INSTITUTO POLITÉCNICO NACIONAL

CENTRO DE INVESTIGACIÓN EN BIOTECNOLOGÍA APLICADA

TESIS

“Estudio de la regulación génica mediada por microRNAs ante estrés por frío en sorgo (*Sorghum bicolor*) “

Pedro Fernando Vera Hernández

Directora de tesis:

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ACTA DE REGISTRO DE TEMA DE TESIS
Y DESIGNACIÓN DE DIRECTOR DE TESIS

México, D.F. a 12 de mayo del 20017

El Colegio de Profesores de Estudios de Posgrado e Investigación de CIBA-Tlaxcala en su sesión ordinaria No. 5 celebrada el día 12 del mes de mayo conoció la solicitud presentada por el(la) alumno(a):

Vera	Hernández	Pedro Fernando
Apellido paterno	Apellido materno	Nombre (s)

Con registro: B 1 5 0 6 7 3

Aspirante de: Doctorado en Ciencias en Biotecnología

1.- Se designa al aspirante el tema de tesis titulado:
Estudio de la regulación génica mediada por microRNAs ante estrés por frío en sorgo (*Sorghum bicolor)*

De manera general el tema abarcará los siguientes aspectos:
Estudio de genes y microRNAs asociados a respuesta a estrés por frío en sorgo
Generación y estudio de líneas transgénicas de plantas sobreexpresoras de algunos microRNAs asociados a respuesta a estrés por frío

2.- Se designa como Director de Tesis al Profesor:
Dra. Flor de Fátima Rosas Cárdenas

3.- El trabajo de investigación base para el desarrollo de la tesina será elaborado por el alumno en:
Centro de Investigación en Biotecnología Aplicada- IPN
que cuenta con los recursos e infraestructura necesarios.

4.- El interesado deberá asistir a los seminarios desarrollados en el área de adscripción del trabajo desde la fecha en que se suscribe la presente hasta la aceptación de la tesis por la Comisión Revisora correspondiente:

Director(a) de Tesis

Dra. Flor de Fátima Rosas Cárdenas

Aspirante

Pedro Fernando Vera Hernández

Presidente del Colegio

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CARTA CESIÓN DE DERECHOS

En la Ciudad de Tepetitla de Lardizabal el día 05 del mesNoviembre del año 2019, el que suscribe Pedro Fernando Vera Hernandez alumno del Programa de Doctorado en Ciencias en Biotecnología con número de registro B150673, adscrito a Centro de Investigación en Biotecnología Aplicada manifiesta que es autor intelectual del presente trabajo de Tesis bajo la dirección de la Dra. Fátima Rosas Cárdenas y cede los derechos del trabajo intitulado **"Estudio de la regulación génica mediada por microRNAs ante estrés por frío en sorgo (*Sorghum bicolor*)"**, al Instituto Politécnico Nacional para su difusión, con fines académicos y de investigación.

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ACTA DE REVISIÓN DE TESIS

En la Ciudad de Tepetitla de Lardizabal siendo las 12 horas del día 25 del mes de Octubre del 2019 se reunieron los miembros de la Comisión Revisora de la Tesis, designada por el Colegio de Profesores de Posgrado de: CIBA-IPN

para examinar la tesis titulada: "Estudio de la regulación génica mediada por microRNAs ante estrés por frío en sorgo (*Sorghum bicolor*)"

por el (la) alumno (a):

Apellido Paterno:	VERA	Apellido Materno:	Hernández	Nombre (s):	Pedro Fernando

Número de registro: B 1 5 0 6 7 3

Aspirante del Programa Académico de Posgrado: Doctorado en Ciencias en Biotecnología

Después de la lectura y revisión individual, así como el análisis e intercambio de opiniones, los miembros de la Comisión manifestaron **APROBAR** ■ **NO APROBAR** □ la tesis, en virtud de los motivos siguientes:

cumple con los requisitos establecidos para poder presentar el examen de grado

Comité tutorial

Dra. Fátima Rosas Cárdenas
Profesor colegiado 12884-EB-17

Dra. Silvia Luna Suárez
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Abstract

As sessile organisms, land plants are continuously confronted with a variety of abiotic stresses that causes significant yield losses in the production of crops, plants have evolved perception and signaling mechanisms to respond and adapt to adverse environmental conditions.

Among the different types of abiotic stress, cold is a limiting factor that affects not only sorghum but many other crops productivity worldwide. Low temperature is a significant determinant of the geographical distribution and productivity of plant species. Low temperature affects the growth, development, and distribution of agronomic species throughout the world. Cold stress causes major crop losses and is a severe threat to sustainability.

The objective of this doctoral thesis is to investigate molecular mechanisms in sorghum to understand better how cold stress signals are perceived and integrated into gene regulation by changes in at multiples levels from microRNAs, transcription factors, transcription of mRNAs and subsequent expression of gene products involved in stress adaptation and tolerance. In the long-term the knowledge generated by this research will help to understand the intricated molecular mechanisms governing stress response under cold situations in order develop more tolerant crops to abiotic stressors, allowing higher yields under challenging conditions to support profitable crops to feed the world's growing population.

This thesis is organized in three main chapters focused on the physiological and molecular responses of sorghum to cold stress. The first chapter consist of a review focused on responses of sorghum to deal with cold stress, such as the photosynthesis adaptation, and growth limitation under low temperatures, the transcription factors involved in cold stress response, and how some genotypes of sorghum are being developed to be cold tolerant as well as the sources of this trait.

For the second chapter, we focused on the response of sorghum to cold stress as the accumulation of free proline (PRO) in leaves. PRO can induce the expression of many genes; it is still unclear whether a more efficient PRO accumulation can be considered a biomarker of tolerance in plants. We evaluated the accumulation of PRO in two genotypes of sorghum with different tolerance to cold stress. In order to explore the cause behind the accumulation of proline under cold stress conditions, we analyzed the Transcription Factors Binding Sites (TFBS) present in the promoter regions in the genes involved in the biosynthesis and degradation of proline in sorghum.

For the third chapter of this thesis, we analyzed selected elements of the antioxidant metabolism, genes and microRNAs transcription in leaves of two varieties of sorghum with contrasting tolerance to cold. We quantified different genes coding for proteins involved in response to low temperatures such as transcription factors, detoxification enzymes and heat shock proteins the upregulation of transcription factor CBF6 was notably high reaching values up to 5 fold changes suggesting that this expression level might be involved in a better adaptation of the plant to cold conditions. We also tested part of the antioxidant metabolism by evaluating the content of phenolic compounds, flavonoids, to see if the radical-scavenging capacity is related to cold tolerance in sorghum.

During the development of this thesis, our project had different branches around molecular mechanisms that plants has developed to deal with abiotic stress, for this reason we included as appendices original articles or book chapters produced during our doctoral studies. The firs attachment is a book chapter in wich we describe techniques for isolation detection and quantification of microRNAs, on the same order of ideas in order to understand the molecular responses to stress it is necessary to quantify many transcript by different techniques such as qPCR, on the next article we describe the validation of housekeeping genes for qPCR normalization data on Amaranth with we presented the most stable reference genes, which will contribute significantly to future gene studies of this species that is characterized by a high degree of tolerance to multiple types of abiotic stressors.

In summary the results generated by this thesis will help to comprehend the molecular mechanisms governing cold stress responses in sorghum and other crops in order to develop more tolerant crops, allowing higher yields under challenging conditions to feed the world's growing population.

Resumen

Las plantas se enfrentan continuamente a una variedad de estés abiótico que causan pérdidas significativas de rendimiento en la producción de cultivos, las plantas han desarrollado mecanismos de percepción y señalización para responder y adaptarse a condiciones ambientales adversas.

Entre los diferentes tipos de estrés abiótico, el frío es un factor limitante que afecta no solo al sorgo sino a la productividad de muchos otros cultivos en todo el mundo. Las bajas temperaturas son determinantes para la distribución geográfica y la productividad de los cultivos. La baja temperatura afecta el crecimiento, desarrollo y distribución de especies agronómicas en todo el mundo. El estrés por frío provoca grandes pérdidas de cultivos y es una grave amenaza para la sostenibilidad.

El objetivo de esta tesis doctoral es investigar los mecanismos moleculares en el sorgo para comprender mejor cómo se perciben e integran las señales de estrés por frío en la regulación génica por cambios en niveles múltiples de microRNAs, factores de transcripción, transcripción de RNAs mensajeros y la posterior expresión de productos genéticos involucrados en adaptación al estrés y tolerancia. A largo plazo, el conocimiento generado por esta investigación ayudará a comprender los complejos mecanismos moleculares que rigen la respuesta al estrés en situaciones de frío para desarrollar cultivos más tolerantes a los estresores abióticos, lo que permite mayores rendimientos en condiciones difíciles para apoyar cultivos rentables para alimentar el crecimiento mundial. población.

Esta tesis está organizada en tres capítulos principales centrados en las respuestas fisiológicas y moleculares del sorgo al estrés por frío. El primer capítulo consiste en una revisión centrada en las respuestas del sorgo para tratar el estrés por frío, como la adaptación de la fotosíntesis y la limitación del crecimiento a bajas temperaturas, los factores de transcripción involucrados en la respuesta al estrés por frío y cómo se están desarrollando algunos genotipos de sorgo para Ser tolerante al frío, así como las fuentes de este rasgo.

El segundo capítulo, se centra en la respuesta metabólica del sorgo al estrés por frío, analizamos la acumulación de metabolitos como prolina libre (PRO) en hojas. PRO puede inducir la expresión de muchos genes; Todavía no está claro si una acumulación de PRO más eficiente puede considerarse un biomarcador de tolerancia en las plantas. Evaluamos la acumulación de PRO en dos genotipos de sorgo con diferente tolerancia al estrés por frío. Para explorar la causa detrás de la acumulación de prolina en condiciones de estrés por frío, analizamos los sitios de unión de factores de transcripción (TFBS) presentes en las regiones promotoras en los genes involucrados en la biosíntesis y degradación de la prolina en el sorgo.
Para el tercer capítulo de esta tesis, analizamos elementos seleccionados del metabolismo antioxidante, la transcripción de genes y microRNAs en dos variedades de sorgo con tolerancia contrastante al frío. Cuantificamos diferentes genes que codifican las proteínas involucradas en la respuesta a bajas temperaturas, tales como factores de transcripción, enzimas de desintoxicación y proteínas de choque térmico, la regulación positiva del factor de transcripción CBF6 fue notablemente alcanzando valores de hasta 5 veces, lo que sugiere que este nivel de expresión podría estar involucrado en una mejor adaptación de la planta al frío. También evaluamos parte del metabolismo antioxidante cuantificando el contenido de compuestos fenólicos, flavonoides y su relación con la producción de radicales teniendo como premisa que está relacionada con la tolerancia al frío en el sorgo.

Durante el desarrollo de esta tesis, nuestro proyecto tenía diferentes ramas alrededor de los mecanismos moleculares que las plantas desarrollaron para lidiar con el estrés abiótico, por esta razón incluimos como apéndices artículos originales o capítulos de libros producidos durante nuestros estudios de doctorado. El primer archivo adjunto es un capítulo de libro en el que describimos técnicas para la detección de aislamiento y cuantificación de microARN, en el mismo orden de ideas para comprender las respuestas moleculares al estrés, es necesario cuantificar muchas transcripciones mediante diferentes técnicas como qPCR, en En el siguiente artículo describimos la validación de genes de mantenimiento para los

datos de normalización de qPCR en Amaranto, presentamos los genes de referencia más estables, lo que contribuirá significativamente a futuros estudios genéticos de esta especie que se caracteriza por un alto grado de tolerancia a múltiples tipos de estrés abióticos.

En resumen, los resultados generados por esta tesis ayudarán a comprender los mecanismos moleculares que rigen las respuestas al estrés por frío en el sorgo y otras plantas para desarrollar cultivos más tolerantes, permitiendo mayores rendimientos en condiciones difíciles para alimentar a la creciente población mundial.

Table of contents

Products of doctoral thesis

The following original articles were the product of this project and are included in the doctoral thesis, the articles listed in part I are focused on the main objective of this thesis which is to generate knowledge around the molecular mechanisms of adaptation and tolerance to cold stress in Sorghum and their provable biotechnological applications. The student is the first author of the articles

- **Vera-Hernández, P**., Martínez Núñez, M., Ruiz-Rivas, M., Rosas-Cárdenas. (In process) Responses of sorghum to cold stresses: a review focused on molecular breeding.

- **Vera-Hernández, P**., Ortega Ramírez, M., Martínez Núñez, M., Ruiz-Rivas, M., Rosas-Cárdenas. (2018) Proline as a probable biomarker of cold stress tolerance in Sorghum (*Sorghum bicolor*). *Mexican Journal of Biotechnology 2018, 3(3):77-86* doi: 10.29267/mxjb.2018.3.3.77

- **Vera-Hernández, P**., Martínez Núñez, M., Ruiz-Rivas, M., Rosas-Cárdenas. (In process) Metabolic and molecular responses to low temperatures of Sorghum (*Sorghum bicolor*) in phenotypes with contrasting tolerance to cold stress

The following articles have also been included as a second part of the thesis since they have been developed as multiple branches of the project during the doctoral studies

- **Vera Hernández, P.**, Martínez Núñez, M., Ruiz Rivas, M., Bibbins Martínez, M., Luna Suárez, S., Rosas Cárdenas, F. (2018). Reference genes for RT-qPCR normalization in different tissues, developmental stages and stress conditions of amaranth. *Plant Biology*.

●**Vera Hernández, P.**, Martínez Núñez, M., Ruiz Rivas, M., Bibbins Martínez, M., Luna Suárez, S., Rosas Cárdenas, F. (In process). A cost-effective and sensitive Probed Based Universal (PBU) qPCR method for the quantification of microRNAs in plants.

●**Vera-Hernández P.F**., de Folter S., Rosas-Cárdenas F..F. (2019) Isolation and Detection Methods of Plant miRNAs. In: de Folter S. (eds) Plant MicroRNAs. Methods in Molecular Biology, vol 1932. Humana Press, New York, NY

The following articles were made in collaboration with other colleagues, and the graduate student is mentioned as a co-author, the articles are not attached to this thesis but can be consulted online or upon request.

●Martínez Núñez, M., Ruiz Rivas, M., Bernal Muñoz R., **Vera Hernández P**., Luna Suárez, S., Rosas Cárdenas, F. (2019) The phenological growth stages of different amaranth species grown in restricted spaces based in BBCH code, South African Journal of Botany, ISSN 0254-6299.

●Likai Wang , Zhiyuan Zhang , Fan Zhang , Zhengyao Shao , Bo Zhao , Austin Huang, **Vera-Hernandez F**, Hong Qiao (In press) EIN2-directed histone acetylation requires EIN3-mediated positive feedback regulation in response to ethylene, Proceedings of the National Academy of Sciences

Chapter I: Responses of sorghum to cold stress a review focused on molecular breeding

Sorghum [(Sorghum bicolor L.) Moench] is the fifth most important crop plant, which can be used for human food, livestock feed, bioenergy, or industrial purposes. The crop has an excellent adaptation potential to different types of abiotic stress, such as drought, high salinity, and high temperatures, but it is susceptible to low temperatures compared to other monocotyledonous species. We have reviewed and discussed some of the researches and developments focused on the mechanisms, gene regulation and signal transductions determining sorghum cold tolerance to help to improve the understanding of the nature of such trait.

Cold tolerance; a rare trait in sorghum

It is hard to determine when and where the domestication of sorghum occurred, but it is hypothesized that was in north-eastern Africa [1] under hot, dry conditions; Therefore, it has been well adapted to drought and high-temperature conditions in which other cereals do not grow, however, sorghum a very sensitive crop at low temperatures compared to most monocotyledonous species.

The gradual expansion to higher altitude areas has led to the occurrence of genotypes better adapted to cold climates, and its introduction in other parts of the world has resulted in the development of more cold-tolerant varieties as well as early maturation and insensitive photoperiod varieties that can grow under this adverse conditions.

Among the different types of abiotic stress, cold is a limiting factor that affects not only sorghum but many other crops productivity worldwide. Low temperature is a major determinant of the geographical distribution and productivity of plant species. The cold temperatures restrict sorghum production temporally, by reducing the window periods for cultivation in places where the crop is already farmed; and

spatially, since cannot grow in colder regions. In this paper we reviewed and discussed some of the research focused on the physiological and genetic nature of cold tolerance.

Sorghum is a crop adapted mainly to warm areas, where minimum average temperatures during the growing season are generally maintained above 18 ° C. However, a minimum amount of sorghum has been grown in the colder highlands of China and some sites in Africa [2,3]. These sorghums are cold tolerant and can be adapted and cultivated in high lands where rainfall is scarce and erratic, and growth conditions are generally less suitable for sorghum cultivation [4].

The term "cold tolerance" is used to describe the ability of sorghum genotypes to germinate, grow, and produce seed satisfactorily under conditions of low temperatures, but above freezing [5].

The genome of sorghum has been sequenced (~730 Mb) [6] is arranged into 10 chromosome pairs or $2n = 20$. In parallel with the analysis of cold stress-induced transcriptome profiling [7], analysis of single nucleotide polymorphisms (SNPs) [7,8], and analysis of changes in contents metabolites such as sugars, lipids, proline [9] provides an excellent tool for breeders to improve stress tolerance of this critical crop.

Adverse effects of cold stress exposure that affects yields in sorghum

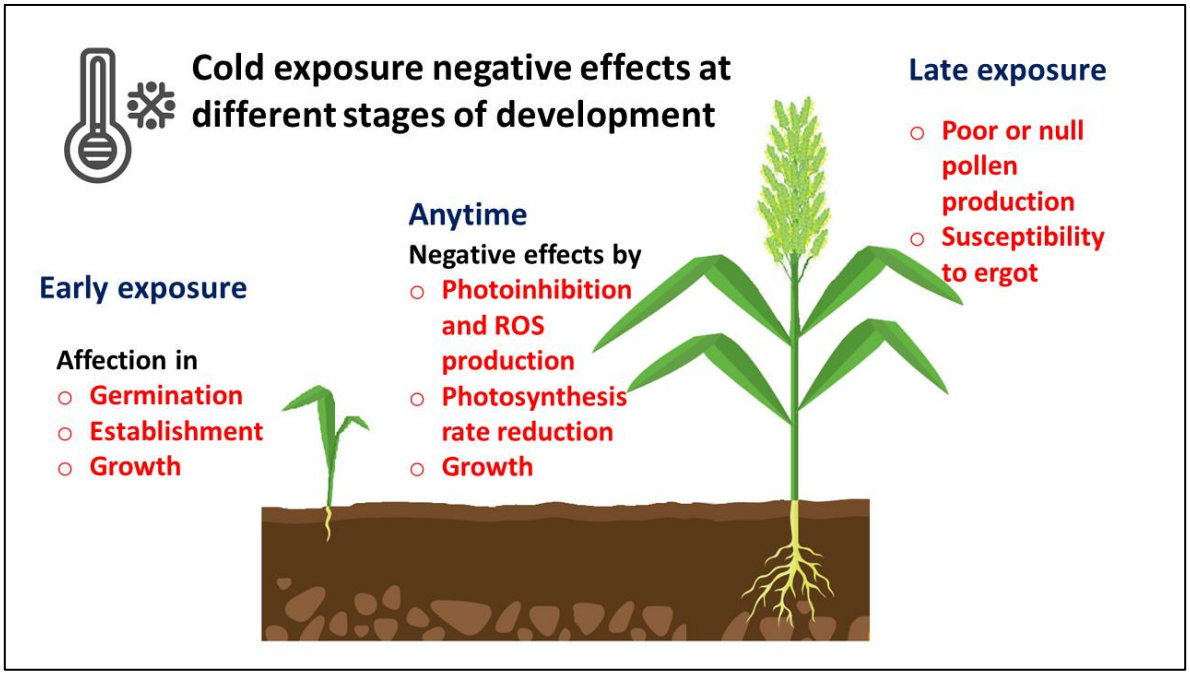
Depending on the phenological state, susceptible sorghums are exposed to cold, one or more of the following characteristics appear:

(i) Early cold exposure. When sorghum is sown at the beginning of the spring, it expresses poor or no germination. Early exposure to cold stress negatively affects germination establishment and growth, reducing sorghum biomass production and yield [2,3,8,10]. In this context, the development of early exposure cold-tolerant sorghum varieties allows a sooner sown to take advantage of spring moisture and permit two cropping cycles per year increasing total production.

(ii) Continuous or late exposure to cold stress. Susceptible genotypes can sometimes grow up to vegetative penological stages, but reproductive stages are affected, in some cases, flowering does not occur, and in some other genotypes reproductive stages appear, but pollen production is affected or even abolished [5]. More specifically, the young microspore stage during atherogenesis is especially susceptible, and male sterility is observed causing massive yield loss. In this same scenario, late or absent production of pollen during flowering under cold conditions favors ergot (*Claviceps Africana*) infection in cold susceptible varieties[11].

Most of the research has been focused on the development of early-season tolerant genotypes. Empirically, cold tolerance in sorghum has been measured by different traits under low temperatures conditions such as germination[12], or seedling vigor [13]. More recent studies suggest the use of methods based on a rank-summation index of common traits like percentage, emergence index, shoot and root dry weight, seedling height, and vigor score during growth under low-temperature stress [14].

By planting short-life varieties and manipulating the sowing date, severe damage can be avoided in the early cold exposure conditions; however, the development of resistant varieties is necessary to deal with late or prolonged exposure to cold.



Photosynthesis reduction to chilling temperatures

C4 photosynthesis has a higher potential efficiency of light and water use than C3 photosynthesis. However, only a few C4 plants can maintain photosynthetically competent leaves at chilling temperatures (<15°C) [15].

The capacity for active photosynthesis during exposure to low temperatures is essential for sorghum or any other crop bred for cold tolerance. In C4 metabolism, Pyruvate Phosphate Dikinase (PPDK) has a critical function for the carboxylation of pyruvate to phosphoenolpyruvate (PEP). A sorghum extract of this enzyme was very sensitive to cold treatment, after 8 min on ice, the enzyme lost about half of its activity, while other C4 species such as *Spartina pectinate*, and *Amaranthus hybridus*, showed a higher cold tolerance [15]. However, there are some C4 plants adapted to cold conditions, such as the grass *Miscanthus giganteus* [16], which has an elevated level of expression of Rubisco and Pyruvate Phosphate Dikinase (PPDK) under cold stress, contrasting to maize and other cold susceptible C4 plants

when exposed to low temperatures. Rubisco reduced activity under low temperatures is another limitation to carbon assimilation in C4 compared with C3 plants photosynthetic rates [17].

Reduction in growth

Reduction in sorghum development can be explained by the susceptibility of its C4 photosynthetic machinery to low temperatures, but also by the upregulation of the expression of DREB1/CBF, necessary to activate cold responsive genes, but at the same time causing growth inhibition in Arabidopsis and rice [18] The mechanism of the growth inhibition involves the stimulation of the expression of Gibberellin inactivating enzymes [19] in a process to promote survival or escape from adverse environmental conditions.

Photoinhibition and production of Reactive Oxygen Species (ROS)

Chilling, coupled with light, can lead to photoinhibition, a phenomenon where the transference of energy from chlorophyll to oxygen is unbalanced, producing toxic ROS [20]. ROS causes oxidative damage to membranes by lipid peroxidation, proteins and DNA [21,22].

Sorghum has developed mechanisms to prevent the production of ROS, and once formed, to detoxify and repair the damage. Primary, sorghum can reduce light absorption by two ways; photoreceptors can sense bright light and move the chloroplast away from the light [23,24]; also some sorghum carotenoid pigments such as zeaxanthin and antheraxanthin can dissipate the excess of absorbed light energy [25] and inhibiting lipid peroxidation [26]. Second, sorghum has an

antioxidant machinery to scavenge ROS composed by; enzymatic constituents such as superoxide dismutases, catalases, and glutathione reductases; as well as antioxidants compounds like ascorbic acid, and flavonoids [27].

The phenolic compounds content is modified during chilling stress [28,29], and there is an association between cold tolerance and higher basal concentration of such compounds in other cultivars [29] Proline usually play a role as an osmolyte but can also work as a powerful antioxidant [21], and a higher proline accumulation during cold stress might be related a better tolerance [9].

Physiological responses to cold stress

Stomata aperture and closing

Cold-sensitive plants usually have low leaf water potentials, while cold-tolerant plants preserve water potentials by closing their stomata and preventing transpiration water loss during chilling [30]; therefore stomatal conductance and transpiration rates have been proposed as features used for marker assisted selection [31] .

Metabolic changes in response to cold

Nitrogenous compounds

Certain amino acids and amine compounds adjust their metabolism during cold stress, particularly those associated with proline biosynthesis [32], there are two biosynthesizes pathways glutamate and ornithine, a Transcription Factors Binding Sites (TFBS) analysis found the presence of a Low Temperature Response Element (LTR) in the promoter region of ɗ- ornithine amino transferase (ɗ-OAT), suggesting

that the Orn pathway might be response to cold responses[9]. GABA (c-aminobutyric acid) is an amine metabolite associated with cryoprotection in other monocots [33].

Sugars

Sugars are an energy source, but also represent carbon precursors, substrates for polymers, storage and signaling molecules important during cold stress. Trehalose, together with its precursor trehalose-6-phosphate, are disaccharides whit a protective role in proteins and membranes during abiotic stress [34,35]. The overexpression of Alpha-trehalose-phosphate synthase (TSP1) in sorghum made the transformants tolerate high salinity as well as the development of higher root growth and biomass [36], but tolerance under cold stress has not been tested yet.

Functional proteins

Heat shock proteins (HSP) function as chaperons and protect proteins from the harmful effect of different types of abiotic stress besides heat, including cold [37,38]; A transcriptomic analysis showed that the HSP Sb03g027330 was highly abundant under stress cold stress [7]. Glutathione-S-transferases (GSTs) have functions in detoxification of xenobiotic compounds, and ROS; they are also abundant under cold stress in sorghum [7]. Late Embryogenesis Abundant (LEA) proteins are essential for membrane stabilization when the cytoplasm becomes dehydrated [39], there is no evidence of accumulation of this proteins during cold stress in experimental conditions tested, but there is a genetic potential since this proteins are accumulated

in drought stress [40] and might be expressed under cold conditions by a crosstalk signaling process with ABA [41].

Molecular responses to cold stress

Transcription factors involved in activation of cold stress responsive genes

Transcription Factors (TFs) are a class of proteins that bind to cis-regulatory DNA sequences responsible for either positively or negatively influence the transcription of specific genes, determining whether a particular gene will be turned "on" or "off" in an organism [42]. Dehydration Responsive Element binding factors (DREB), are members of the APETALA 2/Ethylene-Responsive Element (AP2/ERF) family, which include many critical regulatory and stress responding genes [43,44]. DREBs induce the expression of functional target genes involved in abiotic stresses.

Genes coding for DREB proteins regulates the transcription of a large number of genes involved in the plant response to cold stresses. However, the regulation of DREB genes is not well understood in sorghum. Bioinformatic analysis showed that the motifs within sorghum DREB promoters are mainly involved in abscisic acid-, light- and calcium-mediated regulation [45].

CBF/DREB and ethylene response factor (ERF) proteins share evolutionary and structural relationships [43,46]; In sorghum, both families have groups that contain an LWSY motif at the C-terminus which respond to cold treatment, suggesting that such motif may be involved in cold plant tolerance [47].

Chromatin remodeling and epigenetic memory

The chromatin remodeling derived from epigenetic changes during cold acclimatization has not been studied yet in sorghum, nevertheless there are some clues about epigenetic memory to cold stress; sorghum exposed to photoinhibition treatment changes in the levels of the carotenoids then restored during a recovery period and when rephotoinhibited, the plants showed better protection against photoinhibition [25].

Cold acclimatization is a process conserved between species where many genes are induced [48]. Histone marks like acetylation and deacetylation are central for activation and repression during cold acclimation, the HOS15 gene product that works as histone deacetylator, specifically interacts with histone H4 during cold acclimatization in *A. thaliana* [49,50].

Sources of cold stress tolerance and genomic mapping

Some sources of cold tolerance in sorghum have been identified [51–53]. One of the most studied sources of cold tolerances is Chinese landraces, which under controlled and field cold conditions show a higher emergence and seedling vigor compared to commercial lines (21).

Some undesirable characteristics are linked to cold-tolerant sorghums, particularly grain tannins and high length [54]. Ideally, breeders get the same yield as the commercial hybrids on the outsprint and conserve the desired tolerance making use of marker-assisted selection tools; Quantitative Trait Locus (QTLs) are the most extensive tool for marker-assisted selection. A QTL is a specific chromosomal region

or genetic locus in which particular sequences of bases in DNA markers are statistically associated with variation in a trait that in this case, contribute to cold tolerance.

In sorghum co-localization of different QTLs close to each other are known as hot spots, and highly heritable genes map irregularly to the ten sorghum chromosomes, and several are physically clustered together in chromosomes 1, 2, 3, 4, and 6 (figure 2).

Among different features associated to a better tolerance to cold stress in sorghum, we can list; anthocyanin levels [55] which has been suggested to have photoinhibition a protective function in chilling exposure [56]; survival to cold conditions [8,57]; early vigour ,rate and time of germination under cold environment [8,53,58];chlorophyll content [59], transpiration [31], stomatal conduction [31] , under cold conditions.

Selection for early and late cold tolerance under field conditions is sometimes the most affordable way to select cold-tolerant genotypes which can germinate, grow and develop under challenging circumstances where sensitive genotypes cannot; nevertheless, erratic climatic conditions might misperceive selection for cold temperature stress, also, the polygenic nature of col-tolerance complicates field evaluation due to the presence of genotype×environment interactions. controlled conditions and recommended for further research to evaluate greenhouse screening procedures for cold tolerance in combination with field evaluation under multi-environment testing.

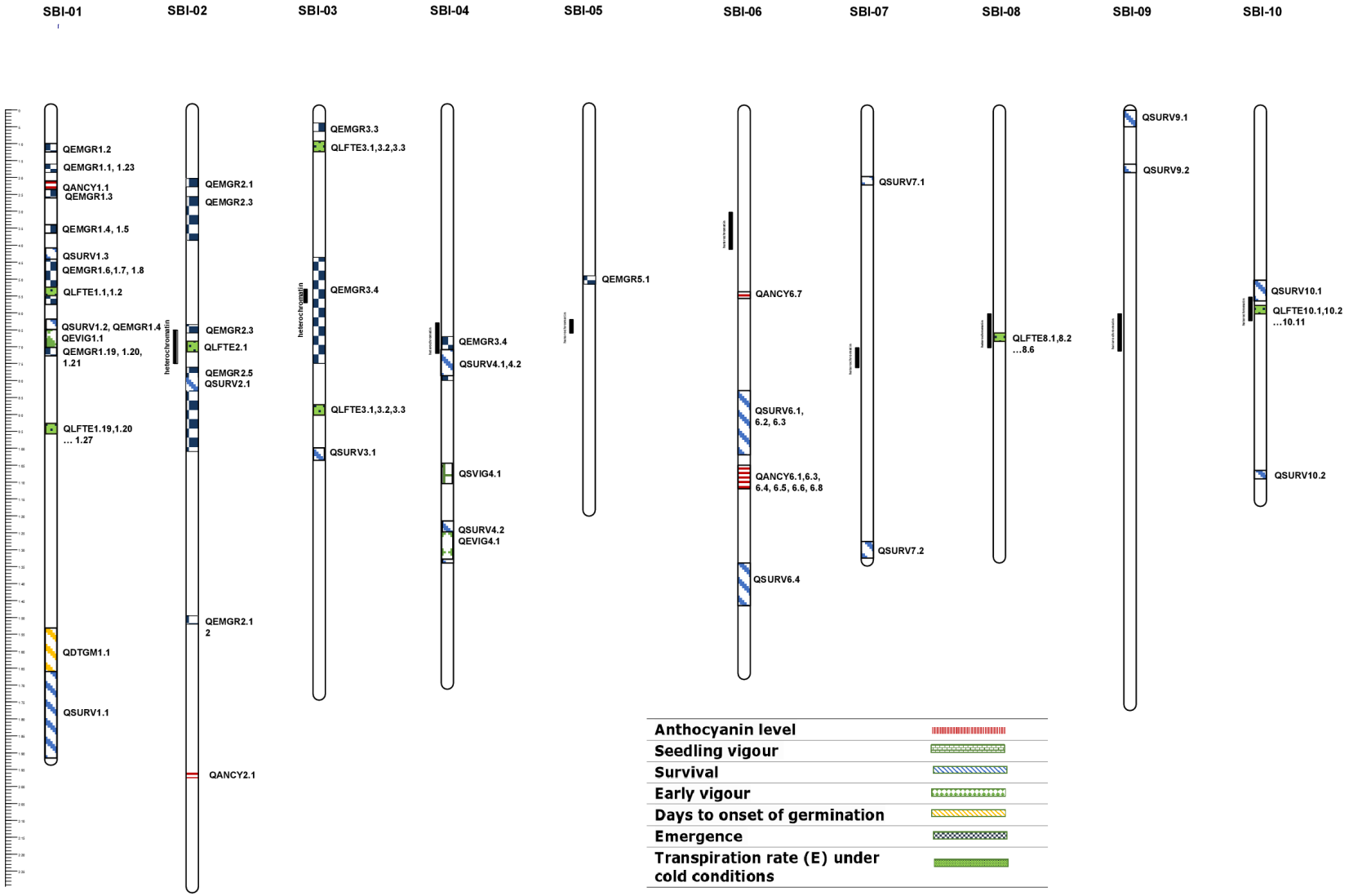


Figure 2. QTL mapping for cold tolerance related traits represented in the consensus linkage map described in Mace et al 2009. Annotations for different traits are shown in the table. The bar on the left hand side shows the distance in centiMorgans from the top of each chromosome. Heterochromatic regions are indicated by a bar to the left of each chromosome.

Conclusions and perspectives

Understanding the physiological and biochemical response mechanisms to low temperatures in Sorghum can contribute to selecting tolerant genotypes and improving crop management. Therefore, the objective of this work was to review and discuss the physiological and genetic nature and function of sorghum cold tolerance.

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Chapter II: Proline as a probable biomarker of cold stress tolerance in Sorghum (Sorghum bicolor)

ABSTRACT

Plants have developed physiological and molecular mechanisms to support and adapt to adverse environments. One response to abiotic stress is the accumulation of free proline (PRO). It has been described that the concentration of PRO increases under conditions of osmotic stress, such as heat, salinity or drought. The role of PRO during cold stress regulation is increasingly studied, it is known that it can induce the expression of many genes, which have the proline-responsive element (PRE) in their promoters, nevertheless due to the complexity of interactions between stress factors and various molecular, biochemical and physiological phenomena it is still unclear whether a more efficient PRO accumulation can be considered a biomarker of tolerance in plants . In the present work, we evaluated the accumulation of PRO in two genotypes of sorghum with contrasting tolerance to cold stress, and we identify a statistically significant difference in its accumulation. Additionally, the increase in PRO concentration correlates with a lower accumulation of reactive species of oxygen (ROS) in leaves. To explore the cause behind the accumulation of proline under cold stress conditions, we identified the Transcription Factors Binding Sites (TFBS) present in the promoter regions in the genes involved in the biosynthesis and degradation of proline in sorghum and other important crops, finding that the untranslated 3 'region P5CS gene contains different TFBS which are essential in transcription of such gene under conditions of osmotic stress. On the other hand, we also found TFBS that could allow the activation of genes involved in proline biosynthesis through the ornithine pathway under cold stress conditions, suggesting that ornithine route can be activated under cold stress conditions.

Introduction

Sorghum is a crop adapted mainly to warm areas. However, a minimum amount of sorghum has been grown in the colder highlands. These sorghums are cold tolerant and can grow on the high ground less suitable for sorghum cultivation. The term "cold tolerance" is used to describe the ability of sorghum genotypes to germinate, grow and produce seed satisfactorily under conditions of low temperatures, but above freezing (Singh, 1985). When susceptible sorghums are cultivated in cold conditions one or more of the following features happens: reduced, or no germination (Tiryaki & Andrews, 2001), the predisposition of sorghum to diseases (McLaren &

Wehner, 1992), and female or male sterility which leads to decreased seed production (Downes & Marshall, 1971). Sorghum responds to adverse conditions with a series of morphological, physiological, biochemical and molecular changes, regulated by multiple signaling pathways in response to stress. Drought, salinity and cold are types of abiotic stress that lead to cellular dehydration, which causes osmotic stress. Osmotic stress also causes the production of reactive oxygen species (ROS).

Adaptation to osmotic stress is a complicated process, involving numerous changes that include decreased growth, changes in gene expression, increased levels of abscisic acid (ABA), accumulation of compatible solutes, and protective proteins, adjustment in ion transport and increases in antioxidant levels. Between various osmolytes accumulated during osmotic stress conditions, proline is the most widely studied. Proline has multifunctional roles not only functioning as a compatible osmolyte, but it can also contribute to scavenging reactive oxygen species (ROS), stabilizing subcellular structures, modulating cell redox homeostasis, supplying energy, and functioning as a signaling molecule to interact with other metabolic pathways under stress conditions.

In higher plants, there are two pathways in proline biosynthesis using Glutamate (Glu) or Ornithine (Orn) as precursor (Figure 3). In the first pathway, Glu is phosphorylated and converted to Δ1-pyrroline-5-carboxylate (P5C) by Δ1-pyrroline-5-carboxylate synthetase (P5CS). P5C is then reduced to proline by Δ1-pyrroline-5-carboxylate reductase (P5CR). In the Orn pathway, In the second pathway, Orn is transaminated to P5C by ornithine-δ-aminotransferase (δ-OAT), and P5C is converted to proline by P5CR. A transcript abundance analysis in M. truncatula suggested that both ornithine and glutamate biosynthesis pathways contribute to the osmotic stress-induced proline accumulation (Armengaud et al., 2004). Two P5CS genes are present in Arabidopsis thaliana and also in Sorghum bicolor, and it is probable that one of them is more active under osmotic stress.

There are only a few reports showing a direct role of Pro in response to cold stress; Duncan et al. (1987) showed that exogenous proline, and inducers of proline accumulation, increase the cold tolerance of regenerable maize callus (Duncan &

Widholm, 1987). Wang et al. (2014) generated Osa-miR319b overexpressors transgenic rice lines (OE), observing a phenotype with a higher tolerance to cold stress in comparison with wild-type plants (WT). They determined the proline content of OE and WT plants under cold stress and control conditions, finding that the accumulation of PRO in OE plants was significantly higher than that in WT plants (Wang et al., 2014). The aim of this article was to find evidence of correlations between proline contents and cold tolerance in sorghum lines with contrasting tolerance and help to confirm the function of proline during the cold response in sorghum.

Materials and methods

Organisms and exposure to stress

Two genotypes of sorghum with contrasting cold tolerance were used. The R19 genotype was kindly donated by Dr. Leopoldo Mendoza Onofre from Montecillo campus of Colegio de Posgraduados, who developed this genotype from selective crosses between different sorghum lines with cold tolerance (Cisneros López et al., 2007; J Osuna-Ortega, 2003; Mendoza-Onofre et al., 2017). On the other hand, the variety TX430 is susceptible to cold and is also a genotype that has been successfully transformed (Wu et al., 2014; Wu & Zhao, 2017). The culture of both genotypes was done during the early spring of 20016 in a substrate composed of peat moss, perlite, vermiculite (3,1,1), the development of the plants was monitored until they reached the phenological phases V1, V3, V5 and HB (Vanderlip and Reeves, 1972) under semi-controlled conditions in greenhouse. Three biological replicas were used per test and two technical replicas, the plants were subjected to cold conditions (5 °C, for 48hrs) while the same number of biological replicates were kept in the greenhouse and served as control. After exposure to cold tissues were collected and were used for the quantification of ROS and proline.

Quantification of proline

For the quantification of free proline in sorghum leaves, the technique described by Carillo (Carillo & Gibon, 2011) was reproduced. Briefly, proline was extracted from leaf tissue using 90% ethanol, then reacted with an acidic solution of ninhydrin, and quantified at 520 nm. For each measurement, its respective calibration curve was generated with a proline standard with known concentrations of 1-0.4-0.2-0.1-0.04 mM.

Quantification of ROS

The technique described by Juszczak (Juszczak & Baier, 2014), was used for ROS quantification. For this purpose, a photographic record under the same conditions of lighting and exposure was made using an SLT-A37K model camera (Sony, Japan), later the images were analyzed by the software ImageJ (National Institutes of Health, USA) to quantify the percentage of the leaf area and the intensity of spots of ROS revealed by the previous staining.

Identification of TFBS in promoters.

The promoter regions (1000 nucleotides of the 3' untranslated regions) of genes involved in the synthesis and degradation of proline (Figure 2A) were identified in sorghum genome (NCBI accession NZ_ABXC00000000), using the PlantTFBS program (Megraw & Hatzigeorgiou, 2010).

Results

In order to quantify the accumulation of ROS in sorghum leaves of the varieties TX430 and R19, they were stained with 3'-Diaminobenzidine (DAB) as described elsewhere (Jambunathan, 2010) the brown stained areas are the places where ROS accumulate, the images were taken in three biological replicas under the same conditions of staining and illumination, Figure 3 depicts specimens in the phenological phase of 5 leaves which percentage of the area with brown deposits coincides with the median of population. The lower image represents the same photograph analyzed with the ImageJ software following the technique described by Juszczak, each pixel quantified as leaf area of the leaf with DAB deposits is shown in red color, later the image was transformed to 8 bits to determine the intensity

mean of the spots on a scale of 256. The rate of ROS accumulation in leaves was calculated as the result of multiplying the percentage of leaf area by the average intensity of the spots (Figure 1). Figure 2B shows the means of the index of the leaf area identified with ROS +/- EE. The asterisks indicate the minimum statistically significant difference (*P <0.05) between the varieties TX430 (susceptible to cold) and R19 (tolerant to cold).

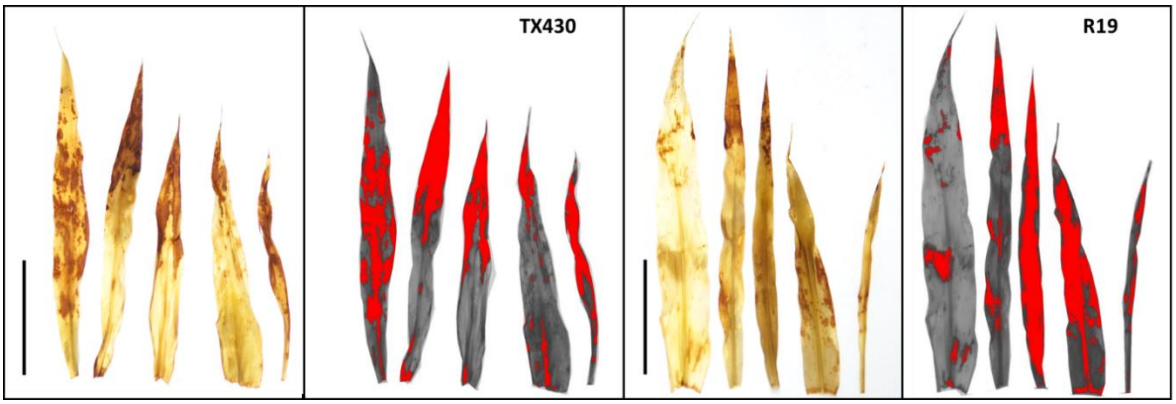


Figure 1. Leaves of sorghum after cold treatment stained with DAB from varieties TX430 (Panel A) and R19 (Panel C) at the phenological state V5, the brown stained areas are the places where ROS accumulate. The images represent the median of the index of the leaf area identified with ROS for each variety. The pictures were further digital analyzed to determine the amount of ROS accumulated (panels C and D) using parameters described by Juszczak, each pixel quantified as leaf area with DAB deposits is shown in red color, later the image was transformed to 8 bits to determine the intensity mean of the spots. The bar scale represents 5 cm.

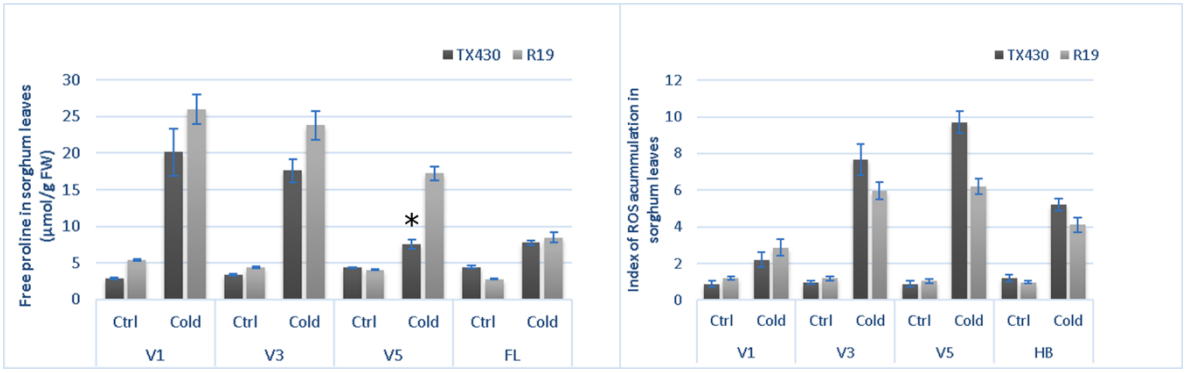
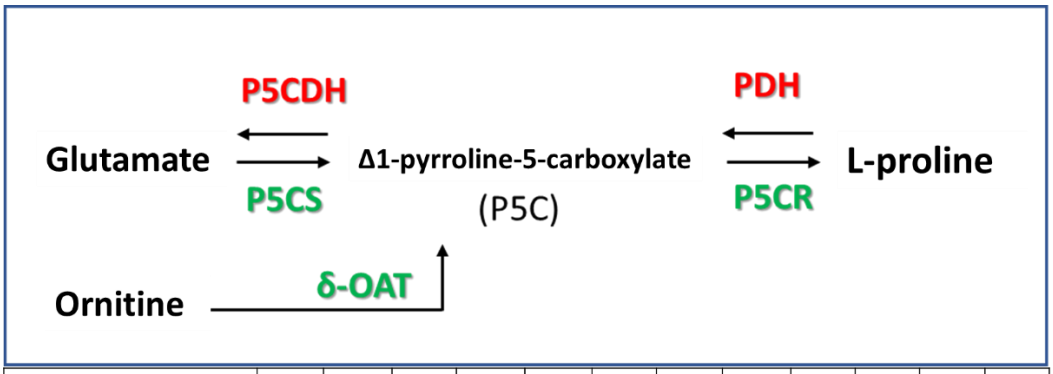


Figure 2 . Accumulation of free proline and ROS in sorghum leaves after exposure to cold treatment at different phenological states. Figure 2A depicts the means of the index of the leaf area identified with ROS +/- EE. Figure 2B shows the means of free proline concentration (umol / g of fresh weight) +/- SE. Juvenile phenological states, V1, V3, V5 and flag leaft of sorghum plants where evaluated. For bought figures means of three biological and two technical replicates of each variety and phenological state are plotted, the asterisks indicate the minimum statistically significant difference (*P <0.05) between the varieties TX430 and R19 at V5 phenological state determinated by a T student test.

The results obtained for the accumulation of free proline in sorghum leaves at the phenological phases V1, V3, V5, and FL are shown in figure 2A. The means of free proline concentration (umol / g of fresh weight) +/- SE of 3 biological and twos technical replicates are plotted. The asterisk indicates the statistically significant difference (*P <0.05) between varieties TX430 (susceptible to cold) and R19 (tolerant to cold) determined by a T student test.

The TFBS found in the promoter regions of the genes related to proline biosynthesis and degradation are shown in Figure 3.



Gene/TFBS	a	b	c	d	e	f	g	h	i	j	k	l
P5CS1	x	x	x	x	x	x	x					
P5CS2		x		x	x							
P5CR		x	x	x	x	x	x	x				
PDH		x	x	x	x							
P5CHD			x									
δ-OAT				x					x	x	x	x

Figure 3. Codifying genes involved in proline biosynthesis and degradation, and TFBS´s found on those genes. Figure 3A depicts the two pathways in proline biosynthesis using Glutamate (Glu) or Ornithine (Orn) as precursor, enzimes involved in proline biosintesys are shown in green and enzimes involved in proline degradation are shown in red. Figure 3B swows genes involved in proline biosynthesis and degradation and their Transcription Factor Binding Sites(TFBS) predicted by bioinformatic analysis. TBFS´s found on their promoters were a) W box, b)G box, c) ABRE d) TATA, e) CCAAT box, f) CCAAT box g) MBS, h) circadian, i) ARE, j) AP-2-like, k) LTR l) HSE m)Auxin response.

Discussion

Previously Ercoli et al. (2004) have reported that the phenological stages prior to the 8 leaf phase are the periods of development where sorghum is more susceptible to cold stress (Ercoli et al., 2004), based on this reference we decided to limit our experiments to an early stages study period, in addition this represents advantages to the study model: the period for obtaining the plants is smaller, likewise, not being in a vegetative and non-reproductive period, there are fewer variables that might regulate the process of seed generation that is opposed to the assembly of responses to stress. During some vegetative phases, the proline content in response

to cold stress was higher in plants of the variety R19 compared to the variety TX430. Only during the phenological phase of five leaves, a statistically significant difference was observed. Although in some of these phases the difference did not reach a level of statistical significance if it is considered as a biologically representative difference, that is, the difference is not noticeably higher, but it is constant. In this regard, it has been reported that the accumulation of proline in transgenic plants (Surender Reddy et al., 2015) can mitigate significantly the damage caused by salinity. Moreover, in wildtype plants, it has been observed that genotypes with a higher tolerance to drought stress accumulate a higher concentration of proline on tolerant plants compared to susceptible plants (Sivaramakrishnan et al., 1988). In contrast, reactive oxygen species accumulation was consistently higher in plants of the variety TX430 compared to the variety R19 reaching a more considerable difference in the phenological phase of five leaves. The above suggests that the R19 variety undergoes less oxidative damage when exposed to cold compared to the TX430 variety, this may be related to a higher accumulation of proline. Although the levels are not significantly different and could be related to a more complex series of regulatory processes in the plant such as the down-regulation of oxidative metabolism, the production of detoxifying enzymes such as glutathione-S transferase, the production of antioxidant metabolites that reduce the damage caused by ROS as well as the combination of some of the mentioned mechanisms. Some of the TFBS present in the promoter regions of both isoforms of P5CS are related to osmotic stress such as the Abscisic Acid Responsive Element (ARE), this data correlates to information obtained by Armengaud (Armengaud et al., 2004) where he found these both isoforms of genes are more abundant during osmotic stress periods, on the other hand the $^{\delta}$OAT promoter region contains regulatory elements essential in the regulation of the expression of during extreme conditions such as the Heat stress element (HSE) or the Low-Temperature response element LTR, suggesting that the Orn pathway might be necessary for response to cold and heat stress.

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Capther 3: Metabolic and molecular changes in response to low temperatures in Sorghum phenotypes with contrastig tolerance to cold stress

Abstract

This paper describes the changes found in selected elements of the antioxidant metabolism, genes and microRNAs transcription of two sorghum varieties (Sorghum bicolor) with a different degree of tolerance to cold stress during various stages of development.

The qPCR analyses showed that the cold tolerant genotype of sorghum had a notably higher upregulation of transcription factor CBF6 when exposed to a cold treatment suggesting that this higher expression level might be involved in a better adaptation of the plant to cold conditions. Both genotypes displayed similar long messenger and microRNAs patterns, characterized by an upregulation of as transcription factors, detoxification enzymes, and heat shock proteins as well as the rise in transcription levels of microRNAs miR172, miR394 and miR398, and downregulation of miR408 and miR166.

We also measured changes in concentration of some compounds related to the antioxidant metabolism; antioxidant activity, flavonoids and phenolic compounds was found to be higher in cold tolerant plants on unexposed plants and reached similar values under chilling conditions suggesting that a stronger reducing power and radical-scavenging capacity is a characteristic of more tolerant plants.

Introduction

Sorghum (*Sorghum bicolor*) is a susceptible crop at low temperatures compared to some other monocotyledonous species. The gradual expansion to higher altitude areas has led to the occurrence of genotypes better adapted to cold climates, and its introduction in other parts of the world has resulted in the development of tolerant variant [1,2]

Among the different types of abiotic of stress, cold is a limiting factor that affects sorghum and many other crops productivity worldwide, affecting the growth, development, and distribution of agronomic species [3].

Low temperature affects photosynthesis rate and disrupts the redox balance leading to oxidative stress and production of reactive oxygen species (ROS) [4]. ROS under normal conditions function as signaling molecules, but their excessive production in can lead to damage in proteins, nucleic acids and lipids [5]. The synthesis of antioxidants products such as phenolic compounds and antioxidant enzymes protects cells against the adverse effects of ROS and improves the resistance of plants to stressors[6].

Reductions in leaf photosynthetic rate are also related to instability of pyruvate phosphate dikinase (PPDK) under cold conditions, [8,9] However, there are other C4 cultivars adapted to cold conditions, such as *Miscanthus giganteus* and some species of the genus Amaranthus[11], for which an increase in Rubisco and PPDK content has been reported [9].

Some genes with predicted functions related to the synthesis of carotenoids, phytohormones, thioredoxin, and antioxidants have been previously described to be probably related to a more efficient response to cold stress [7].

The purpose of this study is to compare changes in the expression of some transcription factors, genes, and microRNAs which govern the responses to cold stress in sorghum; such responses lead to the accumulation of phenolic compounds and other metabolites involved in antioxidant activity and secondary metabolism, that might be involved in different degrees of tolerance to low temperatures.

Materials and methods

Plant material and Growth conditions

Dr. Mendoza Onofre kindly donated the tolerant VA-620 sorghum germplasm, this hybrid has shown good adaptation to the climatic conditions of the Central High Valleys of Mexico[7–9], where no other sorghum is able to grow, and produce flowers, pollen, and seeds successfully [10]. This variety is currently registered in the Plant Variety Rights Gazette of Mexico under the accession number SOG-273-210916.

Thirty-six plants per genotype were used in the experiment. The seeds disinfected in 5% commercial bleach solution for 5 min and then rinsed with distilled water, then were sown in individual plastic pots of 220 cm3 (with drain holes in the bottom) in a greenhouse. The photoperiod was set to 16 h using supplemental light, and air temperatures were semi-controlled about to 28 °C/ 20 °C for day and night, respectively. Plants were grown in Lambert LM-1 Mix soil (Lambert Peat Moss) and fertilized with Peters Excel Cal-Mag Fertilizer (15-5-15) every ten days.

Twenty days after emergence, plants were transferred to each of two growth chambers. Growth chamber was set to conditions of 50% air relative humidity, 28 °C day /20 °C night temperatures, and a photoperiod of 16 h, when introduced from the greenhouse, plants were gradually adapted for 7 days.

Leaves tissue samples were collected from 30-day-old plants during four temperature treatment periods: control or non-stress (28 °C/20 °C for 7 d), cold stress 12 hrs. (8 °C for 12 hrs. at night), cold stress 24 hrs. (8 °C night /15 °C day), and cold stress 48 hrs. (8 °C night /15 °C day), during cold stress treatments photoperiod of was changed to 12 hrs. Each biological replicate was considered as a pool of equal amounts of tissues from three plants exposed to each treatment, for all experiments employed three biological replicates and two technical replicates.

RNA isolation and quality controls

The samples were grounded to a fine powder with mortar and pestle in liquid nitrogen, and 50 mg were used for RNA isolation. Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. The integrity of RNA samples was judged by agarose gel electrophoresis. Low molecular weight RNA fraction was enriched employing to the protocol previosuly described and later used for quantification of microRNAs. The RNA concentration of each sample was measured using UVIS Drop UVS-99 (Avans, Taiwan). Samples with 260/280 ratio between 1.8 and 2.1 and 260/230 ratio of around 2 or slightly above were employed for the analysis.

Selection of genes and microRNAs and primer design

The primer sequences and transcript information are given in Table 1. Specific primers for the amplification of cold-responsive genes were designed for SYBR

Green-based RT-qPCR and synthesized by T4 oligo (Mexico). The amplicon length ranged from 70 to 193 bp to. In the melting curve was observed a single peak of fluorescence, indicating that a unique and specific fragment was amplified during ***RT-qPCR.***

RT-qPCR conditions

First-strand cDNA was synthesized with 1 µg of total RNA in a final reaction volume of 20 µl, using M-MLV Reverse Transcriptase (Sigma-Aldrich, USA) according to the manufacturer's instructions. The RT-qPCR mixture contained 4 µl of diluted cDNA (corresponding to 1 ng of starting amount of RNA), 5 µl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and 400 nM of each gene-specific primer in a final volume of 10 µl. RT-qPCRs with no template controls were also performed for each primer pair. The Real-Time PCR reactions were performed employing StepOnePlus Real-Time PCR System and software (Applied Biosystems, USA). All the RT-qPCRs were performed under following conditions: 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C in 96-well optical reaction plates (Applied Biosystems, USA). The specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 cycles and agarose gel electrophoresis. Two technical and three biological replicates of each sample were used for Real-Time PCR analysis.

Analysis of gene expression variation

The expression level of genes in each reaction was determined by the cycle threshold Ct (the cycle at which fluorescence from reaction exceeds a crossing point automatically set by the StepOne software). To analyze the expression variation of the genes, by the double delta Ct method [15]

Total phenols and flavonoid extraction and quantification

Total phenolic compounds were extracted considering Agbangnan observations for a higher yield extraction [11]. Fresh sorghum leaves were frozen in liquid nitrogen and ground to powder in a mortar with pestle, 0.5 g of homogenized solid were accurately weighted directly in 15 ml falcon tubes, extraction was made in different steps, first 10 volumes (w/v) of neutral ethanol 50% were added to each sample and were placed in a vortex during 8 min, the sample was centrifugated at 13,000 RPM 10 minutes and supernatant was collected into a 15 ml falcon tube, this step was repeated 2 times with neutral 50% ethanol, and two times with acidified ethanol 50% pH 2, in order to hydrolyze components phenolic compounds from carbohydrates or proteins, a final extraction was made adding 10 volumes of neutral 50% ethanol and the mixture was heated to 60 °C during 2 hours, all the supernatants were mixed and distilled water was added until 60 volumes.

The polyphenolic content was determinate using a microplate method [12]. Galic acid was prepared in five 1 in 2 dilutions, from 960 µg ml-1 down to 60 µg ml-1. The Folin-Ciocalteu (FC) reagent was diluted 1:10 with de-ionized water, while sodium carbonate was prepared as a 1 M solution

The polyphenolic content of the extracts, and the gallic acid reference standard serial dilutions were tested with the FC reagent using a MicroMTP assay. 10 µl of diluted extracts or standard were pipetted in triplicate in wells of a MTP. The repeated volumes of FC reagent (100 µL) and sodium carbonate (80 µL) were transferred to each well. The mixtures were incubated at room temperature for 30 minutes and

then analyzed at 630 nm on the MTP reader. Total phenolic values were expressed in terms of gallic acid equivalents (µg gFW-1)

Flavonoid content determination

The flavonoid content was quantified using a colorimetric assay [13]. Briefly, 0.1 ml of the extracts were diluted with distilled water up to 1 ml, and subsequently, 0.050 ml of sodium nitrate solution (5%) was added and allowed to react for 5 min. Then 10% aluminum chloride solution (0.1 ml) was added. Finally, after 6 min, 0.5 ml of 1 M sodium hydroxide and 1 ml of distilled water were added into the mixture. The absorbance of the mixture was immediately recorded at 510 nm and the flavonoid content was expressed as mg of catechin equivalents (CEs) per 100 g of sample. All the tests were carried out in triplicate.

Results

Analysis of gene expression variation.

We measured the variation in the expression of 13 genes previously identified as cold responsive in sorghum [14], shows the relative gene expression with respect to a control not exposed to cold, normalized against the constitutive genes EF-1α and PP2A obtained by qRT-PCR and analyzed by the double delta CT method.

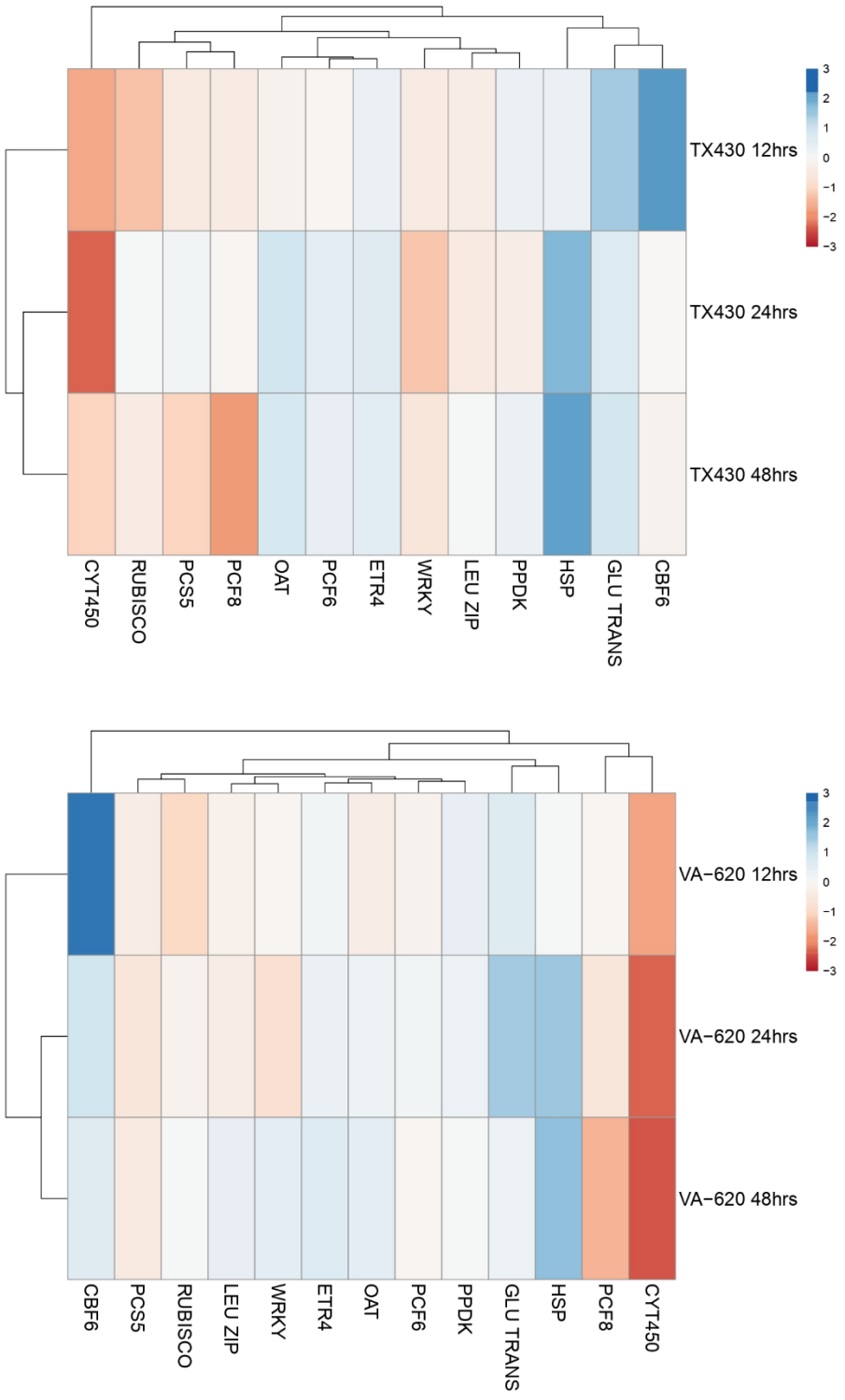


Figure 1. Analysis of gene expression variation of 13 cold tolerance related genes, in leaves from VA620 and TX-430 varieties of sorghum after cold treatment, untreated plants gene expression levels were used to normalize the relative gene expression. Columns are clustered using maximum distance and average linkage

We analyzed by a qPCR technique based on a universal TaqMan probe the change in the expression of 14 conserved microRNAs identified as cold responsive in other species. **Fig. 2** shows a hierarchical clustering and heatmap based on the expression of microRNAs in both varieties of sorghum at different times of exposure to cold stress, data is normalized against non exposed plants, data were obtained by qPCR and analyzed by double delta CT method using u6 and SNOR14 as reference housekeeping genes.

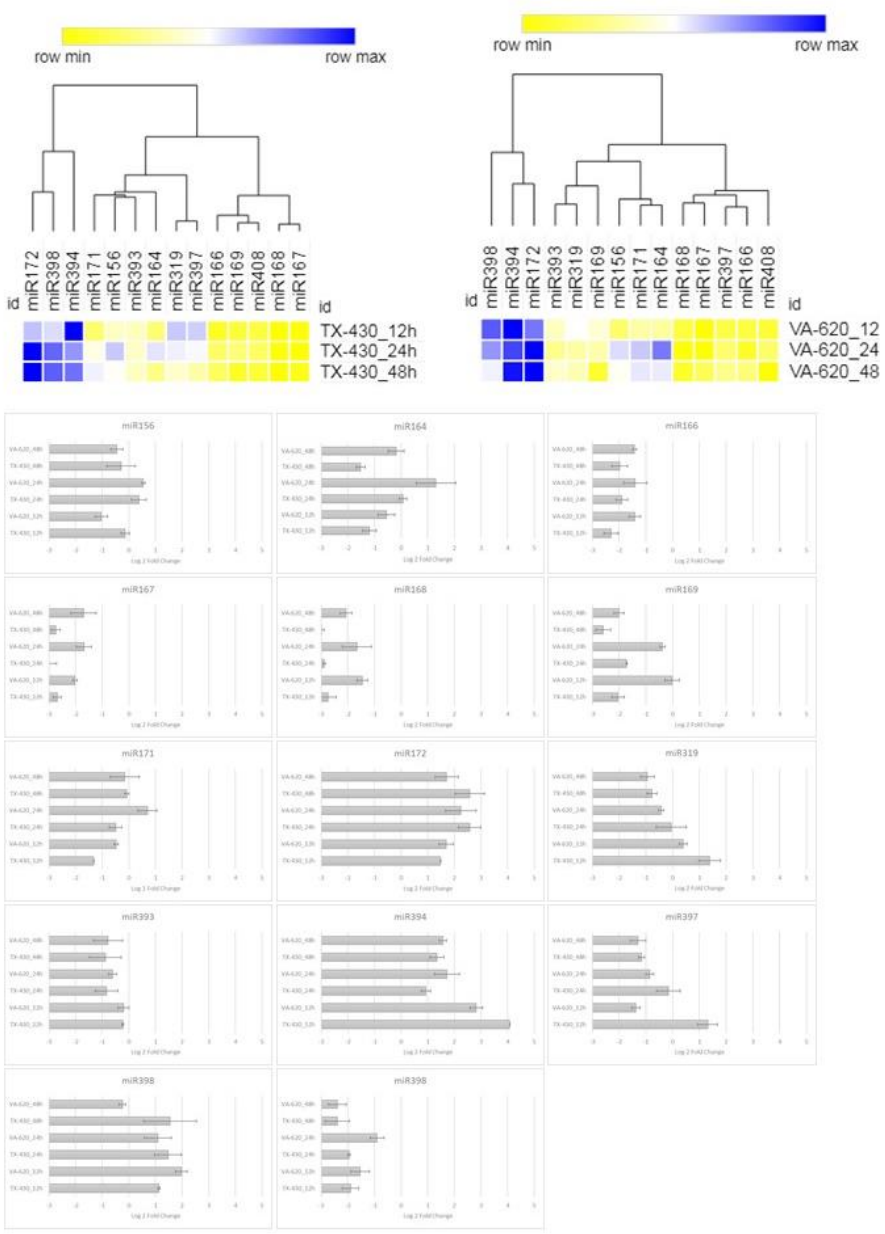
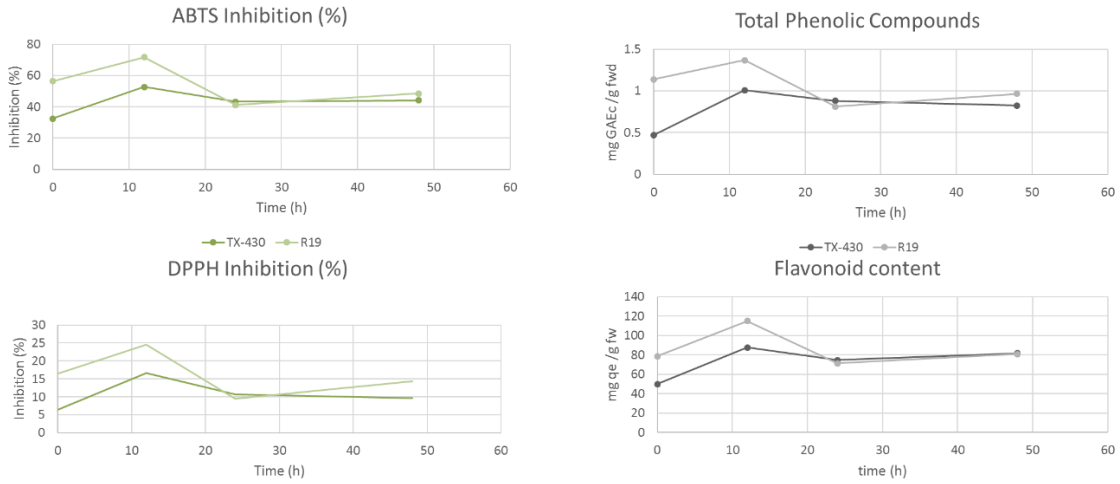


Figure 2 Analysis of microRNAs expression variation. Hierarchical clustering and heatmap based on the expression of 14 conserved microRNAs identified as cold responsive in other species b) Detailed Log2 fold change expression variation of each miRNA

Determination of antioxidant content and activity. We analyzed and compared the changes in quantities of phenolic compounds and the fluctuations in the antioxidant activity of extracts from the leaves of two varieties of sorghum, which presented different degrees of tolerance to cold (Fig. 3).



Discussion

We analyzed the changes in selected elements of the antioxidant metabolism, genes and microRNAs transcription in leaves of two sorghum varieties (Sorghum bicolor) with a different degree of tolerance to cold stress.

Our results have shown that both varieties of sorghum display a similar pattern of microRNA transcription levels characterized by an upregulation of microRNAs miR172, miR394 and miR398 and down regulation of miR408 and miR166.

Most of the genes involved in the responses to low temperatures such as transcription factors, detoxification enzymes and heat shock proteins showed also similar behavior under exposure to low temperatures, nevertheless the upregulation of transcription factor CBF6 higher in tolerant plans suggesting that this expression level might be involved in a better adaptation of the plant to cold conditions.

CBF/DREB1 genes encode a family of transcriptional activators that play an important role in cold tolerance and cold acclimation. In Arabidopsis the expression of the CBF regulon leads to an increase tolerance to cold [15,16] and the overexpression of CBF1, CBF2, or CBF3 in transgenic plants results in an increased tolerance without exposing plants to low temperature [17]. Therefore; a significantly higher expression of the CBF6 transcript in tolerant sorghum genotype, at the shortest time for tissue collecting in this experiment, might be involved in a better cold acclimation leading to the induction of CBF-targeted cold-regulated genes.

We also found that the gene *CYP79*, that codes for a Cytochrome P450, was down regulated under cold stress in both varieties and the suppression of its expression was higher in cold tolerant variety. Cytochrome P450 family proteins are involved in involved in plant hormone biosynthesis and degradation and lipid metabolism[18], its down regulation has also been observed in other plants under cold treatments [19,20]. Also, CYP enzymes are versatile catalysts for a wide range of in substrate oxidation reactions, due to their oxidation capacity can generate ROS and increase oxidative stress [21]. It is still unclear if the role of the downregulation of cytochromes during cold treatment is related to a network of regulation of hormone biosynthesis, or to prevent oxidative stress, or a combination of both processes.

Proline is an important osmolyte that can work as an antioxidant [5], we have previously reported that higher proline accumulation during cold stress might be related a better tolerance, and also the presence of a Low-Temperature Response element LTR in the promoter region of the δOAT gene, suggesting that the Orn pathway might be preferential in response to cold [22]. In this sense, we found that δOAT was slightly induced while at the same time P5CS2 was downregulated suggesting that the accumulation proline is via Orn pathway.

miR319 is a microRNA which regulation under cold stress has been studied in sugarcane [23] Thiebaut and cols. have suggested to be used as a marker for selection of cold-tolerant sugarcane cultivars; in contrast our results showed no differential expression of miR319 under our experimental conditions of exposure to cold, nevertheless we found that gene *PCF8* a TCP transcription factor target or miR319 has a downregulation that is stronger across the time; Over expression and RNA interference transgenic lines in rice resulted in more susceptible and more tolerant phenotypes under cold stress respectively [24]; so it is suggested that the better capacity to repress the expression of TCP genes might be involved in a better tolerance to cold stress.

We found that mir394 was upregulated in both varieties of sorghum, but cold-tolerant plants maintained a high accumulation of this microRNAs along the time while the susceptible genotype dropped its expression. miR394a overexpressing Arabidopsis

transgenic plants and, its correspondence target gene LCR loss of function mutant, showed more tolerance to cold stress [25].

Upregulation of miR172 had a similar pattern in both varieties of sorghum. miR172 might play a central role in response to cold stress, especially in the regulation of CK2 and the circadian rhythm [26], however the differences in cold tolerance between analyzed genotypes might be related to a different regulation point.

miR408 was downregulated in both varieties, its function in tune lignification of the cell wall by targeting laccase encoding genes might be related modification of the pattern the growth during cold stress and also changes in its expression has been previously reported [28]. miR172 is another microRNA important in modulating the plant development during cold stress and was also upregulated in both varieties, it reduces the translation of four AP2 (APETALA) transcription factors leading to a temporal reduction in growth in a survival strategy of the plants.

The analyses have shown that the more-tolerant variety was characterized by a higher content of phenolic compounds, and stronger antioxidant activity. However, the cold stress caused a decrease in the concentration of the phenolics and decreased the scavenging capacity in the leaves of both varieties. The phenolic compounds content is modified during chilling stress in other cultivars [27,29], and there is an association between cold tolerance and higher basal concentration of such compounds in other cultivars [29] among different phenolic compounds flavonoids are determinant in cold acclimation and tolerance [30] and can be induced by cold and also by other type of abiotic stress, its concentration is consistent with antioxidant activity so one or more compounds of this family might be involved in a better cold tolerance.

The protection of cell components from oxidative damage derived from cold is linked to a poly-nature mechanisms through which miR394, and CBF transcription upregulation and cytochromes and miR408 downregulation figure to be very important for regulation at different levels, further research is needed to understand the functional mechanisms that link microRNAs, transcripts, detoxifying enzymes and antioxidant metabolites to plant freezing tolerance.

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Gene	Gene description	Function	Length (pb)	Genebank accesion	FW RV	Primer
P5CS2	Sorghum bicolor delta-1-pyrroline-5-carboxylate synthetase 2	Proline biosynthesis	147	GQ377720	FW	ACAGGAAGCTTGTCAATAGCAG
					RV	GCTGGGAAGAGGATACATCAAG
PCF6	Transcription Factor	TCP family transcription factor	193	XM002466299	FW	CAAGTATGGCAATGCGCCTC
					RV	GGACTGAAGGGTCCCCCTAT
PCF8	Transcription Factor	TCP family transcription factor	133	XM002442504	FW	CGGCCATCCAGTTCTACGAC
					RV	GGAAGTGCGAAGGAGCAGTC
CBF6 (DREB1A)	Dehydration-responsive element-binding protein 1A	Binds to the 11bp GCC box of the ethylene response element (ERE)	142	XM002462647	FW	TAGCAGGACCGACCAAAGGC
					RV	CTCCGAAGAGGTCGAGGCTG
ETR4	Ethylene-responsive transcription factor 4	Key regulatory in plant responses to abiotic stresses	70	XM002445176	FW	TCACTGGACCTCAGCCTGG
					RV	CCATGAACAGCTGGTAGGGC
CYP79	Cytochrome P450	Oxidase enzyme in electron transfer chains	187	Sb10g022470	FW	TAGTTTAGCCGCGGGATGTC
					RV	AGGAGGTCAAGGCTCAGCTA
HSP	Heat shock protein binding	Interact with a heat shock protein, in response to heat shock	72	Sb03g027330	FW	GAAGGTGAGGTGGGAAGATTGC
					RV	CTTCTTATGAGTGGCGATCCTACG
PP2A*	Serine/threonine-protein phosphatase PP2A-1 catalytic subunit	Reference internal gene for qPCR data normalization	138	XM002453490	FW	AACCCGCAAAACCCCAGACTA
					RV	TACAGGTCGGGCTCATGGAAC
EIF4α*	Eukaryotic initiation factor 4A	Reference internal gene for qPCR data normalization	144	XM002451491	FW	CAACTTTGTCACCCGCGATGA
					RV	TCCAGAAACCTTAGCAGCCCA
WRKY51	WRKY transcription factor	Key regulators of many processes in plants	140	*XM002447011*	FW	CGGCCATGTCTAGTCGTTCC
					RV	GTTTTTGCGGCATCGAAGGT
RUBISCO	CCDB-24911-G11 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Carbon dioxide fixation through the reductive pentose phosphate cycle	87	MG227985	FW	ATCTTGGCAGCATTCCGAGT
					RV	ACCAGTAGAAGATTCCGCAGC
PPDK	Pyruvate, phosphate dikinase 1	Participates in pyruvate metabolism and carbon fixation	183	XM_021448366	FW	CTCAACTTTCCGCAGACCCT
					RV	GGCTTCTGAAGGCAGATGGT
OAT	Ornithine aminotransferase mitochondrial	Arginine and proline metabolism	157	XM_002464129	FW	GTACCCATCTCGCCCCAAAA
					RV	TCACAGTGCAAGGGAATGCT

Table 1. List of genes and its correspondence primers used to quantify their expression by Sybr green qPCR

General Conclusions

Understanding the physiological and biochemical nature of responses to low temperatures in Sorghum can contribute to selecting tolerant genotypes and improving crop management.

Proline usually play a role as an osmolyte but can also work as a powerful antioxidant, and a higher proline accumulation during cold stress might be related a better tolerance. We observed that genotypes with a higher tolerance to cold accumulate a higher concentration of proline compared to susceptible plants. We analyzed Transcription Factors Binding Sites (TFBS) and found the presence of a Low Temperature Response Element (LTR) in the promoter region of ɗ- ornithine amino transferase (ɗ-OAT), suggesting that the Orn pathway might be response to cold response

Sorghum has developed mechanisms to prevent the production of ROS, and once formed, to detoxify and repair the damage. The protection of cell components from oxidative damage derived from cold is linked to a poly-nature mechanism through which miR394, and CBF transcription upregulation and cytochromes and miR408 downregulation figure to be very important for regulation at different levels.

Most of the genes involved in the responses to low temperatures such as transcription factors, detoxification enzymes and heat shock proteins showed also similar behavior under exposure to low temperatures, nevertheless the upregulation of transcription factor CBF6 higher in tolerant plans suggesting that this expression level might be involved in a better adaptation of the plant to cold conditions.

The phenolic compounds content is modified during cold treatments and we found and higher basal concentration of such compounds in other cultivars in tolerant genotype.

Further research is needed to understand the functional mechanisms that link microRNAs, transcripts, detoxifying enzymes and antioxidant metabolites to plant freezing tolerance however the results obtained during this doctoral thesis marks some clues about the molecular mechanisms of sorghum to deal with cold stress.

Appendices I: Reference genes for RT-qPCR normalization in different tissues, developmental stages and stress conditions of amaranth

This chapter presents the most stable reference genes for normalization of gene expression analysis in amaranth, which will contribute significantly to future gene studies of this species.

Studies of gene expression are significant for the identification of genes that participate in different biological processes. RT-qPCR requires accurate normalization of data to avoid the misinterpretation of experimental data. In this sense, the selection of reference genes is critical for gene expression analysis.

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RESEARCH PAPER

Reference genes for RT-qPCR normalisation in different tissues, developmental stages and stress conditions of amaranth

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Keywords
Amaranth; gene expression; normalisation; quantitative real-time PCR; reference genes.

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ABSTRACT

- Studies of gene expression are very important for the identification of genes that participate in different biological processes. Currently, reverse transcription quantitative real-time PCR (RT-qPCR) is a high-throughput, sensitive and widely used method for gene expression analysis. Nevertheless, RT-qPCR requires precise normalisation of data to avoid the misinterpretation of experimental data. In this sense, the selection of reference genes is critical for gene expression analysis. At this time, several studies focus on the selection of reference genes in several species. However, the identification and validation of reference genes for the normalisation of RT-qPCR have not been described in amaranth.
- A set of seven housekeeping genes were analysed using RT-qPCR, to determine the most stable reference genes in amaranth for normalisation of gene expression analysis. Transcript stability and gene expression level of candidate reference genes were analysed in different tissues, at different developmental stages and under different types of stress. The data were compared using the geNorm, NormFinder and Bestkeeper statistical methods.
- The reference genes optimum for normalisation of data varied with respect to treatment. The results indicate that *AhyMDH, AhyGAPDH, AhyEF-1α* and *AhyACT* would be optimum for accurate normalisation of experimental data, when all treatment are analysed in the same experiment.
- This study presents the most stable reference genes for normalisation of gene expression analysis in amaranth, which will contribute significantly to future gene studies of this species.

INTRODUCTION

Amaranth consumption has many health benefits, among which are its antihypertensive (de la Rosa *et al.* 2010; Lado *et al.* 2015), antimicrobial (Lipkin *et al.* 2005), antitumor (Ielisieieva *et al.* 2006) and cholesterol-lowering (Berger *et al.* 2003; Shin *et al.* 2004; Pasko *et al.* 2011; Chmelik *et al.* 2013) effects. Amaranth has evolved complex mechanisms to tolerate extremely adverse growing conditions; moreover, it resists different types of biotic and abiotic stress (Delano-Frier *et al.* 2011; Huerta-Ocampo *et al.* 2014; Massange-Sanchez *et al.* 2016; Palmeros-Suarez *et al.* 2017), is easy to grow in agriculturally marginal lands and has high potential for economic exploitation (Emokaro *et al.* 2007; Mlakar *et al.* 2010; Janssen *et al.* 2017). *Amaranthus hypocondriacus* has a diploid karyotype (2n = 32; Radwan *et al.* 2014). A draft genome of *A. hypochondriacus var.* Plainsman that covers 377 Mb of the estimated genome size of 466 Mb has been assembled (Clouse *et al.* 2016) and has been the focus of various transcriptomic studies (Riggins *et al.* 2010; Delano-Frier *et al.* 2011; Liu *et al.* 2014). Some of the libraries available on NCBI databases on amaranth are derived from experiments conducted on plants exposed to either different types of stress, such as drought, water stress, bacteria, salt stress and insect herbivores (Accession PRJNA65409); different development stages or experiments conducted on various tissues (accession PRJNA263128). The draft transcriptome of amaranth has been assembled, mapped and functionally annotated, and is available on public databases (Clouse *et al.* 2016).

A large number of genes are involved in processes such as plant development, differentiation or mounting responses that enable tolerance to different types of biotic and abiotic stress. Amaranth has evolved various mechanisms to tolerate some biotic and abiotic stresses, such as the capacity to increase expression of choline monooxygenase (CMO), which catalyses the synthesis of glycine betaine in response to salinity and drought (Russell *et al.* 1998); up-regulation of transcription factors; DNA-binding of One Zinc Finger 1 (*DOF1*) and Mini Zinc Finger 1 (*MIF1*). These involve a coordinated response comprising osmolyte accumulation, expression of proteins that reduce damage by reactive oxygen species and regulation of transcription factors related to plant growth control (Huerta-Ocampo *et al.* 2011). However, the molecular mechanisms behind these processes are not entirely understood. Transcriptional analysis with reverse transcription quantitative real-time PCR (RT-qPCR) is useful for functional interpretation of genes and can improve the quantification of gene expression profiles. RT-qPCR has become the preferred method for gene expression quantification. Currently, some studies focus on the

selection of reference genes in several organisms (*e.g.* Bevitori *et al.* 2014; Ferdous *et al.* 2015; Singh *et al.* 2015; Li *et al.* 2017); however, the identification of reference genes for the normalisation of RT-qPCR has not been described in amaranth. This study evaluated genes to determine the most stable reference genes and analysed transcript stability of these genes in different tissues, developmental stages and under different types of stress in amaranth.

MATERIAL AND METHODS

Plant growth conditions

Amaranth seeds (*A. hypocondriacus* var. Gabriela) were surface sterilised with 10% sodium hypochlorite solution ($NaClO_4$) and sown in polystyrene trays with 1'' × 1'' × 2.5'' wells, at the end of winter in February 2016. Each well was filled with a sterile substrate composed of Perlite, vermiculite and peat moss (i3:1:1 v/v). Germination trays were kept under semi-controlled greenhouse conditions at the Center for Research in Applied Biotechnology, National Polytechnic Institute of Mexico in Tlaxcala at 2,260 m a.s.l. (19°16'53.2"N, 98°21'57.3" W).

Plant treatment and tissue collection

The different experimental conditions used in this study are shown in Table 1. Briefly, 60-day-old amaranth plants were dissected to obtain leaf, stem, root and panicle tissues samples for further analysis. To gather tissues from various development stages of the amaranth life cycle, plants were collected at different stages of development, corresponding to the opening of cotyledons, five to six leaves, apical inflorescence and seed development (Martínez-Núñez *et al.* in process). For abiotic stress treatments, 30-day-old seedlings were transferred to environmental chambers. Cold treatment was performed at 4 °C compared to the control temperature of 25 °C, with tissue then collected after 48 h of cold treatment. Heat treatment was performed at 42 °C for 48 h. For drought stress, irrigation was suspended until a relative humidity of 8% (after 72 h) was achieved in the substrate, after which the control was regularly irrigated, and the foliar tissue samples then collected. For biotic stress treatment, aphids identified as *Macrosiphum* sp. were collected in the field, with 20-day-old seedlings then transferred to a contention chamber under greenhouse conditions. The amaranth plants were infested with approximately five aphids per plant; after 15 days, the foliar tissue was collected. Three biological replicates were used for each experiment, while samples were collected and immediately frozen in liquid nitrogen and stored at −80 °C.

Table 1. Plant tissue and treatments used for RT-qPCR normalisation. The table present tissues and treatment conditions used in each experimental set for RT-qPCR analysis.

Group	Tissue	Collection time (Days post-germination)	Treatment
Different Tissue	Leaves	60 days	–
	Stem	60 days	–
	Root	60 days	–
	Panicle	60 days	–
Different stages	Aerial tissue	3 days	–
	Aerial tissue	43 days	–
	Aerial tissue	60 days	–
	Aerial tissue	90 days	–
Different types of stress	Aerial tissue	32 days	48 h at 4 °C
	Aerial tissue	32 days	48 h at 42 °C
	Aerial tissue	33 days	Not irrigation for 72 h
	Aerial tissue	32 days	25 °C with normal irrigation
	Aerial tissue	35 days	15 days of exposition at *Macrosiphum sp*
	Aerial tissue	32 days	Normal conditions

Isolation of RNA and quality controls

The samples were ground to a fine powder with a pestle and mortar in liquid nitrogen, and 50 mg used for RNA isolation. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of the RNA samples was judged using agarose gel electrophoresis. The concentration of each sample was measured using UVIS Drop UVS-99 (Avans, Taipei, Taiwan). Samples with a 260/280 ratio of between 1.8 and 2.1 and a 260/230 ratio of approximately 2 or slightly above were used for the analysis.

Selection of candidate reference genes and primer design

Arabidopsis thaliana and *Beta vulgaris* genes were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank) and used as query sequences to retrieve homologous genes from *A. hypochondriacus*. Seven commonly employed candidate reference genes, actin (*AhyACT*), ß-tubulin (*Ahyß-TUB*), glyceraldehyde 3-phosphate dehydrogenase (*AhyGAPDH*), S-adenosylmethionine decarboxylase (*AhySAMDC*), elongation factor 1-alpha (*AhyEF-1α*), 18S ribosomal RNA (*Ahy18S-rRNA*) and malate deshydrogenase (*AhyMDH*) were selected (Table 2). Based on the gene sequence obtained from the transcriptome assembly database (Clouse *et al.* 2016) available at Phytozome (phytozome.jgi.doe.gov), primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) under default parameters (Table 2).

Reverse transcription quantitiative real-time PCR conditions

First-strand cDNA was synthesised with 1 µg total RNA in a final reaction volume of 20 µl, using M-MLV Reverse Transcriptase (Sigma-Aldrich, St. Louis, MI, USA) and according to the manufacturer's instructions. The RT-qPCR mixture contained 4 µl diluted cDNA (corresponding to a 1 ng starting amount of RNA), 5 µl 2 × Power SYBR Green PCR Master Mix (Applied Biosystems) and 400 nM of each gene-specific primer in a final volume of 10 µl. RT-qPCRs with no template controls were also performed for each primer pair. Real-Time PCR reactions were performed with the StepOnePlus Real-Time PCR System and software (Applied Biosystems). All RT-qPCRs were performed under the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C in 96-well optical reaction plates (Applied Biosystems). The specificity of amplicons was verified *via* melting curve analysis (60 to 95 °C) after 40 cycles and agarose gel electrophoresis. Two technical and three biological replicates of each sample were used for the qPCR analysis.

Table 2. Candidate reference genes for RT-qPCR normalisation in amaranth. Gene information, including amplicon length, primers and Tm for seven candidate reference genes.

Gene name	Symbol	Gene length	Amplicon length (bp)	Tm (°C)	Locus (phytozome)		Primer sequence
Actin	*AhyACT*	1134	178	79.1	AHYPO_019031	FW	CGTGACCTGACTGATTACCTTA
						RV	GCTCGTAGTTCTTCTCAATGGC
ß-Tubulin	*Ahyß-TUB*	1069	81	76.8	AHYPO_019789	FW	GGAAGGAATGGACGAGATGG
						RV	TCTTGATACTGCTGATACTCTGC
Glyceraldehyde 3-phosphate dehydrogenase	*AhyGAPDH*	1383	195	80.1	AHYPO_013553	FW	TCAAGGAGGAATCCGAGGGC
						RV	AGTCAACAACACGGGAACTG
S-Adenosylmethionine decarboxylase	*AhySAMDC*	2591	195	81.0	AHYPO_016008	FW	GCTCCGTGCAATCCCACCTA
						RV	CCCATCACAAAGGCCTTGCT
Elongation Factor 1-alpha	*AhyEF-1A*	1377	224	80.4	AHYPO_001308	FW	ACTGTGCTATCCTCATTATTG
						RV	GTTGTAACCGACCTTCTTC
18S ribosomal RNA	*Ahy18S*	1630	109	81.0	AH006866*	FW	CCATAAACGATGCCGACCAG
						RV	AGCCTTGCGACCATACTCC
Malate Dehydrogenase	*AhyMDH*	1539	136	78.5	AHYPO_021284	FW	TGCTCCCAACTGCAAGGTTC
						RV	ACCAAGTGCCCTGTTGTGAT

*Genebank accession for *Amaranthus caudatus* 18S ribosomal RNA. Actin oligonucleotides were reported in Massange-Sanchez *et al.* (2016).

Efficiency of PCR

A series of six-five-fold dilutions (1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125) of cDNA from *A. hypocondriacus* were used to generate the standard curves. The PCR efficiency (E) and correlation coefficient (R^2) were determined for each gene using the linear regression model. PCR efficiency of between 90 and 110% and R^2 >0.99 was considered acceptable.

Analysis of gene expression variation

The expression level of genes in each reaction was determined using the cycle threshold (Cq; cycle at which fluorescence from the reaction exceeds a crossing point automatically set by the StepOne software). To analyse expression variation of the candidate reference genes and determine the best reference genes, the Excel-based methods, geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004), BestKeeper (Pfaffl *et al.* 2004) and the online tool RefFinder (http://leonxie.esy.es/RefFinder) were used. The raw data were directly used with the BestKeeper and RefFinder methods, while for geNorm and NormFinder methods, Cq values were converted into relative quantity values (RQ) *via* the formula $RQ = E^{-\Delta Cq}$, where E is the validated amplification efficiency of each gene, and ΔCq is the difference between the Cq value and the minimum Cq of each gene among the samples. RefFinder enables assessment of the most stable reference gene by comparing the three Excel methods plus the comparative and Delta CT method (Silver *et al.* 2006). Although RefFinder analyses raw data, assuming 100% efficiency for all genes, the outputs were compared to data obtained by the original software, given that PCR efficiencies are not considered, and they been reported to lead to overestimation of differences between groups (De Spiegelaere *et al.* 2015).

RESULTS

Specificity of primers and efficiency of reference genes

The genes *AhyACT*, *Ahyß-TUB*, *AhyGAPDH*, *AhySAMDC*, *AhyEF-1α*, *Ahy18S-rRNA* and *AhyMDH* were used for the RT-qPCR analysis. The primer sequences and transcript information are given in Table 2. Specific primers for the amplification of potential reference genes were designed for SYBR green-based RT-qPCR and synthesised using T4 oligo (Mexico). The amplicon length ranged from 81 to 224 bp (Table 2), with corresponding unique bands observed on agarose electrophoresis gel (Fig. 1A). A single peak of fluorescence was observed on the melting curve (Fig. 1B), indicating that a unique and specific fragment was amplified during RT-qPCR. The R^2, which shows how the data fit the standard curve, was then calculated. The PCR amplification efficiencies (E) for every gene, which is another parameter that gives information about the reaction and involves experimental factors such as length, secondary structure and GC content of the amplicon, was also calculated. The E of RT-qPCR reactions varied from 1.84 to 2.06 and the R^2 were > 0.9932 (Fig. 2). These results meet the standard ($R^2 > 0.99$, and $1.8 < E < 2.2$) established by Ramakers *et al.* (2003).

Expression profiles of candidate reference genes

Measurement of the expression level of all samples showed some variations among the seven reference genes (Fig. 3). Descriptive statistics were calculated using Minitab 17 Statistical Software (Minitab, State College, USA) for each of the tested genes. The Cq values for the seven genes ranged from 18.52 to 27.85, the majority of these values were between 20 and 25 in all tested samples (Fig. 3). *AhyMDH* had a lower SD, whereas *Ahy18S-rRNA* had the highest SD.

Expression stability of candidate reference genes

The most suitable reference genes were evaluated using RefFinder, geNorm, NormFinder and BestKeeper. The stability ranking of candidate reference genes in 36 individual samples was calculated and identified as 'Total', with the 36 samples divided into three groups denominated as: 'Different types of stress', 'Different tissues' and, 'Different stages of development'. The results obtained with every method are summarised in Table 3.

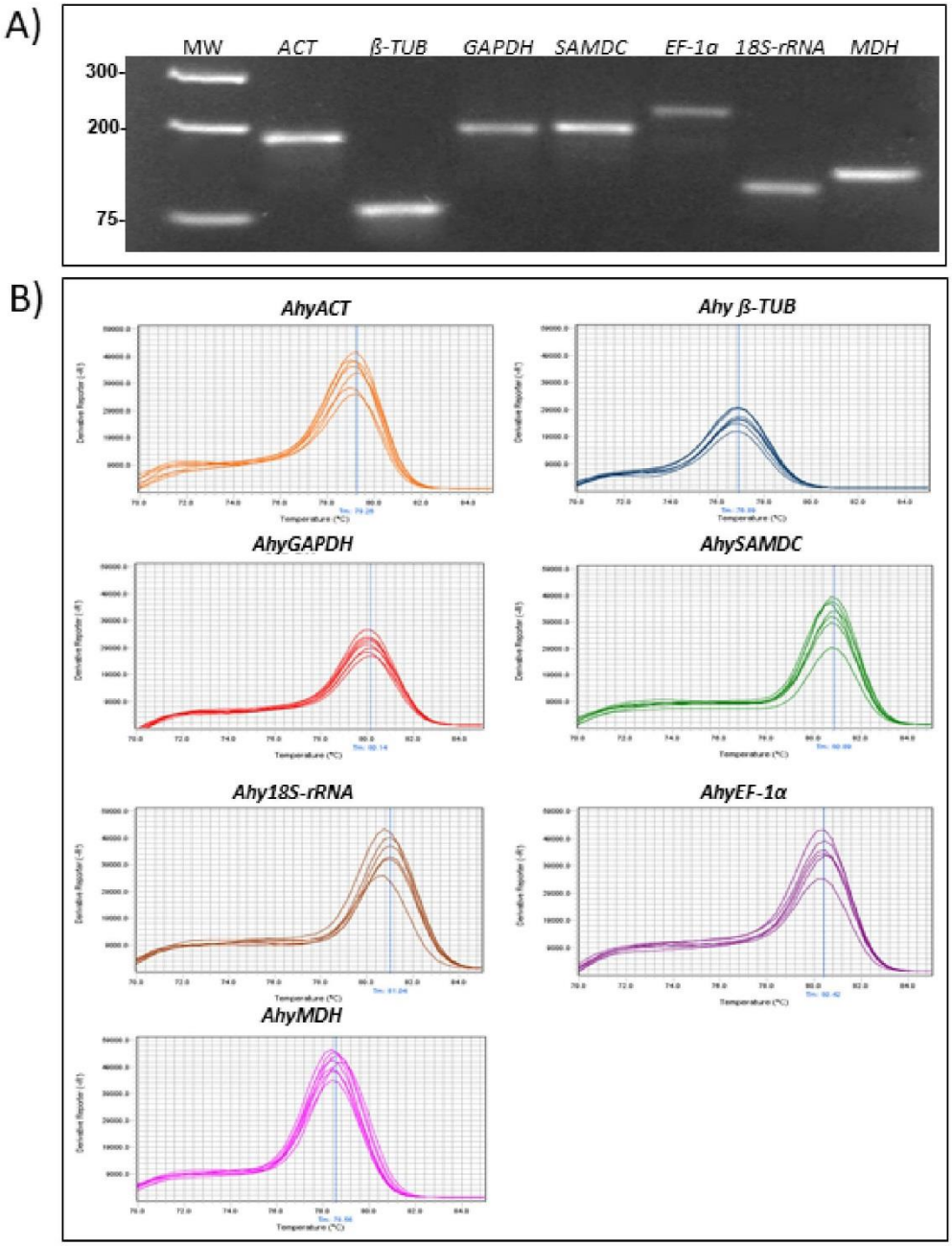

Fig. 1. Specificity of primers and efficiency of reference genes. (A) Electrophoretic analysis of RT-PCR products. Amplicon lengths are indicated in Table 2. Equal amounts of cDNA from all samples were used as template. PCR products were observed with 2.5% agarose gel electrophoresis, 8 cm length, 1X TAE, 7 V/cm, 110 min, and stained with SYBR Gold Nucleic Acid Gel stain. M, GeneRuler 1 kb Plus DNA ladder (ThermoFisher, Waltham, MA, USA). (B) Melting curves of candidate reference genes. Melting curves were generated by heating the amplicon from 70 to 85 °C using the derivative method.

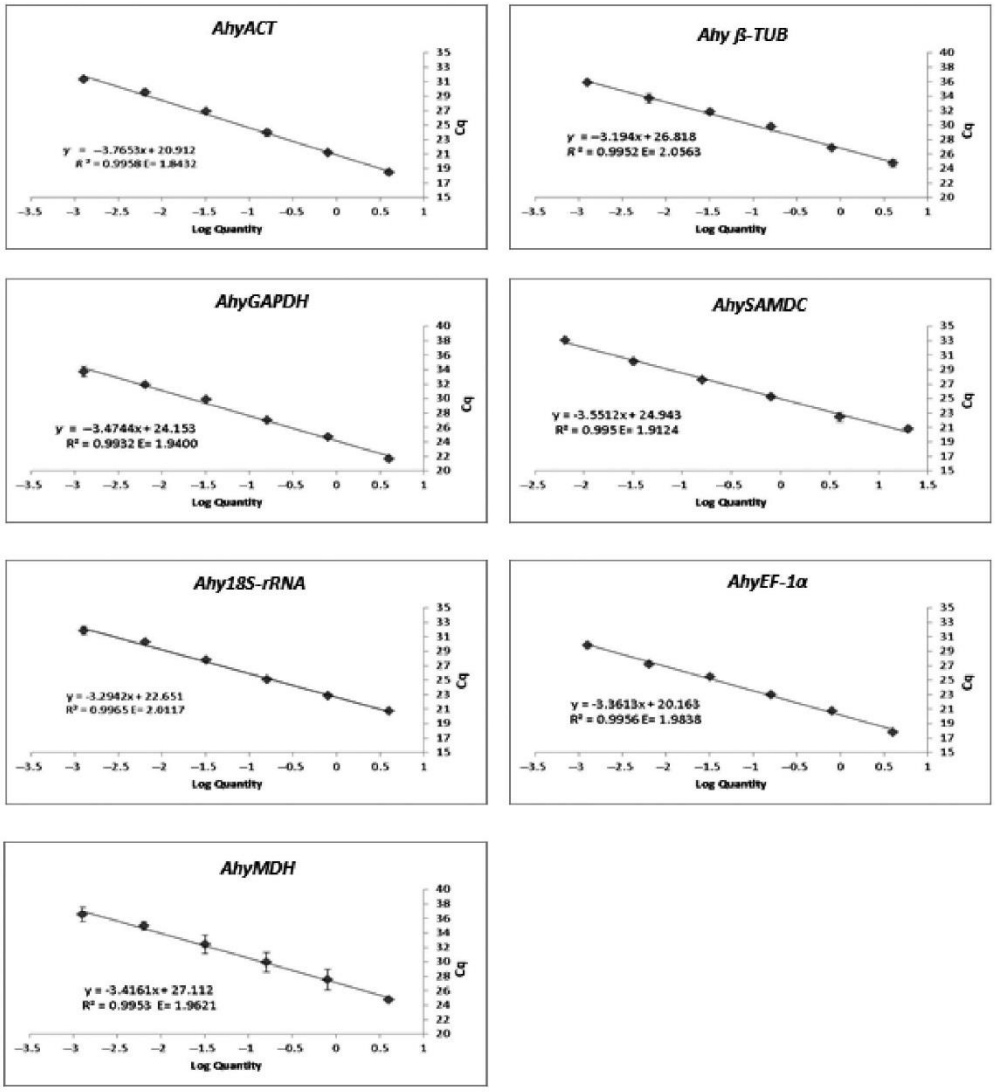

Fig. 2. PCR efficiency and correlation coefficient. The logarithm of each known concentration of starting RNA in the dilution series (*x*-axis) was plotted against mean of the Cq value for used concentration (*y*-axis). Slope (efficiency) and correlation coefficient were obtained *via* linear regression of the standard curve.

As the algorithms used employ different approaches to determine the most stable reference gene, it was found that ranking differed depending on the method employed. Therefore, selection of the most reliable gene for normalisation will depend on the characteristics of every experiment.

Analysis with RefFinder

The RefFinder is a user-friendly tool that integrates four different calculations obtained using gene stability methods. The online RefFinder tool integrates Bestkeeper, NormFinder and geNorm algorithms and combines them into a 'compressive ranking', with outputs from RefFinder and original software obtained from the total samples analysed (Table 4). It has been reported that RefFinder results may be inaccurate because PCR efficiencies are not considered and should only be considered as a guide for deciding which gene to use to normalise PCR data (De Spiegelaere *et al.* 2015). Moreover, results obtained with the original software (NormFinder) are different to those obtained in RefFinder, not only because of the efficiency values

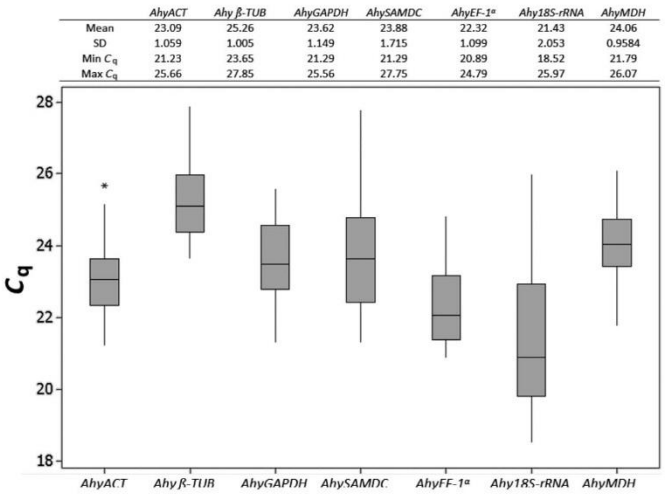

Fig. 3. Descriptive statistics and distribution of Cq- alues of the seven candidate reference genes across all samples in RT-qPCR analysis. (A) Descriptive statistics calculated using Minitab 17. SD, mean and minimum and maximum Cq values for a total of 36 samples. (B) Distribution of Cq values analysed for each gene in all samples. For each gene, distribution of the Cq values is boxplotted from all 72 raw Cq values obtained from the 36 duplicate conditions of amaranth. The borders of the box represent the 25th and the 75th percentiles. The line that divides the box corresponds to the median, while whiskers indicate the highest and lowest Cq values, with the exception of atypical values, which are represented by asterisks.

but also because the original program is able to calculate inter- and intragroup variation, while RefFinder does not allow the user to define groups.

Analysis with GeNorm

The expression stability value (M) for each reference gene was calculated using the geNorm algorithm. A lower M-value indicates higher stability. For the 'Total', 'Different tissues' and 'Different stages of development' groups, *AhyACT/AhyMDH* was the most stable pair, while *AhyGAPDH/AhyMDH* was the most stable pair in the 'Different types of stress' group and *Ahy18S-rRNA* and *AhySAMDC* was the least stable (Fig. 4). Based on calculations of pair-wise variation (V), the geNorm algorithm can determine the optimal number of reference genes for each experimental condition (Fig. 5). It is generally

Table 3. Expression stability and ranking of candidate reference genes using different software for each experimental set. The genes were analysed with BestKeeper, geNorm and NormFinder. Genes were ranked in terms of values obtained from each algorithm used in each group; M, expression stability and SD were used. Normfinder and Genorm used the M, while Bestkeeper used SD.

			Ranking of gene expression						
Group	Method		1	2	3	4	5	6	7
Total	BestKeeper	Gene	*AhyMDH*	*AhyACT*	*Ahyß-TUB*	*AhyEF-1α*	*AhyGAPDH*	*AhySAMDC*	*Ahy18S-rRNA*
		SD value	0.729	0.814	0.826	0.902	0.977	1.336	1.707
	Normfinder	Gene	*AhyMDH*	*AhyGAPDH*	*AhyACT*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.086	0.134	0.142	0.172	0.215	0.242	0.339
	Genorm	Gene	*AhyACT| AhyMDH*		*AhyGAPDH*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.77		0.83	0.87	0.92	1.11	1.32
Different types of stress	BestKeeper	Gene	*AhyEF-1α*	*Ahyß-TUB*	*AhyMDH*	*AhyACT*	*AhyGAPDH*	*AhySAMDC*	*Ahy18S-rRNA*
		SD alue	0.741	0.751	0.802	0.852	1.071	1.121	1.756
	Normfinder	Gene	*AhyMDH*	*AhyGAPDH*	*AhyACT*	*AhyEF-1α*	*AhySAMDC*	*Ahyß-TUB*	*Ahy18S-rRNA*
		M Value	0.105	0.151	0.310	0.317	0.348	0.461	0.723
	Genorm	Gene	*AhyGAPDH| AhyMDH*		*AhyEF-1α*	*AhyACT*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.597		0.827	0.872	0.997	1.16	1.353
Different tissue	BestKeeper	Gene	*AhyMDH*	*AhyACT*	*AhySAMDC*	*Ahyß-TUB*	*AhyEF-1α*	*AhyGAPDH*	*Ahy18S-rRNA*
		SD value	0.66	0.79	0.85	0.87	1.03	1.06	1.39
	Normfinder	Gene	*AhyMDH*	*AhyACT*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*AhyGAPDH*	*Ahy18S-rRNA*
		M value	0.137	0.160	0.184	0.262	0.270	0.467	0.664
	Genorm	Gene	*AhyACT| AhyMDH*		*AhyEF-1α*	*Ahy ß-TUB*	*AhySAMDC*	*AhyGAPDH*	*Ahy18S-rRNA*
		M value	0.678		0.802	0.841	0.915	1.016	1.297
Different stages of development	BestKeeper	Gene	*AhyMDH*	*AhyGAPDH*	*AhyACT*	*Ahyß-TUB*	*AhyEF-1α*	*Ahy18S-rRNA*	*AhySAMDC*
		SD value	0.58	0.65	0.68	0.68	0.84	1.52	1.76
	Normfinder	Gene	*AhyACT*	*AhyMDH*	*AhyGAPDH*	*Ahyß-TUB*	*AhyEF-1α*	*Ahy18S-rRNA*	*AhySAMDC*
		M value	0.062	0.072	0.202	0.286	0.351	0.397	0.595
	Genorm	Gene	*AhyACT| AhyMDH*		*AhyGAPDH*	*AhyEF-1α*	*Ahyß-TUB*	*Ahy18S-rRNA*	*AhySAMDC*
		M value	0.375		0.45	0.57	0.625	0.933	1.081

Table 4. Comparison of ranking of candidate reference genes using RefFinder and original software. The genes were analysed with BestKeeper, geNorm and NormFinder using the web-based tool Refinder and original software. M (expression stability value) and SD were used. *and ΔCT method was not compared.

			Ranking of gene expression						
	Method		1	2	3	4	5	6	7
RefFinder	Δ CT[(a)]	Gene	*AhyMDH*	*AhyACT*	*AhyEF-1α*	*AhyGAPDH*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		Value	1.09	1.2	1.23	1.24	1.36	1.58	1.87
	BestKeeper	Gene	*AhyMDH*	*AhyACT*	*Ahyß-TUB*	*AhyEF-1α*	*AhyGAPDH*	*AhySAMDC*	*Ahy18S-rRNA*
		SD value	0.73	0.81	0.83	0.9	0.98	1.34	1.71
	Normfinder	Gene	*AhyMDH*	*AhyACT*	*AhyGAPDH*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.306	0.684	0.761	0.763	1.035	1.259	1.683
	Genorm	Gene	*AhyACT* | *AhyMDH*		*AhyEF-1α*	*AhyGAPDH*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.846		0.871	0.912	0.953	1.164	1.367
	Recommended comprehensive ranking	Gene	*AhyMDH*	*AhyACT*	*AhyEF-1α*	*AhyGAPDH*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		Value	1	1.68	3.46	3.94	4.4	6	7
Original software	BestKeeper	Gene	*AhyMDH*	*AhyACT*	*Ahyß-TUB*	*AhyEF-1α*	*AhyGAPDH*	*AhySAMDC*	*Ahy18S-rRNA*
		SD value	0.729	0.814	0.826	0.902	0.977	1.336	1.707
	Normfinder	Gene	*AhyMDH*	*AhyGAPDH*	*AhyACT*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.086	0.134	0.142	0.172	0.215	0.242	0.339
	Genorm	Gene	*AhyACT*| *AhyMDH*		*AhyGAPDH*	*AhyEF-1α*	*Ahyß-TUB*	*AhySAMDC*	*Ahy18S-rRNA*
		M value	0.77		0.83	0.87	0.92	1.11	1.32

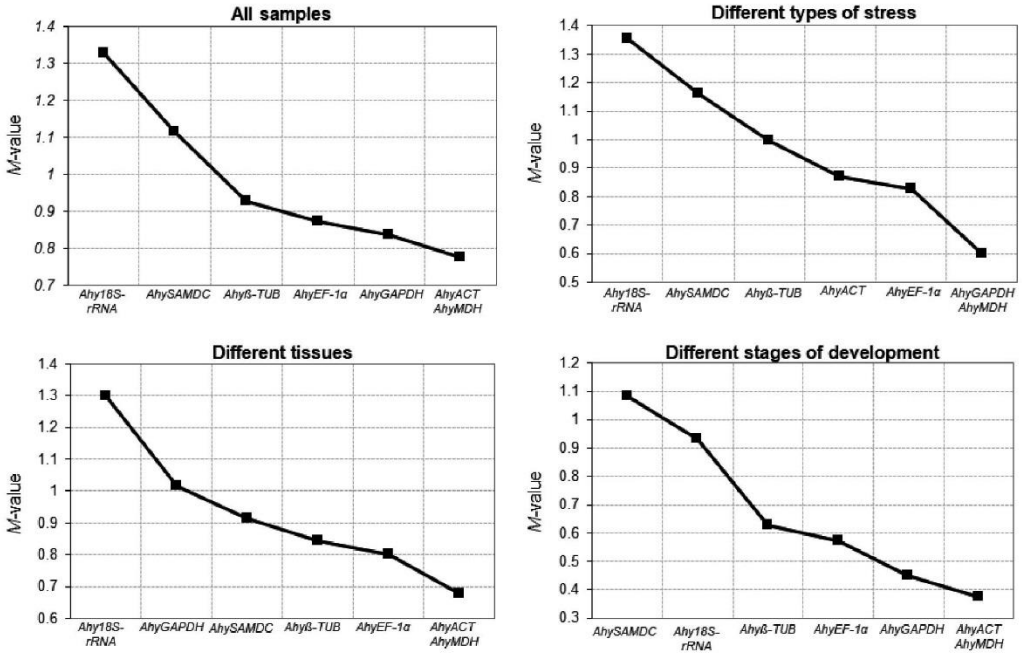

Fig. 4. Gene expression stability and ranking of six reference genes based on geNorm. Expression stability value (M) for each gene was obtained and graphed. The lower the M value, the more stable the gene.

assumed that 0.15 is a cut-off value for determining optimal number of reference genes as Vandesompele *et al.* (2002) decided to take this as a cut-off value, since the inclusion of an additional control gene made no significant contribution to the normalisation factor (NFn + 1) calculated using their data. However, 0.15 must not be taken as a strict cut-off value but rather as a guide value, depending on the volume of genes and samples tested (Singh *et al.* 2015). The use of only four reference genes in the group 'Different stages of development' from the data obtained in this study met this cut-off value (Fig. 5).

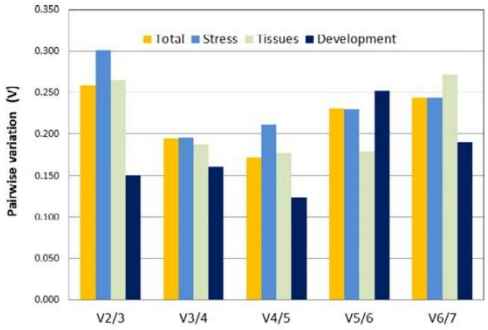

Fig. 5. Analysis of minimum number of reference genes required for RT-qPCR normalisation. Pair-wise variation value (V) was calculated using geNorm to determine the minimum number of reference genes required for normalisation in each experimental set. The graph shows variation in stability with the sequential addition of a subsequent reference gene, starting with the most stably expressed pair of genes (V2/3). From the graph, it can be inferred whether the addition of another gene would have an effect on stability.

However, according to the Primer Design geNorm kit Handbook (Primerdesign 2016), the use of the three best reference genes, which in most conditions include *AhyMDH, AhyACT, AhyEF-1α* and *AhyGAPDH,* is a valid normalisation strategy that results in accurate and reliable normalisation of data. These data were not obtained using the RefFinder output.

Analysis with NormFinder

NormFinder calculates gene expression stability by comparing variation between user-defined sample groups (Andersen *et al.* 2004). This study analysed all the samples, identifying results as 'Total' in Table 4. The same three groups were also defined, as in geNorm analysis, with each group subjected to further analysis, and subgroups defined as the three mean Cq values (of two technical replicates each) corresponding to three individual biological replicates for each condition tested. NormFinder calculates intra- and intergroup variations, considering genes with the lowest variation as stable. Analysis of groups of data found similar results to geNorm, with *AhyMDH/AhyACT* the most stable genes in the 'Different types of tissues' and 'Different stages of development' groups, while *AhyGAPDH/AhyMDH* were the most stable pair in 'Different types of stress' group. However, normFinder results differ from geNorm in the 'Total' group, with very different results obtained between the samples, while intergroup variation calculation is a crucial feature in NormFinder analysis. Similar to geNorm, NormFinder found that the least stable genes are, generally, *AhySAMDC* and *Ahy18S-rRNA*.

Analysis with Bestkeeper

The lower of the SD and CV values computed by BestKeeper software ranked *AhyMDH* as the most stable and thus the best reference gene, when all samples were analysed against each other. While these are similar to findings obtained with geNorm and NormFinder, some of the outputs obtained with this method showed discrepancies. For example, some genes ranked as less stable, *e.g. AhySAMDC and Ahyβ-TUB*, were ranked as more stable than other genes considered as stable, *e.g. AhyGAPDH* (Table 4).

DISCUSSION

The use of appropriate reference genes can correct inaccuracies in terms of the amount of RNA loaded into the reaction and the efficiency of the reverse transcription, with the intention of obtaining real differential expression of the target genes in experimental treatments. However, the direct transfer of reference genes to non-model plants is limited as not all the genes are expressed in the same manner in different species. While the use of only one housekeeping gene for normalisation of RT-PCR data is a common practice, it has been widely demonstrated that results of this common practice constitute erroneous fold expression calculations (Vandesompele *et al.* 2002; Chan *et al.* 2014).

Chan *et al.* (2014) evaluated methods for identifying reference genes and found that NormFinder and geNorm were consistent with each other in obtaining the highest correlation ($R^2 = 0.987$; Chan *et al.* 2014). Hence it enables suggestions to be made for normalisation of PCR data. For PCR data from amaranth exposed in the same experiment to different stress conditions, with different tissues and different stages of development, it is highly recommended to use the four most stable genes (*AhyMDH, AhyGAPDH, AhyEF-1α* and *AhyACT)* to validate normalisation of data.

Data obtained from samples exposed to different types of stress had the highest M values (*i.e.* they are less stable under these conditions). These findings suggest the use of three reference genes, *AhyGAPDH, AhyMDH* and either *AhyEF-1α* or *AhyACT*, when working with samples exposed to biotic or abiotic stress. Furthermore, given that they present similar stability values, the inclusion of a fourth gene would not make a significant contribution to accurate normalisation of PCR data.

Finally, data from samples of different tissues or different stages of development (only one condition per experiment) had low M values (*i.e.* they are more stable under these conditions). This finding suggests use of the pair of genes *AhyACT* and *AhyMDH* as optimal for normalisation of data, while inclusion of a third gene, in terms of development stage, would not make a contribution; however, when working with different tissues, addition of *AhyEF-1α* would be required for accurate normalisation.

Moreover, the results indicate that *Ahy18S-rRNA* and *AhySAMDC* were, in most cases, inappropriate for use as a control in *A. hypocondriacus* and should be avoided. In summary, this study provides useful information about various reference genes for RT-qPCR studies in amaranth, which will contribute significantly to future gene studies in this valuable species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appendices II: Book chapter Isolation and detection of plant microRNAs

Chapter 8

Isolation and Detection Methods of Plant miRNAs

Pedro Fernando Vera-Hernández, Stefan de Folter, and Flor de Fátima Rosas-Cárdenas

Abstract

Small RNAs (sRNAs) are RNAs of low abundance in organisms. Among sRNAs, miRNAs are included and represent approximately 10% of the total number of sRNAs. The isolation of sRNAs is critical for miRNA detection and analysis. The precipitation of low-molecular-weight (LMW) RNAs from total RNA extracts has allowed enrichment of sRNAs. Here, we describe a simple method to isolate sRNAs from different plant species. The main advantage of this method is that it does not need first an extraction of total RNA and it is not based on TRIzol® reagent. This method has been successfully used for miRNA analyses by Northern blot assay and RT-qPCR (these techniques are as well described in this chapter), as well as sRNA library preparation.

Key words sRNA extraction, Plant miRNAs, miRNA analysis, miRNA detection

1 Introduction

To identify and study miRNAs, efficient protocols to isolate sRNAs are necessary. The extraction of total RNA by various methods and subsequently enrichment of small RNAs have been reported [1–5]. The method described here does not need first an extraction of total RNA. One of the main advantages of this method is its simplicity and low cost; it makes use of accessible and low-cost reagents, in contrast to other commercially available methods using the TRIzol® reagent in column-based kits, and in a few steps, good quality of sRNA can be obtained. Previously, we demonstrated that this sRNA extraction method works well for plant tissues with high polysaccharide content such as from cactus, agave, banana, and tomato but also of plant tissues with lower polysaccharide contents such as from *Arabidopsis* and tobacco [6, 7]. To date, it has been demonstrated that the method is very versatile and has been tested for different plant species like chickpea [8], Wuweizi [9], *Monotropa* [10], pear [11], soy, and corn [12]. Moreover, it has been demonstrated that the method also

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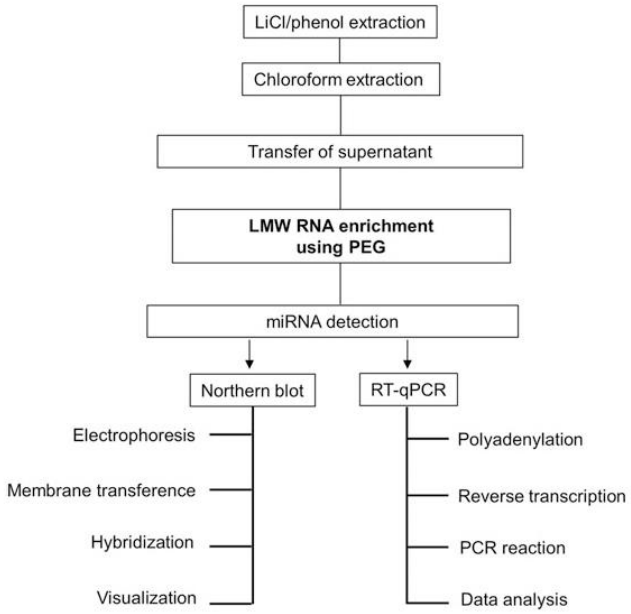

Fig. 1 General procedure of isolation and detection of plant miRNAs

works for animal species such as zebrafish [13]. The sRNA samples obtained by this method have been successfully used in Northern blot assays [6] and for small RNA library preparations [8–11]. Furthermore, we demonstrated that it is possible to quantify the miRNAs by qPCR assay using this protocol.

In summary, here we provide a detailed protocol for sRNA isolation (Fig. 1), which works well for plant tissues of many species, including of tissues with high polysaccharide content (Fig. 2a). Besides SDS-PAGE gel electrophoresis (Fig. 2a), standard agarose gel electrophoresis may be sufficient to judge the quality of enriched sRNA (or low-molecular-weight (LMW) RNA) samples (Fig. 2b). Furthermore, this low-cost simple method is suitable for downstream assays such as Northern blot hybridization (Fig. 2c) and miRNA quantification by qPCR assay (Fig. 2d).

2 Materials

Prepare all solutions using sterile deionized water. Prepare and store all reagents at room temperature (unless indicated otherwise). Slice and grind the tissue samples to a fine powder in a mortar with liquid nitrogen, and store at −80 °C until further use.

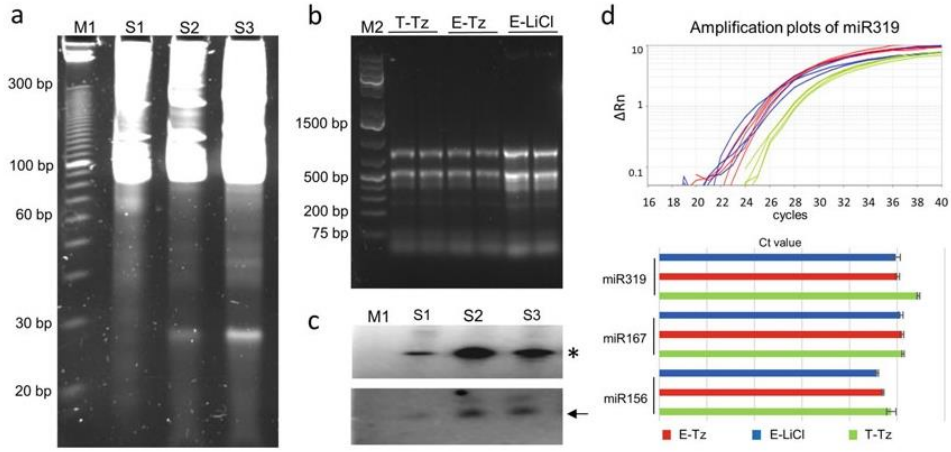

Fig. 2 Visualization of LMW RNAs isolated using different methods and detection of miRNA expression by Northern blot and RT-qPCR assays. (**a**) Electrophoresis in SDS-PAGE gel; 2 μg of each sample was loaded on a 12.5% polyacrylamide gel. M1, 10 bp DNA ladder; S1, *Arabidopsis* leaves; S2, young floral buds; S3, floral buds. (**b**) Electrophoresis in 2% agarose gel of RNA samples from *Arabidopsis* obtained using different sRNA isolation protocols: T-Tz, obtained by TRIzol reagent; E-Tz, RNA obtained by TRIzol reagent and then enriched using PEG; and E-LiCl, sRNA obtained by the method described here. 1 μL of each sample was loaded on the gel. M2, molecular ladder 1 kb plus. (**c**) Northern blot assay for miR159a and for the U6 small nucleolar RNA. Arrow indicates the signal of miR159a, and the asterisk indicates the signal of U6. (**d**) Comparison between Ct (cycle threshold) values for mature miRNA quantification from RNA samples obtained using different sRNA isolation protocols. 1 ng of RNA of each sample was used as input for the RT reaction. The top graph shows the amplification plots for the quantification of miR319, in samples processed by different isolation methods. The graph below shows the average Ct values obtained for the quantification of miR319, miR167, and miR156 from RNA samples from *Arabidopsis* obtained using the E-TZ, E-LiCl, and T-Tz extraction method

2.1 Small RNA Extraction

1. LiCl extraction buffer: 100 mM Tris–HCl pH 9.5, 1% SDS, 100 mM lithium chloride (LiCl), and 10 mM EDTA. Prepare as follows: add about 100 mL water to a bottle, add 3 g of Tris, mix, and adjust pH to 9.5 with HCl. Subsequently, add 0.25 g of SDS, 1.06 g of LiCl, and 0.73 g of EDTA. Mix and adjust to 250 mL with water. Autoclave and store at 4 °C.
2. Phenol pH 8.0. Store at 4 °C.
3. Chloroform-isoamyl alcohol (24:1; v/v). Store at 4 °C.
4. 5 M sodium chloride: dissolve 29.2 g of NaCl in 100 mL of water. Autoclave the solution.
5. 40% polyethylene glycol 8000 (PEG 8000): dissolve 40 g of PEG 8000 in deionized water, and adjust the volume to 100 ml. Filter-sterilize the solution.
6. Phenol-chloroform-isoamyl alcohol (25:24:1; v/v/v). Store at 4 °C.
7. 5 M sodium acetate: dissolve 24.6 g of NaOAc in 50 mL of deionized water. Adjust the pH to 5.2 with glacial acetic acid.

Adjust the final volume to 100 mL with deionized water, and filter-sterilize.

8. Absolute ethanol. Store at 4 °C.
9. Microcentrifuge.

2.2 Small RNA Analysis by Polyacrylamide Gel Electrophoresis

1. 20% ammonium persulfate (APS): dissolve 1 g in 5 mL water. Store at −20 °C.
2. Acrylamide (acrylamide/bisacrylamide 19:1; Bio-Rad).
3. 12% polyacrylamide stock solution, for 12 mL of solution: mix 2.5 mL of 5× TBE, 5.25 g of urea, 3.75 mL of acrylamide, and 3.75 mL of water. Store at 4 °C (*see* **Note 1**).
4. N,N,N′,N′-Tetramethylethylenediamine (TEMED). Store at 4 °C.
5. Vertical electrophoresis gel system (Bio-Rad).
6. Isopropanol. Store at 4 °C.
7. 5× TBE stock: 450 mM Tris-borate and 10 mM EDTA pH 8.3. Prepare as follows: in an appropriate-sized beaker, dissolve 54 g Tris, 27.5 g boric acid, and 3.7 g of EDTA disodium salt in approximately 750 mL of deionized water. Once dissolved, adjust the pH with concentrated HCl, followed by adjusting to 1 l with deionized water (*see* **Note 2**).
8. 0.5× TBE: mix 100 mL of 5× TBE and 900 mL of distilled deionized water.
9. Loading buffer: mix 3.92 mL of deionized formamide 98%, 80 µL of 0.5 M EDTA, 4 mg of xylene cyanol, and 4 mg of bromophenol blue, and adjust the final volume to 4 mL with deionized water. Store at −20 °C.
10. 10 bp DNA ladder.
11. Staining solution: 0.001% SYBR Gold, mix 1 µL of SYBR Gold Nucleic Acid Gel Stain (10,000× in DMSO; Invitrogen) in 100 mL 0.5× TBE buffer.

2.3 Northern Blot Hybridization Analysis

1. Semi-dry trans-blot system (Bio-Rad).
2. Mini-PROTEAN Tetra Cell (Bio-Rad).
3. Storm 860 Molecular Imager System (Amersham Biosciences).
4. Neutral nylon membrane (Hybond-NX, GE Healthcare).
5. Rapid-hyb Buffer (GE Healthcare).
6. 3MM Whatman chromatography paper.
7. 0.5× TBE: mix 100 mL of 5× TBE and 900 mL of distilled deionized water.
8. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich).

9. EDC fixation solution, immediately before use: dissolve 0.753 g of EDC in 10 mL of water, add 245 µL of 12.5 M methylimidazole, and add 150 µL of 1 M HCl.
10. Fragment 5′-AGGGGCCATGCTAATCTTCTC-3, U6 small nucleolar RNA.
11. 20× SSC stock: dissolve 87.6 g NaCl and 44.12 g trisodium in water, adjust to pH 7 with HCl, and adjust to 0.5 l. Autoclave and store at room temperature.
12. Wash solution, 2× SSC: mix 20 mL of 20× SSC solution with 180 mL of deionized water.

2.4 Small RNA Analysis by Agarose Gel Electrophoresis

1. Molecular biology grade standard fusion point agarose.
2. 0.5× TBE: mix 100 mL of 5× TBE and 900 mL of distilled deionized water.
3. 6× loading buffer: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol.
4. Molecular ladder 1 kb plus.
5. Horizontal electrophoresis system (Bio-Rad).

2.5 Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

1. *E. coli* poly(A) polymerase (5 units/µL).
2. Deoxynucleotide solution mix (dNTP; each at 10 mM).
3. MMLV reverse transcriptase kit (Sigma-Aldrich).
4. One-Step TaqProbe qRT-PCR-ROX® master mix (Applied Biological Materials).
5. Primer reverse universal: oligonucleotide 5′-CAGTGCAGGG TCCGAGGT-3′.
6. Universal probe: FAM-CAGAGCCACCTGGGCAATTT-TA MRA.
7. Primers to amplify miR156: oligonucleotide 5′-GTGCAGGG TCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTG TGCTC-3′ was used as reverse primer, and the oligonucleotide 5′-GCCCCTGACAGAAGAGAGT-3′ was used as forward primer.
8. Primers to amplify miR319: oligonucleotide 5′-GTGCAGGG TCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTA GGGAG-3′ was used as reverse primer, and the oligonucleotide 5′-GCGATTGGACTGAAGGGAG-3′ was used as forward primer.
9. Primers to amplify miR167: oligonucleotide 5′-GTGCAGGG TCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTT AGATC was used as reverse primer, and the oligonucleotide 5′-CGCTGAAGCTGCCAGCAT-3′ was used as forward primer.
10. Real-time PCR machine.

3 Methods

3.1 Small RNA Extraction

1. Put 10–100 mg of pulverized frozen tissue in a 1.5 mL microcentrifuge tube (*see* **Note 3**).
2. Add 500 µL of LiCl extraction buffer, and add 500 µL of phenol pH 8.0 to each microcentrifuge tube.
3. Mix well using a vortex for 1 min, incubate the tubes at 60 °C for 5 min, and centrifuge at maximum speed in a microcentrifuge at 4 °C for 10 min.
4. Transfer the supernatant (upper phase) to a new microcentrifuge tube, add 600 µL of chloroform-isoamyl alcohol (24:1; v/v), mix using a vortex for 1 min, and centrifuge at maximum speed at 4 °C for 10 min (*see* **Note 4**).
5. Transfer the supernatant to a new microcentrifuge tube, and incubate at 65 °C for 15 min.
6. Add 50 µL of 5 M NaCl and 63 µL of 40% PEG 8000 (w/v), mix using a vortex for 1 min, followed by incubation on ice for at least 30 min, and centrifuge at maximum speed at 4 °C for 10 min.
7. Transfer supernatant to a new microcentrifuge tube, add 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1; v/v/v), mix for 1 min, and centrifuge at maximum speed at 4 °C for 10 min (*see* **Notes 4** and **5**).
8. Transfer supernatant to a new microcentrifuge tube, and precipitate LMW RNAs by adding 50 µL of 3 M sodium acetate pH 5.2 and 1200 µL of absolute ethanol, and incubate at −20 °C overnight (*see* **Note 6**).
9. Centrifuge tubes at maximum speed at 4 °C for 10 min, discard the supernatant, dry the pellet, and resuspend in 20 µL RNase-free water (*see* **Note 7**). Small RNA samples can be put on ice when used directly or stored at −20 °C for later use.

3.2 Small RNA Analysis by Polyacrylamide Gel Electrophoresis

1. Assemble the electrophoresis system; use 1.5 mm spacers (*see* **Note 8**).
2. Prepare 12% polyacrylamide gels: mix 12% polyacrylamide stock solution, 125 µL of 10% APS, and 12.5 µL of TEMED (*see* **Note 9**). Add this acrylamide solution in the vertical electrophoresis gel system. Let the polyacrylamide polymerize for at least 30 min and then remove the combs (*see* **Note 10**).
3. Pre-run the gel(s) in 0.5× TBE buffer at 90 V for 2 h in the vertical electrophoresis gel system (Bio-Rad) (*see* **Note 11**).
4. Prepare the samples. For 2 µg of LMW RNA (i.e., sRNA), add 0.3 vol (v/v) loading buffer (adjust all samples to the same volume by adding water). Incubate the samples at 65 °C for

5 min to denature the RNA, and then immediately place the tubes on ice for at least 1 min.

5. Before loading each sample in the gel, wash each gel slot with 0.5× TBE using a syringe.
6. Load the samples in the gel (fill empty slots with loading buffer), and run in 0.5× TBE buffer at 90 V for around 2 h (until bromophenol blue of the loading buffer reaches the end of the gel).
7. When the dye front reaches the end of the gel, turn off the power supply. Separate the gel plates with the help of a spatula or similar tool. Remove the upper gel part where the slots were for more easy handling in subsequent steps.
8. Rinse the gel carefully with deionized water to separate it from the glass plate.
9. Stain the gel in 15 mL 0.5× TBE buffer with 0.001% SYBR Gold for 15 min. Afterward, rinse with RNase-free water for 5 min, and subsequently visualize the results under the correct light source (e.g., UV light) (Fig. 2a) (*see* **Note 12**).

3.3 Northern Blot Hybridization Analysis

This protocol is described using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) and based on the Pall and Hamilton protocol with some modifications [14].

1. Cut the nylon membrane to the size of the gel, and cut six sheets of 3MM Whatman paper.
2. Pre-wet three sheets of 3MM Whatman paper with distilled water, and place on the positive electrode. Pre-wet the nylon membrane with 0.5× TBE buffer, and place on top of the 3MM Whatman papers, place the gel on top of the nylon membrane, and place the other three sheets of 3MM Whatman paper (pre-wet with distilled water) on top of the gel. Run the transfer at 10 V for 1 h (*see* **Note 13**).
3. Separate the membrane from the gel, rinse the membrane in 0.5× TBE buffer, and air-dry the membrane at room temperature.
4. Cut a sheet of 3MM Whatman paper of the same size as the membrane, place the membrane on a flat surface (e.g., in a tray or box), and put on top a sheet of 3MM Whatman paper, pour 12 mL of freshly made EDC fixation solution over the membrane with the 3MM Whatman paper, and incubate at 60 °C for 30 min.
5. Subsequently, rinse the membrane twice with RNase-free water. Let the membrane dry, and use directly or store at −20 °C for later use.

6. Pre-hybridize with 15 mL hybridization solution at 42 °C for 1.5 h with constant agitation (*see* **Note 14**).
7. Prepare the radioactive labeled probe: 4 μL of 100 μM oligonucleotide is labeled with 1 μL of T4 kinase (10 U/μL), 1 μL of [γ-32P] ATP (10 mCi/ml), 4 μL of forward buffer, and 10 μL of water (*see* **Notes 15** and **16**).
8. Add the 20 μL of labeled probe of interest to the membrane(s), and incubate at 42 °C for 3–24 h with constant agitation (*see* **Notes 14** and **17**).
9. Wash the membrane twice with wash solution (first for 4 min and then a second time for 2 min) at room temperature.
10. Put the membrane(s) on a flat surface with absorbent paper to remove the excess of wash solution.
11. Pack/seal the membrane(s) with transparent plastic foil.
12. Place the membrane(s) in an exposure cassette, and place a storage phosphor screen, and close the exposure cassette.
13. Expose the membrane(s) to a storage phosphor screen for ~48 h at room temperature (*see* **Note 18**).
14. Scan the phosphor screen with the Storm 860 scanner (Amersham Biosciences) to analyze the U6 control and the miRNA signals (Fig. 2c).

3.4 Small RNA Analysis by Agarose Gel Electrophoresis

1. Make a 2% agarose gel: add 0.6 g agarose in 30 mL of 1× TBE buffer, and dissolve by heating in microwave. Then chill to approximately 60 °C, add 0.6 μL of SYBR Gold staining solution (10,000× stock), mix gently to avoid the formation of air bubbles, and pour the gel using a comb that will form the wells. Assemble the gel in the tank, and add enough 1× TBE running buffer to cover the gel (*see* **Note 19**).
2. Add 0.5–3× vol (v/v) loading buffer to the samples and mix (*see* **Note 20**).
3. Load the gel, and perform the electrophoresis at 5–6 V/cm until the bromophenol blue (the faster-migrating dye) has migrated at least 2–3 cm into the gel or as far as 2/3 the length of the gel.
4. Visualize the RNA in the gel on a UV transilluminator (Fig. 2b) (*see* **Note 21**).

3.5 Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Quantification of plant microRNAs can be made using the S-Poly (T) method [15].

1. For the polyadenylation reaction: mix 500 ng of enriched sRNA, 1 μL of 10× reaction buffer, 1 μL of 10 mM ATP, and 1 unit of poly(A) polymerase, in a reaction volume of 10 μL.

Incubate the reaction at 37 °C for 40 min, followed by heat inactivation at 65 °C for 5 min.

2. Mix 2 μL of the polyadenylation reaction product with 1 μL of 0.5 μM RT primer, 0.5 μL of 10 mM dNTP, 1 μL of 10× MMLV reaction buffer, and 50 units of MMLV Reverse Transcriptase, in a total reaction volume of 10 μL.
3. Incubate at 42 °C for 60 min for synthesis of cDNA, followed by an incubation at 85 °C for 5 min to heat-inactivate the enzyme.
4. For RT-qPCR use a universal probe in a total reaction volume of 10 μL that contains 0.1 μL of RT product(s) (equivalent to 1 ng of starting sRNA), 5 μL of 2× One-Step TaqProbe qRT-PCR-ROX® master mix, 0.3 μL of 10 μM forward primer, 0.3 μL of 10 μM universal reverse primer, and 0.3 μL of 10 μM universal probe. The PCR program is as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s (*see* **Note 22**).
5. Analyze the RT-qPCR results (Fig. 2d).

4 Notes

1. Heat the solution for 30 s in the microwave to dissolve the urea and chill on ice. Unpolymerized acrylamide is neurotoxic, and care should be taken to avoid skin contact; therefore, it is necessary to work with gloves and clean with much soap and water all materials used.
2. Add a large magnetic stir bar, and place slurry on stir plate on high to facilitate mixing and dissolving of chemicals. Heat to facilitate dissolving.
3. The RNA integrity is affected by temperature; it is recommended to cool the microcentrifuge tubes where the tissue will be placed for extraction by holding a few seconds in liquid nitrogen. The amount and handling of the tissue are critical for a successful extraction. The tissue amount depends on the type of plant tissue, for example, for tissues with lots of water, use around 100 mg and not only 10 mg.
4. We recommended to transfer the upper phase to a new microcentrifuge tube previously chilled and place each sample on ice until all samples are ready and subsequently add the chloroform-isoamyl alcohol (24:1; v/v).
5. The supernatant contains low-molecular-weight RNA (i.e., small RNAs), and the pellet consists of high-molecular-weight RNA and DNA mainly.

6. The samples may be incubated less time at −20 °C to precipitate the LMW RNA, but we incubate them not less than 3 h.
7. After discarding the supernatant, give a spin to the microcentrifuge tubes, remove the possible residual supernatant with a pipette, and repeat this process when necessary. Optionally, if some drops of solution remained in the microcentrifuge tubes, dry carefully with strips of 3MM Whatman chromatography paper, but avoid touching the pellet. Finally, dry at room temperature for not more than 10 min.
8. Wait at least 5 min, and observe the water level in the vertical electrophoresis gel system to ensure there is no leakage. Normally we use combs that give ten slots, but others may be used as well.
9. Add APS and TEMED just before pouring the gel(s) in the gel cassette, to prevent too early polymerization. The solution may be placed on ice to delay polymerization.
10. Gels may be stored at 4 °C. Polymerization time affects the quality of the run, and we noted that gels prepared 1 or 2 days previous to their use showed improved band definition.
11. Pre-running of the gel(s) is to remove ammonium persulfate (APS) residues.
12. We use SYBR Gold; however, other types of staining may be used.
13. When adding each layer, roll out all air bubbles using a glass pipette. The critical issue is to ensure that the membrane is tightly and uniformly in contact with the gel. Note, the membrane is in between the gel and the positive electrode.
14. Rapid-hyb™ Buffer (GE Healthcare) is optimized for use in a wide range of hybridization temperatures (42 °C–70 °C).
15. The U6 small nucleolar RNA was used as a positive control.
16. Wear lab coat, gloves, and safety spectacles when handling. For the handling of the radioactive probe, follow the manufacturer's safety guide (http://www.perkinelmer.com/lab-solutions/resources/docs/TCH_Phosphorus32.pdf).
17. Hybridization time and temperature depend on the expression level of each sRNA or miRNA and can be adjusted.
18. We use the Storm 860 Molecular Imager System; however, other systems may be used.
19. You can add the SYBR Gold in the agarose gel or prepare the staining solution with 0.5× TBE buffer with 0.001% SYBR Gold and stain for 15 min, and rinse with RNase-free water for 5 min.
20. Generally, 200 ng of RNA is enough to visualize the different fractions in the RNA isolation(s).

21. Native agarose gel electrophoresis may be sufficient to judge the quality of the enriched sRNA isolation(s), by inspecting the rRNA bands 28S and 18S, and also the band corresponding to the fraction of small RNA. Bands might not be as sharp as in denaturing gels, neither as resolved as in a polyacrylamide gel; nevertheless they are good enough to visualize the multiple bands representing the different fractions.
22. We use the TaqProbe qRT-PCR-ROX® master mix for these experiments and a StepOnePlus Real-Time PCR System (Applied Biosystems); however, other brands and passive dyes may be used depending on the specifications of each device. The probe was synthesized as an oligonucleotide with fluorescein (FAM) as a 5′ reporter and tetramethylrhodamine (TAMRA) as a 3′ label; however, other dark quenchers and fluorophores may be used. Please refer to your PCR machine manual to check for compatibility.

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