*

BIBLIOGRAFÍA

Aguado-Santacruz, G. A.; Cabrera-Ponce, J. L.; Olalde-Portugal, V.; Sanchez-Gonzalez, M. R.; Marquez-Guzman, M. R. and Herrera-Estrella, L. 2001a. Tissue culture and plant regeneration of blue grama grass *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. In Vitro Cell. Dev. Biol. Plant 37:182–189.

Aguado-Santacruz, G.A. 2006. Genetic manipulation of plants for increased drought tolerance. In: Genetic manipulation of plants for increased drought tolerance. In: Advances in Agricultural and Food Biotechnology (347 p.). Guevara-González, R.G. and Torres-Pacheco, I. (eds). Research Signpost. Kerala, India. pp. 71-97.

Aguado-Santacruz, G.A. and García-Moya, E. 1998. Environmental factors and community dynamics at the southermost part of the North American Graminetum. I. On the contribution of climatic factors to temporal variation in species composition. Plant Ecol. 135:13-29

Aguado-Santacruz, G.A., Rascón-Cruz, Q., Cabrera-Ponce, J.L., Martínez-Hernández, A., Olalde-Portugal, V. and Herrera-Estrella, L. 2002. Transgenic plants of blue grama grass, Bouteloua gracilis [H.B.K.] Lag. ex Steud., from microprojectile bombardment of highly chlorophyllous embryogenic cells. Theor. App.Gen. 104:763-771.

Alcocer-Ruthling, M., Robberecht, R. and Thill, D.C. 1989. The response of Bouteloua scorpioides to water stress at two phenological stages. Bot. Gaz. 150:454-461.

Bohnert, H.J. and Shen, B. 1999. Transformation and compatible solutes. Sci. Hort. 78:237-260.

Boyer, J.S. 1982. Plant productivity and environment. Science 218:443-448.

Brouquisse, R. Weigel, P., Rhodes, D., Yocum, C.F. and Hanson, A.D. 1989. Evidence for a ferredoxine-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiol. 90:322-329.

Chang, C.C., Locy, R.D., Smeda, R., Sahi, S.V. and Singh, N.K. 1997. Photoautotrophic tobacco cells adapted to grow at high salinity. Plant Cell Rep. 16:495-502.

Chaves, M.M. 1991. Effects of water deficits on carbon assimilation. J. Exp. Bot. 234:1-16.

Daniell, H. 2002. Molecular strategies for gene containment in transgenic crops. Nat. Biotechnol. 20:581-843.

Delgado-Sánchez, P. 2007. Genes aislados de Bouteloua gracilis bajo déficit hídrico obtenidos por medio de SSH, son diferencialmente expresados en estrés abiótico. 2007. Tesis Maestría. Instituto Potosino de Investigación Científica y Tecnológica. 38 p.

Edmeades, G.O., Bolanos, J., Laffite, H.R., Rajaram, S., Pfeiffer, W. and Fischer, R.A. 1989. Traditional approaches to breeding for drought resistance in cereals. In: Baker, F.W.G. (ed). Drought resistance in cereals. ICSU and CABL, Walllingford, U.K. pp 27-52.

Ellis, R.J. 1984. Principles of chloroplast biogenesis. In: Chloroplast biogenesis. Ellis, R.J. (ed). Cambridge University Press. pp. 1-9.

Emanuelsson, O. and Von Heijne, G. 2001. Prediction of organellar targeting signals. Biochim. Biophys. Acta 1541:114-119.

García-Valenzuela, X., García-Moya, E., Rascón-Cruz, Q., Herrera-Estrella, L. and Aguado-Santacruz, G.A. 2005. Chlorophyll accumulation is enhanced by osmotic stress in graminaceous chlorophyllic cells. J. Plant Physiol. 162:650-661.

Goldschimdt-Clermont, M. 1998. Coordination of nuclear and chloroplast gene expression in plant cells. Int. Rev. Cytol. 177:115-180.

Grant, C.M., Quinn, K.A. and Dawes, I.W. 1999. Differential protein S-thiolation of glyceraldehyde-3-phosphate dehydrogenase isoenzymes influences sensitivity to

oxidative stress. Mol. Cell. Biol. 19:2650-2656.

Griffiths, H. and Parry, M.A.J. 2002. Plant responses to water stress. Ann. Bot. 89:801-802.

Havard-Duclos, B. 1979. Las Plantas Forrajeras Tropicales. Blume Distribuidora. 380 p.

Hayashi, H.A., Mustardy, L., Deshnium, P., Ida, M. and Murata, N. 1997. Transformation of Arabidopsis thaliana with the codA gene for choline oxydase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J. 12:133-142.

Hippler, M., Klein, J., Fink, A., Allinger, T. and Hoerth, P. 2001. Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from Chlamydomonas reinhardtii. Plant J. 28:595-606.

Hoover, M.M., Hein, M.A., Dayton, W.A. and Erlanson, C.O. 1948. The Main Grasses for Farm and Home. In: Grass. Yearbook of Agriculture. Stefferud, A. (ed). USA Dept. Agr. 892 p.

Kaiser, W.M. 1987. Effects of water deficit on photosynthetic capacity. Physiol. Plant. 71:142-149.

Kubis, S., Baldwin, A., Patel, R., Razzaq, A., Dupree, P. and Lilley, K. 2003. The Arabidopsis ppi1 mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. Plant cell 15:1859-1871.

Locy, R.D., Chang, C. C., Nielsen, B.L. and Singh, N.K. 1996. Photosynthesis in salt-adapted heterotrophic tobacco cells and regenerated plants. Plant Physiol. 110:321-328.

Lonosky, P.M., Zhang, X., Honavar, V.S., Dobbs, D.L., Fu, A. and Rodermel, S.T. 2004. A proteomic analysis of maize chloroplast biogenesis. Bioinformatics 134:560-574.

Maluf, M. P., Saab, I. N., Wurtzel, E. T. Sachs, M. M. 1997. The viviparous12 maize mutant is deficient in abscisic acid, carotenoids, and chlorophyll synthesis. J. Exp. Bot. 48:1259-1268.

Martin, W. and Herrmann, R.G. 1998. Gene transfer from organelles to the nucleus: how much, what happens, and why? Plant Physiol. 118:9-17.

Martínez-Castillo, A. 2007. Análisis de la expresión genética de células clorofílicas de una gramínea altamente tolerante a la sequía bajo condiciones de estrés osmótico. Tesis Licenciatura. Instituto Tecnológico de Orizaba. 107 p.

Moreno-Gómez, B. 2006. Efecto de la salinidad sobre la fisiología de células clorofílicas de una gramínea altamente tolerante a la sequía. Tesis de licenciatura. Facultad de Ciencias Químicas. Universidad Veracruzana. Orizaba, Ver. 108 p.

Moreno-Gómez, B. 2009. Análisis Fisiológico y Proteómico de la Respuesta al Estrés Salino en Células Clorofílicas en Suspensión de Bouteloua gracilis. Tesis de Maestría en Ciencias en Ingeniería Bioquímica. Instituto Tecnológico de Celaya. Celaya, Gto. 102 p.

Newell, E. and Rienits, K. G. 1975. Chlorophyll synthesis in a yellow mutant of wheat. Australian J. of Plant Physiol. 2:543-552

Nieuwhof, M. and Van de Dijk, S.J. 1988. Differences between genotypes of tomato (Lycopersicon esculentum Mill.) in net photosynthesis, light absorption by leaves, chlorophyll content and specific leaf fresh weight under low-energy conditions. Neth. J. Agri. Sci. 36:396-399.

Noctor, G. and Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:249-279.

Oelmuller, R. 1989. Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. Photochem. Photobiol. 49:229-239.

Osborne, C. P. 2008. Atmosphere, ecology and evolution: what drove the Miocene espansion of C4 grasslands?. J. Ecol. 96: 35-45

Peters, J.L., Schreuder, M.E.L., Verduin, S.J.W. and Kendrick, R,E, 1992. Physiological characterization of a high pigment mutant of tomato. Photochem. Photobiol. 56:75-82.

Phee, B-K., Cho, J-H., Park S., Jung, J.H., Lee, Y-H., Jeon, J-S., Bhoo, S.H. and Hahn, T-R. 2004. Proteomics analysis of the responses of Arabidopsis chloroplast proteins to high light stress. Proteomics 4:3560-3568.

Rhodes, D. and Hanson, A.D. 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:357-384.

Robinson, S.P. and Jones, G.P. 1986. Accumulation of glycinebetaine provides osmotic adjustment during salt stress. Aust. J. Plant Physiol. 13:659-668.

Rossel, J.B., Wilson, I.W. and Pogson, B.J. 2002. Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol. 130:1109-1120.

Ruiz-Espinoza, E. 2004. Optimización y análisis de proteínas hidrosolubles por electroforesis bidimensional en cultivos celulares de Bouteloua gracilis (H.B.K.) Lag. ex Steud. sometidos a choque osmótico. Tesis Profesional. Instituto Tecnológico de Orizaba. Orizaba, Ver. 77 p.

Russell, D.A. and Sachs, M.M. 1989. Differential expression and sequence analysis of the maize glyceraldehyde-3-phosphate dehydrogenase gene family. Plant Cell 1:793-803.

Rzedowski, J. 1975. An ecological and phytogeographical analysis of the grasslands of México. Taxon. 24:67-80.

Sakamoto, A. and Murata, N. 2000. Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. J.

Exp. Bot. 51:81-88.

Schuster, G., Lisitsky, I. and Klaff, P. 1999. Polyadenylation and degradation of mRNA in the chloroplast. Plant Physiol. 120:937-944.

Sims, P.L., Lang'at, R.K. and Hyder, D.N. 1973. Developmental morphology of blue grama and sand bluestem. J. Range Manage. 26:340-344.

Skriver, K. and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2:503-512.

Taylor, W.C. 1989. Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:211-233.

Tourneux, C. and Peltier, G. 1995. Effect of water deficit on photosynthetic oxygen exchange measured using 18O2 and mass spectrometry in Solanum tuberosum L. leaf discs. Planta 195:570-577.

van Wijk, K.J. 2004. Plastid proteomics. Plant physiology and biochemistry 42:963-977.

Vázquez, H.B. 1988. Respuestas demográficas y esfuerzo reproductivo de Bouteloua gracilis bajo pastoreo continuo y exclusión en "El Gran Tunal", San Luis Potosí. Tesis Profesional. Escuela de Agronomía. Universidad Autónoma de San Luis Potosí. San Luis Potosí, S.L.P. 65 p.

Von Heijne, G., Steppuhn, J. and Herrmann, R.G. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. Eur. J. Biochem. 180:535-545.

Weigel, P., Weretilnyk, E.A. and Hanson, A.D. 1986. Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol. 82:753-759.

Westgate, M.E. and Boyer, J.S. 1985. Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. Planta 164:540-549.

CAPÍTULO II

EFFICIENT PROTOCOL FOR HYDROPHOBIC AND HYDROPHYLIC PROTEIN SOLUBILIZATION OF FREE CELLS FROM PLANTS FOR TWO-DIMENSIONAL ELECTROPHORESIS ANALYSIS[1]

Aguado-Santacruz, G.A.[1] Moreno-Gómez, B.*[2], García-Moya, E.[2], Conde-Martínez, F. V.[2], Robledo-Paz, A.[3] and Delgado-Alvarado, A.[4]

[1]/ División de Estudios de Postgrado e Investigación. Instituto Tecnológico de Roque, Km 8 Carretera Celaya-Juventino Rosas, Roque, Celaya, Guanajuato 38110, México.

[2]/ Postgrado en Botánica, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México

[3]/ Postgrado en Genética, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México.

[4]/ Postgrado en Estrategias para el Desarrollo Agrícola Regional, Colegio de Postgraduados, Campus Puebla. Km 125.5 Carretera Carretera Federal México-Puebla, Santiago Momoxpan, San Pedro Cholula, Puebla 72760, México..

***Corresponding author:**

Blanca Moreno-Gómez

Postgrado en Botánica

Colegio de Postgraduados

Km 36.5 Carretera México-Texcoco

Montecillo Texcoco, Edo México C.P. 56230, México

moreno.blanca@colpos.mx

Abstract

Two dimensional electrophoresis (2-DE) remains as the principal technology for resolving complex protein mixtures, prior to mass spectrometry characterization. Protein extraction and solubilization are the most important steps for revealing a proteome by 2-DE. For any kind of tissue, urea/thiourea-based protein solubilization buffers in combination with detergents and reducing agents are usually employed for maximizing solubilization and resolution of different types of proteins. Because protein composition in plants is species-specific and dependent on growing conditions, development of efficient protocols for improving the proteomic analysis in studies focusing to study the response of plants (whole plants and free cells) to environmental factors is always required. Our study system model, chlorophyllic free cells of blue grama grass *Bouteloua gracilis,* can be used as a unique model for studying the response of grasses to water and osmotic stress at cellular level, due to they are the only one chlorophyllic free cells in the Gramineae family. Using these blue grama grass cells, we tested 16 different solubilizations buffers containing different chaotropes, zwitterionic and nonionic detergents, and reducing agents for maximizing both the number of spots and spot resolution in 2-D gels. The most efficient solubilization buffer developed in this work functioned well in a wide pH range utilized in proteomic studies (3–10). This buffer contained two chaotropic agents (7 M urea and 2 M thiourea), two reducing agents [100 mM DTT (dithiothreitol) and 2 mM TBP (tributyl phosphate)], two zwitterionic detergents [2.65% CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfo-nate), 2% ASB 14 (amidesulfobetaine-14)] and a nonionic detergent (2% Triton X-100).

Keywords: proteomics, 2-D gel electrophoresis, proteome, free cells, proteins, solubilization buffer, reducing agents, zwitterionic detergent.

Introduction

Two-dimensional gel electrophoresis (2-DE) technique, in combination with Mass Spectrometry (MS), still remains as one of the most important methods for revealing complex protein profiles (Rabilloud 2002), despite all the described and known limitations pertaining reproducibility and difficulty in efficient separation of low abundance proteins, low and high molecular weight proteins and highly acidic, basic and hydrophobic proteins (Gygi et al 2000; Wall et al 2000).

Protein extraction has become the most important step in proteome studies. However, to reach a satisfactory 2-DE resolution and a good representation of the proteome, a high-quality protein solubilization is required (Görg et al 2004). Because each plant sample has unique features related to protein composition and growing conditions, an appropriate protocol is always required (Shaw and Riederer 2003). Currently, the most popular sample solubilization buffer is based on O'Farrell's lysis buffer and modifications thereof (9 M urea, 2–4% CHAPS [3-3(cholamidolpropyl)-dimethylammonio-1-propane sulfo-nate], 1% DTT (dithiothreitol), and 2% (v/v) carrier ampholytes; O'Farrell 1975). Unfortunately, this urea-based lysis buffer is not ideal for solubilization of all proteins, particularly for membrane or other highly hydrophobic proteins. Improvement in the solubilization of hydrophobic proteins has been achieved using thiourea (Rabilloud 1998) and new zwitterionic detergents such as sulfobetaines (Chevallet et al 1998).

Different techniques have been employed for the protein extraction of diverse plant samples such as whole plants, organs, tissues, somatic embryos and calluses. For example, leaves were used in sugarcane and maize (Zhou et al 2012; Wu et al 2012), somatic embryos in coffee (*Coffea Arabica*) and cyclamen (*Cyclamen persicum*) (Tonietto et al 2012; Bian et al 2009), and calluses in vanilla (*Vanilla planifolia*) (Chin Tan et al 2013) for protein analysis. In this study, chlorophyllous cell cultures of blue grama grass, established by Aguado-Santacruz et al in 2001, were used for developing improved protein solubilization protocol. This cell culture contains high chlorophyll levels and well-developed chloroplasts, representing a unique model

for studying photosynthesis and analyzing the effects of different stresses such as osmotic, cold or heat on chloroplast development and photosynthesis (García-Valenzuela et al 2005; Aguado-Santacruz et al 2011). In the work presented here, this cell model was utilized for optimizing samples for 2-D gel electrophoresis analysis by testing 16 solubilization buffers, containing two chaotropes (7 M urea and 2 M thiourea), and different concentrations of zwitterionic and nonionic detergents, and reducing agents. Our results showed that the most efficient buffer for solubilizing the total protein of the chlorophyllic cells contained, besides the two chaotropic agents, two reducing agents (100 mM DTT [dithiothreitol] and 2 mM TBP [tributyl phosphate]), two zwitterionic detergents (2.65% CHAPS [3-3(cholamidolpropyl)-dimethylammonio-1-propane sulfonate], 2% ASB 14 [amidesulfobetaine-14]) and a nonionic detergent (2% Triton X-100). This optimized buffer worked well in the wide pH range normally utilized in proteomic studies (3–10).

Materials and Methods

Sample preparation

A chlorophyllic cell suspension (`TADH-XO´) was obtained by culturing shoot tips of blue grama as described before by Aguado-Santacruz et al 2001 and García-Valenzuela et al 2005. This chlorophyllic cell line was routinely grown in liquid propagation medium (PM), which consisted of basal salts and vitamins of the MS medium (Murashige and Skoog 1962), 3% sucrose, 1 mg l^{-1} 2,4-D (2,4 dichlorophenoxyacetic acid), 2 mg l^{-1} BAP (benzylaminopurine) and 40 mg l^{-1} adenine (Aguado-Santacruz et al 2001). An initial inoculum of chlorophyllic cells was prepared by culturing 1 g fresh weight cells into 50 ml of PM. After growing for eight days, 125 ml-Erlenmeyer flasks containing 25 ml of PM were inoculated with 500 mg fresh weight of green cells. These flasks were agitated at 95 r.p.m. on a gyratory shaker in a growth chamber (33±1^{o}C and 33.8% relative humidity) under continuous light (photon flux density = 77 μmol s^{-1} m^{-2}) provided by 30 watts fluorescent daylight lamps (General Electric, model F30T8-D). After six days, chlorophyllic cells were

harvested using a vacuum filtration system and then frozen in liquid nitrogen for further protein extraction.

Protein extraction

Three hundred and fifty mg fresh weight frozen cells were suspended in 800 μl of the extraction buffer cited in Issacson et al (2006). This extraction buffer was composed of 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 50 mM EDTA. Immediately before use, a reducing agent (β-mercaptoethanol) and a cocktail protease inhibitor (Complete mini, EDTA-free, 11836170001, Roche Diagnostic, Germany) were added to the extraction buffer to final concentrations of 2% (v/v) and 1 mM, respectively. After the cells were mixed with the extraction buffer, 800 μl cold phenol saturated with Tris-HCl, pH 7.5, were added. The mixture was stirred on a vortex mixer for 30 min at 4^{o}C and later centrifuged at 5,000 x g for 30 min at 4^{o}C. The upper phenolic phase was collected in a new tube, and then 800 μl of the extraction buffer were newly added to the remnant pellet in order to repeat the extraction process two more times. To precipitate the protein, 5 volumes of cold 0.1 M ammonium acetate in methanol were added to the collected phenolic phase, which was then stored at -20^{o}C overnight. Washing steps were carried out two times with methanol and once with acetone. Finally, the samples were air dried for five min. Subsequently, 150 μl of each solubilization buffers tested were added to the samples. Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ).

Solubilization buffers

In this analysis, 16 different solubilization buffers methods were tested for protein solubilization of chlorophyllic cells of blue grama (Table 1) by one dimension SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Formulation of these new buffers were based on the published protocols by Di-Ciero et al (2004),

Gu-Kang et al (2004), Hye-Kyoung et al (2005), Natarajan et al (2005), Parker (2006), Rabilloud et al (2007), Martins et al (2007) and Rabilloud (2009).

One dimension SDS-PAGE analysis was carried out with Laemmli electrophoresis running buffer (Laemmli 1970), loading 100 μg protein per lane and using 5 μl protein ladder of 220 kDa (Benchmarck^{TM}10747-012 Life Technologies, Gaithers-burg, Md). Gels were elaborated using 12% acrylamide and the samples were run at 100 V for approximately 3 h and then stained with PhastGel Blue Coomassie R-350 (PhastGel Blue^{TM} 17051-801 Amersham Bioscience, Piscataway, NJ). All one dimension SDS-PAGE gel separations were repeated at least five times for each solubilization buffer. The most efficient buffers were selected in terms of intensity and number of bands by GelQuant Software (Biosistematica, Tavistock, Devon, UK) and then the samples prepared with these buffers were utilized for running two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis (2-DE)

Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ). Solubilized samples containing 1500 μg of protein were applied to 18 cm IPG gel strips with a pH separation range of 3–10 (GE Life Sciences, Piscataway, NJ). Taking into account the maximal volume permitted by strip, we used 18 cm IPG gel strips. After 14 h of rehydration, isoelectric focusing (IEF) was carried out at 22^{o}C, first applying 100 V for 2 h, later 250 V for one hour, subsequently 0.5 h at 500 V, 0.5 h at 1000 V, and finally 8000 V for 13 h in an IPGphor apparatus (GE Life Sciences, Piscataway, NJ), maintaining a limiting current of 50 mA per strip. First dimension strips were subjected to the standard reduction step with 0.05% DTT, (15 min), and the alkylation step with 1.25% 2-iodoacetamide (IAA, 15 min) prior to running the second dimension electrophoresis. The second dimension (SDS-PAGE) was performed on 12% polyacrylamide gels using an Ettan DALTsix electrophoresis unit connected to a refrigerating system (GE Life Sciences, Piscataway, NJ). The electrophoresis was carried out for 1 h at 100 V, and later on at a constant current of

30 mA until the dye front reached the bottom of the gel and the proteins were detected by a colloidal Coomassie blue staining (PhastGel Blue Coomassie R-350). Five independent samples prepared with each solubilization buffer were run and then the resulting spots were analyzed (Table 2).

2-DE image analysis

The stained gels were scanned by transmittance at 300 dpi of resolution using Lab Scan software (GE Life Sciences, Piscataway, NJ). Images were saved as *.mel files. The software ImageMaster 2D platinum version 6.0 (GE Life Sciences, Piscataway, NJ) was used for spot detection, counting and quantification.

Statistical analysis

For high reliability, the same parameters, based on the default settings, were applied to each set of gels stained simultaneously in the same tray. After calculating the standard deviation (SD) and coefficient of variation (CV), the statistical significance of the variation in PhastGel Blue Coomassie R-350-stained spot intensity across three replicates was calculated (t-test, $p < 0.05$).

Results and Discussion

In this research, we formulated and compared 16 different buffers for protein solubilization of chlorophyllous free cells. Efficiency of protein solubilization was measured in terms of number of spots, spot resolution and intensity in 2D–PAGE analysis. The protein extraction process utilized in this work was based on the phenol protocol described by Isaacson et al (2006). This extraction process has been reported as efficient in proteomic research (*e.g.* Grosse et al 2008; Cilia et al 2009; Kelley et al 2010) and in previous work with our chlorophyllous cells, in which comparatively greater protein yields were obtained (up to 2.78 μg mg^{-1} fresh weight) using this phenol-based protocol.

All of the 16 solubilization buffers contained 7 M urea and 2 M thiourea as chaotropic agents, while the differences among them were related to the type (s) and concentration (s) of the reducing agents and detergents incorporated (Table 1). One dimension SDS-PAGE analysis revealed differences in the intensity and number of bands as a function of the buffer employed. Buffers B9, B10 and B11 revealed higher number of bands (Table 1), but the bands obtained with B2, B7 and B16 showed higher intensity. Consequently, we only choose the plant samples processed with six buffers (B2, B7, B9, B10, B11 and B16) for further bidimensional electrophoresis analysis. Before 2-DE analysis, the total protein content quantified in samples processed with buffers B2, B7, B9, B10, B11 and B16 was 8.1, 12.3, 17.9, 13.5, 15.9 and 39.6 $\mu g\ \mu l^{-1}$, respectively, showing that B16 buffer yielded the highest protein concentration. Di-Ciero et al (2004) mentioned that one of the best detergents to solubilize hydrophobic proteins is ASB 14 (amidesulfobetaine-14) followed by CHAPS and finally Triton X-100. Because hydrophobic proteins are grouped into basic pH (Marmagne et al 2004), we decided to analyze two-dimensional electrophoresis in a pH separation range of 3–10.

Overall, the effectiveness of each solubilization buffer can be easily observed by the number and abundance of spots revealed between the isoelectric points 3 to 10 and the molecular weight values within 10 and 220 kDa (Figure 1, Table 2). 768 protein spots were revealed in the gel loaded with the sample treated with B16 buffer consisting of a 7 M urea/2 M thiourea-based buffer combined with the reducing agents, DTT (dithiothreitol) and TBP (tributyl phosphate), at concentrations of 100 mM and 2 mM, respectively, zwitterionic detergents, CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfonate) and ASB 14 (amidesulfobetaine-14), at a concentrations of 2.65% and 2.00%, respectively, and the nonionic detergent Triton X-100 at concentration of 2.00%. Other studies (Chevallet et al 1998; Cordwell et al 2000; Molloy et al 2001; Leimgruber et al 2002; Di-Ciero et al 2004) have also shown the effectiveness of these sulfobetaines detergents for 2-DE analysis. Our results indicate that the method based on B16 buffer enriched the samples for membrane proteins, which represented 32.6% of the total protein (Figure 1). This greater efficiency is the result of the combined action of the different components integrating

the buffer. Urea is a chaotropic agent that is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and denaturation. Likewise, thiourea is an organosulfur compound first utilized in proteomic studies by Rabilloud in 1998, which is better suited for breaking hydrophobic interactions; inconveniently, its usefulness is somewhat limited due to its poor solubility in water.

On the other hand, detergents, such as SDS, are utilized to prevent hydrophobic interactions between the hydrophobic protein domains, which result in protein loss due to aggregation and precipitation. Because the anionic detergent SDS is one of the most efficient surfactants, solubilization of proteins in SDS solutions has been recommended for protein solubilization (Boucherie et al 1995; Harder et al 1999). Additionally, zwitterionic detergents, such as CHAPS, and sulfobetaines (SB 3–10 or ASB 14) perform better and have been shown to solubilize -in combination with urea and thiourea chaotropes- several integral membrane proteins (Santoni et al 2000; Molloy 2000).

Since reduction and prevention of re-oxidation of disulfide bonds is also a critical step of the sample preparation procedure, reducing agents are necessary for cleaving intermolecular disulfide bonds to achieve complete protein unfolding. The most commonly used reductants are dithiothreitol (DTT) and dithioerythritol (DTE), which are applied in excess (100 mM). However, DTT and DTE are not well suited for the reduction and solubilization of proteins containing high cysteine content. Herbert et al (1998) have proposed tributylphosphine (TBP) in lower concentrations (2 mM) as an alternative to DTT. Our data show that the solubilization of acid and basic proteins is better achieved when the combination of TBP, ASB 14, CHAPS, and Triton X-100 is considered, making the protein solubilization particularly efficient in free cells of the blue grama grass.

Finally, we expect this efficient and improved protocol for obtaining protein samples with satisfactory quality for 2-D analysis be instrumental in analyzing the cellular response to biotic and abiotic stresses in the chlorophyllous free cells as in similar plant models. Our research group has used PEG 8000 to study the effect of osmotic stress on these free cells. PEG has been demonstrated in the past to be

imperfect with respect to permeability (Lawlor 1970; Yaniv and Werker 1983; Newton et al 1990) and several reports suggest that it may be taken up by plants (Mexal et al 1975; Woolley 1963) causing detrimental effects other than osmotic ones (Lagerwerff et al 1961; Lawlor 1970; Leshem 1966). However other reports suggest that PEG can be used as an efficient non-penetrating osmoticum (Appelgate 1960; Mexal et al 1975). For future research, we tested the full protocol in free cells treated with 14% PEG 8000 obtaining a good resolution of proteins (data not show).

Table 1. Efficiency of different buffers for solubilizing the total protein of chlorophyllous free cells of blue grama as measured by the number and intensity of bands revealed by one dimensional electrophoresis SDS-PAGE. All one dimension SDS-PAGE gel separations were repeated at least five times for each solubilization buffer.

	Reducing agent (mM)		Detergent (%)			pH stabilizer (mM)		
Buffer*	DTT	TBP	CHAPS	Triton X-100	ASB 14	K_2CO_3	Average No. of bands	Average band intensity
B1	20		2.65				32	530259
B2	50		2.65				33	612440
B3	100		2.65				26	243278
B4		2	2.65				24	286875
B5		4	2.65				33	471767
B6		5	2.65				34	463486
B7		6	2.65				36	664791
B8		4	2.65			5	36	447155
B9		6	2.65			5	54	790717
B10	100	2	2.65				57	671710
B11		4	2.65	1.35		5	40	713803
B12		4	4.00			5	49	292469
B13		6	2.65		2.00	5	26	486422
B14	100	2	2.65		2.00		24	251293
B15		6	2.00		2.00	5	39	635765
B16	100	2	2.65	2.00	2.00		41	728598

*All buffers contained 7 M urea and 2 M thiourea as chaotropic agents.

Table 2. Number of protein spots revealed in 2-DE profiles of free cells of blue grama using six solubilization buffers for sample preparation. Five independent samples prepared with each solubilization buffer were run.

Buffer	Composition	No. of acid protein spots	No. of basic protein spots	Standard deviation (SD)	Coefficient of variation (CV)
B2	7 M urea, 2 M thiourea, 2.65% CHAPS, 50 mM DTT	228	29	3.51	0.013
B7	7 M urea, 2 M thiourea, 2.65% CHAPS, 6 mM TBP	289	29	4.09	0.012
B9	7 M urea, 2 M thiourea, 2.65% CHAPS, 6 mM TBP, 5 mM K_2CO_3	180	38	3.40	0.015
B10	7 M urea, 2 M thiourea, 2.65% CHAPS, 100 mM DTT, 2 mM TBP	368	119	6.76	0.013
B11	7 M urea, 2 M thiourea, 2.65% CHAPS, 4 mM TBP, 5 mM K_2CO_3, 1.35% Triton X-100	444	99	5.50	0.010
B16	7 M urea, 2 M thiourea, 2.65% CHAPS, 100 mM DTT, 2 mM TBP, 2% Triton X-100, 2% ASB 14	517	251	8.50	0.011

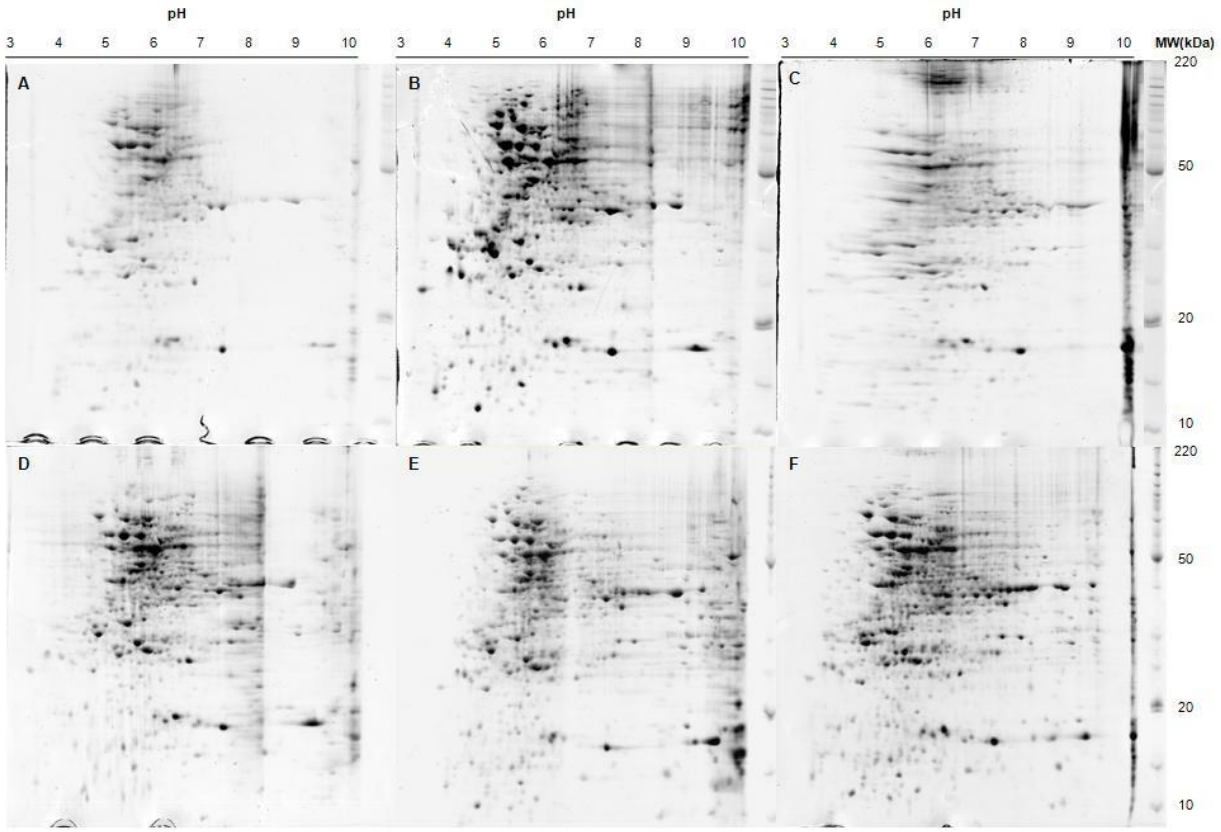

Figure 1. Two-dimensional electrophoresis of chlorophyllic cells proteins from blue grama extracted, using various methods: (A) B2, (B) B7, (C) B9, (D) B10, (E) B11, and (F) B16 buffers. Protein (1500 μg) was run used 18 cm IPG strips (pH 3–10) in first dimension, and the second-dimension run used 12% SDS–PAGE. Gels were stained with PhastGel Blue Coomassie R-350. Five independent samples prepared with each solubilization buffer were run.

Acknowledgements

We are grateful to the Consejo Nacional de Ciencia y Tecnología (CONACyT) for their financial support through Project 82390 and to INIFAP and CONACyT for student funding.

References

Aguado-Santacruz GA, Cabrera-Ponce JL, Ramírez-Chávez E, Rascón-Cruz Q, Herrera-Estrella L, Olalde-Portugal V (2001) Establishment, characterization and plant regeneration from highly chlorophyllous embryogenic cell cultures of blue grama grass, Bouteloua gracilis (H.B.K.) Lag ex. Steud. Plant Cell Rep 20:131-136.

Aguado-Santacruz, GA, Betancourt-Guerra DA, Siqueiros-Cedón T, Arévalo-Gallegos S, Rivera-Chavira BE, Nevarez-Moorillon GV, Moreno-Gómez B, Rascón-Cruz Q (2011) Comparison of the structure and organization of the rrna operons of Bouteloua gracilis and Zea mays. Can J Plant Sci 91:107-116.

Appelgate HG (1960) Freezing-point depressions of Hoagland´s carbowax systems. Nature 186:232-233.

Bian F, Zheng C, Qu F, Gong X, You C (2010) Proteomic analysis of somatic embryogenesis in *Cyclamen persicum* Mill. Plant Mol Biol Rep 28:22-31.

Boucherie H, Dujardin G, Kermorgant M, Monribot C, Slonimski P, Perrot M (1995) Two-dimensional protein map of *Saccharomyces cerevisiae:* construction of a gene-protein index. Yeast 11:601-613.

Chevallet M, Santoni V, Poinas A, Rouqié D, Fuchs, A, Kieffer S, Rossignol M, Lunardi J, Garin J, Rabilloud T (1998) New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19:1901-1909.

Chin Tan B, Foan Chin C, Liddell S, Alderson P (2013) Proteomic analysis of callus development in Vanilla planifolia. Plant Mol Biol Rep 31:1220-1229.

Cilia M, Fish T, Yang X, Mclaughlin M, Thannhauser TW, Gray S (2009) A Comparison of Protein Extraction Methods Suitable for Gel-Based Proteomic Studies of Aphid Proteins. J Biomol Tech 20:201-215.

Cordwell SJ, Noumens AS, Verrills NM, Basseal DJ, Walsh BJ (2000) Subproteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional electrophoresis gels. Electrophoresis 21:1094-1103.

Di-Ciero L, Bellato CM, Meinhardt LW, Ferrari F, Castellari RR, Marangoni S, Novello JC (2004) Assessment of four different detergents used to extract membrane proteins from Xilella fastidiosa by two-dimensional electrophoresis. Braz J Microbiol 35:269-274.

García-Valenzuela X, García-Moya E, Rascón-Cruz Q, Herrera-Estrella L, Aguado-Santacruz GA (2005) Chlorophyll accumulation is enhanced by osmotic stress in gramineous chlorophyllic cells. J Plant Physiol 162:650-661.

Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665-3685.

Grosse C, Heinekamp T, Kniemeyer O, Gehrke A, Brakhage AA (2008) Protein kinase a regulates growth, sporulation, and pigment formation in *Aspergillus fumigatus.* Appl Environ Microbiol 74:4923-4933.

Gu Kang J, Jae Pyo Y, Won Cho J, Haeng Cho M (2004) Comparative proteome analysis of differentially expressed proteins induced by K+ deficiency in Arabidopsis thaliana. Proteomics 4:3549-3559.

Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R (2000) Evaluation of two-dimensional gelelectrophoresis-based proteome analysis technology. Proc Natl Acad Sci USA 97: 9390-9395.

Harder A, Wildgruber R, Nawrocki A, Fey SJ, Larsen, PM, Görg A (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. Electrophoresis 20:826-829.

Herbert B, Molloy MP, Gooley, AA, Walsh BJ, Bryson, WG, Williams KL (1998) Improved protein solubilization in 2-D electrophoresis using tributyl phosphine as reducing agent. Electrophoresis 19:845-851.

Hye-Kyoung K, Yokoyama R, Nishitani K (2005) A proteomic approach to apoplastic proteins involved in cell wall regeneration in protoplasts of Arabidopsis suspension-cultured cells. Plant Cell Physiol 46:843-857.

Isaacson T, Damasceno CM, Saravanan RS, He Y, Catalá C, Saladié M, Rose JKC (2006) Sample extraction techniques for enhanced proteomic analysis of plant tissues. Nat Protocol 1:769-774.

Kelley BS, Lee S-J, Damasceno CMB, Chakravarthy S, Kim B-D, Martin GB, Rose JKC (2010) A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. Plant J 62:357-366.

Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227:680-685.

Lagerwerff JV, Ogata G, Eagle HE (1961) Control of osmotic pressure of culture solutions with polyethylene glycol. Science 133: 1486-1487.

Lawlor DW (1970) Absorption of polyethylene glycol by plants and their effects on plant growth. New Phytol 69:501-513.

Leimgruber RM, Malone JP, Radabaugh MR, LaPorte ML, Violand BN, Monahan JB (2002) Development of improved cell lysis, solubilization and imaging approaches for proteomic analyses. Proteomics 2:135-144.

Leshem B (1966). Toxic effects of carbowaxes (polyethylene glycols) on *Pinus halapensis* Mill. seedlings. Plant Soil 24:322-324.

Marmagne A, Aude-Rouet M, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. Mol cell proteomics 3:675-691.

Martins D, Menezes-Oliveira B, Dosantos-Farias A, Oka-Horiuchi RS, Crepaldi-Domingues C, De-Paula E, Marangoni S, Farid-Gattaz W, Dias-Neto E, Camillo-Novello J (2007) The use of ASB-14 in combination with CHAPS is the best for

solubilization of human brain proteins for two-dimensional gel electrophoresis. Brief funct genomics proteomics 6:70-75.

Mexal J, Fisher JT, Osteryoung J, Reid CPP (1975) Oxigen availability in polyethylene glycol solutions and its implications in plant-water relations. Plant Physiol 55:20-24.

Molloy MP (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal Biochem 280:1-10.

Molloy MP, Phadke ND, Maddock JR, Andrews PC (2001) Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. Electrophoresis 22:1686-1696.

Murashige T, Skoog FA (1962) Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

Natarajan S, Xu C, Caperna TJ, Garrett WM (2005) Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Anal Biochem 342:214-220.

Newton RJ, Puryear JD, Bhaskaran S, Smith RH (1990) Polyethylene glycol content of osmotically stressed callus culture. J Plant Physiol 135:646-652.

O'Farrel PH (1975) High resolution two-dimensional electrophoresis of proteins. The J Biol Chem 250:4007-4021.

Parker R, Flowers TJ, Moore AL, Harpham NVJ (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J Exp Bot 57:1109-1118.

Rabilloud T (1998) Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis 19:758-760.

Rabilloud T (2002) Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics 2:3-10.

Rabilloud T (2009) Detergents and chaotropes for protein solubilization before two-dimensional electrophoresis. Method Mol Biol 528:259-267.

Rabilloud T, Adessi C, Giraudel A, Lunardi J (2007) Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18:307-316.

Santoni V, Molloy M, Rabilloud T (2000) Membrane proteins and proteomics: Un amour impossible? Electrophoresis 21:1054-1070.

Shaw MM, Riederer BM (2003) Sample preparation for two-dimensional gel electrophoresis. Proteomics 3:1408-17.

Tonietto A, Hiromi SJ, Batista TJ, M. de Souza E, O. Pedrosa F, Franco OL, Mehta A (2012) Proteomic analysis of developing somatic embryos of Coffea Arabica. Plant Mol Biol Rep 30:1393-1399.

Wall DB, Kachman MT, Gong SY, Hinderer R, Parus S, Misek DE, Hanash SM, Lubman DM (2000) Isoelectric focusing nonporous RP HPLC: a two-dimensional liquid-phase separation method for mapping of cellular proteins with identification using MALDI-TOF mass spectrometry. Anal Chem 72:1099-1111.

Widholm JM (1992) Properties and uses of photoautotrophic plant cell cultures. Int Rev Cytol 132:109-175.

Woolley DG (1963) Effects of nutrition, osmotic pressure, and temperature of the nutrient solution on plant growth and chemical composition. I. Spring wheat at the four and six leaf stage. Can J Plant Sci 43:44-50.

Wu L, Zu X, Wang X, Sun A, Zhang J, Wang S, Chen Y (2013) Comparative proteomic analysis of the effects of salicylic acid and abscisic acid on maize (Zea mays L.) leaves. Plant Mol Biol Rep 31:507-516.

Yaniv Z, Werker E (1983) Absorption and secretion of polyethylene glycol by Solanaceous plants. J Exp Bot 34:1577-1584.

Zhou G, Yang L-T, Li Y-R, Zou C-L, Huang L-P, Qiu L-H, Huang X, Kumar Srivastava M (2012) Proteomic analysis of osmotic stress-responsive proteins in sugarcane leaves. Plant Mol Biol Rep 30:349-359.

CAPÍTULO III

PROTEOMIC ANALYSIS OF THE CHLOROPLAST RESPONSE OF *Bouteloua gracilis* FREE CELLS TO OSMOTIC STRESS

Moreno-Gómez, B.[2], García-Moya, E.[2], Conde-Martínez, F.V.[2], Robledo-Paz, A.[3], Delgado-Alvarado, A.[4] and Aguado-Santacruz, G.A.[1]

[1]/ División de Estudios de Postgrado e Investigación. Instituto Tecnológico de Roque, Km 8 Carretera Celaya-Juventino Rosas, Roque, Celaya, Guanajuato 38110, México.

[2]/ Postgrado en Botánica, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México

[3]/ Postgrado en Genética, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México.

[4]/ Postgrado en Estrategias para el Desarrollo Agrícola Regional, Colegio de Postgraduados, Campus Puebla. Km 125.5 Carretera Carretera Federal México-Puebla, Santiago Momoxpan, San Pedro Cholula, Puebla 72760, México.

***Corresponding author:**

Moreno-Gómez, B.

Abstract

Protein metabolism and expression play important role in plant adaptation to water stress. Plants can respond defensively to this stress for a limited period changing their gene expression profiles. Chloroplasts are plant cell organelles of cyanobacterial origin. They perform essential metabolic and biosynthetic functions of global significance, including photosynthesis and amino acid synthesis. Most of the proteins that constitute the functional chloroplast are encoded in the nuclear genome and imported into the chloroplast after translation in the cytosol. Since protein targeting is difficult to predict, many nuclear encoded plastid proteins are still to be discovered. Several proteome studies of plant cell organelles have been reported, including chloroplasts and mitochondria. Chloroplasts are of particular interest for plant biologists because of their complex biochemical pathways for essential metabolic functions. In the light of the multiplicity of the molecular, biochemical and physiological functions occurring in the chloroplast, it is expected that the analysis of its proteome will therefore provide new insights into pathway compartmentalization and protein sorting, particularly when plant cells grown and develop under suboptimal environmental conditons. The objectives of this study were to obtain an optimized protocol for isolation and purification of chloroplasts from chlorophyllic cell suspensions of *Bouteloua gracilis* and examine the chloroplast proteomic response to osmotic stress induced by polyethylene glycol (PEG) in the chlorophyllic cells to identify chloroplast proteins associated with stress tolerance. Chloroplasts were isolated from these cell suspension and both soluble and insoluble proteins were extracted and subjected to two-dimensional (2-D) gel electrophoresis. The resolved proteins were subsequently identified by matrix assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS) and a comparative database analysis. 68 protein spots, which were identified as responsive to osmotic stress were then selected for analysis and 43 of these were successfully identified using MALDITOF-MS analysis. 22 of the 43 identified proteins were found to decrease their expression levels during osmotic stress, while 16 were found as proteins that up-regulate their expression levels in response to osmotic stress. Most of the proteins that were down-regulated in response to osmotic stress are involved in

photosynthesis pathways On the other hand, many of the 16 up-regulated proteins were identified as previously well-known osmotic stress-related proteins, such as superoxide dismutase (SOD), heat shock proteins (HSPs) and ascorbate peroxidase (APX). Five novel proteins that were more highly expressed during periods of osmotic stress, but had no clear functional relationship to these conditions, were also identified in this study.

Keywords: *Bouteloua gracilis*, Chloroplast protein, Osmotic stress, MALDI-TOF-MS

Introduction

Plastids constitute a paradigm for organelle proteomics because they have several interesting properties. From an evolutionary perspective, chloroplasts arose from cyanobacteria through endosymbiosis (Goksùyr, 1967). They are semiautonomous organelles, which depend to a large extent on nucleus-encoded proteins that are imported into the plastid after translation in the cytosol. Plastids perform essential cellular functions and harbour a complex biosynthetic machinery for nitrogen fixation, amino acid biosynthesis, sulphur metabolism, isoprenoid biosynthesis, and gene expression (Staehelin and Newcomb, 2000). Despite their importance for cellular function and development, the current understanding of plastid metabolic functions is far from being complete. After thirteen years of chloroplast proteome research, there is now a realistic estimate of the potential contribution of proteomics approaches to chloroplast research. Probably the most valuable information that has been obtained during this time is new information about subcellular protein localization (van Wijk and Baginsky, 2011). Currently, existing prediction tools fail to predict a substantial number of the plastid localized proteins and appear to be less reliable than suggested in the literature. Proteomics can help defining novel protein sorting rules and improving existing prediction algorithms for protein localization. It is expected that the importance of this aspect will increase over the years to come as more and more proteome data become available for different plastid types. Another aspect of importance is the discovery of hypothetical, putative or unknown proteins. Proteomics data demonstrated that a significant number of the proteins that constitute the plastid proteome have one of the above annotations. Although significant progress has been made to establish the proteome of higher plant plastids, the proteins identified to date represent only a small fraction of the complete chloroplast proteome. Much of the progress has been driven by technology development and improved genomics resources. The main difference between proteomic technologies today and 10 years ago is the much improved sensitivity (routinely at 1–50 fmol), the accelerated duty cycle (now tandem mass spectrometry [MS/MS] scans within a few hundred ms), the improved mass accuracy (down to a few ppm for peptides), and the increased resolution (up to 100,000) of the latest generation mass spectrometers. Furthermore

coupling of nano-liquid chromatography (LC) with MS/MS is now routine, and split-free nano-LC systems now deliver low flow rates for nanospray ionization with excellent reproducibility (van Wijk and Baginsky, 2011). Also important is the availability of improved software tools for the reliable identification of peptides based on MS/ MS spectra along with statistically sound estimates of false discovery rates in large data sets. With the maturation of proteomics work flows, quantitative information for plastid proteins became available. These new technologies, in combination with the availability of multiple sequenced plant genomes, now allow for answering more comprehensive and sophisticated questions as compared with a decade ago (Baginsky, 2009; Gstaiger and Aebersold, 2009; Schulze and Usadel, 2010; Walther and Mann, 2010; van Wijk and Baginsky, 2011).

Water deficit is a significant problem in agricultural production, including perennial grasses (Xu and Huang, 2010). Plant adaption to water stress may be accomplished through changes at the molecular, cellular, and physiological levels. Physiological studies have demonstrated that changes in water relation, nutrient uptake, hormonal metabolism, carbon metabolism, and antioxidant metabolism play important roles in drought tolerance (Bray, 1997; Xu and Huang, 2010). Transcriptomic studies have revealed that the expression of a wide range of genes is regulated in response to water deficit (Kreps et al., 2002; Seki et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). A study in *Arabidopsis* showed that 1008 mRNAs were up-regulated in response to water deficit (Kreps et al., 2002). Although RNA and DNA microarrays are powerful tools in the detection of gene expression, limited knowledge of stress-responsive protein expression remains a major gap in understanding biological functions of genes and the linkage between gene expression and physiological functions. Therefore, comprehensive profiling of stress-responsive proteins is important for further understanding the molecular mechanisms controlling plant drought tolerance (Xu and Huang, 2010).

Proteomics, the study of global changes in proteins, offers a powerful approach to discovering the genes and pathways that are crucial for stress responsiveness and

tolerance. The identification and characterization of stress-responsive proteins and their corresponding genes has proven to be of immense practical values. Recently, proteomic-based technologies have been successfully applied, to the systematic study of the proteomic responses in many plant species to a wide range of abiotic stresses, including water stress (Salekdeh et al., 2002; Phee et al., 2004; Plomion et al., 2006; Gazanchian et al., 2007; Hajheidari et al., 2007; Ingle et al., 2007; Kottapalli et al., 2007; Xu and Huang, 2010; Muneer et al., 2014). These studies indicated that abiotic stress altered the abundance of proteins involved in carbohydrate and energy metabolism, cellular detoxification, protein degradation and processing, signal transduction, and cell wall strengthening (Muneer et al., 2014). However, very limited information is available on proteomic responses of perennial grasses to water stress, and even more limited are the studies on chloroplast proteomics in grasses. Perennial grasses may express stress-responsive proteins associated with long-term adaptation or stress survival, as perennial grasses must endure stringent environmental conditions or persist through the tensions periods, unlike annual crops which produce seeds and may die in the case of severe drought (DaCosta and Huang, 2009). Thus, developing long-term adaptation mechanisms is critical for the survival of perennial grasses in water-limiting environments. A better understanding of proteomic responses to water stress in perennial grass species is vital for the development of breeding or biotechnology strategies to improve global plant growth and productivity, and to reduce water use in areas with limited rainfall or irrigation. Investigation of stress-responsive proteins in tolerant cultivar in comparison to sensitive cultivar may identify specific proteins related to stress tolerance in grass. Fu et al. (2007) reported that drought tolerance of creeping bentgrass was improved by overexpression of LEA3 genes encoding dehydrin proteins whereas that, in bermudagrass was found exogenous polyamines as protective effect in response to salt and drought stresses (Shi et al., 2013).

Using *Bouteloua gracilis* as the source plant material, Aguado-Santacruz et al (2001) developed the first and unique chlorophyllic cell suspension in Poaceae, Physiological, biochemical and molecular investigations have been carried out using

the cell model to solve fundamental questions about the plant cell response to osmotic stress (Aguado-Santacruz et al., 2001; Aguado-Santacruz et al., 2002; García-Valenzuela et al., 2005; Aguado-Santacruz et al., 2006). The objective of this study was to obtain a reliable protocol for chloroplast purification of chlorophyllic cell suspensión of *Bouteloua gracilis* and decifrate the chloroplast proteome of the osmotic stress response of *Bouteloua gracilis* at the cell level using the chlorophyllic suspension model.

Materials and Methods

Growth and osmotic treatment of suspension cells of *Bouteloua gracilis*

Chlorophyllic suspension cells of *Bouteloua gracilis* were used to carry out this study. An initial inoculum of chlorophyllic cells was prepared by culturing 1 g fresh weight cells into 50 ml of propagation medium (PM) containing basal salts and vitamins of the MS medium (Murashige and Skoog 1962), 3% sucrose, 1 mg l^{-1} 2,4-D (2,4 dichlorophenoxyacetic acid), 2 mg l^{-1} BAP (benzylaminopurine) and 40 mg l^{-1} adenine (Aguado-Santacruz et al 2001). After growing the cells for eight days in a growth chamber (33±1°C and 33.8% relative humidity) under continuous light (photon flux density = 77 µmol $s^{-}1$ $m^{-}2$) provided by 30 watts fluorescent daylight lamps (General Electric, model F30T8-D), 125 ml Erlenmeyer flasks containing 25 ml of PM were inoculated with 500 mg fresh weight of green cells (García-Valenzuela et al., 2005). Cells of these flasks were grown under the same environmental conditions employed for cultivation of the initial inoculum. After 10 days, chlorophyllic cells were harvested by vacuum filtration system and immediately employed for isolation and purification of chloroplast and for protein extraction.

Isolation of intact chloroplasts

10 day suspension cells of *Bouteloua gracilis* were collected by a vaccum filtration system and used to isolate intact chloroplasts a modification of the method proposed by Kubis et al. (2008). Before starting the purification process of chloroplasts, a

Percoll gradient was prepared (Kubis et al., 2008). Briefly, 10 g of fresh cells are weighed and placed in a precooled mortar. Cells were homogenized with 20 mL of extraction buffer (EB) containing Sorbitol 1 M, Trycine 0.5 M, EDTA 0.25 M, $NaHCO_3$ 0.25 M and, BSA 0.25%. Subsequently the cells were filtered using a steril and folded gauze cloth for obtaining a filtrate in a 100 mL beaker. The collected material was newly filtered using firstly a 100 μm nylon filter and then a 60 μm nylon filter. The filtrate was centrifuged using a precooled 250 mL Nalgene tube for 10 min at $1,000g_{max}$ (brake on) at 4°C; this is equivalent to 3,000 rpm in a SLA-1500 rotor of a Sorvall RC6 centrifuge, with both acceleration and deceleration set to 7. The supernadant was resuspend carefully in residual aprox 500 μL. The resuspended homogenate was transfered onto the top of the preformed Percoll gradient, using 1 mL cutted tips Gilson pipet tip (lacking 5 mm from the fine end for increasing aperture size); pipetting in this step was done very carefully and slowly to avoid disturbing the gradient. To separate the intact chloroplasts from broken chloroplasts and other debris, the Percoll gradient containg the filtrate was centrifuged in a swing-out rotor at $7,800g_{max}$ for 10 min (brake off) at 4°C; this is equivalent to 7,000 rpm in an HB-6 rotor of a Sorvall RC6 centrifuge, with acceleration set to 7 and deceleration set to 2. After centrifugation, the tube was removed carefully and placed in ice. The lower green band in the gradient contains intact chloroplasts, whereas the upper band contains broken chloroplasts. Broken chloroplasts are removed and discarded by pipetting, and the intact chloroplasts are recovered using a 1 mL cutted tips and transferred to a precooled 30 mL nalgene tube. The volumen of recovered intact chloroplast can range from 2 to 6 mL. Finally, 25 mL of resuspension buffer was added to wash off the Percoll gradient.

Preparation of chloroplast proteins

For the isolation of chloroplast proteins, three hundred and fifty mg fresh weight of isolated chloroplasts were suspended in 800 µl of the extraction buffer as described by Aguado-Santacruz et al. (2015). This extraction buffer is composed of 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 50 mM EDTA. Immediately before using, a reducing agent (β-mercaptoethanol) and a cocktail protease inhibitor

(Complete mini, EDTA-free, 11836170001, Roche Diagnostic, Germany) were added to the extraction buffer to final concentrations of 2% (v/v) and 1 mM, respectively. After the cells were mixed with the extraction buffer, 800 µl cold phenol saturated with Tris-HCl, pH 7.5, were added. The mixture was stirred using a vortex mixer for 30 min at 4°C and later centrifuged at 5,000 x g for 30 min at 4°C. The upper phenolic phase was collected in a new tube, and then 800 µl of the extraction buffer were newly added to the remnant pellet in order to repeat the extraction process two more times. To precipitate the protein, 5 volumes of cold 0.1 M ammonium acetate in methanol were added to the collected phenolic phase which was then stored at -20^{o}C overnight. Washing steps were carried out two times with methanol and once with acetone. Finally, the samples were air dried for five min. Subsequently, 150 µl of solubilization buffer were added to the samples. Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ).

2-D gel electrophoresis and isoelectric focusing

For full denaturation, protein samples were incubated in a solubilization buffer (Aguado-Santacruz et al., 2015) that contains two chaotropic agents, 7 M urea and 2 M thiourea; two reducing agents [100 mM DTT (dithiothreitol) and 2 mM TBP (tributyl phosphate)]; two zwitterionic detergents [2.65% CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfo-nate), 2% ASB 14 (amidesulfobetaine-14)] and a nonionic detergent (2% Triton X-100).

Two-dimensional gel electrophoresis (2-DE)

Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ). Solubilized samples containing 1000 µg of protein were applied to 18 cm IPG gel strips with a pH separation range of 3–10 (GE Life Sciences, Piscataway, NJ); we used this size of strips because of the high concentration of protein per µl obtained. After 14 h of rehydration, isoelectric focusing (IEF) was carried out at 22°C, applying 100 V for 2 h, 250 V for one hour, 500 V for 0.5 h, 1000 V for 0.5 h, and finally 8000 V for 13 h in an IPGphor apparatus (GE Life Sciences, Piscataway, NJ), maintaining a limiting current of 50 mA per strip. First dimension

strips were subjected to the standard reduction step with 0.05% DTT for 15 min and the alkylation step with 1.25% 2-iodoacetamide (IAA) for 15 min) prior to running the second dimension electrophoresis. The second dimension (SDS-PAGE) was performed in on 12% polyacrylamide gels using an Ettan DALTsix electrophoresis unit connected to a refrigerating system (GE Life Sciences, Piscataway, NJ). The electrophoresis was carried out for 1 h at 100 V, and later on at a constant current of 30 mA until the dye front reached the bottom of the gel and the proteins were detected by colloidal Coomassie blue staining (PhastGel Blue Coomassie R-350). Five independent samples prepared with each solubilization buffer were run and then, the resulting spots were analyzed.

2-DE Image analysis and protein identification

The stained gels were scanned by transmittance at a resolution of 300 dpi using Lab Scan software (GE Life Sciences, Piscataway, NJ). Images were saved as *.mel files. The software ImageMaster 2D platinum version 6.0 (GE Life Sciences, Piscataway, NJ) was used for spot detection, counting and quantification. The target protein spots were automaticall excised from the stained gels and digested with trypsin using a Spot Handling Workstation (Amersham Biosciences). The protein gel pieces were placed into the 96-well plate and washed with 1:1 mixture of 100 mL of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 20 min at 37°C. The washed solution was drained and the gel pieces were then washed four times with water and incubated in 100 mL of 100 mM ammonium bicarbonate for 20 min at 37°C. Subsequently, 75% acetonitrile was added and the samples dried for 20 min at 40°C. Trypsin digestion was carried out as follows: sequencing-grade porcine trypsin (Promega, Madison, WI, USA) was suspended in 20 mM ammonium bicarbonate at a concentration of 20 mg/mL and 10 mL aliquots were used to rehydrate the dried gel pieces. The trypsin digestion was carried out for 2 h at 37°C. Peptides were extracted from the digest as follows: 50 mL of 50% acetonitrile containing 0.1% TFA was added to each well and incubated for 20 min at 37°C and then the supernatants were transferred to new 96-well plates. The extracts were concentrated for 2 h at 40°C. A solution of peptides was mixed with the same volumen of a matrix solution consisting

of saturated a-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile containing 0.1% TFA. After the peptides were co-crystallized with CHCA by evaporating the organic solvents and then the tryptic-digested peptide masses were measured using an Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences). All MALDI spectra were internally calibrated using a standard peptide mixture (angiotensin III and adrenocorticotropic hormone fragment 18-39, Amersham Biosciences). Peptide mass fingerprint data were matched to the NCBI database using Profound program under 50 ppm mass tolerance.

Results and Discussion

Chloroplast isolation and purification

Chloroplasts are ubiquitous in plants and algae, and perform numerous essential functions including the biosynthesis of amino acids, lipids, nucleotides, hormones, vitamins, and secondary metabolites, as well as oxygenic photosynthesis (Lopez-Juez and Pyke, 2005; Leister, 2003). Chloroplasts are therefore important sites for the production of organic matter and oxygen, and so provide the fuels essential for all higher forms of life (Nelson and Ben-Shem, 2004). In recent years purification protocols of chloroplasts have been described for different plants including *Pisum sativum* (Peltier et al., 2000), *Brassica napus* (Jain et al 2008), *Zea mays* (Lonozky et al., 2004), *Solanum lycopersicum* (Muneer et al., 2014), *Araucaria angustifolia* and *Pinus patula* (Vieira et al., 2014). However, most studies of chloroplast proteomics have focused on Arabidopsis because of its importance as a molecular model (Kleffmann et al., 2004; Phee et al., 2004; Seigneurin-Berny et al., 2008; Kubis et al., 2008; Grabsztunowicz and Jackowski, 2015).

Here we described a simple, rapid and reliable method for isolating chloroplasts from the chlorophyllic cell model of *Bouteloua gracilis*. The samples prepared using the methodology described here are mostly intact, and exhibit minimal contamination from other cellular compartments. To illustrate these points, we analyzed a typical chloroplast preparation using our protocol by light microscopy (Fig. 1)

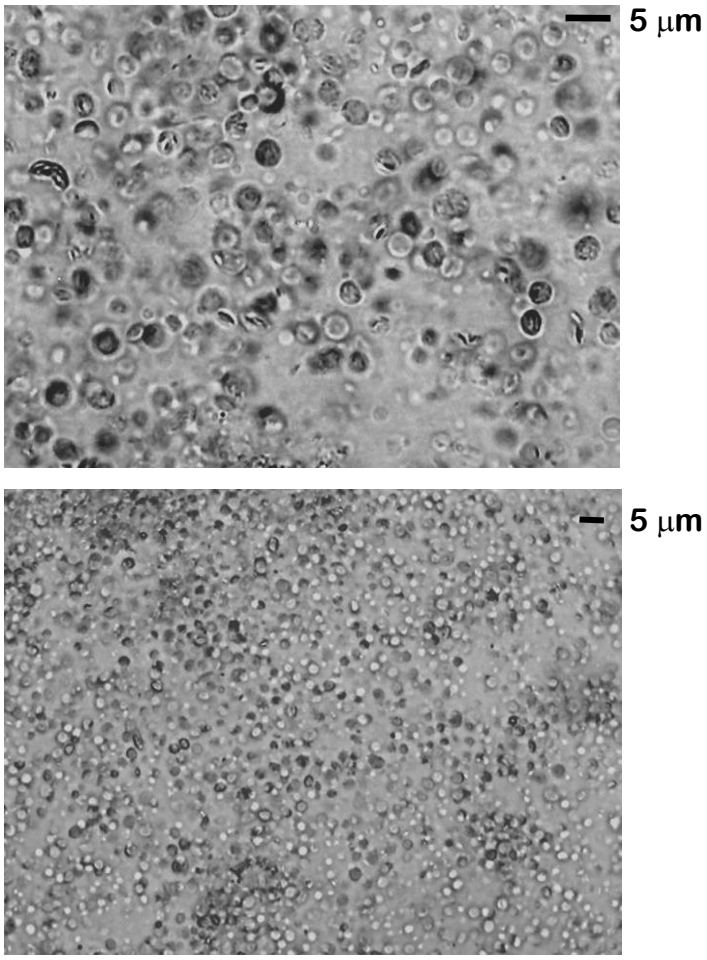

Figure 1. Light micrograph of chloroplasts isolated by a modification of the method of Kubis et al. (2008). Chloroplasts were isolated from 10-day-old suspension cells of *Bouteloua gracilis.*

We tested our protocol in various plants varying the conditions for Percoll purification of chloroplast. From these tests it was evident that *Bouteloua gracilis* chloroplasts were more fragile than chloroplasts purified from spinach or pea leaves. Therefore, all steps of the procedure should be performed by combining carefulness and rapidity. The optimal osmoticum concentration for chloroplast isolation was 1 M sorbitol and this concentration has to be maintained during the entire purification procedure, combined with Trycine 0.5 M, EDTA 0.25 M, $NaHCO_3$ 0.25 M and, BSA 0.25%.

Chloroplast proteomics in osmotic stress

Some physiological studies on the cellular response to osmotic stress have been performed using these cell suspensions of *Bouteloua gracilis* (Aguado-Santacruz et al., 2001; Aguado-Santacruz et al., 2005; Aguado-Santacruz et al., 2006), so we considered very important to address important questions derived from these studies from a proteomics perspective. First, the chlorophyllic cell suspensions of *Bouteloua gracilis* treated with 14% and 21% PEG 8000 respond to osmotic stress by increasing their chlorophyll content according to the strength of the osmotic tension applied (Aguado-Santacruz et al., 2005). Although previous studies in chlorophyllic cells of dicots had documented an increased chlorophyll production under saline stress (Winicov and Button, 1991; Locy et al., 1996; Chang et al., 1997), the study of Aguado-Santacruz et al. (2005) was the first report showing (a proportionally) increase in chlorophyll accumulation in response to osmotic stress in graminaceous chlorophyllic cells.

Thus, high-resolution difference gel electrophoresis was used to detect stress responsive proteins in the chlorophyllic cell suspension of *Bouteloua gracilis* under osmotic stress induced by PEG-8000. A representative gel image is shown in Fig. 2 where the expression of some protein spots altered by PEG-induced water stress. A total of 43 protein spots were successfully identified using MALDITOF-MS analysis. 22 of the 43 identified proteins were found to decrease their expression levels during osmotic stress, while 16 of the candidate proteins showed up-regulated expression levels under osmotic stress. Most of the proteins that were downregulated during osmotic stress are involved in photosynthesis pathways. On the other hand, many of the 16 up-regulated proteins were identified as previously well-known osmotic stress-related proteins, such as superoxide dismutase (SOD), heat shock proteins (HSPs) and ascorbate peroxidase (APX). Five novel proteins that were more highly expressed during periods of osmotic stress, but had no clear functional relationship to these conditions, were also identified in this study (Table 1).

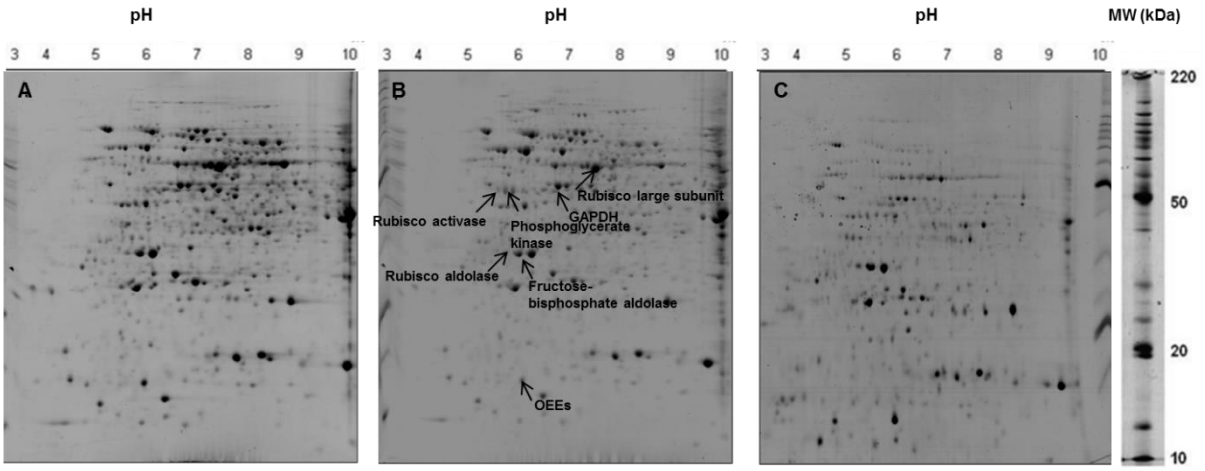

Figure 2. 2-D electrophoresis maps comparing *Bouteloua gracilis* chloroplast protein isolated from normal, 14 and 21% PEG-8000. A) Chloroplast proteins in normal conditions, B) Chloroplast proteins in 14% PEG conditions, C) Chloroplast protein in 21% PEG conditions. 1 mg protein was applied to each IPG strip (18 cm, pH 3-10). For separation of the in the second dimension, a 12% SDS-PAGE was carried out.

The studies of chloroplast proteins in response to water stress have shown that photosynthetic inhibition is one of the primary detrimental effects of water deficit (Lawlor and Cornic, 2002; Kottapalli et al., 2009). Water deficit may limit photosynthesis through stomatal limitation and/or metabolic limitation (Flexas et al., 2004, 2006). However, the more remarkable physiological response of this particular chlorophyll cell line to osmotic stress is an increased chlorophyll content. As reported before by García-Valenzuela et al. (2005), in our study, osmotic stress also increased chlorophyll accumulation in the chlorophyllic system according to the strength of the osmotic stress applied. In the present study, PEG-induced osmotic stress resulted in decline in the abundance of very important proteins involved in all of the three phases of the dark reaction of photosynthesis (carbon fixation, reduction and regeneration), including Rubisco large subunit and Rubisco activase, and Rubisco large subunit, Rubisco activase, chloroplastic aldolase and chloroplastic glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The chloroplast GAPDH is a key enzyme that catalyzes the reduction of 3-phosphoglycerate to triose phosphate, a key step in photosynthesis linking the photochemical events of the thylakoid membranes to the carbon metabolism. Chloroplastic aldolase is an important enzyme in controlling the

Ribulose 1,5-bisphosphate regeneration rate in photosynthesis (Iwaki et al., 1991). Rubisco and Rubisco activase control carbon fixation. Downregulation of Rubisco activase protein and reduction in its enzyme activity has also been reported in other plant species in response to water stress (Costa et al., 1998; Lawlor and Cornic, 2002; Ingle et al., 2007; Kottapalli et al., 2009). Reduced expression of the Rubisco large subunit, Rubisco activase, chloroplastic aldolase and chloroplastic GAPDH indicate that osmotic stress may impose metabolic limitations for photosynthesis in creeping bentgrass o blue grama grass by reducing the abundance of proteins catalyzing carbon fixation, reduction, and regeneration processes. Oxygen evolving enhancer proteins (OEEs) consist of three subunits, OEE 1, OEE 2 and OEE 3. These are nuclear-encoded chloroplast proteins, that are peripherally bound to photosystem II (PSII) on the lumenal side of the thylakoid membrane. In the present study, the abundance of OEE 2 increased under osmotic stress. Previous studies in other plant species also reported that the expression level of OEE is increased by drought stress (Gazanchian et al., 2007) and salinity stress (Murota et al., 1994; Abbasi and Komatsu, 2004; Xu et al., 2010). It is known that OEE 2 and OEE 3 can be easily removed from PSII complex under osmotic stress (Murota et al., 1994). The enhancement of abundance of OEE2 under water stress might be due to an acceleration of the dissociation of these proteins from the PSII complex, suggesting drought-induced damages in PSII system. Previous experiments with tobacco cells grown under saline stress have shown that this enhanced chlorophyll content is precisely due to increased thylakoid number (Chang et al., 1997). These data suggest that the abundance of OEE2 can be related to the osmotic stress-mediated increase the chlorophyll content of this chlorophyllic cell suspension cells under osmotic stress.

Table 1. Effects of PEG-induced osmotic stress on chloroplast protein abundance in suspension cells of *Bouteloua gracilis*

Spot no.	Protein name	Fold change	Category
Decreased			
1-3	ATPase, beta subunit	-2.5	Energy
4	Rubisco large subunit	-3.5	Energy

5	phosphoglycerate kinase	-3.2	Energy
6	fructose-bisphosphate aldolase	-3.1	Energy
7	Rubisco aldolase	-2.9	Energy
8	Rubisco activase	-3.2	Energy
9	ATPase a-subunit	-2.2	Energy
10	ATP synthase CF1 g-chain 1	-2.7	Energy
11	50S ribosomal protein L4	-2.5	Energy
12,13	Light-harvesting complex I	-2.4	Energy
14	Putative fructose bisphosphate aldolase	-2.4	Energy
15	Photosystem II oxygen-evolving complex 33	-2.5	Energy
16	Chloroplastic aldolase	-2.5	Energy
17	Oxygen-evolving complex protein 1	-2.6	Energy
18	Methionine synthase	-2.8	Metabolism
19	Photosystem II oxygen-evolving complex 23	-2.1	Energy
20	50S Ribosomal protein L	-3.1	Energy
21	Fructose-biphosphate (FBP) aldolase	-2.8	Energy
22	Dehydration induced protein-related	-3.5	Unclear classification
Increased			
23	Heat shock protein 70 (dnaK)	+2.1	Protein destination/Storage
24	Glycerol-3-phosphate acyltransferase	+2.5	Energy
25	Putative monodehydroascorbate reductase	+2.2	Disease/Defense
26	Beta-d-glucan exohydrolase	+2.9	Metabolism
27	Fe-superoxide dismutase	+2.9	Disease/Defense
28	Cytochrome f	+2.8	Energy
29	Monodehydroascorbate reductase	+2.2	Disease/Defense
30	Oxygen-evolving enhancer (OEE)	+3.5	Energy
31	Cytochrome b6-f complex iron-sulfur subunit	+3.1	Energy
32	Beta-d-glucan exohydrolase	+3.0	Metabolism
33	Carbonic anhydrase	+2.6	Energy
34	Cyclophilin A-2	+2.4	Protein destination/Storage
35	Glutathione-S-transferase	+2.3	Energy
36	Ascorbate peroxidase (APX)	+2.8	Disease/Defense
37	Catalase-1	+2.9	Disease/Defense
38	Carbonic anhydrase	+3.2	Energy

Unknown protein		
39	Unknown protein	-2.8
40	Unknown protein	-2.6
41	Unknown protein	+2.5
42	Unknown protein	+2.7
43	Unknown protein	+2.5

Other studies have previously reported changes in protein expression related to respiration metabolism under water stress. In *Bouteloua gracilis*, PEG-induced water stress resulted in the reduction of the intensity of protein spots identified as phosphoglycerate kinase, fructose-bisphosphate aldolase, malate dehydrogenase, and enolase. Phosphoglycerate kinase is a transferase enzyme which transfers a phosphate group from 1,3-biphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. Malate dehydrogenase catalyzes the interconversion of malate and oxaloacetate. Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of 2-phosphoglycerate to phosphoenolpyruvate. All of these enzymes catalyze reactions in glycolysis or the citric acid cycle in the respiration metabolism. Plomion et al. (2006) also reported that the abundance of proteins for respiration was reduced under drought stress. The decline in proteins involved in respiration suggests that respiratory activity may be down-regulated for conservation of carbon in relation to reduced photosynthesis under drought stress (Huang and Fu, 2000).

Drought stress often causes accumulation of reactive oxygen species such as singlet oxygen, superoxide radical, hydroxyl radical and hydrogen peroxide in plant cells (Apel and Hirt, 2004). Antioxidant enzymes, such as ascorbate peroxidase (APX), catalase, and glutathione-S-transferase (GST) may be activated to scavenge these toxic compounds during the early phase of plant adaptation to drought stress (Zhang and Kirkham, 1994). The increased expression of GSTs has been identified in several proteomics or transcription analyses of plants that were exposed to different stresses (Sappl et al., 2004; Smith et al., 2004; Roth et al., 2006; Gazanchian et al., 2007; Yang et al., 2007). Hajheidari et al. (2007) reported that drought increased GST

in a water stress tolerant cultivar of wheat while it showed reduced leves in a sensitive cultivar. In the present study, the expression of catalase and GST was increased under osmotic stress. The increased accumulation of APX, catalase and GST in this study indicated that osmotic stress-induced oxidative stress in blue grama grass and different antioxidant enzymes are up-regulated as a mechanism to counteract the damage proked by oxidative stress.

Certainly, definition of the main factors (and organelle interactions) involved in the global cell response to osmotic stress is not easy, however this study provides important information concerning the participation of the chloroplast proteome to osmotic stressl. It is important to highlight that this work is one of the few studies focused to define the participation of the chloroplast proteins in the cell response to osmotic stress. We expect that as more information emerges about the chloroplast proteome in response to osmotic stress we can be able to solve intriguing and fundamental questions of the participation of these organells in the context of the whole plant with important repercussions on our capacity to alter the genome of the plants for increasing their water stress tolerance.

Acknowledgements

We are grateful to the Consejo Nacional de Ciencia y Tecnología (CONACyT) for their financial support through Project 82390 and to INIFAP and CONACyT for student funding.

References

Abbasi FM, Komatsu S. A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics 4:2072–81.

Aguado-Santacruz, G. A.; Cabrera-Ponce, J. L.; Olalde-Portugal, V.; Sanchez-Gonzalez, M. R.; Marquez-Guzman, M. R. and Herrera-Estrella, L. 2001. Tissue

culture and plant regeneration of blue grama grass Bouteloua gracilis (H.B.K.) Lag. ex Steud. In Vitro Cell Dev Biol Plant 37:182–189.

Aguado-Santacruz, G.A. 2006. Genetic manipulation of plants for increased drought tolerance. In: Genetic manipulation of plants for increased drought tolerance. In: Advances in Agricultural and Food Biotechnology (347 p.). Guevara-González, R.G. and Torres-Pacheco, I. (eds). Research Signpost. Kerala, India. pp. 71-97.

Aguado-Santacruz, G.A. and García-Moya, E. 1998. Environmental factors and community dynamics at the southermost part of the North American Graminetum. I. On the contribution of climatic factors to temporal variation in species composition. Plant Ecol 135:13-29.

Aguado-Santacruz, G.A., Moreno-Gómez, B., García-Moya, E., Conde-Martínez, V., Robledo-Paz, A. and Delgado-Alvarado, A. 2015. Efficient protocol for hydrophobic and hydrophilic protein solubilization of free cells from plants for two-dimensional electrophoresis analysis. Plant Mol Biol Rep. 33:1579-1585.

Aguado-Santacruz, G.A., Rascón-Cruz, Q., Cabrera-Ponce, J.L., Martínez-Hernández, A., Olalde-Portugal, V. and Herrera-Estrella, L. 2002. Transgenic plants of blue grama grass, Bouteloua gracilis [H.B.K.] Lag. ex Steud., from microprojectile bombardment of highly chlorophyllous embryogenic cells. Theor App Gen 104:763-771.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–99.

Baginsky, S. and Gruissem W. 2004. Chloroplast proteomics: potentials and challenges. J Exp Bot 55:1213-220.

Bray, E.A. 1997. Plant responses to water deficit. Trends Plant Sci 2:48–54.

Chang, C-C., Locy, R.D., Smeda, R., Sahi, S.V. and Singh, N.K. 1997. Photoautotrophic tobacco cells adapted to grow at high salinity. Plant Cell Rep 16:495–502.

Costa, P., Bahrman, N., Frigerio, J.M., Kremer, A. and Plomion, C. 1998. Water-deficit-responsive proteins in maritime pine. Plant Mol Biol. 38:587–96.

DaCosta, M. and Huang, B. 2009. Physiological adaptations of perennial grasses to drought stress. In: Barrera ED, Smith WK, editors. Perspectives in biophysical plant ecophysiology. Universidad Nacional Autónoma de México; 2009. p. 169–90.

Flexas, J., Bota, J., Galmes, J., Medrano, H. and Ribas-Carbo, M. 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol Plant 127:343–52.

Flexas, J., Bota, J., Loreto, F., Cornic, G. and Sharkey, T.D. 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol 6:269–79.

Fu, D., Huang, B. and Xiao, Y. 2007. Overexpression of barley hva1 gene in creeping bentgrass for improving drought tolerance. Plant Cell Rep. 26:467–77.

García-Valenzuela, X., García-Moya, E., Rascón-Cruz, Q., Herrera-Estrella, L. and Aguado-Santacruz, G.A. 2005. Chlorophyll accumulation is enhanced by osmotic stress in gramineous chlorophyllic cells. J Plant Physiol 162:650-661.

Gazanchian, A., Hajheidari, M., Sima, N.K. and Salekdeh, G.H. 2007. Proteome response of *Elymus elongatum* to severe water stress and recovery. J Exp Bot 58:291–300.

Goksùyr, J. 1967. Evolution of eucaryotic cells. Nature 214: 1161.

Grabsztunowicz, M. and Jackowski, G. 2015. Isolation of intact and pure chloroplasts from leaves of Arabidopsis thaliana plants acclimated to low irradiance for studies on Rubisco regulation. Acta Soc Bot Pol 82:91–95.

Gstaiger M, Aebersold R (2009) Applying mass spectrometry-based proteomics to genetics, genomics and network biology. Nat Rev Genet 10: 617–627.

Hajheidari, M., Eivazi, A., Buchanan, B.B., Wong, J.H., Majidi, I. and Salekdeh, G.H. Proteomics uncovers a role for redox in drought tolerance in wheat. Proteome Res 6:1451–60.

Huang, B. and Fu, J. 2000. Photosynthesis, respiration, and carbon allocation of two cool-season perennial grasses in response to surface soil drying. Plant Soil. 227:17–26.

Ingle, R.A., Schmidt, U.G., Farrant, J.M., Thomson, J.A. and Mundree, S.G. 2007. Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscose*. Plant Cell Environ 30:435–46.

Iwaki, T., Wadano, A., Yokota, A. and Himeno, M. 1991. Aldolase—an important enzyme in controlling the ribulose 1,5-bisphosphate regeneration rate in photosynthesis. Plant Cell Physiol 32:1083–91.

Jain, R., Katavic, V., Agrawal, G.K., Guzov, V.M. and Thelen, J.J. 2008. Purification and proteomic characterization of plastids from *Brassica napus* developing embryos. Proteomics. 8: 3397–3405.

Kleffmann, T., Russenberger, D. Zychlinski, A.V. Wayne, C. Kimmen, S. Wilhelm, G. and Baginsky, S. 2004. The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biology 14:354–362.

Kottapalli, K.R., Rakwal, R., Shibato, J. and Burow, G., Tissue, D., Burke, J. 2009. Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. Plant Cell Environ 32:380–407.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X. and Harper, J.F. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol 130:2129–41.

Kubis, S.E., Lilley, K.S. and Jarvis, P. 2008. Isolation and preparation of chloroplasts from Arabidopsis thaliana plants. Meth Mol Biol 425: 171-186.

Lawlor, D.W. and Cornic, G. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ 25:275–94.

Leister, D. 2003. Chloroplast research in the genomic age. Trends Genet 19:47-56.

Locy, R.D., Chang, C-C., Nielsen, B.L. and Singh, N.K. 1996. Photosynthesis in salt-adapted heterotrophic tobacco cells and regenerated plants. Plant Physiol. 110:321–8.

Lonosky, P.M. Zhang, X., Vasant, G.H. Dobbs, D.L. Fu, A. and Rodermel. S.R. 2004. A Proteomic Analysis of Maize Chloroplast Biogenesis. Plant Physiol 134:560-574.

Lopez-Juez, E. and Pyke, K.A. 2005. Plastids unleashed: their development and their integration in plant development. Int. J Dev Biol 49:557-577.

Muneer, S., Gyeong, Y.P., Manivannan, A., Soundararajan, P. and Ryong, B.J. 2014. Physiological and Proteomic Analysis in Chloroplasts of *Solanum lycopersicum* L. under Silicon Efficiency and Salinity Stress. Int J Mol Sci 15: 21803-21824.

Murota, K., Ohshita, Y., Watanabe, A., Aso, S., Sato, F. and Yamada, Y. 1994. Changes related to salt tolerance in thylakoid membranes of photoautotrophically cultured green tobacco cells. Plant Cell Physiol: 35:107–13.

Nelson, N. and Ben-Shem, A. 2004. The complex architecture of oxygenic photosynthesis. Nat Rev Mol Cell Biol 5:971-982.

Peltier, J.B. Friso, G. Kalume, D.L., Roepstorff, P. Nilsson, F. Adamska, I. and van Wijk, K.J. 2000. Proteomics of the Chloroplast: Systematic Identification and Targeting Analysis of Lumenal and Peripheral Thylakoid Proteins. The Plant Cell 12:319–341.

Phee, B-K., Cho, J-H., Park, S., Jung, J.H., Lee, Y-H., Jeon, J-S., Bhoo, S.H. and Hahn T-R. 2004. Proteomic analysis of the response of *Arabidopsis* chloroplast proteins to high light stress. Proteomics. 4: 3560–3568.

Plomion, C., Lalanne, C., Claverol, S., Meddour, H., Kohler, A. and Bogeat-Triboulot, M. 2006. Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. Proteomics 6:6509–27.

Roth, U., Roepenack-Lahaye, E. and Clemens, S. 2006. Proteome changes in Arabidopsis thaliana roots upon exposure to Cd2+. J Exp Bot 57:4003–13.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. 2002. Proteomic analysis of rice leaves during drought stress and recovery. Proteomics. 2:1131–45.

Sappl, P.G., Onate-Sanchez, L., Singh, K.B. and Millar, A.H. 2004. Proteomic analysis of glutathione S-transferases of Arabidopsis thaliana reveals differential salicylic acid-induced expression of the plant-specific phi and tau classes. Plant Mol Biol. 54:205–19.

Schulze, W.X. and Usadel, B. 2010. Quantitation in mass-spectrometry-based proteomics. Annu Rev Plant Biol 61: 491–516.

Seigneurin-Berny, D., Dorne, A.J., Joyard, J. and Rolland, N. 2008. Percoll-purified and photosynthetically active chloroplasts from *Arabidopsis thaliana* leaves. Plant Physiol Biochem 46:951–955.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M. and Oono, Y. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31:279–92.

Shi, H., Ye, T. and Chan, Z. 2013. Comparative Proteomic and Physiological Analyses Reveal the Protective Effect of Exogenous Polyamines in the Bermudagrass (*Cynodon dactylon*) Response to Salt and Drought Stresses. J. Proteome Res 12: 4951–4964.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 2007. Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–7.

Smith, A.P., DeRidder, B.P., Guo, W.J., Seeley, E.H., Regnier, F.E. and Goldsbrough PB. 2004. Proteomic analysis of Arabidopsis glutathione S-transferases from benoxacor- and coppertreated seedlings. J Biol Chem 279:26098–104.

Staehelin, L.A. and Newcomb, E.H. 2000. Membrane structure and membraneous organelles. In: Buchanan B, Gruissem W, Jones R, eds. Biochemistry and molecular biology of plants. Rockville, Maryland: Ame Soc Plant Physiol, 358-410.

van Wijk, K.J. and Baginsky, S. 2011. Plastid Proteomics in Higher Plants: Current State and Future Goals. Plant Physiol.155: 1578–1588.

Vieira, L.dN., Faoro, H., Fraga, H.P.dF., Rogalski, M., and de Souza, E.M. 2014. An Improved Protocol for Intact Chloroplasts and cpDNA Isolation in Conifers. PLoS ONE 9: e84792

Walther, T.C. and Mann, M. 2010. Mass spectrometry-based proteomics in cell biology. J Cell Biol 190: 491–500.

Winicov, I. and Button, J.D. 1991. Accumulation of photosynthesis gene transcripts in response to sodium chloride by salt-tolerant alfalfa cells. Planta. 183:478–83.

Xu, C. and Huang, B. 2010. Differential proteomic responses to water stress induced by PEG in two creeping bentgrass cultivars differing in stress tolerance. J Plant Physiol 167:1477–1485.

Xu, C., Sibicky, T. and Huang, B. 2010. Proteomic profiling and identification of differential salt responsive proteins in leaves and roots in two cultivars of creeping bentgrass differing in salinity tolerance. Plant Cell Rep. 29:595–615.

Yang, Q., Wang, Y., Zhang, J., Shi, W., Qian, C. and Peng, X. 2007. Identification of aluminum responsive proteins in rice roots by a proteomic approach: cysteine synthase as a key player in Al response. Proteomics. 7:737–49.

Zhang, J., Kirkham, M.B. 1994. Drought stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol. 35:785–91.

CAPITULO IV

A NOVEL "YELP" CHLOROPHYLL-DEFICIENT MUTANT OF *Bouteloua gracilis:* PROTEOMIC APPROACH

Moreno-Gómez, B.*[2], García-Moya, E.[2], Conde-Martínez, F. V.[2], Robledo-Paz, A.[3] and Delgado-Alvarado, A.[4], Aguado-Santacruz, G.A.[1]

[1]/ División de Estudios de Postgrado e Investigación. Instituto Tecnológico de Roque, Km 8 Carretera Celaya-Juventino Rosas, Roque, Celaya, Guanajuato 38110, México.

[2]/ Postgrado en Botánica, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México

[3]/ Postgrado en Genética, Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Edo. México 56230, México.

[4]/ Postgrado en Estrategias para el Desarrollo Agrícola Regional, Colegio de Postgraduados, Campus Puebla. Km 125.5 Carretera Carretera Federal México-Puebla, Santiago Momoxpan, San Pedro Cholula, Puebla 72760, México.

***Autor de correspondencia**

Moreno-Gómez, B.

Abstrac

“YELP” is a yellow spontaneous stable mutant of the wild-type chlorophyllic cell line of *Bouteloua gracilis*, which is chlorophyll-deficient but able to develop etioplast-like plastids in the presence of light. In order to reveal the genetic and biochemical basis of this mutation, 2-DE was used to investigate the difference of the chloroplast protein expression patterns between ‘YELP’ and its parental wild type chlorophyllic cell line ‘TADH-XO’. From the results of 2-DE gels analysis, approximately 750 spots were detected on each gel, and 55 spots were expressed differently at least two-fold between the mutant and the wild type cells lines. Using MALDI-TOF/TOF MS, 40 of 55 spots were identified, which could be categorized into three classes: carbon metabolism, energy metabolism and defense/stress response. Compared to the parental wild-type highly chlorophyllic cell line ‘TADH-XO’, the expression of ascorbate peroxidase and malate dehydrogenase was up-regulated in ‘YELP’, whereas, other proteins as the large subunit of the Ribulose 1,5-bisphosphate carboxylase/oxygenase and the Oxygen-evolving enhancer protein (OEE) was down-regulated in ‘YELP’. Because of their participation in practically all fundamental metabolic processes of the cell, particularly, photosynthesis and free radical scavenging, the results of our study are explained on the basis of the presence of green, well-developed and functional chloroplasts.

Keywords: *Bouteloua gracilis*, albine mutant, chlorophyll biosynthesis, proteomics

Introduction

Mutants altered in the chlorophyll biosynthesis have been reported in many high plants such as rice (Wu and Zhang et al., 2007), tomato (Terry, 1997) and *Arabidopsis thaliana* (Kumar and Soll, 2000; Eckardt et al., 2007). The characterization of mutants is a great challenge because many genes are involved in the mutation. In spite of these difficulties to characterize mutants with atypical production of photosynthesis pigments, many studies have permitted to identify different genes associated with the chlorophyll synthesis and/or chloroplast development. Mutants altered in any step of the chlorophyll synthesis or chloroplast biogenesis represent important experimental models for studying the biochemistry, physiology and genetics of these biological processes. These mutants can be obtained spontaneously or can be induced from plant tissue or cells cultured *in vitro* (Phillips et al., 1994). Albinism is a common problem encountered in interspecific crosses and tissue culture experiments including anther culture and generation of doubled haploids. It is characterized by partial or complete loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes. This pigment loss, in turn, impairs photosynthesis and the plants eventually die at a young stage without reaching maturity (Kumari et al., 2009). In the last years, many chloroplast-related mutants with alterations in leaf pigment content and composition, chlorophyll fluorescence, plastid-to-nucleus signaling, and plastid division were investigated (Leister, 2003). The mutated genes encode enzymes (Carol et al., 1999; Wu et al., 1999), components involved in protein translocation (Settles et al., 1997; Walker et al., 1999; Klimyuk et al., 1999), proteolysis (Chen et al., 2000), protein complex assembly (Sundberg et al., 1997), metal ion homeostasis (Curie et al., 2001), thylakoid biogenesis (Meurer et al., 1998; Kroll et al., 2001), mRNA processing and translation (Fisk et al., 1999; Felder et al., 2001), xanthophyll cycle (Niyogi et al., 1998), porphyrin metabolism and transport (Mochizuki et al., 2001; Møller et al., 2001) and different subunits of the photosynthetic apparatus (Li et al., 2000; Varotto et al., 2000; Munekage et al., 2001). Cereal genetic stocks offer a unique collection of chloroplast- deficient mutants, most of which have been genetically and biochemically

characterized (Henningsen et al., 1993; Stern et al., 2004). The barley albostrians mutant lacking plastid ribosomes was used to elucidate the influence of plastids on mitochondrial gene expression revealing that impaired plastids enhance transcript levels of mitochondrial genes in green tissues (Hedtke et al., 1999). Furthermore, the same mutant was used to study the regulation of tetrapyrrole biosynthesis pathway and its involvement in the chloroplast–nucleus interaction (Yaronskaya et al., 2003). The barley TIGRINA-d gene, ortholog to the *Arabidopsis* Flu gene, was found to be involved in feedback regulation of tetrapyrrole biosynthesis leading to suppression of the protochlorophyllide (Pchlide) accumulation in the dark (Lee et al., 2003). Other barley mutants were used to elucidate some key steps of chlorophyll biosynthesis, for instance, xantha-l encodes a mutated form of Mg-protoporphyrin IX monomethyl ester cyclase (Rzeznicka et al., 2005), while xantha-f, -g, and -h carry genetic lesions at three distinct loci encoding the three Mg-chelatase subunits (Jensen et al., 1996). Photosynthetic maize mutants have revealed the function of a pentatricopeptide repeat (PPR) protein involved in chloroplast biogenesis, suggesting a role for PPR proteins in the modulation of organellar gene expression in plants. Indeed, PPR4 protein is required for the accumulation of the trans-spliced chloroplast rps12 mRNA and, consequently, for the accumulation of plastid ribosomes (Schmitz-Linneweber et al., 2006). Finally, the barley vir-zb63 mutant characterized by a constitutively reduced plastoquinone pool has allowed the recognition of the important role of plastoquinone in post-transcriptional regulation of photosynthetic genes (Frigerio et al., 2007).

In 2005, we obtained a spontaneous chlorophyll-deficient mutant of the wild type chlorophyllic cell line which as named as ‘*YELP*‘ (Yellow Etioplast-Like Plastids), which is characterized, among other alterations, by chlorophyll loss and lower growth rate. In the last five years, the cell ultrastructure and physiological characters of YELP have been studied (Jimenez-Francisco, 2013). It was proved that the yellow color is related to very low levels of photosynthesis pigments (chlorophyll and carotenoids) electronic microscopy analysis revealed remarkable differences in plastid

ultraestructure. Plastids of YELP line were heterogeneous and smaller, with abnormal and poorly developed membrane systems (Jimenez-Francisco, 2013).

Proteome analysis at the level of cellular structures represents a powerful analytical strategy. One of the key potentials of this approach is the capability to enhance the understanding of the biochemical machinery for subsequently functional studies (Hou et al., 2009). The progress of proteomics and related technologies over the last decade is based on two major developments the progress of proteomics and its related technologies (AGI, 2000; Aebersold and Mann, 2003; Ephritikhine et al., 2004; Baginsky and Wilhelm, 2004). 2-DE gel eletrophoretics (2-DE gels) is a classical method for proteomics analysis, which is based on isoelectric point (pI) and Mass for separation of the protein profiles. However, the shortcomings of this technique have to be considered in analyzing differential proteins patterns, particularly the complexity for to analyzing proteins with low abundance and solubility (Santoni et al., 2000). Biological mass spectrometry (MS) is the key technique used for proteome research. The successful combination of 2-DE and MS/MS analysis allowed the comparison of theoretical isoelectric point and molecular mass with the experimentally determined values for each of the pI (Baginsky and Gruissem, 2004). The aim of this work was to analyze and compare the complete proteome of the wild-type highly chlorophyllic cell line 'TADH-XO' and the chlorophyll-deficient line 'YELP' of *Bouteloua gracilis*, exploring some of the causals of the differences between the two cell lines.

Materials and Methods

The mutant yellow cell culture used in this study was obtained from a green cell line generated by us previously (García-Valenzuela et al., 2005). A yellowish cell clump was spontaneously formed within the green cell masses and then it was isolated and cultured on solid medium having the same components of the medium employed for growing the wild-type, green cells (Aguado-Santacruz et al., 2001). After four months, these cells were transferred to liquid medium and subcultured every 10 d.

To characterize this mutant yellow cell line, growth was evaluated for both the green, wild-type line and the yellow mutant cell line in batch cultures for 12 d. As starting material, mutant and wild type cells were collected 8 d after inoculation into fresh liquid medium using a filtration system (Figure 1). Twenty-five Erlenmeyer flasks (125 mL capacity; 25 flasks per treatment) were filled with 24 mL of growing medium MPC and then inoculated with 0.4 g of green or yellow cells. Inoculated flasks were kept on a shaker at 90 r.p.m. in a growth chamber with continuos photon flux of 77 μmol m^{-2} s^{-1} provided by cool fluorescent lamps, temperature of 30 ±1°C and a relative humidity of 33.8%. After 8 d the cells were collected and prepared for protein extraction and 2-D electrophoresis.

Preparation of chloroplast proteins

For the isolation of proteins, 350 mg fresh weight were suspended in 800 µl of the extraction buffer cited in Aguado-Santacruz et al. (2015). This extraction buffer was composed of 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 50 mM EDTA. Immediately before using this buffer, a reducing agent (β-mercaptoethanol) and a cocktail protease inhibitor (Complete mini, EDTA-free, 11836170001, Roche Diagnostic, Germany) were added to the extraction buffer to final concentrations of 2% (v/v) and 1 mM, respectively. After the cells were mixed with the extraction buffer, 800 µl cold phenol saturated with Tris-HCl, pH 7.5, were added. The mixture was stirred on a vortex mixer for 30 min at 4°C and later centrifuged at 5,000 x g for 30 min at 4°C. The upper phenolic phase was collected in a new tube, and then 800 µl of the extraction buffer were newly added to the remnant pellet in order to repeat the extraction process two more times. To precipitate the protein, 5 volumes of cold 0.1 M ammonium acetate in methanol were added to the collected phenolic phase, which was then stored at -20°C overnight. Washing steps were carried out two times with methanol and once with acetone. Finally, the samples were air dried for five min. Subsequently, 150 µl of solubilization buffer were added to the samples. Total protein

was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ).

2-D gel electrophoresis and isoelectric focusing

For full denaturation, protein samples were incubated in a solubilization buffer (Aguado-Santacruz et al., 2015) containing two chaotropic agents (7 M urea and 2 M thiourea), two reducing agents [100 mM DTT (dithiothreitol) and 2 mM TBP (tributyl phosphate)], two zwitterionic detergents [2.65% CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfo-nate), 2% ASB 14 (amidesulfobetaine-14)] and a nonionic detergent (2% Triton X-100).

Two-dimensional gel electrophoresis (2-DE)

Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ). Solubilized samples containing 1200 µg of protein were applied to 18 cm IPG gel strips with a pH separation range of 3–10 (GE Life Sciences, Piscataway, NJ). Due to the concentration of sample per µl, were used 18 cm IPG gel strips. After 14 h of rehydration, isoelectric focusing (IEF) was carried out at 22^{o}C, first applying 100 V for 2 h, later 250 V for one hour, subsequently 0.5 h at 500 V, 0.5 h at 1000 V, and finally 8000 V for 13 h in an IPGphor apparatus (GE Life Sciences, Piscataway, NJ), maintaining a limiting current of 50 mA per strip. First dimension strips were subjected to the standard reduction step with 0.05% DTT, (15 min), and the alkylation step with 1.25% 2-iodoacetamide (IAA, 15 min) prior to running the second dimension electrophoresis. The second dimension (SDS-PAGE) was performed on 12% polyacrylamide gels using an Ettan DALTsix electrophoresis unit connected to a refrigerating system (GE Life Sciences, Piscataway, NJ). The electrophoresis was carried out for 1 h at 100 V, and later on at a constant current of 30 mA until the dye front reached the bottom of the gel and the proteins were detected by colloidal Coomassie blue staining (PhastGel Blue Coomassie R-350).

Five independent samples prepared with each sample were run and then the resulting spots were analyzed.

2-DE Image analysis and protein identification

The stained gels were scanned by transmittance at 300 dpi of resolution using Lab Scan software (GE Life Sciences, Piscataway, NJ). Images were saved as *.mel files. The software ImageMaster 2D platinum version 6.0 (GE Life Sciences, Piscataway, NJ) was used for spot detection, counting and quantification. The target protein spots were automaticall excised from the stained gels and digested with trypsin using a Spot Handling Workstation (Amersham Biosciences). The protein gel pieces were placed into the 96-well plate and washed with 1:1 mixture of 100 mL of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 20 min at 37°C. The washing solution was drained and the protein gel pieces were then washed four times with water and incubated in 100 mL of 100 mM ammonium bicarbonate for 20 min at 37°C. 75% acetonitrile was added and dried for 20 min at 40°C. Trypsin digestion was carried out as follows: sequencing-grade porcine trypsin (Promega, Madison, WI, USA) was suspended in 20 mM ammonium bicarbonate at a concentration of 20 mg/mL and then 10 mL aliquots were used to rehydrate the dried gel pieces. The trypsin digestion was carried out for 2 h at 37^{o}C. Peptides were extracted from the digest as follows: 50 mL of 50% acetonitrile containing 0.1% TFA was added to each well and incubated for 20 min at 37°C and then the supernatants were transferred to new 96-well plates. The extracts were concentrated for 2 h at 40°C. A solution of peptides was mixed with the same volumen of a matrix solution consisting of saturated a-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile containing 0.1% TFA. After the peptides were co-crystallized with CHCA by evaporating organic solvents, tryptic-digested peptide masses were measured using an Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences). All MALDI spectra were internally calibrated using a standard peptide mixture consisting of angiotensin III and adrenocorticotropic hormone fragment 18-39 (Amersham Biosciences). Peptide mass fingerprint data were matched to the NCBI database using Profound program under 50 ppm mass tolerance.

Results and Discussion

Differential protein expression

Results from 2-DE gels showed differential protein expression patterns between wild type, highly chlorophyllic and mutant chlorophyll-deficient cells (Fig. 1). When analyzed with ImageMaster 2D platinum version 6.0 (GE Life Sciences, Piscataway, NJ) the image gels showed approximately 750 protein spots that were reproducibly detected in gels with Coomassie blue staining (PhastGel Blue Coomassie R-350). From these, only 40 spots were differentially expressed more than two-fold between green and yellow cells. Among these spots, we found 17 proteins that were up-regulated and 23 that were down-regulated in the YELP mutant as compared to the wild type parental line 'TADH-XO' (Fig. 2).

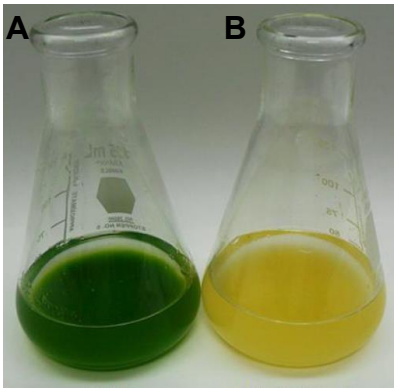

Figure 1. Suspension cells of *Bouteloua gracilis*. A) TADH-XO, wild-type highly chlorophyllic cells. B) YELP, chlorophyll-deficient cells possessing etioplast-like plastids.

Functional identification of the proteins

Most of the down-regulated proteins in YELP were related to carbohydrate and energy metabolism (Table 1). We could predicate that the metabolism system of the YELP cells have been influenced apparently. The 40 proteins identified by MALDI-

TOF/TOF-MS can be classified into functional groups related to carbohydrate metabolism, energy metabolism and defense/stress response (Table 1). Large subunit, Beta subunit of Rubisco, and Rubisco elongation factor TU (spots 3, 2 and 7, respectively), Phosphoglycerate kinase (spot 8), Oxygen-evolving enhancer protein 2 (OEE2; spots 14 and 18), Phosphoribulokinases (spots 9 and 10) and Fructose-bisphosphate aldolase (spot 12) are the main enzymes and proteins related to the carbohydrate metabolism. Certainly, deficiency of these proteins had a profund impact on the metabolism and membranes structure of the plastids of YELP, which show a development apparently arrested at the etioplast stage, despite being expose to continuos light (Jimenez-Francisco, 2013). ATP synthase CF1 beta subunit (spots 4 and 5) and malate dehydrogenase, cytoplasmic (spot 11) belong to energy metabolism protein group. Our results showed that expression of the ATP synthase CF1 beta subunit was significantly decreased in the 'YELP' mutant compared to the TADH-XO cell line (Figure 2; Table 1).

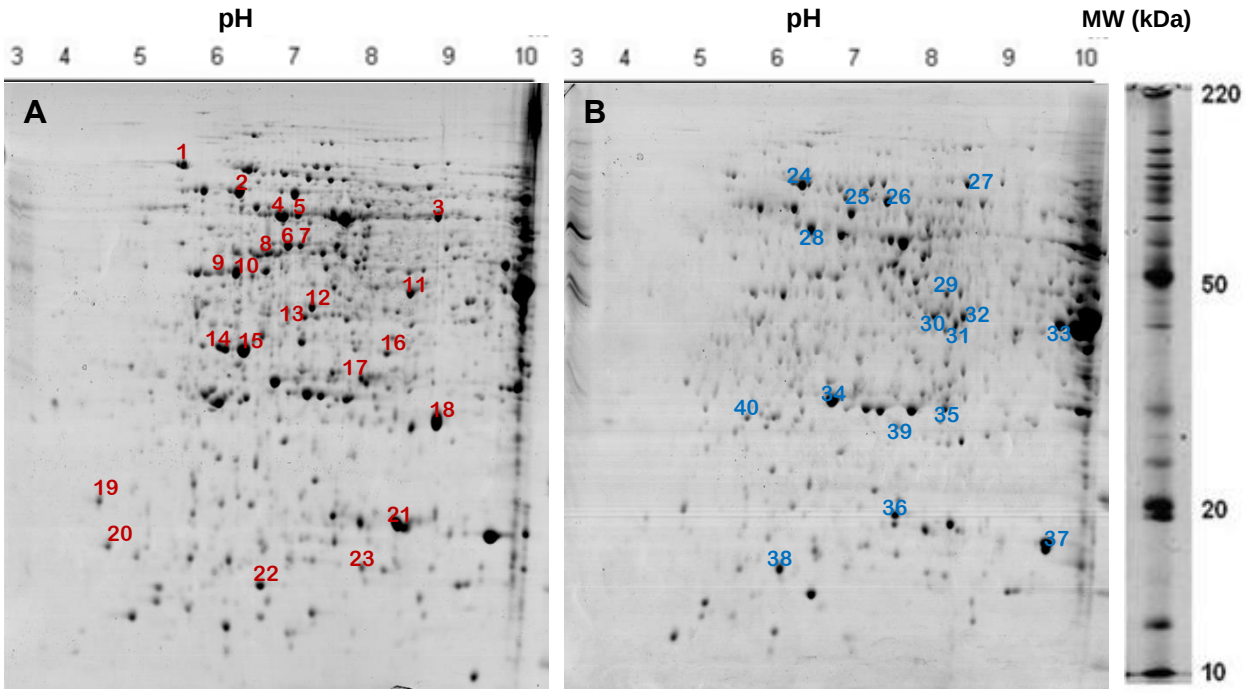

Figure 2. 2-D analysis of 40 spots selected from *Bouteloua gracilis* proteome. A) TADH-XO, wild-type highly chlorophyllic cells. B) YELP, chlorophyll-

deficient. Red color spots indicate down-regulated proteins in YELP. Blue color spots indicate up-regulated proteins in YELP.

A Os04g0434800 chloroplastic ascorbate peroxidase (spot 16), classified within the defense/response stress protein group was found to be down-regulated in 'YELP', whereas Hsp70 (spot 24) and another ascorbate peroxidase (spot 35) both included also in the protective proteins were up-regulated in 'YELP' (Fig. 2). Our study showed that 17 proteins were up-regulated in 'YELP' which were mainly related to the defense/response stress and energy metabolism.

Table 1. Protein identification of spots on 2-DE gels down and up regulated in YELP

Spot no.	Protein name	Category
Decreased		
1	Unknow	
2	Rubisco subunit binding protein beta subunit	Carbohydrate metabolism
3	Ribulose 1,5-bisphosphate carboxylase/oxidase large subunit	Carbohydrate metabolism
4	ATP synthase CF1 beta subunit	Energy metabolism
5	ATP synthase CF1 beta subunit	Energy metabolism
6	Translational elongation factor Tu	Energy metabolism
7	Rubisco translational elongation factor Tu	Carbohydrate metabolism
8	Phosphoglycerate kinase	Carbohydrate metabolism
9	Phosphoribulokinase	Carbohydrate metabolism
10	Phosphoribulokinase	Carbohydrate metabolism
11	Malate dehydrogenase, cytoplasmic	Carbohydrate metabolism
12	Fructose-bisphosphate aldolase	Carbohydrate metabolism
13	Enoyl-reductase	Carbohydrate metabolism
14	Oxygen E. Enhancer protein (OEE)	Energy metabolism
15	Unknown	
16	Os04g0434800-Chloroplastic ascorbato peroxidase	Defense/stress response
17	Unknown	
18	Oxygen-evolving enhancer protein 2 (OEE2)	Energy metabolism
19	Unknown	
20	Unknown	
21	Unknown	
22	Aldolase	Carbohydrate metabolism
23	Unknown	

Increased		
24	Heat shok protein 70 (hsp70)	Defense/stress response
25	2,3-bisphosphoglycerate	Carbohydrate metabolism
26	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Carbohydrate metabolism
27	Os02g0644100 Putative stress-induced protein sti1	Defense/stress response
28	Os06g0136600 Putative enolase	Carbohydrate metabolism
29	Unknown	
30	Malate dehydrogenase	Carbohydrate metabolism
31	Os01g0880800 Stearoyl-ACP desaturase	Energy metabolism
32	Malate dehydrogenase	Carbohydrate metabolism
33	Cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC4	Carbohydrate metabolism
34	Triosephosphate isomerase	Energy metabolism
35	Ascorbate Peroxidase	Defense/stress response
36	Os08g0139100 Putative DAG protein	Plastid development
37	Unknown	
38	actin-depolymerizing factor 3	Defense/stress response
39	Os12g0106000; ferritin	Plastid development
40	Unknown	

Among all the biochemical reaction occurring at the chloroplast, carbohydrate metabolism is generally considered as the major process. In our study, many proteins taking part in this process showed reduced expression levels in the yellow mutant, such as Rubisco translational elongation factor Tu, Rubisco subunit binding protein beta, Rubisco large subunit, phosphoglycerate kinase, phosphoribulokinase, malate dehydrogenase, fructose-bisphosphate aldolase, enoyl-reductase and aldolase (Table 1), all of them important enzymes of the Carbon Metabolism in chloroplast. Regina et al. (2000) reported that FBPase was related to the heat-induced accumulation in the oat chloroplast and FBPase down-regulated expression is related to the low-temperature. Moreover, in another study carried out in a barley albine mutant was also found that phosphoglycerate kinase activity was strongly down-regulated along with triosephosphate isomerase, and aldolase activity. These findings suggest a specific and strong pertubation of the Calvin cycle enzymes. An exception

was the plastidic FBPase, which participates in the Calvin cycle but its expression was not markedly affected. In contrast to the other Calvin cycle enzymes, this enzyme is also involved in plastidic starch synthesis (Boldt et al., 1992).

On the other hand, energy metabolism is the basis of all the life activities. ATP synthase widely exists both in the mitochondria and chloroplast as the main enzyme of the ATP biosynthetic pathway. As a membrane-bound enzyme complex and ion transporter (Park et al., 2007), it produces ATP from ADP in the presence of a proton gradient across the membrane (Tucker et al., 2001). In the chloroplast, this enzyme mainly participates in the photophosporylation reaction. In our study, ATP synthase expression was significantly down-regulated in 'YELP' as compared to 'TADH-XO'. Its down-regulation in 'YELP' is expected to impact the electron transference and oxidative phosphorylation, leading reduced ATP synthesis. The enzyme above-mentioned participate in the energy metabolism process, but locate at different sites of the pathway, and have dissimilar expression patterns in 'YELP'. We can not explain this, but we hypothesize that the activity of ATPase simulated obviously in YELP could cancel out the damage of the electron transfer by down-regulation of other enzymes. Particular interest in this study is the oxygen-evolving enhancer protein 2 (OEE2), which was down-regulated in 'YELP'. This polypeptide has been implicated in photosynthetic oxygen evolution, and it is associated to the photosystem II complex, the site of oxygen evolution in all higher plants and algae (Mayfield et al., 1987). OEE2 is a nuclear-encoded chloroplast protein, and peripherally bound to photosystem II (PSII) on the lumenal side of the thylakoid membrane. The transit peptide which is the signal directing OEE2 to their appropriate compartment of chloroplast resides in the N-terminal region. The transit peptide region is composed of two domains, one with a chloroplast import domain (CID) and the other with a thylakoid transfer domain (TTD). OEEs synthesized in the cytosol translocate across the chloroplast by CID, and intermediate OEEs are yielded. These intermediate proteins subsequently translocate across the thylakoid membrane by TTD (Robinson and Klosgen, 1994), resulting in mature portion of OEEs are located on lumenal side of PSII (Sugihara et al., 2000). Down-regulation of this polypeptide in 'YELP'

suggests that thylakoids are not functional. This is additionally supported by the ultrastructural analysis carried our before by Jimenez-Francisco (2013) in which a disorder in the organization of the membrane structure was evident.

Finally, the heat-inducible proteins termed "heat-shock proteins (Hsps)" constitute an important part of the stress-responsive proteins (Nover, 1991). Hsp70 is reported to act as a molecular chaperon helping in reparation and renaturation of proteins damaged by stress. In addition to heat stress, plant Hsp are also accumulate in response to a large number of stresses, such as heavy metals, oxidative stress, salt, chilling and anoxic conditions (Ahsan et al., 2007). Some reports revealed that the levels of Hsp70 does not change to any extent during stress treatment in maize (Adrian et al., 1998) and rice (Dong et al., 2007). However, in ‘YELP’ the expression of Hsp70 was up-regulated, which suggested that some stress condition maintains turned on the protective machinery of ‘YELP’

2-DE gels proteome analysis showed that the expression of many proteins is affected in the chlorophyll-deficient mutant in comparison to the proteome of the highly chlorophyllic ‘TADH-XO’. However more research should be carried out to define the critical factors leading to the etioplast-like phenotype of the plastids in the ‘YELP’ mutant, which is a fundamental information for explaining the biochemical and physiological basis of chlorophyll synthesis and chloroplast development.

Acknowledgements

We are grateful to the Consejo Nacional de Ciencia y Tecnología (CONACyT) for their financial support through Project 82390 and to INIFAP and CONACyT for student funding.

References

Adrian, A.L., Paul, H.B., Dinakar, B. and Thomas, E.E. 1998. Heat-stress response of maize mitochondria. Plant physiol. 116: 1097-1110.

Aebersold, R. and Mann, M. 2003. Mass spectrometry- based proteomics. Nature 422:198-207.

Aguado-Santacruz, G. A.; Cabrera-Ponce, J. L.; Olalde-Portugal, V.; Sanchez-Gonzalez, M. R.; Marquez-Guzman, M. R. and Herrera-Estrella, L. 2001. Tissue culture and plant regeneration of blue grama grass Bouteloua gracilis (H.B.K.) Lag. ex Steud. In Vitro Cell Dev Biol Plant 37:182–189.

Aguado-Santacruz, G.A., Moreno-Gómez, B., García-Moya, E., Conde-Martínez, V., Robledo-Paz, A. and Delgado-Alvarado, A. 2015. Efficient protocol for hydrophobic and hydrophilic protein solubilization of free cells from plants for two-dimensional electrophoresis analysis. Plant Mol Biol Rep. 33:1579-1585.

Ahsan, N., Lee, D.G., Lee, S.H. and Kang, K.Y. 2007. Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere 67:1182-1193.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408: 796-815.

Baginsky, S. and Gruissem W. 2004. Chloroplast proteomics: potentials and challenges. J Exp Bot 55:1213-220.

Boldt, R., Borner, T. and Schnarrenberger, C. 1992. Repression of the Plastidic lsoenzymes of Aldolase, 3-Phosphoglycerate Kinase, and Triosephosphate Isomerase in the Barley Mutant "albostrians". Plant Physiol. 92:895-900.

Carol, P., Stevenson, D., Bisanz, C., Breitenbach, J., Sandmann, G., Mache, R., Coupland, G. and Kuntz, M. 1999. Mutations in the Arabidopsis gene IMMUTANS cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. Plant Cell 11:57–68

Cheng, D.M., Fan, S.H., Liu, X.L. and Guo, A.G. 2006. Purification and sequence analysis of cDNA coding region for porphobilinogen deaminase from a stage albinism line of wheat. Chin J Biochem Mol Biol 22: 973-978.

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S.L., Briat, J.F. and Walker, E.L. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. Nature 409:346-349.

Dong, G.L., Nagib, A., Lee, S.H., Kang, K.Y., Jeong, D.B., Lee, I.J. and Lee, B.H. 2007. A proteomic approach in analyzing heat-responsive proteins in rice leaves. Proteomics 7: 3369-3383.

Eckardt, N.A. 2007. Thylakoid development from biogenesis to senescence, and ruminations on regulation. Plant Cell 19:1135–1138.

Ephritikhine, G., Ferro, M. and Rolland, N. 2004. Plant membrane proteomics. Plant Physiol Bioch 42: 943-962.

Felder, S. 2001. The nucleus-encoded HCF107 gene of Arabidopsis provides a link between intercistronic RNA processing and the accumulation of translation-competent psbH transcripts in chloroplasts. Plant Cell 13:2127–2141.

Fisk, D.G. 1999. Molecular cloning of the maize gene crp1 reveals similarity between regulators of mitochondrial and chloroplast gene expression.EMBO J 18:2621–2630.

Frigerio, S., Campoli, C., Zorzan, S., Fantoni, L.I., Crosatti, C. and Drepper, F. 2007. Photosynthetic antenna size in higher plants is controlled by the plastoquinone redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 282:29457–29469.

García-Valenzuela, X., García-Moya, E., Rascón-Cruz, Q., Herrera-Estrella, L., Aguado-Santacruz, G.A. 2005. Chlorophyll accumulation is enhanced by osmotic stress in gramineous chlorophyllic cells. J Plant Physiol 162:650-661.

Hou, L. Xu, H., Du, G-Y., Lin, J.T., Duan, M. and Guo, A-G. 2009. Proteome analysis of chloroplast protein in stage albinism line of winter wheat (*Triticum aestivum*) FA85. BMB rep 42:450-455.

Jiménez-Francisco, B. 2013. Fisiología comparada de una línea celular clorofílica y una mutante amarilla del zacate Bouteloua gracilis en condiciones de estrés osmótico. Tesis de maestría. Colegio de Postgraduados. Montecillo Texcoco. pp.122.

Klimyuk, V.I., Persello-Cartieaux, F., Havaux, M., Contard-David, P., Schuenemann, D., Meiherhoff, K., Gouet, P., Jones, J.D., Hoffman, N.E. and Nussaume, L. 1999. A chromodomain protein encoded by the Arabidopsis CAO gene is a plant-specific 25 component of the chloroplast signal recognition particle pathway that is involved in LHCP targeting. Plant Cell. 11:87-99.

Kroll, D., Meierhoff, K., Bechtold, N., Kinoshita, M., Westphal, S., Vothknecht, U.C., Soll, J. and Westhoff, P. 2001. VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. Proc Natl Acad Sci U S A. 98:4238-42.

Kumar, A.M. and Soll, D. 2000. Antisense HEMA1 RNA expression inhibits heme and chlorophyll biosynthesis in Arabidopsis thaliana. Plant Physiol 122: 49-55.

Kumari, M., Clarke, H.J. Samll, I., Kadambot, H.M.S. 2009. Albinism in Plants: A Major Bottleneck in Wide Hybridization, Androgenesis and Doubled Haploid Culture. Crit Rev Plant Sci 28:393-409.

Leister, D. 2003. Chloroplast research in the genomic age. Trends Genet 19:47-56.

Li, X-P., Björkman, O., Shih, C., Grossman, A.R., Rosenquist, M., Jansson, S., Niyogi, K.K. 2000. A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403:391-395.

Mayfield, S.P., Rahire, M., Frank, G., Zuber, H. and Rochaix, J.D. 1987. Expression of the nuclear gene encoding oxygen-evolving enhancer protein 2 is required for high levels of photosynthetic oxygen evolution in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A.84:749-53.

Meurer, J., Grevelding, C., Westhoff, P. and Reiss, B. 1998. The PAC protein affects the maturation of specific chloroplast mRNAs in Arabidopsis thaliana. Mol Gen Genet 258: 342–351.

Møller, S.G., Kunkel, T. and Chua, N.H. 2001. A plastidic ABC protein involved in intercompartmen communication of light signaling. Genes Dev 15: 90-103.

Munekage, Y. 2001. PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110:361–371.

Niyogi, K.K. 1999. Photoprotection revisited: genetic and molecular approaches Annu Rev Plant Physiol Plant Mol Biol 50:333–359.

Nover, L. 1991. Inducers of HSP synthesis: heat shock and chemical stressors; in Heat Shock Response, Nover, L. (ed): PP. 5-40, CRC Press, Florida, USA.

Park, J.E., Kwon, H.J., Kang, Y. and Kim, Y.S. 2007. Proteomic Analysis of O-GlcNAc Modifications Derived from Streptozotocin and Glucosamine Induced β-cell Apoptosis. JBMB 40:1058-1068.

Phillips, R.L., Kaepplert, S.M. and Olhoft, P. 1994. Genetic instability of plant tissue cultures: Breakdown of normal controls. Proceedings of the National Academy of Science of the United States of America 91:5222-5226.

Regina, M. and Shimon, G. 2000. Identification and characterization of a heat-induced isoform of aldolase in oat chloroplast. Plant Mol Biol 44:487-498.

Santoni, V., Molloy, M. and Rabilloud, T. 2000. Membrane proteins and proteomics. Electrophoresis 21:1054-1070.

Settles, A.M., Yonetani, A., Baron, A., Bush, D.R., Cline, K. and Martienssen, R. 1997. Secindependent protein translocation by the maize Hcf106 protein. Science 278: 1467-1470.

Stern, D.B., Hanson, M.R., Barkan, A. 2004. Genetics and genomics of chloroplast biogenesis: maize as a model system. Trends Plant Sci 9:293-301.

Sugihara, K., Hanagata, N., Dubinsky, Z., Baba, S. and Karube, I. 2000. Molecular characterization of cDNA encoding oxygen evolving enhancer protein 1 increased by salt treatment in the mangrove Bruguiera gymnorrhiza. Plant Cell Physiol. 41:1279–1285.

Sundberg, E., Slagter, J.G., Fridborg, I., Cleary, S.P., Robinson, C., Coupland, G. 1997. ALBINO3, an Arabidopsis nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria. Plant Cell 9:717-730.

Terry, M.J. 1997. Phytochrome chromophore deficient mutants. Plant Cell Environ. 20:740-745.

Varotto, C., Pesaresi, P., Meurer, J., Oelmuller, R., Steiner-Lange, S., Salamini, F., Leister, D. 2000. Disruption of the Arabidopsis photosystem I gene psaE1 affects photosynthesis and impairs growth. Plant J 22: 115–124Walker MB, Roy LM, Coleman E, Voelker R, and Barkan A (1999) The maize tha4 gene functions in sec-independent protein transport in chloroplasts and is related to hcf106. J Cell Biol 147: 267-276.

Wu, Z.M. and Zhang, X.B. 2007. A chlorophyll-deficient rice mutant with impaired chlorophy-llide esterification in chlorophyll biosynthesis. Plant Physiol 145:29-40.

Wu, D., Wright, D.A., Wetzel, C., Voytas, D.F. and Rodermel, S. 1999. The IMMUTANS variegation locus of Arabidopsis defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. Plant Cell 11: 43–55

CAPITULO IV

CONCLUSIONES Y DISCUSION GENERAL

En este proyecto de tesis de doctorado se plantearon varias investigaciones para llevar a cabo el trabajo completo titulado “Expresión diferencial de proteínas nucleares y cloroplastídicas de células clorofílicas de *Bouteloua gracilis* en estrés osmótico”. En una primera etapa se realizó la optimización de los protocolos para extracción de proteínas de cloroplasto y de células clorofílicas y mutantes de *Bouteloua gracilis* y separación de estas por electrophoresis bidimensional en geles de poliacrilamida. El cuello de botella en la resolución de proteínas por este método es la composición del bufer de solubilización. En esta primera etapa se formularon y se probaron 16 diferentes bufers de solubilización. La eficiencia de los bufers se determinó por el número de proteínas observadas en los geles, por la resolución y la intensidad de estas proteínas en el análisis de los geles de poliacrilamida. El proceso de extracción de proteínas utilizado en este trabajo se basó en el protocolo de fenol descrito por Issacson et al (2006). Este proceso de extracción fue reportado por otros autores como un protocol eficiente en investigaciones de proteómica (*e.g.* Grosse et al 2008; Cilia et al 2009; Kelley et al 2010) y en un trabajo previo de nuestro equipo de investigación con muestras de células clorofílicas, en el cual se obtuvieron altas cantidades de proteína (arriba de 2.78 μg mg^{-1} de peso fresco) usando este método. Los 16 bufers de solubilización contenían urea 7 M y thiourea 2 M como agentes caotrópicos y la diferencia entre estos bufers se presentó en la concentración de los agentes reductores y detergentes incorporados (Cuadro 1, Capítulo II). Previo al análisis en segunda dimension se realizó un análisis en una sola dimensión. Este primer análisis reveló diferencias entre intensidades y número de bandas y los resultados indicaron que los bufers B9, B10 y B11 mostraron el mayor número de bandas, mientras que los buffers B2, B7 y B16 mostraron la más alta intensidad de las bandas. Los resultados en este análisis nos llevaron a elegir 6 de los 16 bufers para realizar el análisis por electroforesis bidimensional (Cuadro 1, Capítulo II). Debido a que las proteínas hidrofóbicas se agrupan en un pH báscio (Marmagne et al 2004), decidimos analizar las muestras en un rango de pH de 3-10 en

electroforesis bidimensional. En este rango fue fácilmente observable la efectividad de los bufers de solubilización por el número y abundancia de las proteínas en el gel que se encontraron entre los puntos isoeléctricos 3 a 10 y pesos moleculares entre 10 y 220 kDa (Figura 1, Cuadro 2, Capítulo II). Los resultados de este análisis mostraron que el mejor bufer fue el B16 que contenía 7 M de urea, 2 M de thiourea y combinado con los agentes reductores DTT (dithiothreitol) y TBP (tributyl phosphate) a la concentración de 100 mM y 2 mM, respectivamente, dos detergentes zwitterionicos CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfonate) y ASB 14 (amidesulfobetaine-14) a una concentración de 2.65% y 2.00%, respectivamente, y un detergente no ionico Triton X-100 a una concentración de 2.00%. En otros estudios (Chevallet et al 1998; Cordwell et al 2000; Molloy et al 2001; Leimgruber et al 2002; Di-Ciero et al 2004) tambien se mostró la efectividad de estos detergentes del tipo sulfobetainas para análisis en 2-DE. Este bufer fue capaz de resolver 768 proteínas en los geles de poliacrilamida. Nuestros resultados indican que el método basado en el bufer B16 enriqueció las proteínas de membrana en estas muestras, las cuales representan un 32.6% del total de las proteínas (Figura 1, Capítulo II). Esta alta eficiencia es el resultado de la acción combinada de los diferentes componentes del bufer. La urea es un agente caotrópico altamente eficiente en el rompimiento de los enlaces hidrógeno, conduciendo a un desplegamiento de las proteínas y su desnaturalización. Por su parte la tiourea es un compuesto organosulfurado estudiado por primera vez en proteómica (Rabilloud 1998) y es la major opción para romper las interacciones hidrofóbicas, sin embargo no puede ser empleado en altas concentraciones debido a su poca solubilidad en agua. Por su parte, los detergentes tales como SDS, son utilizados para prevenir las interacciones hidrofóbicas entre los dominios de las proteínas hidrofóbicas, lo que resulta en una pérdida de proteína por la agregación y precipitación. Debido a que el detergente aniónico SDS es uno de los surfactantes más eficientes, varios autores recomiendan este detergente para el proceso de solubilización (Boucherie et al 1995; Harder et al 1999). Adicionalmente, los detergentes zwitterionicos, como el CHAPS y las sulfobetaínas (SB 3–10 or ASB 14) llevan a cabo un major proceso de solubilización. Estos compuestos han sido probados eficientemente en combinación

con urea y thiourea para la resolución de proteínas de membrana (Santoni et al 2000; Molloy, 2000). Por otra parte, en estos análisis un paso crítico es la reducción y prevención de la re-oxidación de los enlaces disulfito, en este caso es necesario el empleo de agentes reductores. Los agentes redactores comunmente usados son el DTT y el DTE, los cuales son aplicados en exceso (100 mM). Sin embargo, estos dos no son muy recomendables cuando la muestra tiene alto contenido de proteínas que contienen cisteina. Hebert et al (1998) propuso el TBP (tributylphosphine) en bajas concentraciones como una alternative al DTT. Nuestros datos muestran que el TBP solubiliza eficientemente proteínas ácidas y básicas de las células clorofílicas de *Bouteloa gracilis.*

En una segunda investigación se realizó la optimización de un protocolo para el aislamiento y purificación de cloroplastos intactos y posteriormente se llevó a cabo el análisis en los cambios de expression de las proteínas de cloroplastos de células de *Bouteloua gracilis* sometidas a estrés osmótico con PEG8000. Los cloroplastos son ubicuos en plantas y algas, y llevan a cabo numerosas funciones esenciales, incluyendo la biosíntesis de aminoácidos, lípidos, nucleótidos, hormonas, vitaminas y metabolitos secundarios, así como la fotosíntesis oxigénica (López-Juez y Pyke, 2005; Leister, 2003). Los cloroplastos son por lo tanto los sitios importantes para la producción de materia orgánica y oxígeno, por lo que proporcionan los combustibles esenciales para todas las formas superiores de vida (Nelson y Ben-Shem, 2004). En los últimos años los protocolos de purificación de los cloroplastos se han descrito para diferentes plantas, incluyendo *Pisum sativum* (Peltier et al., 2000), *Brassica napus* (Jain et al., 2008), *Zea mays* (Lonozky et al., 2004), *Solanum lycopersicum* (Munir et al., 2014), *Araucaria angustifolia* y *Pinus patula* (Vieira et al., 2014). Sin embargo, la mayoría de los estudios de proteómica del cloroplasto han sido centrados en Arabidopsis debido a su importancia como modelo molecular (Kleffmann et al, 2004; Phee et al, 2004;. Seigneurin-Berny et al, 2008; Kubis et al., 2008; Grabsztunowicz y Jackowski, 2015). En esta segunda investigación describimos un método simple, rápido y fiable para el aislamiento de cloroplastos con el modelo de células clorofílica de *Bouteloua gracilis*. Las muestras preparadas utilizando la metodología descrita aquí presentan cloroplastos intactos, y exhiben

una contaminación mínima de otros compartimentos celulares. Para ilustrar estos puntos, se analizó una preparación típica de cloroplasto utilizando nuestro protocolo por microscopía de luz (Fig. 1 Capítulo III). Este protocolo fue evaluado en varias plantas empleando diferentes concentraciones de Percoll. A partir de estas pruebas fue evidente que los cloroplastos de *Bouteloua gracilis* eran más frágiles que los cloroplastos purificados a partir de espinaca u otras plantas. Por lo tanto, todos los pasos del procedimiento se deben realizar con sumo cuidado y rapidez. La concentración óptima osmótica para el aislamiento de cloroplastos fue de 1M de sorbitol y esta concentración fue mantenida durante todo el procedimiento de purificación, en combinación con Tricine 0,5 M, EDTA 0,25 M, NaHCO3 0,25 M y BSA 0,25%.

Adicional a la optimización del protocolo de aislamiento de cloroplastos, se llevó a cabo un análisis de expresión de proteínas cloroplastídicas en células clorofílicas tratadas con PEG 8000. Algunos estudios fisiológicos sobre la respuesta celular al estrés osmótico se han realizado utilizando estas suspensiones de células de *Bouteloua gracilis* (Aguado-Santacruz et al., 2001; García-Valenzuela et al., 2005; Aguado-Santacruz et al., 2006), por lo que consideramos muy importante abordar estudios de proteómica. En primer lugar, las suspensiones de células clorofílica de *Bouteloua gracilis* tratadas con 14% y 21% de PEG 8000 responden al estrés osmótico mediante el aumento de su contenido de clorofila de acuerdo con la fuerza de la tensión osmótica aplicada (García-Valenzuela et al., 2005). Aunque estudios previos en células clorofílicas de dicotiledóneas habían documentado un aumento de la producción de clorofila bajo estrés salino (Winicov y Button, 1991; Locy et al., 1996; Chang et al., 1997), el estudio de García-Valenzuela et al. (2005) fue el primer informe que muestra (proporcionalmente) aumento en la acumulación de clorofila en respuesta al estrés osmótico en las células clorofílica de esta gramíneas. Por lo tanto, se utilizó electrophoresis de alta resolución en geles de poliacrilamida para detectar las proteínas de cloroplasto de respuesta de estrés en la suspensión celular clorofílica de *Bouteloua gracilis* bajo estrés osmótico inducido por PEG-8000. Una imagen de los geles representativos se muestra en la Fig. 2 (Capítulo III), donde se observa que la expresión de algunas proteínas es alterada por el estrés osmótico

inducido por PEG 8000. Un total de 43 proteínas se identificaron con éxito utilizando análisis MALDITOF-MS. 22 de las 43 proteínas identificadas se encontraron que disminuyen sus niveles de expresión durante estrés osmótico, mientras que 16 de las proteínas detectadas mostraron niveles incrementados de expresión bajo estrés osmótico. La mayor parte de las proteínas que disminuyen durante el estrés osmótico están involucrados en las vías de sintesis de la fotosíntesis. Por otra parte, muchas de las 16 proteínas incrementadas fueron identificadas como proteínas previamente conocidas relacionadas con el estrés osmótico, tales como la superóxido dismutasa (SOD), proteínas de choque térmico (HSP) y ascorbato peroxidasa (APX). Cinco nuevas proteínas fueron más altamente expresadas durante los períodos de estrés osmótico, pero no tenían relación funcional clara a estas condiciones (Tabla 1, Capítulo III). Los estudios de proteínas de cloroplastos en respuesta al estrés osmótico han demostrado que la inhibición fotosintética es uno de los efectos perjudiciales primarios de déficit de agua (Lawlor y Cornic, 2002; Kottapalli et al., 2009). El déficit de agua puede limitar la fotosíntesis a través del cierre de los estomas y/o limitaciones metabólica (Flexas et al., 2004, 2006). Sin embargo, la respuesta fisiológica más notable de esta línea celular clorofilica en particular al estrés osmótico es un aumento en el contenido de clorofila. Como se informó anteriormente por García-Valenzuela et al. (2005), en nuestro estudio, el estrés osmótico también aumentó la acumulación de clorofila en el sistema clorofílica de acuerdo con la fuerza del estrés osmótico aplicado. En el presente estudio, el estrés osmótico inducido por PEG resultó en una disminución de la abundancia de proteínas muy importantes implicadas en las tres fases de la reacción oscura de la fotosíntesis (la fijación de carbono, reducción y regeneración), incluyendo la subunidad grande de Rubisco, Rubisco activasa, Rubisco aldolase cloroplastídica y Glyceraldehydes-3-fosfato deshidrogenasa (GAPDH). La GAPDH cloroplastídica es una enzima clave que cataliza la reducción de 3-fosfoglicerato a triosa fosfato, un paso clave en la fotosíntesis que une los eventos fotoquímicos de las membranas de tilacoides para el metabolismo de carbono. La Aldolasa cloroplástica es una enzima importante en el control de la tasa de regeneración de 1,5-ribulosa bifosfato en la fotosíntesis (Iwaki et al., 1991). Rubisco y Rubisco activase controlan la fijación de

carbono. La disminución de la Rubisco activasa y la reducción en su actividad también se ha descrito en otras especies de plantas en respuesta al estrés hídrico (Costa et al., 1998; Lawlor y Cornic, 2002; Ingle et al, 2007; Kottapalli et al 2009). La reducción de expresión de la subunidad grande Rubisco, Rubisco activasa, Aldolasa cloroplastídica y GAPDH cloroplastídica indican que el estrés osmótico puede imponer limitaciones metabólicas para la fotosíntesis en varios pastos al reducir la cantidad de proteínas que catalizan la fijación de carbono, reducción, y los procesos de regeneración. Por otro lado, las proteínas potenciadoras de oxígeno en evolución (OEE) constan de tres subunidades, OEE 1, 2 y OEE OEE 3. Estos son proteínas de cloroplastos codificadas en el núcleo, que están periféricamente unidas al fotosistema II (PSII) en el lado luminal de la membrana tilacoide. En el presente estudio, la abundancia de OEE 2 aumentó bajo estrés osmótico. Estudios previos en otras especies de plantas también describen que el nivel de expresión de OEE se incrementa por la sequía (Gazanchian et al., 2007) y el estrés salino (Murota et al., 1994; Abbasi y Komatsu, 2004; Xu et al, 2010). Se sabe que OEE, OEE 2 y 3 se pueden disociar fácilmente del complejo PSII bajo estrés osmótico (Murota et al., 1994). El aumento de la abundancia de OEE2 bajo estrés hídrico podría ser debido a una aceleración de la disociación de estas proteínas del complejo PSII, lo que sugiere daños inducidos por la sequía en el sistema de PSII. Experimentos anteriores con células de tabaco cultivadas bajo estrés salino han demostrado que este aumento de contenido de clorofila se debe precisamente a mayor número de tilacoides (Chang et al., 1997). Estos datos sugieren que la abundancia de OEE2 puede estar relacionado con el aumento del estrés osmótico mediada por el contenido de clorofila de estas células cloofílicas en suspension.

Otros estudios descritos anteriormente han presentado cambios en la expresión de proteínas relacionadas con el metabolismo de la respiración bajo estrés hídrico. En *Bouteloua gracilis*, el estrés hídrico inducido por PEG8000 resultó en la reducción de la intensidad de las proteína identificadas como Fosfoglicerato quinasa, Fructosa bifosfato aldolasa, Malato deshidrogenasa, y Enolasa. Fosfoglicerato quinasa es una enzima transferasa que transfiere un grupo fosfato a partir de 1,3-biphosphoglycerate a ADP, ATP y la formación de 3-fosfoglicerato. Malato deshidrogenasa cataliza la

interconversión del malato y oxalacetato. Enolasa, también conocida como Deshidratasa fosfopiruvato, es una metaloenzima responsable de la catálisis de 2-fosfoglicerato a fosfoenolpiruvato. Todas estas enzimas catalizan reacciones en la glucólisis o el ciclo del ácido cítrico en el metabolismo de la respiración. Plomion et al. (2006) también reportaron que la abundancia de proteínas que participan en el proceso de respiración se redujo en condiciones de sequía. La disminución de las proteínas implicadas en la respiración sugiere que la actividad respiratoria puede ser sub-regulada para la conservación de carbono en relación a la reducción de la fotosíntesis bajo estrés por sequía (Huang y Fu, 2000). Ciertamente, no es fácil definir los factores principales implicados en la respuesta celular al estrés osmótico, sin embargo, este estudio proporciona información importante sobre la participación del proteoma del cloroplasto para estrés osmótico. Es importante destacar que este trabajo es uno de los pocos estudios enfocados en definir la participación de las proteínas de cloroplastos en la respuesta celular al estrés osmótico. Esperamos que a medida que emerge más información sobre el proteoma del cloroplasto en respuesta a estrés osmótico podamos ser capaces de resolver preguntas intrigantes y fundamentales de la participación de estos organelos en la panta complete con importantes repercusiones en nuestra capacidad para alterar el genoma de las plantas y aumentar su tolerancia al estrés hídrico.

Bibliografía

Abbasi FM, Komatsu S. A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics 4:2072–81.

Aguado-Santacruz, G. A.; Cabrera-Ponce, J. L.; Olalde-Portugal, V.; Sanchez-Gonzalez, M. R.; Marquez-Guzman, M. R. and Herrera-Estrella, L. 2001. Tissue culture and plant regeneration of blue grama grass Bouteloua gracilis (H.B.K.) Lag. ex Steud. In Vitro Cell Dev Biol Plant 37:182–189.

Boucherie H, Dujardin G, Kermorgant M, Monribot C, Slonimski P, Perrot M (1995) Two-dimensional protein map of *Saccharomyces cerevisiae:* construction of a gene-protein index. Yeast 11:601-613.

Chang, C-C., Locy, R.D., Smeda, R., Sahi, S.V. and Singh, N.K. 1997. Photoautotrophic tobacco cells adapted to grow at high salinity. Plant Cell Rep 16:495–502.

Chevallet M, Santoni V, Poinas A, Rouqié D, Fuchs, A, Kieffer S, Rossignol M, Lunardi J, Garin J, Rabilloud T (1998) New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19:1901-1909.

Cilia M, Fish T, Yang X, Mclaughlin M, Thannhauser TW, Gray S (2009) A Comparison of Protein Extraction Methods Suitable for Gel-Based Proteomic Studies of Aphid Proteins. J Biomol Tech 20:201-215.

Cordwell SJ, Noumens AS, Verrills NM, Basseal DJ, Walsh BJ (2000) Subproteomics based upon protein cellular location and relative solubilities in conjunction with composite two-dimensional electrophoresis gels. Electrophoresis 21:1094-1103.

Costa, P., Bahrman, N., Frigerio, J.M., Kremer, A. and Plomion, C. 1998. Water-deficit-responsive proteins in maritime pine. Plant Mol Biol. 38:587–96.

Di-Ciero L, Bellato CM, Meinhardt LW, Ferrari F, Castellari RR, Marangoni S, Novello JC (2004) Assessment of four different detergents used to extract membrane proteins from Xilella fastidiosa by two-dimensional electrophoresis. Braz J Microbiol 35:269-274.

Flexas, J., Bota, J., Galmes, J., Medrano, H. and Ribas-Carbo, M. 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol Plant 127:343–52.

Flexas, J., Bota, J., Loreto, F., Cornic, G. and Sharkey, T.D. 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol 6:269–79.

García-Valenzuela, X., García-Moya, E., Rascón-Cruz, Q., Herrera-Estrella, L. and Aguado-Santacruz, G.A. 2005. Chlorophyll accumulation is enhanced by osmotic stress in gramineous chlorophyllic cells. J Plant Physiol 162:650-661.

Gazanchian, A., Hajheidari, M., Sima, N.K. and Salekdeh, G.H. 2007. Proteome response of *Elymus elongatum* to severe water stress and recovery. J Exp Bot 58:291–300.

Grabsztunowicz, M. and Jackowski, G. 2015. Isolation of intact and pure chloroplasts from leaves of Arabidopsis thaliana plants acclimated to low irradiance for studies on Rubisco regulation. Acta Soc Bot Pol 82:91–95.

Grosse C, Heinekamp T, Kniemeyer O, Gehrke A, Brakhage AA (2008) Protein kinase a regulates growth, sporulation, and pigment formation in *Aspergillus fumigatus.* Appl Environ Microbiol 74:4923-4933.

Harder A, Wildgruber R, Nawrocki A, Fey SJ, Larsen, PM, Görg A (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. Electrophoresis 20:826-829.

Herbert B, Molloy MP, Gooley, AA, Walsh BJ, Bryson, WG, Williams KL (1998) Improved protein solubilization in 2-D electrophoresis using tributyl phosphine as reducing agent. Electrophoresis 19:845-851.

Huang, B. and Fu, J. 2000. Photosynthesis, respiration, and carbon allocation of two cool-season perennial grasses in response to surface soil drying. Plant Soil. 227:17–26.

Ingle, R.A., Schmidt, U.G., Farrant, J.M., Thomson, J.A. and Mundree, S.G. 2007. Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscose*. Plant Cell Environ 30:435–46.

Isaacson T, Damasceno CM, Saravanan RS, He Y, Catalá C, Saladié M, Rose JKC (2006) Sample extraction techniques for enhanced proteomic analysis of plant tissues. Nat Protocol 1:769-774.

Iwaki, T., Wadano, A., Yokota, A. and Himeno, M. 1991. Aldolase—an important enzyme in controlling the ribulose 1,5-bisphosphate regeneration rate in photosynthesis. Plant Cell Physiol 32:1083–91.

Jain, R., Katavic, V., Agrawal, G.K., Guzov, V.M. and Thelen, J.J. 2008. Purification and proteomic characterization of plastids from *Brassica napus* developing embryos. Proteomics. 8: 3397–3405.

Kelley BS, Lee S-J, Damasceno CMB, Chakravarthy S, Kim B-D, Martin GB, Rose JKC (2010) A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. Plant J 62:357-366.

Kleffmann, T., Russenberger, D. Zychlinski, A.V. Wayne, C. Kimmen, S. Wilhelm, G. and Baginsky, S. 2004. The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biology 14:354–362.

Kottapalli, K.R., Rakwal, R., Shibato, J. and Burow, G., Tissue, D., Burke, J. 2009. Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. Plant Cell Environ 32:380–407.

Kubis, S.E., Lilley, K.S. and Jarvis, P. 2008. Isolation and preparation of chloroplasts from Arabidopsis thaliana plants. Meth Mol Biol 425: 171-186.

Lawlor, D.W. and Cornic, G. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ 25:275–94.

Leimgruber RM, Malone JP, Radabaugh MR, LaPorte ML, Violand BN, Monahan JB (2002) Development of improved cell lysis, solubilization and imaging approaches for proteomic analyses. Proteomics 2:135-144.

Leister, D. 2003. Chloroplast research in the genomic age. Trends Genet 19:47-56.

Locy, R.D., Chang, C-C., Nielsen, B.L. and Singh, N.K. 1996. Photosynthesis in salt-adapted heterotrophic tobacco cells and regenerated plants. Plant Physiol. 110:321–8.

Lonosky, P.M. Zhang, X., Vasant, G.H. Dobbs, D.L. Fu, A. and Rodermel. S.R. 2004. A Proteomic Analysis of Maize Chloroplast Biogenesis. Plant Physiol 134:560-574.

Lopez-Juez, E. and Pyke, K.A. 2005. Plastids unleashed: their development and their integration in plant development. Int. J Dev Biol 49:557-577.

Marmagne A, Aude-Rouet M, Ferro M, Rolland N, Alcon C, Joyard J, Garin J, Barbier-Brygoo H, Ephritikhine G (2004) Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. Mol cell proteomics 3:675-691.

Molloy MP, Phadke ND, Maddock JR, Andrews PC (2001) Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. Electrophoresis 22:1686-1696.

Murota, K., Ohshita, Y., Watanabe, A., Aso, S., Sato, F. and Yamada, Y. 1994. Changes related to salt tolerance in thylakoid membranes of photoautotrophically cultured green tobacco cells. Plant Cell Physiol: 35:107–13.

Nelson, N. and Ben-Shem, A. 2004. The complex architecture of oxygenic photosynthesis. Nat Rev Mol Cell Biol 5:971-982.

Peltier, J.B. Friso, G. Kalume, D.L., Roepstorff, P. Nilsson, F. Adamska, I. and van Wijk, K.J. 2000. Proteomics of the Chloroplast: Systematic Identification and Targeting Analysis of Lumenal and Peripheral Thylakoid Proteins. The Plant Cell 12:319–341.

Phee, B-K., Cho, J-H., Park, S., Jung, J.H., Lee, Y-H., Jeon, J-S., Bhoo, S.H. and Hahn T-R. 2004. Proteomic analysis of the response of *Arabidopsis* chloroplast proteins to high light stress. Proteomics. 4: 3560–3568.

Plomion, C., Lalanne, C., Claverol, S., Meddour, H., Kohler, A. and Bogeat-Triboulot, M. 2006. Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. Proteomics 6:6509–27.

Rabilloud T (1998) Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis 19:758-760.

Santoni V, Molloy M, Rabilloud T (2000) Membrane proteins and proteomics: Un amour impossible? Electrophoresis 21:1054-1070.

Seigneurin-Berny, D., Dorne, A.J., Joyard, J. and Rolland, N. 2008. Percoll-purified and photosynthetically active chloroplasts from *Arabidopsis thaliana* leaves. Plant Physiol Biochem 46:951–955.

Vieira, L.dN., Faoro, H., Fraga, H.P.dF., Rogalski, M., and de Souza, E.M. 2014. An Improved Protocol for Intact Chloroplasts and cpDNA Isolation in Conifers. PLoS ONE 9: e84792.

Winicov, I. and Button, J.D. 1991. Accumulation of photosynthesis gene transcripts in response to sodium chloride by salt-tolerant alfalfa cells. Planta. 183:478–83.

Xu, C., Sibicky, T. and Huang, B. 2010. Proteomic profiling and identification of differential salt responsive proteins in leaves and roots in two cultivars of creeping bentgrass differing in salinity tolerance. Plant Cell Rep. 29:595–615.