Centro de Investigación en Alimentación y Desarrollo, A.C.

ANÁLISIS MOLECULAR DE TALLOS DE *Capsicum annuum* L. EN RESPUESTA A ESTRÉS ABIÓTICO POR BAJA TEMPERATURA

Por:

JESÚS CHRISTIAN GRIMALDI OLIVAS

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Dra. Josefina León Félix
Directora de Tesis

Dra. María Claudia Villicaña Torres
Integrante de Comité de Tesis

Dr. José Basilio Heredia
Integrante de Comité de Tesis

Dra. Melina López Meyer
Integrante de Comité de Tesis

ABRAHAM CRUZ M.

Dr. Abraham Cruz Mendívil
Integrante de Comité de Tesis

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CONTENIDO

RESUMEN

El pimiento morrón (*Capsicum annuum*) es uno de los cultivos más importantes a nivel mundial. No obstante, la producción de este cultivo puede ser alterada por distintos estreses abióticos, como las bajas temperaturas. Este estrés induce una serie de cambios bioquímicos, morfológicos y moleculares que impactan en la composición lipídica de la membrana, los pigmentos fotosintéticos, la acumulación de azúcares libres, prolina, y el metabolismo secundario. Estos eventos moleculares se han estudiado a mayor profundidad gracias a las ciencias ómicas. La integración de datos de diferentes ómicas ha sido una herramienta muy utilizada para la identificación de vías metabólicas claves alteradas durante la exposición a diversos tipos de estrés abiótico. El presente estudio tuvo como objetivo estudiar las bases moleculares de los mecanismos de respuesta a estrés por baja temperatura en tallos de pimiento morrón, mediante el uso de herramientas ómicas tales como la genómica, proteómica, transcriptómica y metabolómica con la integración de las dos últimas. Para ello, se realizó la secuenciación masiva del ARN (RNA seq) y se analizaron los perfiles transcriptómicos para identificar los genes de expresión diferencial, los perfiles proteómicos y metabolómicos se obtuvieron mediante cromatografía líquida acoplada a espectrometría de masas, y la integración de datos se realizó en la base de datos de vías metabólicas de la planta utilizando el proteóma y metabolóma. encontrando que el estrés por baja temperatura en tallos de pimiento morrón indujo la expresión diferncial de genes relacionados a las vías metabólicas de biosíntesis de flavonoides, biosíntesis de alcaloides, vías relacionadas al ritmo circadiano, de señalización de MAPK y de transducción de señales de hormonas vegetales. También se observó la acumulación de proteínas relacionadas a la respuesta al ion Cadmio, al proceso biosintético del piridoxal fosfato, el desarrollo de filomas, al transporte de endosomas tardíos a vacuolas y al ensamblaje del signalosoma COP9. De igual manera se identificó una alteración en las vías metabólicas de biosíntesis de flavonoides, de fenilpropanoides, de arginina, la oxidación del piruvato y el ciclo del ácido cítrico (TCA). Los datos generados en esta investigación proporcionan información sobre los genes y las rutas metabólicas involucradas en la respuesta de los tallos de pimiento morrón al estrés por baja temperatura. Siendo las proteínas y metabolitos identificadas de gran apoyo en las vías metabólicas relacionadas en osmoprotección, respuesta antioxidante y respiración celular.

Palabras claves: *Capsicum annuum*, transcriptómica, proteómica, metabolómica, integración de datos ómicos

ABSTRACT

Bell pepper (*Capsicum annuum* L.) is one of the most important crops worldwide. However, the production of this crop can be altered by different abiotic stresses, such as low temperatures. This stress induces a series of biochemical, morphological, and molecular changes that impact the lipid composition of the membrane, photosynthetic pigments, the accumulation of free sugars, proline, and secondary metabolism. These molecular events have been studied in greater depth thanks to omics sciences. Integrating data from different omics has been a widely used tool for identifying critical metabolic pathways altered during exposure to different types of abiotic stress. The present study aimed to study the molecular basis of the response mechanisms to low-temperature stress in bell pepper stems through the use of omics tools such as genomics, proteomics, transcriptomics, and metabolomics with the integration of the last two. For this purpose, massive RNA sequencing (RNA seq) was performed, and transcriptomic profiles were analyzed to identify differentially expressed genes. Proteomic and metabolomic profiles were obtained by liquid chromatography coupled to mass spectrometry, and data integration was performed in the plant's metabolic pathways database using the proteome and metabolome. They are finding that low-temperature stress in bell pepper stems induced the differential expression of genes related to the metabolic pathways of flavonoid biosynthesis, alkaloid biosynthesis, pathways related to the circadian rhythm, MAPK signaling, and plant hormone signal transduction. The accumulation of proteins related to the response to the cadmium ion, the biosynthetic process of pyridoxal phosphate, the development of phyllomes, the transport of late endosomes to vacuoles, and the assembly of the COP9 signalosome was also observed. Likewise, an alteration was identified in the metabolic pathways of flavonoid biosynthesis, phenylpropanoids, arginine, pyruvate oxidation, and the citric acid cycle (TCA). The data generated in this research provide information on the genes and metabolic pathways involved in the response of bell pepper stems to low-temperature stress. The proteins and metabolites identified greatly support the metabolic pathways of osmoprotection, antioxidant response, and cellular respiration.

Key words: *Capsicum annuum,* Transcriptomics, Proteomics, Metabolomics and Omics data integration

1. SINOPSIS

1.1 Justificación

Estudios previos realizados en *Capsicum annuum* nos han proporcionado información relevante sobre algunos de los mecanismos moleculares implicados en la respuesta a estrés por baja temperatura ocasionando cambios bioquímicos, morfológicos y moleculares que impactan la composición lipídica de la membrana, los pigmentos fotosintéticos, la acumulación de azúcares libres y prolina, y el metabolismo secundario. Estos eventos moleculares se han estudiado a mayor profundidad gracias a las ciencias ómicas. La integración de datos de diferentes ómicas ha sido una herramienta muy utilizada para la identificación de vías metabólicas claves alteradas durante la exposición a diversos tipos de estrés abiótico. Sin embargo, los mecanismos de respuesta a estrés por baja temperatura en *C. annuum* aún son escasos además de que se centran principalmente en hojas y frutos, dejando por un lado los tallos. Por lo anteriormente mencionado es necesario realizar mayores estudios a nivel transcripcional, proteómico y metabolómico en tallos de *C. annuum* que nos permita enriquecer la información que se tiene en las bases de datos de pimiento en respuesta a estrés abiótico y a su vez nos permita identificar las vías metabólicas centrales en respuesta a estrés por baja temperatura.

1.2 Antecedentes

El cultivo de pimiento morrón (*C. annuum*) juega un papel muy importante en el sector agrícola de México, para el año 2023 ocupo el segundo lugar a nivel nacional, resultando atractivo para la producción en condiciones de invernadero debido a la gran demanda que se tiene en los mercados internacionales siendo una gran alternativa para producirlo todo el año y obtener mejores precios en el mercado (Castillo *et al.*, 2017). En el año 2023, la producción de pimiento morrón a nivel mundial fue de 38 millones de toneladas. En cuento la producción mundial, China se posiciona en

primer lugar con una producción de 16 millones de toneladas, mientras que México se posicionó en segundo lugar con una producción de 3 millones de toneladas (FAOSTAT 2023).

C. annuum es una planta herbácea perenne que pertenece a la familia *Solanaceae*, junto con la berenjena, la papa y el tomate, y es una de las hortalizas con mayor producción a nivel global. Así mismo *C. annuum* es considerado un buen objeto de estudio en la investigación científica debido a su valor nutricional, sus aplicaciones en la biotecnología, su utilidad como modelo para estudiar la respuesta a estreses abióticos, la genética de plantas, y su capacidad para proporcionar una comprensión profunda de los procesos biológicos a través de enfoques ómicos, además de que en la actualidad se encuentran en las bases de datos tanto su genoma, transcriptoma y proteoma así mismo se han identificado un gran número de metabolitos importantes en diversas vías metabólicas (Kong *et al.,* 2019; Morales-Merida *et al.,* 2021; Zhang *et al.,* 2022b)

1.2.1. Respuesta a Estrés Abiótico en Plantas

Los factores abióticos son componentes fundamentales del medio ambiente que determinan la distribución y productividad de las plantas. En la naturaleza, las plantas se enfrentan constantemente a condiciones ambientales abióticas adversas como la sequía, el calor, el frío, las deficiencias de nutrientes y el exceso de sal o niveles de metales tóxicos en el suelo (Zhu 2016). Estos estreses abióticos limitan la utilización mundial de las tierras cultivables y afectan negativamente a la productividad de los cultivos. Por lo tanto, comprender cómo las plantas perciben las señales de estrés y se adaptan a las condiciones ambientales adversas es fundamental para la seguridad alimentaria mundial (Mittler 2006).

Para resistir el estrés ambiental, las plantas han desarrollado vías regulatorias interconectadas que les permiten responder y adaptarse a sus entornos de manera oportuna. Las condiciones de estrés abiótico afectan muchos aspectos de la fisiología de las plantas y causan cambios generalizados en los procesos celulares. Algunos de los cambios son respuestas no adaptativas que simplemente reflejan el daño infligido por un factor estresante, como los cambios perjudiciales en la fluidez de la membrana y la estructura de la proteína causados por el estrés por calor o frío, y las interrupciones en la cinética de las enzimas y las interacciones moleculares causadas por iones

tóxicos. Sin embargo, muchos de los cambios son respuestas adaptativas que conducen a una mayor resistencia al estrés y, por lo tanto, son objetivos potenciales para la mejora de los cultivos. Los procesos implicados en la respuesta adaptativa incluyen la reparación del daño inducido por el estrés, el reequilibrio de la homeostasis celular y el ajuste del crecimiento a niveles adecuados para la condición de estrés particular (Bailey-Serres *et al.,* 2019; Zhang *et al.,* 2020)

Numerosos estudios genéticos, bioquímicos y moleculares han identificado muchos de los factores que regulan las respuestas al estrés abiótico, y ahora está claro que el estrés abiótico provoca respuestas multinivel, que involucran la detección de estrés, la transducción de señales, la transcripción, el procesamiento de transcripciones, la traducción y las modificaciones de proteínas postraduccionales (Koyro *et al.,* 2012; Zhu 2016).

1.2.2. Respuesta a Estrés por Baja Temperatura en Plantas

El estrés por bajas temperaturas afecta el crecimiento y desarrollo de las plantas, el rendimiento, la calidad y la distribución geográfica (Cao *et al.,* 2022; Zhang *et al.,* 2022a). Las bajas temperaturas de las plantas se pueden clasificar en daños por frío (CI) que va de 0 ° a 15 ° C y por heladas (FI), por debajo de 0 °C. Diverdas investigaciones han demostrado que temperaturas que inducen daño por frío, provocan el estrés por frío de la planta, caracterizado por un menor crecimiento de la planta, deshidratación, marchitamiento de las hojas, baja acumulación de materia seca de órganos vegetativos como rizomas y hojas, y disminución del rendimiento o la calidad (Zhang *et al.,* 2011). Desde el punto de vista bioquímico, el estrés por frío da lugar a una mala fluidez de la membrana celular, disociación de complejos proteicos, sobreabundancia de las especies ROS, reducción de la actividad de varias enzimas y reducción de la tasa fotosintética (Zhang *et al.,* 2016). Además, el estrés de CI afecta a la actividad de las helicasas de ARN, reduciendo la estabilidad de la estructura secundaria del ARN en consecuencia, bajas tasas de transcripción y traducción (Owttrim 2013). Por otro lado, las temperaturas de daño por heladas (FI) causan daños severos a las plantas como: congelación celular, cristalización, daño mecánico directo, deshidratación celular extrema, cambios en la osmolalidad y apoptosis (Song *et al.,* 2018). A pesar del efecto del estrés por frío, las plantas adquieren naturalmente la aclimatación al frío a través de la variación genética y la

selección natural. Los estudios han revelado que las células vegetales se aclimatan al estrés por frío ajustando la membrana plasmática, la sustancia protectora osmótica intracelular, el sistema redox y la tasa de fotosíntesis. Además, la mejora de las redes de regulación molecular, como la expresión génica y las modificaciones, que mantienen el equilibrio metabólico entre la síntesis de materia vegetal y el consumo de energía (Ding *et al.*, 2019). Estos eventos moleculares se han estudiado con mayor rapidez en las diferentes partes anatómicas de las plantas gracias a los avances tecnológicos en las últimas décadas facilitado el estudio a gran escala de muchos genes, proteínas y metabolitos, estas herramientas son conocidas como ciencias ómicas , (Morrison *et al.*, 2006). Cabe mencionar que la integración de datos de diferentes ómicas ha sido una herramienta muy utilizada para la identificación de vías metabólicas claves alteradas durante la exposición a diversos tipos de estrés abiótico (Jamil *et al.*, 2020; Chao *et al.*, 2023).

1.2.3. Ciencias Ómicas

1.2.3.1. Genómica. La genómica se refiere al estudio del conjunto completo de ADN de un organismo, incluidos todos sus genes, es decir, el "genoma". Con el advenimiento de la tecnología de secuenciación de última generación, la adquisición de datos a escala genómica nunca ha sido tan fácil, expandiendo nuestra capacidad para analizar y comprender genomas completos y disminuyendo la brecha existente entre genotipo y fenotipo. El estudio del genoma ayuda a los investigadores a entender la interacción de los genes entre sí y con el entorno. La genética y la genómica suenan igual, pero tienen distinciones específicas. La genética es el estudio de la herencia, o cómo las características de los organismos vivos se transmiten de una generación a la siguiente a través del ADN. Diversos estudios que se centran en un número específico y limitado de genes, o parte de genes con función conocida, para comprender cómo estos influyen en particular rasgos de interés. En la actualidad, la tecnología de alto rendimiento y los avances en biología computacional han cambiado este paradigma permitiendo el estudio de organismos en términos de la estructura del genoma, abordando cuestiones biológicas a una escala de todo el genoma. (Vailati-Riboni et al., 2017).

1.2.3.2. Transcriptómica. El transcriptoma es el ARN total (es decir, ARNm, ARN no codificante, ARNr, ARNt, entre otros) expresado por una célula o tejido, lo que representa una instantánea del metabolismo celular. La era del transcriptoma comenzó cuando Schena et al. (1995) desarrolló la tecnología "micro array" utilizando el sintetizador de ADN de chorro de tinta, lo que permite el análisis de un conjunto predeterminado (de cientos a miles) de ARNm celular a gran escala. Sin embargo, la reciente introducción de la tecnología de secuenciación de ADN de próxima generación (NGS) de alto rendimiento ha revolucionado la transcriptómica al permitir el análisis de ARN a través de la secuenciación de ADNc a escala masiva (RNAseq) (Voelkerding et al. 2009). Esta tecnología eliminó varios desafíos planteados por tecnologías como microarreglos, incluido el rango dinámico limitado de detección, al tiempo que proporciona mayor conocimiento de los aspectos cualitativos, y no solo cuantitativos, de la transcripción: (1) sitios de inicio de la transcripción, (2) transcripciones con sentido y (3) antisentido eventos de corte y empalme nativos alterados y (4) fusión de genes. Como también proporciona información detallada sobre la porción de ARN no codificante del ARN total, RNAseq ha permitido la comprensión de mecanismos reguladores complejos (por ejemplo, la epigenética) (Vailati-Riboni et al., 2017).

1.2.3.3. Proteotómica. El término "proteoma" se definió como la caracterización y cuantificación de todos conjuntos de proteínas en una célula, órgano u organismo en un momento específico y fue acuñado por Wasinger y col. (1995). Por tanto, un análisis proteómico nos proporciona la cantidad de proteínas presentes de una célula o tejido en un momento determinado, lo que facilita el descubrimiento de nuevos biomarcadores, identificación y localización de modificaciones postraduccionales y estudio de interacciones proteína-proteína (Chandramouli y Qian 2009). Las técnicas poderosas tienen se ha establecido para identificar y cuantificar diferencialmente especies de proteínas de complejos muestras biológicas y proteómica está siendo adoptada por investigadores ganaderos (Lippolis y Reinhardt 2008; Sauerwein et al., 2014). El núcleo de la proteómica moderna es la espectrometría de masas (MS) (Aebersold y Mann 2003), una técnica en la que todos los compuestos químicos de una muestra se ionizan y las moléculas cargadas (iones) resultantes se analizan de acuerdo con sus relaciones de masa a carga (m / z). Para una separación previa simple de mezclas de proteínas complejas antes Análisis de MS electroforesis en gel de poliacrilamida unidimensional o bidimensional (1D-PAGE, 2D-PAGE) se utiliza a menudo. Pero

para mejorar aún más la automatización en el proceso y crear un flujo de análisis de canalización, se utilizan diferentes tipos de cromatografía líquida (LC o HPLC) para complementar o sustituir la separación basada en gel. técnicas. La identificación de las proteínas entre tratamientos o afecciones se realiza mediante comparación con una base de datos de proteínas "digeridas in silico", lo que significa que el crudo los datos se comparan directamente con datos generados teóricamente a partir de bases de datos de proteínas. La cuantificación confiable de la proteína identificada también es posible con varios Métodos de cuantificación basados en EM que incluyen etiquetado químico, metabólico, enzimático y sin etiquetado (May et al. 2011). Los avances proteómicos hicieron posible la cuantificación absoluta de proteínas a través del AQUA (cuantificación absoluta de proteínas), QConCat (proteínas artificiales compuestas de péptidos concatenados) y estándares de proteínas para enfoques de cuantificación absoluta (PSAQ) (Rivers et al., 2007; Brun et al., 2007).

1.2.3.4. Metabolómica. La metabolómica consiste en el perfil global de metabolitos en una muestra. Se puede realizar un análisis metabolómico en una variedad de factores biológicos. fluidos y tipos de tejidos y pueden utilizar varias plataformas tecnológicas diferentes. La metabolómica suele utilizar análisis de alta resolución junto con herramientas estadísticas como el análisis de componentes principales (PCA) y mínimos cuadrados parciales. (PLS) para obtener una imagen integrada del metabolóma (Zhang et al., 2012). Como una de las técnicas analíticas espectroscópicas más comunes, nuclear magnética La resonancia magnética (RMN) puede identificar de forma única y cuantificar simultáneamente una amplia gama de compuestos orgánicos en el rango micro-molar, proporcionando información imparcial sobre los perfiles de metabolitos. El amplio espectro de moléculas detectables por este enfoque incluye péptidos, aminoácidos, ácidos nucleicos, carbohidratos, orgánicos ácidos, vitaminas, polifenoles, alcaloides y especies inorgánicas. Aplicación de MS está ganando un mayor interés en la metabolómica de alto rendimiento, a menudo junto con otras técnicas como la cromatografía (GE-MS, LC-MS, UPLS-MS) o las técnicas electroforéticas (CE-MS). Debido a su alta sensibilidad y amplia gama de metabolitos cubiertos, la EM se ha convertido en la técnica de elección en muchos estudios de micrófonos metabólicos (Zhang et al., 2012).

1.2.4. Integración de Datos Ómicos

Para entender un fenómeno biológico, necesitamos así un enfoque holístico que integre todas las facetas e interacciones de un sistema biológico, así como recoja y analice estos datos complejos jerárquicos lo más a fondo posible. Por esta razón, las ciencias biológicas sólo han hecho progresos considerables desde la era de la omics y el big data. Las tecnologías de alto rendimiento y los datos de secuenciación de nueva generación (NGS) permiten modelar sistemas biológicos para comprender los complejos mecanismos moleculares subyacentes. Como corresponsales de los niveles de flujo de información en dogma central, los big data biológicos también son multinivel y a menudo se conocen como datos multiómicos (es decir, genómica, transcriptómica, epigenómica, proteómica, metabolómica). Al combinar estos "omics", el complejo big data biológico puede abordarse para revelar las relaciones entre entidades biológicas e identificar biomarcadores que caracterizan los sistemas biológicos (Santiago-Rodriguez and Hollister 2021; Scossa *et al.,* 2021). Los sistemas biológicos dependen de la transferencia de información desde los ácidos nucleicos hacia las proteínas y metabolitos para dar forma a la función y el fenotipo. Esto se conoce como la cascada ómicas, y los estudios impulsados por las omics están facilitando una nueva comprensión más holística de la biología de sistemas. Esto reconoce que la respuesta a diversos tipos de estrés resulta de procesos complejos y/o heterogéneos, y un enfoque combinado puede ser más poderosa que los las ómicas de manera individuales. Estudios recientes también sugieren que los valores atípicos en conjuntos de datos multi-ómicos pueden señalar moléculas clave en respuesta a diversos estreses, mismos que pueden ser de gran apoyo en el manejo agronómico de las plantas ante condiciones adversas favoreciendo una mejor interpretación biológica (Picard *et al.,* 2021; Scossa *et al.,* 2021).

1.2.4.1. Integración basada en la correlación. Se centra en encontrar vínculos correlativos entre elementos de un conjunto de datos y del otro, de diversas maneras: correlación de Pearson y Spearman, prueba gamma de Goodman, modelos lineales robustos y correlaciones parciales. La correlación a menudo falla debido a escalas diferentes de los datos. Los análisis basados en correlación son útiles para la integración de datos ómicos cuando hay una falta de conocimiento

bioquímico, por lo que son ampliamente utilizados para la integración de datos multiómicos. Estos enfoques pueden proporcionar una visión limitada en casos de sistemas altamente multicolineales, por lo que se utilizan los modelos gráficos gaussianos, la correlación parcial y las redes bayesianas, ya que tienen la capacidad de desacoplarse directamente de asociaciones de variables indirectas (Cavill et al., 2016).

1.2.4.2. Integración basada en concatenación. Se basa en concatenar las tablas de datos producidas por cada tecnología ómica en una única tabla de datos, para posteriormente realizar un análisis integrado. Al obtener los datos de tecnologías muy diferentes, se introducirá un sesgo que favorece el conjunto de datos más grande, con diferentes varianzas. Por todo ello, la obtención resultados entre la integración de dos ómicas, como puede ser la la metabolómica y la transcriptómica, no es sencilla (Spicker et al., 2008).

1.2.4.3. Integración multivariante. Son métodos más complejos. Trata de utilizar un conjunto de datos para predecir aspectos de otro conjunto de datos o para encontrar las asociaciones de "covarianza" entre los dos conjuntos de datos(Krumsiek et al., 2016) . Los conjuntos de datos se usan de forma no concatenada, manteniendo los conjuntos en bloques o dimensiones separadas dentro del modelo. Las dos técnicas multivariantes más comunes son PCA y PLS. Se utilizan para conjuntos de datos con altos niveles de colinealidad, como es el caso con los datos ómicos, donde muchos genes o metabolitos tendrán perfiles similares (Griffin et al., 2004).

1.2.4.4. Técnicas de integración basada en vías. *Son métodos que intentan integrar los datos utilizando el conocimiento biológico existente a través de vías metabólicas predefinidas en bases de datos, representando conexiones complejas entre diversos tipos de componentes (genes, proteínas, metabolito…). Asignan, de forma automatizada, los metabolitos y transcritos medidos, a redes y encuentran aquellos donde hay evidencia estadística de un cambio significativo en su comportamiento entre dos condiciones, o una correlación entre el comportamiento de la vía y un punto de interés fenotípico. Tienen la limitación de que, en el caso de conocimiento insuficiente de*

los genes, proteínas y/o interacciones de metabolitos, a menudo se extienden a través de relaciones empíricas o correlaciones entre medidas. En definitiva, cada conjunto de datos ómicos se analiza por separado y se construye un fundamento biológico coherente que explique los fenómenos observados en los perfiles moleculares individuales(Cavill et al., 2011).

1.2.5. Estudios de Integración de Datos Ómicos Basados en Respuesta a Estrés por Baja Temperatura

Lou y col. (2018) realizaron un estudio proteómico y transcriptómico en hojas de níspero expuestas a un estrés por baja temperatura (4° C) Los resultados mostraron 1210 genes expresados diferencialmente (DEG), así mismo se identificaron 300 proteínas diferenciales (DEP). posteriormente los autores análisis de RT-PCR cuantitativa en tiempo real con el fin de validar los resultados obtenidos mostrando que la expresión de genes de D-sorbitol-6-fosfato deshidrogenasa dependiente de NADP, antocianina sintasa y fenilalanina amonio-liasa eran consistentes con el patrón del perfil del transcriptoma, lo que sugirió que estos tres los genes pueden desempeñar un papel vital en la tolerancia al frío del níspero.

Zhao y col. (2019) al realizar un análisis transcriptómico y metabolómico en un cultivar de trigo comercial resistente al invierno (cv. Jing 411). El análisis de transcriptoma reveló que 29.066 genes se expresaron diferencialmente después de la aclimatación en frío en comparación con el control, de los cuales, 745 genes fueron regulados al alza tras el tratamiento de congelación. El Análisis metabolómico detecto 223 metabolitos enriquecidos principalmente durante la aclimatación en frío incluyeron carbohidratos, flavonoides y aminoácidos de los cueles Ocho metabolitos comunes habían alterado la abundancia después del tratamiento de congelación; seis aumentaron y dos disminuyeron. El análisis integrado de estas dos ómicas reveló que las vías de señalización de la fitohormona y la biosíntesis de ácido abscísico (ABA)/ácido jasmónico (JA) se modularon significativamente bajo tratamientos de aclimatación y congelación en frío.

Xu y col. (2020) realizaron un análisis transcriptómico y metabolómico con el fin de identificar los genes y metabolitos expresados específicamente durante la aclimatación por frio (CA) en plantas de tabaco. El análisis transcriptómico reveló 6905 genes expresados diferencialmente (DEG)

durante la CA. Los análisis de anotación funcional y enriquecimiento revelaron que los DEG estaban involucrados principalmente en la transducción de señales, el metabolismo de carbohidratos y la biosíntesis de fenilpropanoides. De igual forma lograron identificar 35 metabolitos modificados significativamente durante la CA mediante una plataforma LC-MS. Metabolitos protectores, como aminoácidos, carbohidratos, intermedios del ciclo del ácido tricarboxílico (TCA) y sustancias relacionadas con fenilpropanoides, fueron los metabolitos encontrados durante la CA.

1.2.6. Estudios de Integración de Datos Ómicos Basados en Respuesta a Estrés por Baja Temperatura en *C. annuum*

En la actualidad son pocos los estudios de integración de datos omicos en respuesta a estrés por baja temperatura en *C. annuum* sin embargo se han identificado cambios a nivel morfológicos, bioquímicos y moleculares en respuesta a estrés por baja temperatura como el estudio realizado por León-Chan y col. (2017) quienes al evaluar hojas de pimiento, en respuesta a baja temperatura (LT), radiación UV-B y la combinación (LT + UV-B), se demostró que en LT presento una reducción del 19% de la clorofila y una mayor acumulación del flavonoide apigenina-7-O-glucósido (A-7-G).

En la actualidad se han utilizado enfoques transcriptómico en las diversas partes anatómicas (tallos, hojas, frutos) de pimiento morrón expuestas a baja temperatura como lo es el estudio realizado por Chan y col. (2020) quienes analizaron la expresión diferencial de varios genes involucrados en la biosíntesis de antocianinas (MYB, F3H, F3’5’H, DFR y ANS) mediante q-PCR en tallos de *C. annuum* después de la exposición a baja temperatura, radiación UV-B y estrés combinado (LT + UV-B). Este estudio mostró que la mayoría de los genes biosintéticos de antocianinas fueron más influenciados por la exposición a baja temperatura que por la radiación UV-B. Por otro lado, Kong et al. 2020, al evaluar frutas de *C. annuum* bajo diferentes condiciones de almacenamiento (4 y 10 °C) durante 0, 5, 10, 15 y 18 días mediante RNA-Seq, identificaron un total de 3863 genes diferencialmente expresados (DEGs), encontrando 1669 DEGs sobreexpresados y 2194 subexpresados. En general, estos genes estaban involucrados en procesos de osmorregulación,

deshidratación, estabilidad de membranas y transducción de señales hormonales vegetales.

Ji y col. (2023) evaluaron hojas de cultivares de *Capsicum annuum* resistentes y susceptibles al frío a temperaturas inferiores a 4 °C expuestas durante 0, 8, 16 y 24 horas. El análisis de RNA-Seq permitió la identificación de 2937 DEGs, que estaban principalmente involucrados en la biosíntesis de flavonoides y el metabolismo de fenilalanina. Para explorar más a fondo este cultivo, Morales-Mérida y col. (2021) realizaron un análisis transcriptómico por RNA-Seq en tallos de plántulas de pimiento morrón sometidas a estrés combinado (LT + UV-B) a 1, 3 y 25 horas de exposición, encontrando 281, 280 y 326 genes diferencialmente expresados a 1, 3 y 25 horas, respectivamente. Estos genes estaban relacionados con la actividad de hidrolasas, respuesta al estrés, respuesta a estímulos, proceso metabólico de carbohidratos y procesos biosintéticos, y también encontraron vías KEGG significativamente enriquecidas relacionadas con el ritmo circadiano de las plantas, biosíntesis de flavonoides y señalización MAPK.

De igual manera se han reportado el uso de enfoques ómicos integrativos en C. annuum en respuesta a estrés por baja temperatura. Zhanget al. (2022b) al integrar el transcriptoma y metabolóma en dos cultivares de pimiento, XS (sensible al frío) y GZ (resistente al frío) identificaron 10.931 genes expresados diferencialmente (DEG) y 657 metabolitos expresados diferencialmente (DEM) en el modo de iones positivos y 390 DEM en el modo de iones negativos en ambos cultivares. La integración de estos datos evidencio que la regulación de la vía ICE (inductor de la expresión de CBF)-CBF (factores de unión a repetición C)-COR (regulada por frío) por la señalización de Ca2+, la señalización de MAPK y la señalización de especies reactivas de oxígeno (ROS) desempeñaban un papel clave en la respuesta al estrés por baja temperatura en pimiento. Xu *et al.,* (2023) realizaron una integración de un perfil proteómico y metabolómico en fruto de pimiento morrón (*Capsicum annuum*) de un cultivar sensible al frío (cv. 129) y un cultivar tolerante al frío (cv. 130) en respuesta a estrés por baja temperatura, observando que los cambios en las proteínas y metabolitos de cv. 130 fueron menores comparación con cv. 129. Los autores proponen que la tolerancia al enfriamiento exhibida por cv. 130 se asocia con la regulación de la homeostasis lipídica de membrana por otros metabolitos, incluido el nivel de azúcares solubles, antioxidantes y polifenoles. Recientemente Morales-Merida *y col.* (2024) evaluó tallos de pimiento expuestos a estrés combinado de baja temperatura y radiación UV-B reportaron un aumento en la acumulación de proteínas relacionadas con la fotosíntesis, el transporte de electrones mitocondriales y la respuesta a un estímulo, también se identificaron flavonoides y sus glucósidos.

Los autores evidenciaron una afectación del metabolismo del carbono, las vías del tetrapirrol y la escopolamina.

El resultado de estas investigaciones ha revelado información significativa sobre los cambios bioquímicos, morfológicos y moleculares que se producen en las distintas partes anatómicas de *C. annuum*, los cuales podrían desempeñar un papel crucial en la respuesta de la planta al estrés por bajas temperaturas. Curiosamente los estudios multiomicos realizados en tallos de pimiento morrón son escasos a pesar de ser un órgano muy importante el cual cumple funciones de fotosíntesis, soporte, transporte y almacenamiento de nutrientes, además se interconecta con todos los órganos de las plantas (Rodriguez and De La Vega 2021). Por lo que es importante estudiar las bases moleculares de los mecanismos de respuesta a estrés por baja temperatura que, a su vez nos permita revelar los procesos metabólicos clave en ante este estrés, mediante la integración de datos ómicos con el fin de incrementar la información en las diferentes bases de datos que nos permitan desarrollar estrategias para un mejor manejo agronómico.

1.3. Hipotesis

- Los genes relacionados con familia de genes de metabolismo antioxidante, factores de transcripción y señalización de hormonas vegetales participan en respuesta estrés por baja temperatura en *C. annuum.*
- Las proteínas implicadas en fotosíntesis, metabolismo de carbohidratos, HSP y biosíntesis de compuestos fenólicos participan en respuesta al estrés por baja temperatura en *C. annuum*.
- Los metabolitos como compuestos fenólicos, carbohidratos aminoácidos y hormonas vegetales son los que participan en respuesta al estrés por baja temperatura en *C. annuum*.
- Las principales vías metabólicas implicadas en respuesta a estrés por baja temperatura en *C. annuum* serán aquellas relacionadas a biosíntesis de fenilpropanoides, metabolismo de aminoácidos y carbohidratos.

1.4. Objetivo General

Estudiar las bases moleculares de los mecanismos de respuesta a estrés por baja temperatura en tallos de pimiento morrón (*Capsicum annuum*)

1.5. Objetivos Específicos

- Identificar los genes de expresión diferencial que participan en el mecanismo de respuesta a estrés por baja temperatura de tallos de *Capsicum annuum*, mediante análisis transcriptómicos.
- Identificar las proteínas de expresión diferencial de *Capsicum annuum* que participan en el mecanismo de respuesta a estrés por baja temperatura, mediante análisis proteómicos.
- Identificar los metabolitos acumulados diferencialmente de *Capsicum annuum* que participan en el mecanismo de respuesta a estrés por baja temperatura, mediante análisis metabolómico.
- Identificar las vías metabólicas clave de *Capsicum annuum* en respuesta a estrés por baja temperatura mediante la integración de datos proteómicos y metabolómicos.

1.6 Sección Integradora del Trabajo

Este trabajo de investigación se dividió en artículos, los cuales se abordaron de la siguiente manera:
Artículo 1. Forma parte del objetivo 1 Para entender mecanismo de respuesta a este estrés abiótico, realizamos un análisis transcriptómico RNA-Seq para obtener el perfil transcriptómico de Capsicum annuum expuesto a estrés por baja temperatura, donde las bibliotecas se construyeron a partir de lecturas de muestras de control y de estrés por baja temperatura, variando en promedio por tratamiento de 22.952.190,5-27.305.327 lecturas emparejadas con un tamaño de 30 a 150 pb. El número de genes expresados diferencialmente (DEGs) para cada tratamiento fue de 388, 417 y 664 en T-17 h, T-22 h y T-41 h, respectivamente, identificando 58 genes regulados al alza y 169

genes regulados a la baja compartidos entre los tres tiempos de exposición. Asimismo, se identificaron 23 DEGs que codifican TFs en T-17 h, 30 DEGs en T-22 h y 47 DEGs en T-42 h, respectivamente. El análisis GO reveló que los DEG estaban implicados en la actividad catalítica, la respuesta al estímulo de la temperatura, la actividad oxidorreductasa, la respuesta al estrés, el transporte de iones fosfato y la respuesta al ácido abscísico. El análisis de vías KEGG identificó que los DEG estaban relacionados con la biosíntesis de flavonoides, la biosíntesis de alcaloides y las vías del ritmo circadiano de las plantas en el caso de los genes regulados al alza, mientras que, en el caso de los genes regulados a la baja, pertenecían a las vías de señalización MAPK y de transducción de señales de hormonas vegetales, presentes en los tres puntos temporales de exposición a bajas temperaturas. La validación del método transcriptómico se realizó mediante la evaluación de cinco DEGs por reacción en cadena de la polimerasa cuantitativa (q-PCR).

Los datos obtenidos en el presente estudio proporcionan nuevos conocimientos sobre los perfiles del transcriptoma del tallo de *C. annuum* en respuesta al estrés por bajas temperaturas. Los datos generados pueden ser útiles para la identificación de genes candidatos clave y mecanismos moleculares implicados en la respuesta a este tipo de estrés.

Artículo 2. Forma parte de los objetivos 2, 3 y 4 tomando en cuenta que el tiempo 41h de oposición a bajas temperaturas mostro una mayor expresión de genes, es por ello que se optó por analizar el proteoma y metabolóma de ese tiempo para posteriormente realizar la integración de dato ómicos con el encontrar las alteraciones en las vías metabólicas de tallos de pimiento morrón causados por la baja temperatura mediante la integración de datos ómicos. Los perfiles proteómicos y metabolómicos se obtuvieron mediante cromatografía líquida acoplada a espectrometría de masas, y la integración de datos se realizó en la base de datos de vías metabólicas de la planta. Identificamos un total de 159 proteínas de expresión diferencial (PED), de las cuales 69 eran regulada a la baja y 83 al alza. En cuanto a los metabolitos se identificaron un total de 60 metabolitos mediante metabolómica dirigida y 16 por metabolómica no dirigida, de los cuales 62 en total presentaron una acumulación diferencial al alza y lo demás no presentaron cambios significativos.

El estrés por baja temperatura indujo una acumulación de proteínas relacionadas con la respuesta al ion de cadmio, el proceso biosintético del piridoxal fosfato, el desarrollo de filomas, el transporte de endosomas tardíos a vacuolas y el ensamblaje del signalosoma COP9. De igual manera, se identificó una alteración en las vías metabólicas de la biosíntesis de flavonoides, la biosíntesis de

fenilpropanoides, la biosíntesis de arginina, la oxidación del piruvato y el ciclo del ácido cítrico (TCA)

Los datos generados en esta investigación proporcionan nuevos conocimientos sobre los perfiles del proteoma y metabolóma del tallo de *C. annuum* en respuesta al estrés por bajas temperaturas. Siendo las proteínas y metabolitos identificadas de gran apoyo en las vías metabólicas relacionadas en osmoprotección, respuesta antioxidante y respiración celular.

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2. TRANSCRIPTOMIC ANALYSIS OF BELL PEPPER (*Capsicum annuum l.)* REVEALING KEY MECHANISMS IN RESPONSE TO LOW TEMPERATURE STRESS

Jesús Christian Grimaldi-Olivas[1], Brandon Estefano Morales-Merida[1], Claudia Villicaña[2], J Basilio Heredia[1], Luis Alberto Lighbourn-Rojas[5], Rubén León-Chan[5], Abraham Cruz-Mendívil[4], Melina López-Meyer[3] and Josefina León-Félix [1]*

[1]Centro de Investigación en Alimentación y Desarrollo (CIAD). Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110, Culiacán, Sinaloa, México

[2]CONAHCYT-Centro de Investigación en Alimentación y Desarrollo (CIAD), Carretera a Eldorado Km 5.5, Campo el Diez, Culiacán, Sinaloa C. P. 80110, México

[3]Instituto Politécnico Nacional (IPN), Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes # 250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[4]CONAHCYT-IPN, CIIDIR Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[5]Laboratorio de Genética, Instituto de Investigación Lightbourn, A. C., Cd. Jiménez, Chihuahua, 33981, México

* For correspondence: ljosefina@ciad.mx

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ORIGINAL ARTICLE

Transcriptomic analysis of bell pepper (*Capsicum annuum* L.) revealing key mechanisms in response to low temperature stress

Jesús Christian Grimaldi-Olivas[1] · Brandon Estefano Morales-Merida[1] · Abraham Cruz-Mendívil[2] · Claudia Villicaña[3] · J. Basilio Heredia[1] · Melina López-Meyer[4] · Rubén León-Chan[5] · Luis Alberto Lightbourn-Rojas[5] · Josefina León-Félix[1]

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Abstract

Background Bell pepper (*Capsicum annuum* L.) is one of the most economically and nutritionally important vegetables worldwide. However, its production can be affected by various abiotic stresses, such as low temperature. This causes various biochemical, morphological and molecular changes affecting membrane lipid composition, photosynthetic pigments, accumulation of free sugars and proline, secondary metabolism, as well as a change in gene expression. However, the mechanism of molecular response to this type of stress has not yet been elucidated.

Methods and results To further investigate the response mechanism to this abiotic stress, we performed an RNA-Seq transcriptomic analysis to obtain the transcriptomic profile of *Capsicum annuum* exposed to low temperature stress, where libraries were constructed from reads of control and low temperature stress samples, varying on average per treatment from 22,952,190.5–27,305,327 paired reads ranging in size from 30 to 150 bp. The number of differentially expressed genes (DEGs) for each treatment was 388, 417 and 664 at T-17 h, T-22 h and T-41 h, respectively, identifying 58 up-regulated genes and 169 down-regulated genes shared among the three exposure times. Likewise, 23 DEGs encoding TFs were identified at T-17 h, 30 DEGs at T-22 h and 47 DEGs at T-42 h, respectively. GO analysis revealed that DEGs were involved in catalytic activity, response to temperature stimulus, oxidoreductase activity, stress response, phosphate ion transport and response to abscisic acid. KEGG pathway analysis identified that DEGs were related to flavonoid biosynthesis, alkaloid biosynthesis and plant circadian rhythm pathways in the case of up-regulated genes, while in the case of down-regulated genes, they pertained to MAPK signaling and plant hormone signal transduction pathways, present at all the three time points of low temperature exposure. Validation of the transcriptomic method was performed by evaluation of five DEGs by quantitative polymerase chain reaction (q-PCR).

Conclusions The data obtained in the present study provide new insights into the transcriptome profiles of *Capsicum annuum* stem in response to low temperature stress. The data generated may be useful for the identification of key candidate genes and molecular mechanisms involved in response to this type of stress.

Keywords Abiotic stress · Low temperature · *Capsicum annuum* · Transcriptomic analysis

✉ Josefina León-Félix
ljosefina@ciad.mx

[1] Laboratorio de Biología Molecular y Genómica Funcional, Centro de Investigación en Alimentación y Desarrollo (CIAD) A.C., Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110 Culiacán, Sinaloa, Mexico

[2] Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), CONAHCYT-Instituto Politécnico Nacional (IPN), Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049 Guasave, Sinaloa, Mexico

[3] Laboratorio de Biología Molecular y Genómica Funcional, CONAHCYT-Centro de Investigación en Alimentación y Desarrollo (CIAD) A.C., Carretera Culiacán-Eldorado Km 5.5, Campo el Diez, C.P. 80110 Culiacán, Sinaloa, Mexico

[4] Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), Instituto Politécnico Nacional (IPN), Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049 Guasave, Sinaloa, Mexico

[5] Laboratorio de Genética, Instituto de Investigación Lightbourn, A. C., C.P. 33981 Ciudad Jiménez, Chihuahua, Mexico

Abbreviations

DEGs	Differential expression genes
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
LT	Low temperature

Introduction

Plants are exposed daily to different types of stress, such as low temperatures, so they develop various strategies to maximize their tolerance to cold and survive, since this stress can affect growth and development. This is why plants opt for various strategies to maximize their tolerance to cold and survive, this process is known as cold acclimation, where various signal transduction mechanisms act to counteract the adverse effects caused by stress [1, 2]. This acclimation triggers physiological changes affecting membrane lipid composition, photosynthetic pigments, accumulation of free sugars and proline, changes in antioxidant levels, as well as extensive rearrangement of gene expression [3–6]. Bell pepper (*Capsicum annuum* L.) is a perennial herbaceous species belonging to the family, such as eggplant, potato and tomato chilli, being one of the most widely produced vegetables in the world. However, bell pepper production can be affected by low temperatures, as its optimum growth temperature ranges from 21 to 27 °C [7–10]. Several investigations confirm that exposure to low temperatures (0–15 °C) induces numerous morphological, biochemical and molecular changes in *C. annuum* [11]. who analyzed the content of chlorophyll, carotenoids and phenolic compounds in response to low temperatures (LT), UV-B radiation and the combination of both (LT + UV-B) in leaves of *C. annuum*, where the LT condition presented a 19% reduction of chlorophyll in bell pepper leaves and a higher accumulation of the flavonoid apigenin-7-O-glucoside (A-7-G), and it was shown that during LT its total reducing capacity (TRC) increased. Previous studies make reference to the fundamental role of the enzymes of the endogenous antioxidant system in the adaptation to low temperatures in sweet peppers, such as ascorbate, glutathione and dehydrogenases, favoring the homeostasis of reactive oxygen species caused by such stress [12].

Technological advances in recent decades have facilitated the large-scale study of many genes, proteins, metabolites; these tools are known as omics [13]. In addition, the study of gene expression by q-PCR and the development of new high-throughput sequencing (NGS) technologies have revolutionized the study of mRNA (transcriptomics) through large-scale cDNA sequencing (RNA-Seq) that allows the identification of differentially expressed genes between different tissues or treatments, with RNA-Seq being a powerful tool to better understand gene expression profiles [14].

Among gene expression studies in *C. annuum*, León et al. [15] analyzed the differential expression of several genes involved in anthocyanin biosynthesis (MYB, F3H, F3'5'H, F3'5'H, F3'5'H, DFR and ANS) by q-PCR in *C. annuum* stem after exposure to low temperature, UV-B radiation and combined stress (LT + UV-B). This study showed that most of the anthocyanin biosynthetic genes were mostly influenced by low temperature exposure than UV-B radiation. On the other hand, Kong et al. [16] by evaluating *C. annuum* fruits under different storage conditions (4 and 10 °C) for 0, 5, 10, 10, 10, 15 and 18 days by RNA-Seq, identified a total of 3863 differentially expressed genes (DEGs), and found 1669 up-regulated DEGs and 2194 down-regulated, in general these genes were involved in osmoregulation processes, dehydration, membrane stability and plant hormone signal transduction. Ji et al. [17] evaluated leaves of cold-resistant and cold-susceptible *Capsicum annuum* cultivars at temperature below 4 °C exposed to 0, 8, 16 and 24 h, RNA-Seq analysis allowed the identification of 2937 DEGs, which were mainly involved in flavonoid biosynthesis and phenylalanine metabolism. To further explore this culture, Morales-Mérida et al. [18] performed an RNA-Seq transcriptomic analysis of bell pepper seedling stem subjected to combined stress (LT + UV-B) at 1, 3 and 25 h of exposure, and found 281, 280 and 326 genes differentially expressed at 1, 3 and 25 h, respectively. These genes were related to hydrolase activity, stress response, stimulus response, carbohydrate metabolic process, and biosynthetic process, and they also found significantly enriched KEGG pathways related to plant circadian rhythm, flavonoid biosynthesis, and MAPK signaling.

Zhang et al. [19] al integrated the transcriptome and metabolome of leaves of a resistant bell pepper cultivar and a cold-susceptible cultivar, where they identified a total of 10,931 differentially expressed genes, 657 (positive ion mode) and 390 (negative ion mode) differentially expressed metabolites (DEGs). Where DEGs were mainly involved in amino acid biosynthesis, plant hormone signal transduction and mitogen-activated protein kinase (MAPK) signaling pathway and DEMs were mainly free polyamines (PA), plant hormones and osmolytes. They also conclude that the main pathways in response to low temperature stress in bell pepper are mediated by the regulation of the ICE-CBF-COR (cold-regulated) pathway, Ca2 + signaling, MAPK signaling and reactive oxygen species (ROS) signaling.

The result of these investigations has provided relevant information on the various biochemical, morphological and molecular changes that occur in the different anatomical parts of *C. annuum* and that could be playing a fundamental role in the response to low temperature stress. The stem is one of the most important organs of the plant, fulfilling diverse functions such as photosynthesis, support, besides being connected to all plant organs [20]. However, at present, only information on the transcriptome of *C. annuum*

stems in response to combined stress (low temperature with UV-B radiation) is available, so further research is needed on *C. annuum* stems exposed to low temperature stress where the gene expression profile during this stress is detailed at the transcriptomic level to better understand the changes attributed during low temperature exposure. Therefore, the objective of this research was to analyze the transcriptomic profile of *C. annuum* stems by RNA-Seq at early, intermediate and late stages of exposure to low temperature stress (17, 22 and 41 h), which will allow us to generate new knowledge of key genes or molecular events in response to this type of stress that in turn will generate useful data for breeding programs allowing better agronomic management of cultivars tolerant to low temperatures.

Materials and methods

Collection to vegetative material

Seeds of commercial bell pepper variety Canon cv. (Zeraim Gedera Syngenta; Israel) were germinated following previously used methodology [11]. Briefly, 28 days after sowing (DDS), seedlings were placed in acclimatization chambers (GC-300TLH, JEIO TECH; South Korea) and handled under normal growth conditions, consisting of a 12 h photoperiod (6:00 to 18:00 h) of PAR radiation (972 µmol m^{-2} s^{-1}), with temperature of 25/20 °C (day/night) and relative humidity of 65% for three days. To carry out the low temperature treatment, the temperature was adjusted to 15/10 °C at 18:00 h on day 30 (Fig. 1). For sampling, stems were collected from 10 bell pepper plants for each treatment as shown in Fig. 1, as a control, seedling stems were taken at 10 h on day 31, which were maintained at a temperature of 25/20 °C (day/night) (control), For the treatments, stems were collected at 11 h on day 31 (17 h of exposure) (T-17 h), at 16 h on day 31 (22 h of exposure) (T-22 h) and at 11 h on day 32 (22 h of exposure) (T-41 h). Two biological replicates were taken with two replicates for each treatment. Tissue was frozen with liquid nitrogen and stored at − 80 °C for further analysis.

Total RNA extraction and library preparation

RNA extraction was carried out from stems of treated and control plants using Trizol reagent methodology (Ambion, Life Technologies, USA). A set of 10 stems was exposed to with liquid nitrogen and 50 to 100 mg to tissue was taken for total RNA isolation; For RNA precipitation, 0.5 ml isopropyl alcohol was replaced with a mixture of 0.30 ml isopropyl alcohol and 0.30 ml saline (0.8 M sodium citrate and 1.2 M NaCl); finally, RNA was washed once with 100% ethyl alcohol and twice with 75% ethyl alcohol (used DEPC water). RNA was then treated with the Turbo DNA free kit (Invitrogen, Life Technologies, USA) to remove genomic DNA, concentration was determined with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was analyzed by agarose gel electrophoresis.

From RNA of acceptable integrity and purity (A260/A280: ≥ 1.8; RIN ≥ 8) cDNA libraries of 30–150 bp and paired ends were generated on Illumina TruSeq library system. The concentration of two libraries was determined by fluorometry on Qubit (Life Technologies). Subsequently,

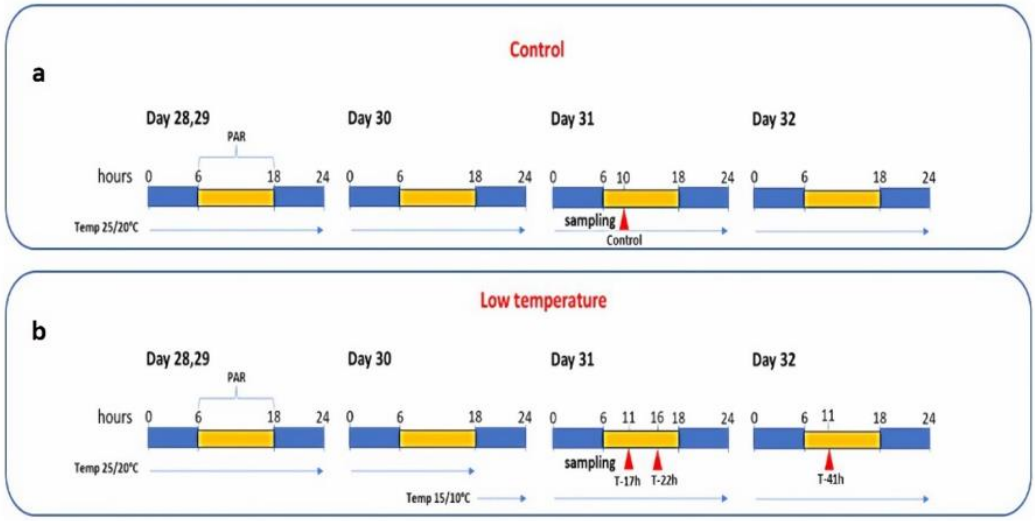

Fig. 1 Diagram describing the application of low temperature to bell pepper plants from day 28 to 32, as well as the sampling times in control (**a**) and low temperature (**b**). Samples were taken at different intervals indicated by a red triangle

libraries were sequenced on the Illumina NextSeq-500 platform according to the sequencing service provider, Laboratorio Nacional de Genómica para la Biodiversidad (LANGEBIO) Unidad CINVESTAV-IPN; Irapuato, Guanajuato, Mexico.

RNA-Seq data processing and identification of differentially expressed genes

Bioinformatics analyses were performed on the Galaxy platform (https://usegalaxy.org/). The quality of raw reads was visualized using FastQC Read Quality reports (Galaxy Version 0.73 + galaxy0), followed by read processing with the Trimmomatic flexible read trimming tool Trimmomatic for Illumina NGS data (Galaxy Version 0.38.0) with the following parameters: read quality of 20 on the Phred scale (SLIDINGWINDOW:4:20) and minimum read length of 20 bp (MINLEN: 20). Subsequently, with the filtered reads, a pseudo-alignment with the reference transcriptome of the hot bell pepper cultivar CM334 (https://plants.ensembl.org/Capsicum_annuum/Info/Index) was performed and transcript abundances were obtained using the Kallisto-quant tool (Galaxy Version 0.46.2 + galaxy0) to quantify RNA-Seq transcript abundances. Differentially expressed genes (DEGs) were determined using DESeq2, where DEGs with ≥ 2 and ≤ 2 fold expression relative to the control and an FDR value ≤ 0.05 were considered. Volcano diagrams and cluster analyses were performed with the tools pheatmap, EnhancedVolcano and in R software (version 4.2.2), Venn diagrams were performed in the BioVenn platform (version 4.2.2). http://www.r-project.org/), Venn diagrams were performed in the BioVenn platform (http://www.biovenn.nl/index.php). Gene annotation, gene ontology and SDR enrichment analysis was performed on the PANTHER classification system platform (http://pantherdb.org/). GO terms were performed with FDR ≤ 0.05. We also performed statistical enrichment of DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG FDR ≤ 0.05) to determine the metabolic pathways in response to stress. The search for FTs was carried out on the iTAK (http://itak.feilab.net/cgi-bin/itak/index.cgi) and PlantTFDB (http://planttfdb.gao-lab.org/) platforms.

Transcriptome validation by real-time PCR (q-PCR)

The cDNA was synthesized from 2 µg of RNA using Superscript III kit (Invitrogen, Life Technologies, USA), quantified with the NanoDrop 2000c spectrophotometer and stored at − 20 °C till further use. Primer efficiency was calculated using two-fold dilution series from 400 to 6.25 ng cDNA. q-PCR was performed in a final volume of 10 µl of reaction mixture containing 1 µl of cDNA (100 ng/µl) plus 9 µl of master mix using a CFX96TM real-time PCR detection system (Bio-Rad, USA). The master mix consisted of 5 µl of SYBR SsoAdvanced Universal SYBR Green Supermix (BioRad, USA). Amplification conditions were performed under the following conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles with denaturation at 95 °C for 10 s, and hybridization temperature (Ta) for each primer set, as described in Supplementary Tables 1, for 30 s. Melting-amplification curves were performed to determine amplification specificity, and agarose gel was used to confirm amplicon size. Gene expression was calculated with the $2^{-\Delta\Delta Cq}$ method using β-TUB as normalizer gene and CFX Manager 3.0 software (BioRad Laboratories, Inc.). For each sample, two biological replicates (n = 2) with two technical replicates (n = 3) were performed. Finally, RNA-Seq data were correlated with q-PCR data using Minitab version 19 software.

Results

RNA sequencing and identification of differentially expressed genes (DEGs)

Libraries were constructed from control and low temperature stress sample reads, ranging on average per treatment from 22,952,190.5, 25,368,406.5, 21,691,733.5 and 27,305,327 for Ctrl, T-17 h, T-22 h, T-41 h samples respectively from paired reads whit size varying from 30 to 150 bp (S1 Table). After filtering the quality of the reads, more than 2 gigabases (GB) of clean reads were obtained with a Q20% higher than 79% (S1 table). Principal component analysis of the identified gene abundances showed a clustering pattern, serving as a quality control for subsequent analyses (Fig. 2). Data obtained from differential expression analysis identified 388 DEGs for T-17 h, where 131 of them were up-regulated and 257 down-regulated (Fig. 3a); for T-22 h, 417 DEGs were identified, of which 126 were up-regulated and 291

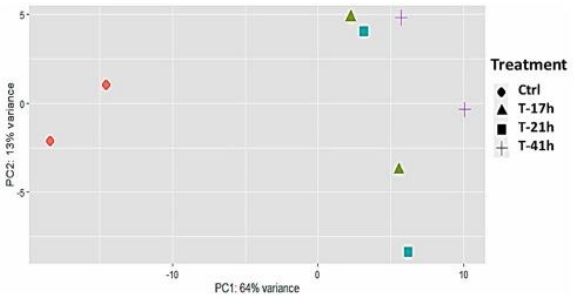

Fig. 2 Principal component analysis (PCA) of gene abundances of all treatments. The PCA plot represents the total abundances considering two biological replicates indicating differences in the transcriptomic profile of each treatment as shown by the corresponding colors and symbols

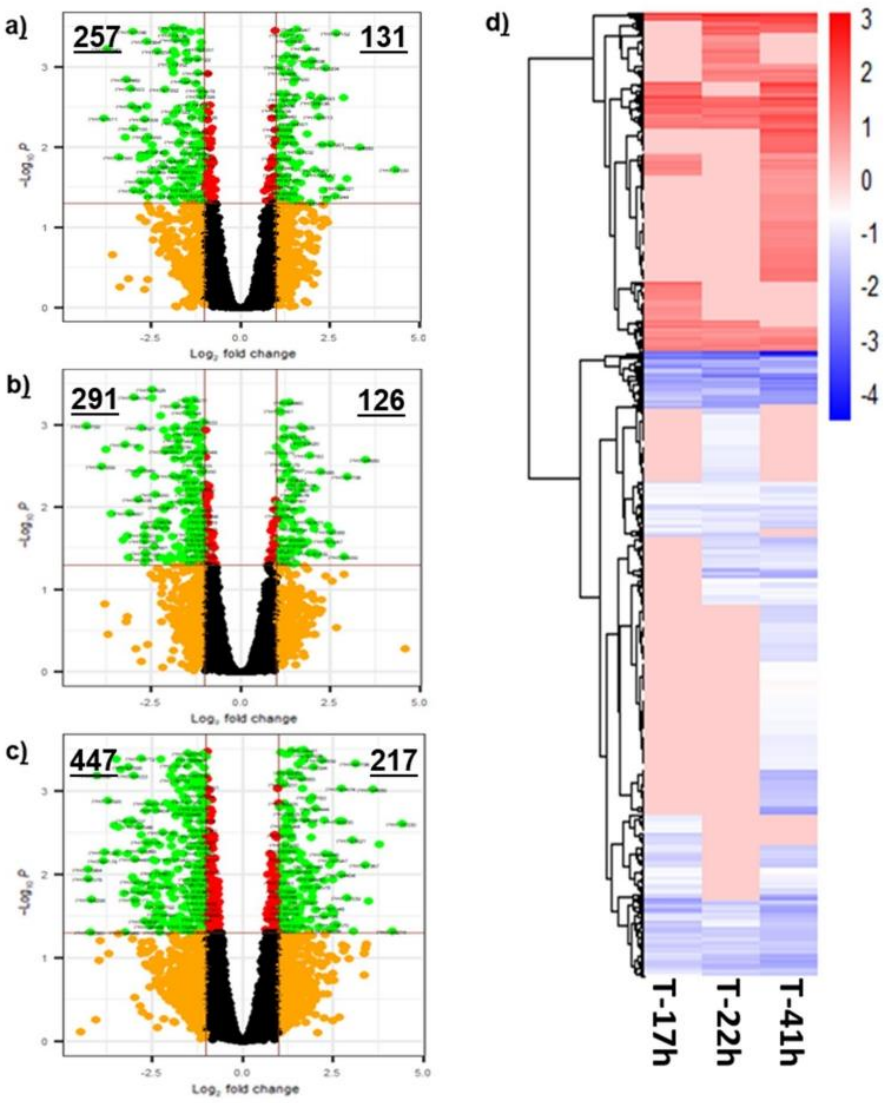

Fig. 3 Volcano plot of differentially expressed genes **a** T-17 h, **b** T-22 h and **c** T-41 h, (FDR values ≤0.05 and LFC (≥2 and ≤2) dots marked in green indicate DEGs, left section are down-regulated and right section are up-regulated genes. **d** hierarchical clustering of DEGs from each low temperature stress exposure time based on values of FDR ≤0.05 and LFC (≥2 and ≤2). Blue to red colors indicate low to high gene expression pattern

down-regulated (Fig. 3b); for T-41 h, 664 DEGs were identified, being 217 up-regulated and 447 down- regulated (Fig. 3c). Gene hierarchy analysis shows that T-41 h had a more notable effect on gene transcription compared to T-17 h and T-22 h, which had a similar expression pattern (Fig. 3d).

Analysis of Venn diagrams allowed us to identify 58 DEGs that remained up-regulated in all the treatments, likewise DEGs that differed significantly in three treatments were identified, of these, 34, 27 and 102 DEGs in T-17 h, T-22 h and T-41 h, respectively (Fig. 4a). As for the down-regulated DEGs, 29, 50 and 171 were found at T-17 h, T-22 h and T-41 h respectively, similarly169 DEGs remained at all times (Fig. 4b). Among the 58 up-regulated genes present at all three times, genes such as glutathione S-transferase, chalcone synthase-1B, chalcone synthase-2 and inositol-3-phosphate synthase and others related to secondary metabolite synthesis and stimulus response were maintained; while the 169 genes down-regulated from the three cold exposure times involved genes related to stimulus response, such as the abscisic acid receptor PYL5/6, as well as genes related to defense such as chitinase and acid endochitinase pcht28 effective against pathogens.

Annotation and GO functional classification of DEGs

DEGs found at all time points were annotated and classified into a total of 41 functional groups including molecular function, biological processes and cellular component (Fig. 5). GO enrichment analysis of T-17 h identified

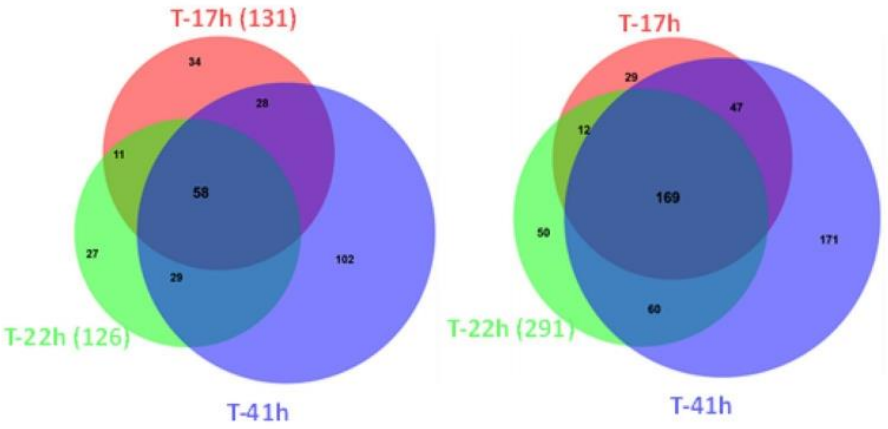

Fig. 4 Venn diagrams showing number of differentially expressed genes at different time intervals, up-regulated (**A**) and down-regulated (**B**) during low temperature stress at 17, 22 and 41 h

significantly enriched categories (FDR ≤ 0.05), 10 categories for molecular function, 15 for cellular processes and three for cellular component, at T-22 h, seven were identified for molecular function, 15 for cellular processes and two for cellular component, in case of T-41 h, 16 categories were identified for molecular function, 20 for cellular processes and six for cellular component. Among the GO functional groups found on all occasions, molecular function, catalytic activity (GO:0003824) and oxidoreductase activity (GO:0016491) was observed, in the case of biological processes, five GO functional groups were seen, response to abscisic acid (GO:0009737), phosphate ion transport (GO:0006817), temperature stimulus (GO:0009266), stimulus (GO: 0050896) stress (GO:0006950), it is worth mentioning that for the cellular component no shared functional groups were found at all time points, however in case of time points 1 and 3, they shared the categories of protein-containing complex (GO:0032991) and cell periphery (GO:0071944), for time points 2 and 3 they shared the categories of cell wall (GO:0005618) and vacuole (GO:0005773) (S3 Table).

In addition to the above categories, other GO functional groups were found significantly enriched at all time points, at T-17 h treatment, the categories ion binding (GO:0043167) with 110 DEGs, lyase activity (GO: 0016829) with 22 DEGs, macromolecule metabolic process (GO: 0043170) with 54 DEGs, response to chemicals (GO:0042221) with 33 DEGs, secondary metabolic process (GO:0019748) with 13 DEGs, chloroplast (GO:0009507) with 26 DEGs were observed and a cold acclimation category was also found with four DEGs. As for the categories found at T-22 h, enzyme inhibitory activity (GO:0004857) with 14 DEG, transmembrane transport (GO:0055085) with 40 DEG, carbohydrate metabolic process (GO:0005975) with 33 DEG, inorganic anion transport (GO:0015698) with 23 DEG and phosphate ion transport (GO:0006817) with 18 DEG were found. Finally, in the T-41 h treatment, the categories ion binding (GO:0043167) with 186 DEG, metal ion binding (GO:0046872) with 102 DEG, transmembrane transporter activity (GO:0022857) with 51 DEG, lyase activity (GO:0016829) with 27 DEG, metabolic processing of macromolecules (GO: 0043170) with 103 DEG, response to chemicals (GO:0042221) with 53 DEG, carbohydrate metabolic processing (GO:0005975) with 50 DEG, inorganic anion transport (GO:0015698) with 27 DEG, cell periphery (GO:0071944) with 69 DEG and cell wall (GO:0005886) with 60 DEG were found.

Stress response was one of the significant categories found, where genes related to antioxidant system (S3 Table), pathogen defense, osmoprotection, plant hormone signaling, ubiquitin conjugation pathway, chaperone activity and transcriptional regulators were found, some of these genes were peroxidase, chitinase, acid endochitinase pcht28, major allergen Pruar 1, abscisic acid and environmental stress inducible protein TAS14, ethylene responsive element binding protein C4, ethylene responsive proteinase inhibitor 1, ethylene responsive transcription factor 5, osmotin-like protein OSML15, dehydrin DHN2, dehydrin-like protein (Dhn), BTB/POZ and TAZ domain-containing protein (2 and 4), 70 kDa heat shock protein, 18. 5 kDa class (I and V), DnaJ-like protein 2, heat stress transcription factor A-2e, BRUTUS zinc finger protein, AP2/ERF and B3 domain-containing transcript. In the category of response to temperature stimulus, the following genes namely: DHN2 dehydrin, heat stress transcription factor A-2e, 18.5 kDa class (I and V) heat shock protein, DnaJ-like protein 2, heat shock protein 90–2, and abscisic acid and environmental stress inducible protein TAS14 were observed (S4 Table). Abscisic acid responsiveness was another category found at all time points, where genes related to plant hormone signaling and plant defense were found, such as abscisic acid receptor PYL1/4/5/5/5/6, abscisic acid and environmental stress inducible protein TAS14, dehydrin DHN2, and major allergen Pruar 1 (S5 Table). Oxidoreductase activity was another category found throughout where genes coding for

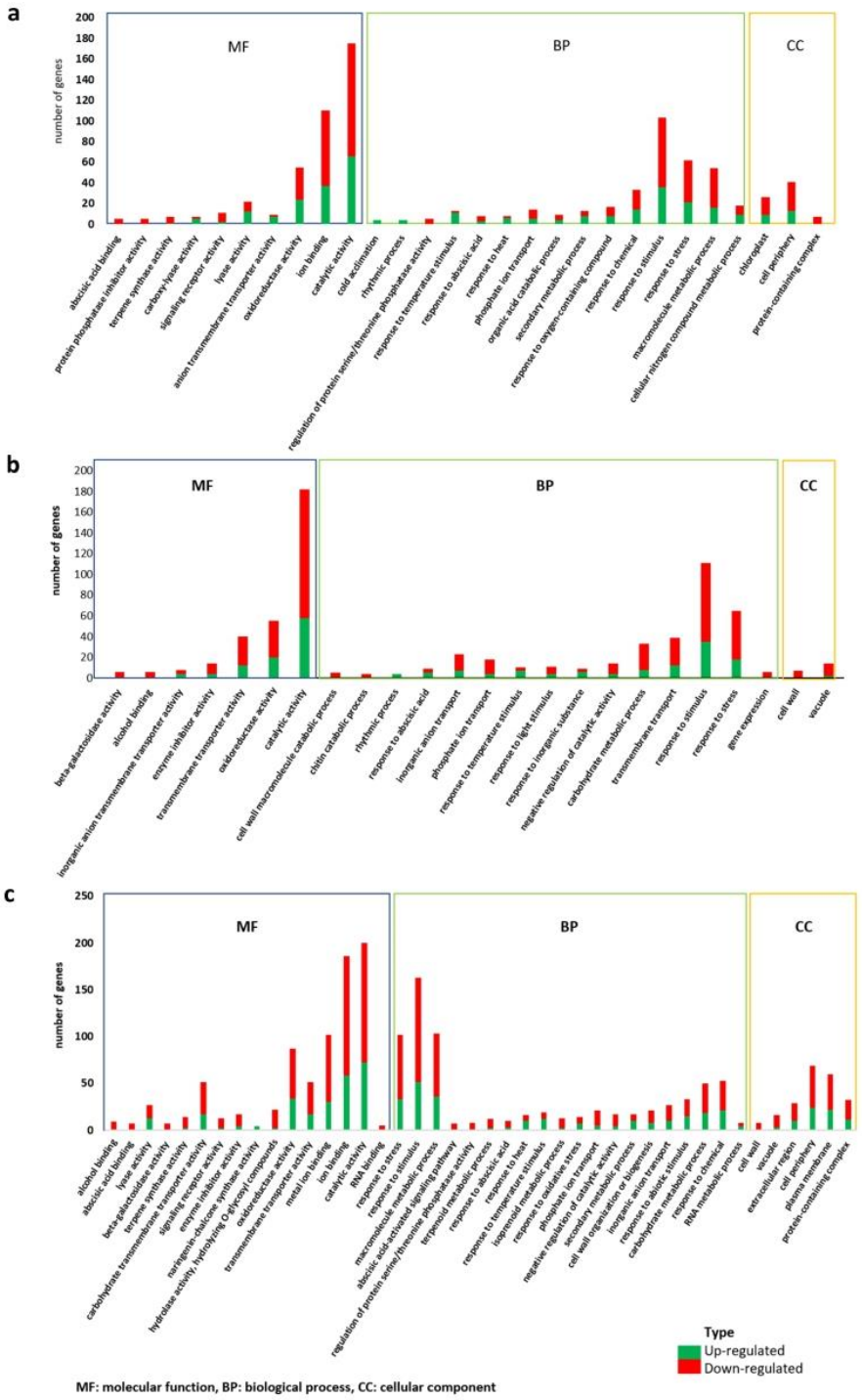

Fig. 5 GO enrichment analysis of differentially expressed genes (DEG) in response to low temperature stress. Significantly enriched GO terms are shown (FDR < 0.05). **a** GO terms at T-17 h; **b** GO terms at T-22 h; **c** GO terms at T-41 h

Lipoxygenase, Omega-6 fatty acid desaturase, endoplasmic reticulum, Delta(8)-fatty acid desaturase, Peroxidase, Cytochrome P450 93B16, Laccase, NADPH-protochlorophyllide oxidoreductase, Dihydrolipoyl dehydrogenase 1, chloroplastic, PGR5-like protein 1B, chloroplastic, Inositol oxygenase, Isoflavone 2′-hydroxylase, alkane hydroxylase MAH1, Flavonoid 3'-monooxygenase, Linolenate hydroperoxide lyase, chloroplastic, involved in lipid metabolism,

antioxidant system, secondary metabolite synthesis, cell wall and chlorophyll biosynthesis (S6 Table).

To further elucidate the metabolic process carried out by DEGs, a KEGG enrichment map was performed, where we could observe that at time point 1 six pathways were enriched, being phenylpropanoid biosynthesis the one with the highest number of DEGs, however, none of them presented a significant enrichment (Fig. 6a), in the case of down-regulated DEGs, out of a total of five enriched pathways, only MAPK signaling pathway was significantly enriched (Fig. 6d). At time point 2, four enriched pathways were found for the up-regulated DEGs, with the endoplasmic reticulum protein processing pathway having the highest number of DEGs, although none of the pathways were significantly enriched (Fig. 6b), as for the pathways of the down-regulated DEGs are concerned, at time point 2 they had four enriched pathways, with the MAPK signaling pathway being the only one that was significantly enriched (Fig. 6e). The up-regulated DEGs at time point 3 enriched five pathways, of which protein processing in the endoplasmic reticulum, circadian rhythm, flavonoid biosynthesis, tropane biosynthesis, and piperidine and pyridine alkaloids were significantly enriched (Fig. 6c), and for down-regulated DEGs at time point 3, a total of five pathways were enriched, with the MAPK signaling pathway and plant hormone signal transduction being the significantly enriched pathways (Fig. 6f).

Validation of differentially expressed genes by q-PCR

To validate the results of the transcriptome evaluated by RNA-Seq, five DEGs were selected, of which three were up-regulated and two were down-regulated during low temperature stress, another parameter taken into account for their evaluation was that they were found within the GO and KEGG categories significantly enriched and their relative expression was evaluated by quantitative real-time PCR (q-PCR) analysis (S7 table). When comparing both expression values (q-PCR versus RNA-Seq), a strong correlation was observed with an $r^2 = 0.8002$ (Fig. 7a). For most of the genes evaluated we observed a higher expression pattern in the q-PCR analysis compared to RNA-Seq, Py2D is one of the genes that was found to be up-regulated, and also showed a higher expression pattern in the q-PCR analysis, the opposite occurs with the BG gene that was down-regulated and a down-regulation could be observed in the RNA-Seq analysis at all exposure times than in the q-PCR analysis (Fig. 7b).

Discussion

Plants, when exposed to abiotic stress conditions such as low temperature, generate changes at the transcriptional level in order to acclimate and respond to stress [21]. In the present study we focused on generating transcriptomic data at three different times of exposure to low temperature, where we could find a total of 388 DEGs for T-17 h, 417 DEGs for T-22 h and 664 DEGs for T-41 h, contrasting our findings with the results obtained by Morales -Merida et al. [18] who when evaluating *C. annuum* seedling stems in response to combined stress of low temperature with UV-B radiation at three exposure times found a total of 281, 280 and 326 DEGs at 1, 3 and 25 h, respectively.

GO and KEGG enrichment analysis is one of the most widely used tools in gene expression analysis. Therefore, we chose to analyze the DEGs found at all time points to observe which categories were significantly enriched. In the present study, it can be observed that in the three times of exposure to low temperatures, the category of biological processes showed greater similarity in all the times points as they remained significantly enriched, response to temperature stimulus, response to abscisic acid, phosphate ion transport, response to stimulus and response to stress. For the molecular function category, oxidoreductase activity and catalytic activity remained significantly enriched. For the analysis of KEGG pathways at all exposure time points, MAKK signaling pathway, plant hormone signal transduction and flavonoid biosynthesis were found at all exposure times, the same pathways have been found previously when investigating the combined stress of low temperature with UV-B radiation [18] and for the first time in stem seedlings exposed to low temperature. Since these categories and pathways were maintained at all exposure time points, the genes involved in them could be a key player in response to this type of stress, although further studies are still required.

Response to temperature stimulus remained up-regulated and the gene coding for heat stress transcription factor A-2e (HSF A-2e) expressed during environmental stress [22]. In rice plants, the two types of gene family (HSF A-1 and A2) have been reported with OsHsfA2c being the only one reported to have a high temperature-dependent induction [23]. Chauhan et al. [24] Analyzed heat shock factors in rice plants during development and exposed to various abiotic stresses using DNA microarray technique and it was shown that OsHsfA2c factor was induced upon various abiotic stresses in shoot tissues. Similarly, it has been shown that the accumulation of HSF A-2 can form a heterodimer with HSF A-1 generating a super activator complex and thus maintain the expression of heat shock proteins (HSP) [25, 26]. Interestingly, HSF A-2e was up-regulated at all three time points of exposure to low temperature stress, so this

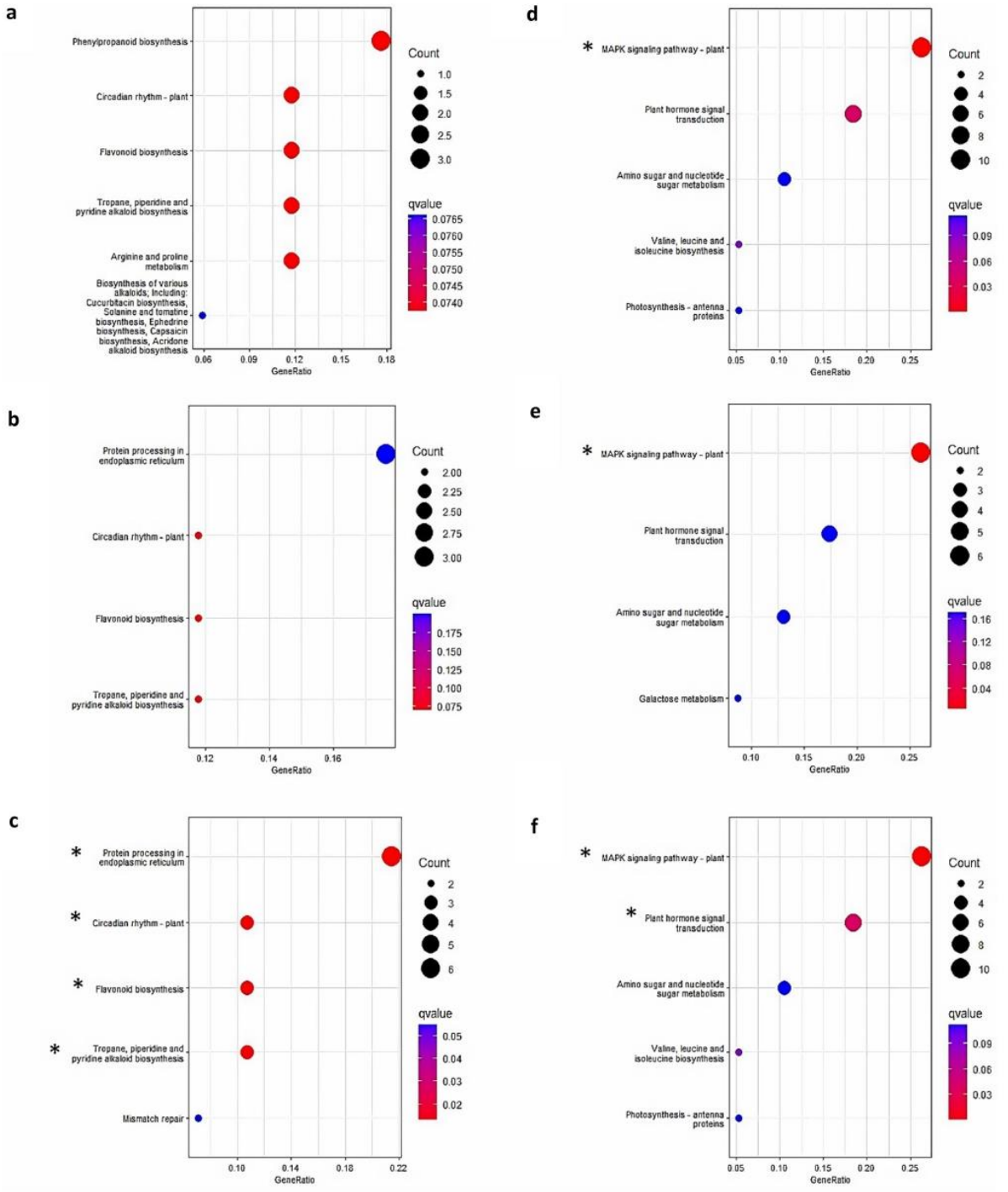

Fig. 6 Enrichment analysis of differentially expressed genes by KEGG. **a** Genes up-regulated at time 1, **b** Genes up-regulated at time 2, **c** Genes up-regulated at time 3, **d** Genes down-regulated at time 1, **e** genes down-regulated at time 2, **f** Genes down-regulated at time 3. The "x" axis indicates the enrichment factor and the "y" axis shows the KEGG pathway. The color of the dot represents the adjusted P-value and the size of the dot represents the number of genes, categories with (*) indicate significant difference (FDR ≤ 0.05)

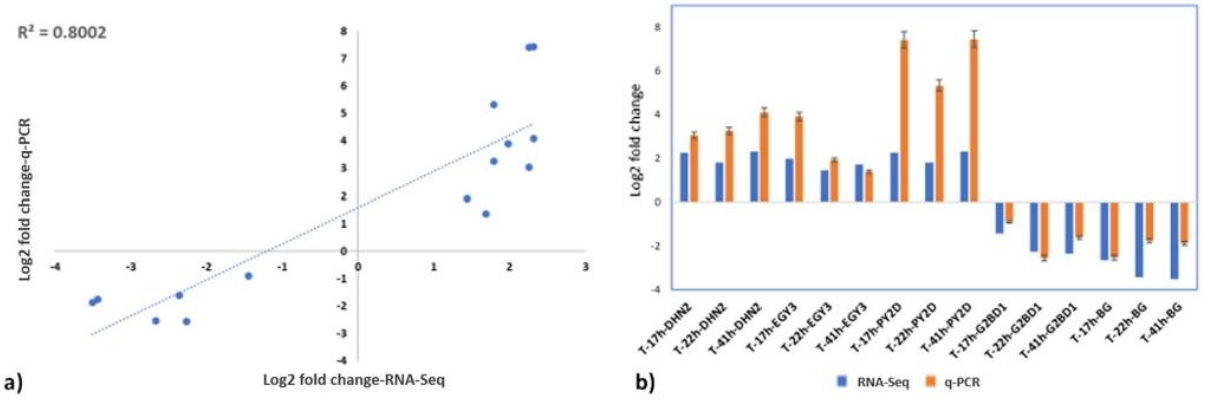

Fig. 7 **a** Close correlation ($r^2 = 0.8002$) of five differentially expressed genes evaluated in two approaches. **b** Comparison of gene expression of five differentially expressed genes

gene could be playing an important role in response to this type of stress.

In the present study, 4 HSP members of the HSP20 family were found, out of which HSP18.5 kDa class I, HSP 22.0 kDa class IV, HSP 17.4 kDa class III up-regulated and HSP 18.8 kDa class V down-regulated were observed. HSP20 or sHSP are characterized by a molecular weight of 12–43 kDa class IV [27, 28]. These HSP20 could be involved in response to low temperature stress, thus being very important in the stabilization of lipid bilayers, providing membrane protection against excessive temperatures [29] It has also been shown that these HSP20 can confer resistance to various abiotic stresses such as drought, salinity and cold stress [22].

Other DEGs that remained up-regulated at all exposure times are gene encoding enzymes of the CYP450 family (CYP 93B16, 72A1, 75B2 (HT1), 93B1) involved in oxidoreductase activity category, which were involved in the oxidative metabolism of endogenous compounds such as phenylpropanoids and cyanogenic glycosides, playing a major role in molecular processes of plant-pathogen interaction [30]. Furthermore, in recent studies, several authors have reported that the CYP450 family is actively involved in the biosynthesis of growth phytoregulators such as gibberellins, abscisic acid and brassinosteroids, [31, 32]. This indicates the relevant importance of CYP450 enzyme complex in low temperature tolerance, as ABA accumulates in large amounts in response to this type of stress [33].

Receptors of the pyrabactin resistance group, pyrabactin-like resistance and ABA receptor regulatory component (PYR/PYL/RCAR) bind ABA and activate the ABA-mediated signaling pathway [34]. However, in our results we found that PYL1, PYL4, PYL5 and PYL6 receptors involved in the abscisic acid response category were down-regulated. Similar results were reported by Chen et al. [35] who when evaluating cotton plants showed diverse expression patterns of GhPYL10 and GhPYL 12 and GhPYL 26 receptors in different plant tissues, however when cotton plants were subjected to drought stress an accumulation of ABA was induced, causing a down-regulation of GhPYL10/12/26 receptors. With this result we can elucidate that when excessive ABA accumulation occurs, the expression of PYL-type receptors is repressed. This leads to the binding of ABA to other types of receptors, favoring the activation of several pathways that could be more directed to respond to this type of stress.

Interestingly, in the same category, we could observe that a gene encoding the dehydrin TAS14 remained up-regulated. TAS14 is induced by ABA and abiotic stresses involved in water loss (desiccation, osmotic stress, salt stress and cold) [36]. Godoy et al. [37] revealed a distinct pattern of TAS14 protein expression by immunolocalization technique in various tissues of tomato plants during salt stress, where TAS14 was localized in provascular tissue of apical parts of shoots, in adventitious root primordia and in differentiated cortical cells of stem and leaves. One of the features of the genes encoding dehydrins is that they can be induced experimentally by exogenous application of ABA in mature embryos or vegetative tissues, as well as in conjunction with increased endogenous ABA production known to occur in plants subjected to water stress [38]. In present study, we were also able to identify genes encoding dehydrins DHN and DHN2 up-regulated at all exposure times within the category of response to temperature stimulus and response to abscisic acid, providing us with information to propose that ABA plays a very important role in response to temperature stress in bell pepper seedling stems.

The enzyme chalcone synthase (CHS) plays a key role in biosynthesis of anthocyanins, which are synthesized through the phenylpropanoid pathway and accumulate mainly in

leaves and stem in response to changes in light intensity and low temperatures [39]. Our investigation found genes encoding chalcone synthase 2 and chalcone synthase 1B, involved in the KEGG pathways of flavonoid biosynthesis, plant circadian rhythm and tropane, piperidine and pyridine alkaloid biosynthesis at all time points of low temperature exposure and only at T-17 h was the phenylpropanoid pathway enriched. Shvarts et al. [40] evaluated anthocyanin accumulation and assessed CHS gene expression in petunia flowers, demonstrating that at moderately low temperatures, the CHS gene was overexpressed in flower corollas and increased anthocyanin accumulation. Similar results were demonstrated in *Arabidopsis thaliana* leaves [39] and ginkgo biloba leaves where an accumulation of flavonoids paralleled CHS gene transcript levels, as well as a correlation between chalcone synthase activity and flavonoid accumulation [41].

Our results allow us to elucidate that the enzyme chalcone synthase plays a very important role in response to low temperature stress, favoring a greater accumulation of secondary metabolites to counteract the effects caused by low temperature stress, as the present study also found genes involved in flavonoid and alkaloid biosynthesis up-regulated in the oxidoreductase activity category upon exposure to low temperature stress (Lycodione synthase, Flavonoid 3′-monooxygenase, and Secologanin synthase) (S6 Table).

The KEGG pathways of plant hormone signal transduction and MAPK signaling pathway were enriched in down-regulated genes at all exposure times, being the genes encoding abscisic acid receptor PYL5, PYL6, acid endochitinase pcht28 and mitogen-activated protein kinase kinase 9 (MKK9).

Several investigations have shown that overexpression of AtPYLs receptor potentiates response to ABA in Arabidopsis plants [41]. Furthermore, exogenous application of ABA caused down-regulation of several AtPYLs (PYR1, PYL1, PYL4, PYL5, PYL6, and PYL8) in whole plant tissues of Arabidopsis. To explain this expression pattern, they introduced negative feedback regulatory mechanisms [42]. Since, ABA can accumulate in large amounts in response to low temperature stress, in present study, accumulation of endogenous ABA could be introducing the negative feedback regulatory mechanism leading to down-regulation of PYL5 and PYL6 [33, 42].

As mentioned above, the gene encoding acid endochitinase pcht28 remained down-regulated at all three exposure times. There are several reports on the reduction of chitinase expression by various plant hormones, heat shock and mycorrhizal development in roots [43]. Therefore, chitinase induction in plants is generally non-specific and enhanced by biotic and abiotic stresses [43, 44]. Leah et al. [45] applied ABA exogenously to barley plants to simulate what occurs in response to salt stress. They were able to observe an inhibitory effect of ABA on the expression of an acid chitinase (class III).

The gene coding for MKK9 protein was another gene that remained down-regulated at all three low temperature exposure times. Gunapati et al. [46] evaluated transgenic cotton and Arabidopsis plants overexpressing NAC2 gene by microarray technique showed that there was increased activation of ABA and JA pathways and suppression of ethylene pathway, leading to suppression of ethylene-responsive genes such as ERF6/ERF1/WRKY33/MPK3/MKK9/ACS6. Both MKK9 and other ethylene response genes, such as ethylene response element binding protein C4, transcription containing AP2/ERF and B3 domains, ethylene response transcription factor 5, ethylene response element binding protein C3, remained down-regulated at all three exposure time points (S2 Table). Based on these results, we can speculate that the ethylene signaling pathway is partially repressed in response to low temperatures. Finally, the relative expression data of five DEGs assessed by q-PCR supported the results obtained by RNA-Seq analysis as a strong correlation was observed with an $r^2 = 0.8002$ (Fig. 7), similar results have been reported by validating with this technique, giving further robustness to our results [47].

TFs in response to low temperature

Transcription factors are involved in several central roles in plant signaling, playing an important role in downstream mechanisms of gene expression regulation. Currently, about 1500 TFs have been identified, of which some of them have been confirmed to play important roles in the low temperature response including NAC, bZIP, MYB, WRKY and AP2/ERF [48, 49]. In our investigation we found a total of 23 DEGs coding for FTs at T-17 h, 30 DEGs at T-22 h

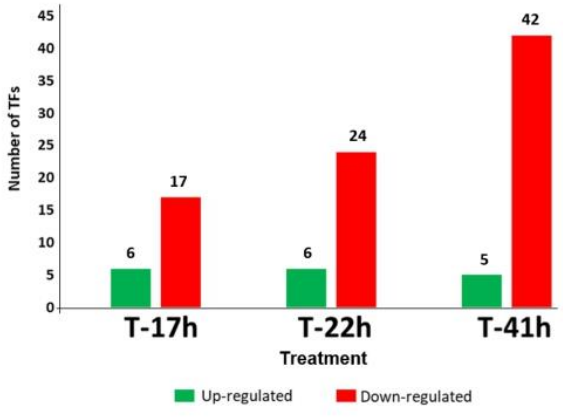

Fig. 8 TFs expressed differentially at the three low temperature exposure times. The "x" axis indicates the treatment and the "y" axis the number of TFs

and 47 DEGs at T-42 h (Fig. 8). Belonging to the bZIP, HD-ZIP, HSF, CO-like, GRF, LBD, MYB, bHLH, NF-YA, B3, C2H2, C3H, ERF, NAC, WRKY, GATA, GRAS and ZF-HD families. Where we could observe only one TF was up-regulated at all times of low temperature exposure which encodes for Heat stress transcription factor A-2e, which was also reported by Ji et al. [17] to evaluate transcriptomes of bell pepper leaves at 0, 4, 8, 8, 16, 24 h of low temperature exposure. Liu et al. [50]. evidenced that in Arabidopsis more than 65% of the genes regulated by heat stress were regulated by HSFA1, as well as it played a very important role in response to other abiotic stresses, however, more research is required to understand the relationship of heat stress factor and low temperature response. In the case of TFs that were down-regulated a total of 16 TFs were shared across all exposure times where MYB, bHLH, WRKY, ERF, NAC and HD-ZIP families were mainly highlighted. Yao et al. [51] in evaluating the ginger genome showed that the MYB TF exhibited diverse regulatory functions in low-temperature and hormonal treatments related to signal transduction. In a study by Kong et al. [16] when evaluating bell pepper fruits exposed to low temperature they found a total of 250 DEGs, which belonged to 41 TF families, demonstrating that the AP2/ERF, MYB and NAC families were involved in low temperature stress in bell pepper fruits, these data coincide with our findings because we found the same TF families in our DEGs, therefore these TFs could be playing a similar role in the regulation of gene expression in stems to what happens in bell pepper fruits, regulating the expression of downstream target genes in response to low temperature stress, however, further research is needed to clarify the regulatory mechanisms of these TFs in response to low temperature.

Conclusion

A general stem transcript profile of bell pepper (*Capsicum annuum* L.) seedlings exposed early, intermediate and late to low temperatures was generated using RNA-seq technology, where we were able to identify differentially expressed genes related to flavonoid biosynthesis pathways and up-regulated plant circadian rhythm and down-regulated genes involved in plant hormone transduction and the MAKK signaling pathway at all exposure time points. In addition, we were able to find HSF, CO-like, NAC, bZIP, MYB, WRKY and AP2/ERF transcription factor families which have been reported to modulate gene expression in response to various abiotic stresses such as low temperature. We were also able to observe that, in response to low temperature, there was greater activation of genes related to ABA signaling compared to ethylene signaling. The data generated in this research provide novel insights at the transcriptional level of the *C. annuum* stem serving as a basis for understanding the molecular mechanisms involved in the resistance of *C. annuum* to low temperature. However, these data are based on transcriptomic analysis, so further research is needed to complement our findings by focusing mainly on structural genes and TFs.

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Authors' contributions JLF, BH and LLR, who designed and coordinated the study. JCGO performed the experimental analysis. BEMM, ACM, JLF, MLM, CV and JCGO analyzed the results. Contribution of reagents/materials/analytical tools: RLC, JLF, LLR and BH. CV, MLM and ACM edited the English grammar of the manuscript. All authors drafted, read and approved the manuscript.

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Data availability Data presented in this study are available on fair request to the corresponding author.

Declarations

Conflict of interest All other authors declare no conflicts of interest.

Ethical approval Not applicable.

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3. INTEGRATED ANALYSIS OF BELL PEPPER (*Capsicum annuum*) PROTEOME AND METABOLOME REVEALS KEY METABOLIC PATHWAYS IN RESPONSE TO LOW TEMPERATURE STRESS

Jesús Christian Grimaldi-Olivas[1], Brandon Estefano Morales-Merida[1], Claudia Villicaña[2], J Basilio Heredia[1], Luis Alberto Lighbourn-Rojas[5], Rubén León-Chan[5], Abraham Cruz-Mendívil[4], Melina López-Meyer[3], José A. Guerrero-Analco [6], Eliel Ruiz-May [6], Juan L. Monribot-Villanueva[6], Esaú Bojórquez-Velázquez[6] and Josefina León-Félix [1]*

[1]Centro de Investigación en Alimentación y Desarrollo (CIAD). Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110, Culiacán, Sinaloa, México

[2]CONAHCYT-Centro de Investigación en Alimentación y Desarrollo (CIAD), Carretera a Eldorado Km 5.5, Campo el Diez, Culiacán, Sinaloa C. P. 80110, México

[3]Instituto Politécnico Nacional (IPN), Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes # 250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[4]CONAHCYT-IPN, CIIDIR Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[5]Laboratorio de Genética, Instituto de Investigación Lightbourn, A. C., Cd. Jiménez, Chihuahua, 33981, México

[6]Red de Estudios Moleculares Avanzados, Instituto de Ecología A. C., Cluster BioMimic®, Carretera Antigua a Coatepec 351, Congregación el Haya, Xalapa 91073, Veracruz, México

* For correspondence: ljosefina@ciad.mx

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Leon Felix, Josefina <ljosefina@ciad.mx>

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2. Integrated analysis of bell pepper (*Capsicum annuum*) proteome and metabolome reveals key metabolic pathways in response to low temperature stress

Jesús Christian Grimaldi-Olivas[1], Brandon Estefano Morales-Merida[1], Claudia Villicaña[2], J Basilio Heredia[1], Luis Alberto Lighbourn-Rojas[5], Rubén León-Chan[5], Abraham Cruz-Mendívil[4], Melina López-Meyer[3], José A. Guerrero-Analco [6], Eliel Ruiz-May [6], Juan L. Monribot-Villanueva[6], Esaú Bojórquez-Velázquez[6] and Josefina León-Félix [1]*

[1]Centro de Investigación en Alimentación y Desarrollo (CIAD). Carretera Culiacán-Eldorado Km 5.5 Col. Campo el Diez, C.P. 80110, Culiacán, Sinaloa, México

[2]CONAHCYT-Centro de Investigación en Alimentación y Desarrollo (CIAD), Carretera a Eldorado Km 5.5, Campo el Diez, Culiacán, Sinaloa C. P. 80110, México

[3]Instituto Politécnico Nacional (IPN), Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes # 250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[4]CONAHCYT-IPN, CIIDIR Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes #250 Col. San Joachin, C.P. 81049, Guasave, Sinaloa, México

[5]Laboratorio de Genética, Instituto de Investigación Lightbourn, A. C., Cd. Jiménez, Chihuahua, 33981, México

[6]Red de Estudios Moleculares Avanzados, Instituto de Ecología A. C., Cluster BioMimic®, Carretera Antigua a Coatepec 351, Congregación el Haya, Xalapa 91073, Veracruz, México

* For correspondence: ljosefina@ciad.mx

Abstract

The bell pepper (*Capsicum annuum*) is one of the most important crops worldwide. However, the production of this crop can be affected by various abiotic stresses, such as low temperatures (LT). This stress induces biochemical, morphological, and molecular changes that impact membrane lipid composition, photosynthetic pigments, free sugar and proline accumulation, and secondary metabolism. These molecular events have been studied in greater depth thanks to omics sciences approaches. Integrating data from different omics has been a widely used tool for identifying key metabolic pathways altered during exposure to various types of biotic and abiotic stress. This study aimed to identify alterations in the metabolic pathways of bell pepper stems caused by LT through multi omics data integration. Proteomic and metabolomic profiles were obtained using liquid chromatography coupled with accurate mass spectrometry, and data integration was performed in the plant metabolic pathway database. Low-temperature stress induced an accumulation of proteins related to response to Cadmium ion, pyridoxal phosphate biosynthetic process, phyllome development, late endosome to vacuole transport, and COP9 signalosome assembly. Similarly, alterations were identified in the metabolic pathways of flavonoid biosynthesis, phenylpropanoid biosynthesis, arginine biosynthesis, pyruvate oxidation, and the citrate cycle. The data generated in this study provide new insights into the proteome and metabolome profiles of *C. annuum* stems in response to LT stress, with identified proteins and metabolites crucial for metabolic pathways related to osmoprotection, antioxidant response, and cellular respiration.

Key message

LT stress has a significant impact on the metabolic pathways of flavonoid biosynthesis, phenylpropanoid biosynthesis, arginine biosynthesis, pyruvate oxidation, and citrate cycle (TCA cycle), generating important compounds to counteract the effects caused by such stress.

Keywords: *Capsicum annuum*; Proteome; Metabolome; Low temperature; Omics data integration

Introduction

The bell pepper (*Capsicum annuum* L.) is a perennial herbaceous plant belonging to the Solanaceae family, along with eggplant (***Solanum melongena*)**, potato (*Solanum tuberosum*), and tomato (*Solanum lycopersicum*). It is one of the world's most widely produced vegetables. However, bell pepper production can be compromised by LT, as its optimal growth range is between 21 and 27 ºC (Bakker andvan-Uffelen 1988 ; Pressman et al. 2006; Hincha and Zuther 2014; Ali et al. 2021). When routinely exposed to various types of stress, such as cold, plants activate mechanisms to enhance their resistance to LT and ensure survival. This process is known as cold acclimation (Ouellet and Charron 2013). During cold acclimation, physiological, biochemical, and molecular changes occur, generating extensive reorganization of gene expression, alteration in cell membrane lipid composition, photosynthetic pigments, accumulation of cryoprotectants such as free sugars and proline, and an increase in antioxidant levels (Doherty et al. 2009; Thomashow 2010; Theocharis et al. 2012; Guo et al. 2018; Bailey-Serres et al. 2019; Zhang et al. 2020). These molecular events have been studied more rapidly in various anatomical parts of plants due to technological advances in recent decades, which have facilitated large-scale studies of many genes, proteins, and metabolites; these tools are known as omics sciences (Morrison et al. 2006). Integrating data from different omics approaches has been a widely used tool for identifying critical metabolic pathways altered during exposure to various types of abiotic stress (Jamil et al. 2020; Chao et al. 2023).

In bell peppers, morphological, biochemical, and molecular changes in response to LT stress have been identified. For example, a study by León-Chan et al. (2017) evaluating bell pepper leaves in response to LT, UV-B radiation, and the combination of LT + UV-B showed a 19% reduction in chlorophyll and a higher accumulation of the flavonoid apigenin-7-O-glucoside (A-7-G) under LT conditions. Similarly, increased expression of genes involved in anthocyanin biosynthesis (*MYB*, *F3H*, *F3'5'H*, *DFR*, and *ANS*) was observed in bell pepper stems exposed to LT compared to UV-B radiation stress and the combined LT + UV-B stress (León-Chang et al. 2020).

Currently, transcriptomic analysis have been applied to various anatomical parts (stems, leaves, fruits) of bell pepper exposed to LT, revealing numerous genes involved in molecular processes

such as osmotic regulation, dehydration, membrane stability, plant hormone signal transduction, flavonoid biosynthesis, phenylalanine metabolism, alkaloid biosynthesis, circadian rhythm, and the Mitogen-activated protein kinase (MAPK) signaling pathway (Kong et al. 2019; Ji et al. 2020; Morales-Merida et al. 2021; Grimaldi-Olivas et al. 2023). Similar reports have used integrative omics approaches to study *C. annuum*'s response to cold stress. Zhang et al. (2022), by integrating transcriptomic and metabolomic data in two bell pepper cultivars, XS (cold-sensitive) and GZ (cold-tolerant), identified 10,931 differentially expressed genes (DEGs) and 657 differentially accumulated metabolites (DAMs) in positive ionization mode, and 390 DAMs in negative ionization mode across both cultivars. This data integration highlighted that the regulation of the ICE (inducer of CBF expression)-CBF (C-repeat binding factors)-COR (cold-regulated) pathway by Ca^{2+} signaling, MAPK signaling, and reactive oxygen species (ROS) signaling plays a crucial role in the cold stress response in bell pepper. In response to LT stress, Xu et al. (2023) integrated proteomic and metabolomic profiles in bell pepper fruit from a cold-sensitive cultivar (cv. 129) and a cold-tolerant cultivar (cv. 130), observing that changes in proteins and metabolites in cv. 130 were less pronounced compared to cv. 129. The authors proposed that cold tolerance exhibited by cv. 130 regulates membrane lipid homeostasis through other metabolites, such as soluble sugars, polyphenol intermediates of glutathione metabolite, folate biosynthesis, thiamine metabolism, and riboflavin metabolism. Recently, Morales-Merida et al. (2024) evaluated bell pepper stems exposed to combined LT and UV-B stress, reporting an increase in the accumulation of proteins related to photosynthesis, mitochondrial electron transport, and response to stimuli, as well as identifying flavonoids and their glycosides. The authors observed perturbations in carbon metabolism, tetrapyrrole pathways, and scopolamine.

The results of these studies have provided significant insights into the biochemical, morphological, and molecular changes occurring in *C. annuum*, which may play a crucial role in the plant's response to LT stress. Notably, multi-omic studies focusing on bell pepper stems are scarce despite their importance in photosynthesis, support, nutrient transport, storage, and connectivity with all plant organs (Rodriguez andDe La Vega 2021). Therefore, the objective of our research was to identify the altered metabolic pathways in response to low-temperature stress through the integration of proteomic and metabolomic data. This approach will enable the identification of key biomolecules, generating valuable data that will facilitate improved agronomic management for the early mitigation of such stress.

Materials and methods

Plant Material Acquisition

Pepper seeds of the commercial variety Canon cv. (Zeraim Gedera Syngenta; Israel) were germinated following the methodology previously described by León-Chan et al. 2017. Subsequently, 28 days after sowing (DAS), the seedlings were transferred to acclimatization chambers (GC-300TLH, JEIO TECH; South Korea) and grown under standard conditions consisting of a 12-hour photoperiod (6:00 AM to 6:00 PM) with photosynthetically active radiation (PAR) of 972 μmol m^{-2} s^{-1}, a temperature regime of 25/20 °C (day/night), and a relative humidity of 65% for three days. To implement the low-temperature treatment, the temperature was adjusted to 15/10 °C at 6:00 PM on day 30. For sampling, stems from 20 pepper plants were collected for control and treatment samples, as illustrated (Figure S1). Control samples were taken from seedlings at 11 AM on day 32, maintained at a temperature of 25/20 °C (day/night) (control), while cold treatment samples were collected 41 hours post-exposure. Three biological replicates were performed for both control and treatment, and the tissue was immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

Protein Extraction

Our general procedure utilized the phenol-acetone extraction approach, as described by Monribot-Villanueva et al. (2022). Stem powder (500 mg) was suspended in 2 mL of absolute phenol, 2 mL of ice-cold extraction buffer [(0.1 M Tris-HCl, pH 8.4, 0.15 M NaCl, 30% sucrose, 1% SDS)], 1:3 (w/w) polyvinylpolypyrrolidone (PVPP), and 20 µL of β-mercaptoethanol. The samples were mixed for 20 minutes and then centrifuged at 3,000 × g for 30 minutes at 4 °C. The supernatant was collected and precipitated overnight with 15 mL of cold acetone at -20 °C. The mixture was then centrifuged at 3,000 × g for 30 minutes at 4 °C, and the acetone was removed. Subsequently, 80% acetone was added to wash the protein pellets, which were washed twice with absolute methanol. The pellets were air-dried in a fume hood and dissolved in 200 µL of dissolution buffer (0.1 M TEAB and 1% SDS). Protein concentration was measured using a BCA protein assay kit (Pierce, Thermo Scientific). Following the manufacturer's instructions (Bio-Rad), SDS-PAGE was performed and stained with Sypro Ruby solution. Molecular masses of the bands were determined using broad-range precision protein standards (Bio-Rad).

Protein Digestion, Prefractionation, Desalting, and Labeling

One hundred µg of pure protein extracts from each sample were reduced for 45 min at 60 °C with 27.5 µL of 0.1 M Tris (2-carboxyethyl) phosphine (TCEP), followed by alkylation with 30 µL of 0.3 M iodoacetamide for 1h at room temperature in the dark. The reduction reaction was then quenched with 32.5 µL of 0.3 M dithiothreitol (DTT) for 10 min. Proteins were precipitated overnight with five volumes of cold acetone. The samples were centrifuged at 10,000 × g for 15 min at 4 °C, and the acetone was removed. The resulting pellets were washed with 80% acetone and then with absolute methanol twice. The pellets were air-dried in a fume hood and dissolved in 100 µL of 50 mM triethylammonium bicarbonate (TEAB) containing 0.1% SDS. Proteins were digested overnight at 37 °C with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) at a 1:30 (w/w) trypsin-to-protein ratio. Additional trypsin was added at a 1:60 (w/w) trypsin-to-protein ratio, and digestion continued for 4h at 37 °C. Tandem Mass Tag (TMT) 6-plex reagents (Thermo Fisher Scientific) were used for labeling in the following order: 126, 127N, and 127C for control samples, and 130N, 130C, and 131 for treatment samples. Protein fractions were separated using high pH reversed-phase liquid chromatography (RPLC, Pierce). Desalting of all fractions was performed using Thermo Scientific C18 cartridges, and samples were dried using a CentriVap vacuum concentrator (Labconco).

Nano LC-MS/MS

Labeled peptides were reconstituted in 0.1% formic acid in LC-MS grade water (solvent A), and 5 µL of this solution was injected onto a nano LC platform (UltiMate 3000 RSLC system, Dionex, Sunnyvale, CA) using a NanoViper C18 trap column (3 µm, 75 µm × 2 cm, Dionex). Peptides were then separated on an EASY spray C-18 RSLC column (2 µm, 75 µm × 25 cm) at a 300 nL/min flow rate. The gradient was as follows: 10 min with solvent A and 0.1% formic acid in 90% acetonitrile (solvent B), 10 min with solvent A, solvent B increased from 7% to 20% over 25 min, held at 20% for 15 min, increased from 20% to 25% over 15 min, increased from 25% to 95% over 20 min, and 8 min with solvent A. The Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific), equipped with an EASY Spray nano ion source, was connected to the nanoLC platform (Thermo Fisher Scientific). The source temperature was set to 280 °C, and the mass spectrometer operated in positive ion mode with a nanospray voltage of 3.5 kV. External calibrants used were

caffeine, Met-Arg-Phe-Ala (MRFA), and Ultramark 1621 (Thermo Fisher Scientific).

Sequential Precursor Selection (SPS)-MS3

The Orbitrap analyzer performed full MS scans at a resolution of 120,000 (FWHM), with a scan range of 350-1500 m/z, AGC target of 2.0e5, maximum injection time of 50 ms, intensity threshold of 5.0e3, dynamic exclusion from 1 to 70s, and a mass tolerance of 10 ppm. Fragmentation parameters included: precursor mass selection range of 400-1200 m/z, ion exclusion width of 18 m/z for low and five m/z for high, CID with 35% collision energy and Q activation of 0.25, AGC target of 1.0e4 with a maximum injection time of 50 ms, TMT tag loss, and detection performed in an ion trap. MS3 spectra were collected using 10 sequential SPS isolation windows. MS3 precursors were fragmented by HCD at 65% collision energy and analyzed using the Orbitrap at a resolution of 60,000 with a scan range of 120-500 m/z, an isolation window of 2 m/z, AGC target of 1.0e5, and a maximum injection time of 120 ms in a microscan.

Data Analysis and Interpretation

MS/MS and (SPS)-MS3 spectra based on decision trees were processed in Proteome Discoverer (v2.4) (PD, Thermo Fisher Scientific) using the Mascot (v2.4.1, Matrix Science), AMANDA (Dorfer et al. 2014), and SEQUEST HT (Eng et al. 1994) search engines. Searches were conducted against translated unigene databases created from the reference proteome of *C. annuum* (UP000222542) in UniProt. The search parameters included complete tryptic specificity, allowing for up to two missed cleavages. Static modifications included carbamidomethylation of cysteine (+57.021 Da) and TMT 6-plex modifications on N-terminal/lysine residues (+229.163 Da). Dynamic modifications included methionine oxidation (+15.995 Da) and deamidation of asparagine/glutamine (+0.984 Da). For the SPS-MS3 method, identification was performed in the lower resolution linear ion trap, applying ±10 ppm and ±0.6 Da tolerances. Peptide hits were filtered using the Percolator algorithm to achieve a maximum false discovery rate (FDR) of 1% (Käll et al. 2007). Quantification of reporter ions for TMT labels was performed with a PD software template at the MS3 level, applying mass tolerances of ±10 ppm for the most confident centroid and a 75% co-isolation filter for precursors. A protein was considered upregulated or downregulated if its t-test *p*-value was <0.05 and the fold change (FC) in relative abundance was >1.41 or <0.7 ($\log_2$ fold change >0.50 or <−0.50), respectively. All graphs were created using the

ggplot2 package (v3.5.1) (Ginestet 2011).

Protein Annotation Methods

Gene Ontology (GO) enrichment analysis was performed on protein sets using Singular Enrichment Analysis on the Kobas-i platform (http://bioinfo.org/kobas/genelist/, accessed December 14, 2023). GO-based biological process clustering was carried out using the Revigo platform (http://revigo.irb.hr/, accessed December 28, 2023), and Voronoi tree maps were generated using the SRplot platform (https://www.bioinformatics.com.cn/plot_basic_voronoi_treemaps_plot_079_en, accessed January 25, 2024). The analysis included $\log_{10}$-transformed absolute FDR values. GO terms were considered highly enriched with an FDR < 0.05.

Metabolite Extraction

Plant samples were ground using a mortar and pestle with the aid of liquid nitrogen and stored at -80 °C until further analysis. The samples (control and treatment) were dried at 40 °C for 24 hours using an Excalibur Parallax Hyperware food dehydrator (Sacramento, CA, USA). Subsequently, 300 mg of each sample was combined with 100 mg of diatomaceous earth and transferred to 10 mL extraction cells. Crude extracts were obtained using an accelerated solvent extraction system (Dionex, ASE 350) as previously described by Morales-Merida et al. (2024). Methanol was used as the solvent for all extractions. The extraction process was conducted at 60 °C with a 5-minute extraction cycle. The rinse solution volume was 30% of the total solvent, and the carrier gas was nitrogen. Aliquots were filtered using 0.2 µm pore size polytetrafluoroethylene (PTFE) membranes and then transferred to 1.5 mL sample vials.

Untargeted Metabolomic Analysis

Untargeted metabolomic analyses were performed on methanolic extracts from stem samples of both control and treatment groups based on the methods described by Monribot-Villanueva et al. (2022). The analyses were performed using an ultra-high-performance liquid chromatograph coupled to a high-resolution mass spectrometer (UPLC-HRMS-QTOF; Class I-Synapt G2-Si, Waters, Milford, MA, USA). Both positive and negative ionization modes were employed. Chromatographic separation was carried out using a Waters Acquity BEH column (1.7 µm, 2.1 ×

50 mm) in reversed-phase mode. The column oven temperature was set to 40 °C, while the sample temperature was maintained at 15 °C. Mobile phases included water (A) and acetonitrile (B), both supplemented with 0.1% formic acid. All solvents used in this study were MS-grade and obtained from SIGMA. Capillary, cone, and source voltages were set to 3000, 40, and 80 V, respectively. Source and desolvation temperatures were set to 100 °C and 20 °C, respectively. The desolvation gas flow rate was 600 L/h, and the nebulizer pressure was 6.5 Bar (650,000 Pa). Leucine-enkephalin was used as a lock mass. The mass spectrometer was calibrated with a mass range of 50-1200 Da using an MS^e method. Collision energy for function 1 was 6 V, while a ramp from 10 to 30 V was used for function 2. The scan time was 0.5 seconds. Metabolomic data were initially processed with MassLynx (Waters™) and MarkerLynx (Waters™). Data analysis was performed using the MetaboAnalyst platform (https://www.metaboanalyst.ca, accessed January 8, 2024), utilizing its various modules. The statistical analysis module was used to identify features that were up-accumulated if they had LFC (log2 fold change) values ≥ 2, and down-accumulated if LFC ≤ -2 and FDR ≤ 0.01. Signals m/z were tentatively identified using the Functional Analysis module. Metabolites showing over-accumulation and down-accumulation in both ionization modes were subsequently linked and analyzed using the Pathway Analysis module.

Targeted Metabolomic Analysis

Phenolic compounds were quantified on methanolic extracts from stem samples of both control and treatment groups groups based on the methods described by Monribot-Villanueva et al. (2022). This was done using an UPLC coupled to a triple quadrupole (-QqQ) mass spectrometer (Agilent Technologies, 1290/6460). Chromatographic separation was achieved with a Zorbax SB-C18 reversed-phase column (Agilent) with dimensions of 1.8 µm and 2.1 × 50 mm. The column oven temperature was set to 40 °C. Mobile phases included water (A) and acetonitrile (B), both supplemented with 0.1% formic acid. All solvents used in this study were MS-grade and obtained from SIGMA. Calibration curves ranging from 0.25 to 17 µM were constructed for each compound (60 compounds). The r^2 values for quadratic regressions were 0.99. Data were processed with MassHunter software (Agilent Technologies). Statistical analysis was performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca, accessed February 5, 2024) to identify LFC and FDR (<0.05). Features were identified as over-accumulated or down-accumulated down-regulated with LFC values ≥ 1 or ≤ 0.5 in treatment/control samples.

Omics Data Integration

Metabolic pathways were constructed using Uniprot annotations (https://www.uniprot.org/, accessed February 20, 2024) and the MetaboAnalyst 6.0 platform (https://plantcyc.org/, accessed February 25, 2024), using Arabidopsis thaliana as the reference. The log_2-fold changes of proteins and metabolites showing significant differences were input into the KEGG Mapper platform (https://www.genome.jp/kegg/mapper/, accessed February 26, 2024) to obtain an overview of cellular omics and visually represent the metabolic pathways (Kanehisa andSato 2020).

Results

Identification of Differentially Expressed Proteins (DEPs)

To identify differentially expressed proteins, proteins were extracted from the stems of *C. annuum* subjected to LT and untreated controls. It was confirmed that both conditions had a similar profile and good protein integrity in SDS-PAGE (Figure S2). Proteomic profiles were obtained using LC-MS/MS. Principal component analysis (PCA) was performed to evaluate the behavior of our biological replicates, revealing an explained variation value in principal component 1 (PC1) of 87.7%, which corresponds to the regulation of protein accumulation under low-temperature stress. This result is crucial for subsequent analyses (Figure 1A). A total of 1,029 proteins were identified, and cutoffcutoff values were applied to identify differentially expressed proteins (DEPs) with fold changes greater than 1.41 (over-accumulated) and less than 0.7 (down-accumulated), with a significance value of $P < 0.05$. A total of 152 DEPs were identified under these parameters, of which 84 were over-accumulated and 71 were down-accumulated (Figure 1B) (Table S1). GO enrichment analysis and clustering of redundant terms were conducted to evaluate the functions of these DEPs.

Additionally, GO enrichment analysis and clustering of redundant terms were performed to assess DEP functions (Table S2). An increase was observed in categories associated with biological processes such as response to cadmium ion, pyridoxal phosphate biosynthetic process, phyllome development, late endosome to vacuole transport, and COP9 signalosome assembly (Figure 1C). Conversely, a negative regulation was found in categories related to response to heat, post-embryonic root development, photosynthesis, miRNA-mediated post-transcriptional regulation, and chromatin organization (Figure 1D).

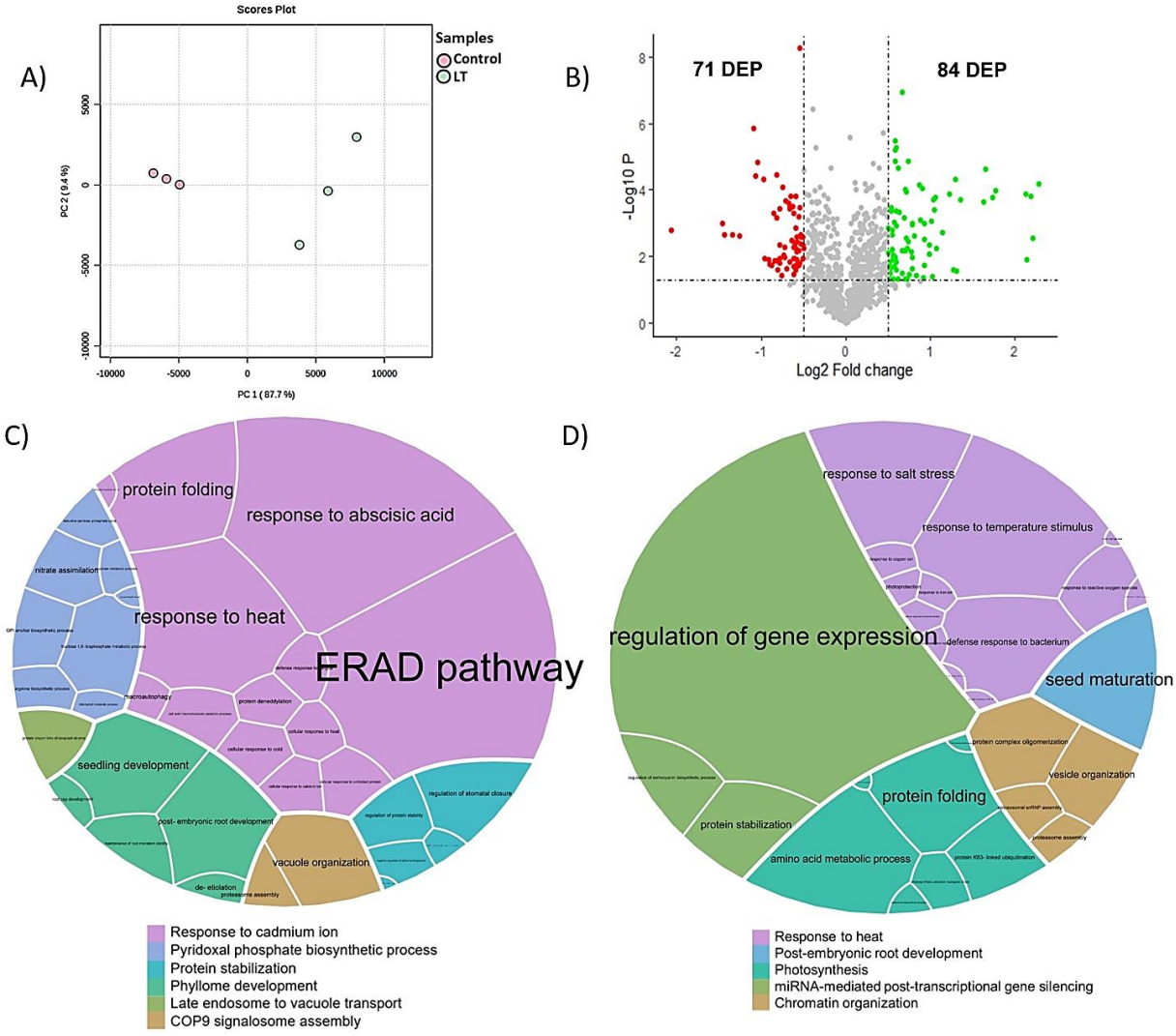

Figure 1. Proteomic Analysis of Pepper Stems in Response to Cold. (A) Principal component analysis of biological replicates for control (light green) and cold-treated (light red) samples. (B) Volcano plot of proteins accumulated in response to cold. Green points indicate over-accumulated proteins, while red points denote down-accumulated proteins. The X-axis represents the logarithmic fold change, and the Y-axis shows the P-value on a -Log_{10} scale. (C) Voronoi diagram of Gene Ontology (GO) analysis of differentially expressed proteins grouped into superclusters, showing over-accumulated proteins. (D) Voronoi diagram of GO analysis for down-accumulated proteins, based on absolute log_{10} P-value.

Untargeted Metabolomic Analysis

Untargeted metabolomic analysis was performed using both positive and negative electrospray ionization modes on the phytochemical profiles obtained from the stems of *C. annuum* in both the control and LT-treated groups. Principal component analysis (PCA) revealed that Principal Component 1 (PC1) explains the 99% of the variation between biological replicates of control versus cold-treated samples, indicating that the phytochemical profiles are distinct under each condition (Figure 2A). To identify over-accumulated metabolites, a cutoff value of LFC ≥ 2 was applied, and 58 metabolites met this criterion. Pathway analysis identified 37 pathways, of which seven showed significant enrichment (Figure 2B, Table S3 and Table S4).

The most significant metabolic pathway was flavone and flavonol biosynthesis, with key metabolites including kaempferol (LFC: 5.88), apigenin (LFC: 5.61), luteolin (LFC: 6.10), kaempferol 3-O-rhamnoside (LFC: 5.69), and quercetin 3-O-rhamnoside, 7-O-glucoside (LFC: 5.51). This was followed by the argininebiosynthesis pathway, including glutamic acid (LFC: 5.29), glutamine (LFC: 5.09), N-acetyl-L-glutamate 5-semialdehyde (LFC: 6.02), and L-arginine (LFC: 5.51). Other significant pathways included Vitamin B6 metabolism (glutamine [LFC: 5.09] and 4-hydroxy-L-threonine [LFC: 6.01]), Nitrogen metabolism (glutamine [LFC: 5.09] and glutamic acid [LFC: 5.29]), Purine metabolism (adenosine phosphosulfate [LFC: 5.74], glutamine [LFC: 5.09], and adenine [LFC: 5.91]), Flavonoid biosynthesis (apigenin [LFC: 5.61], luteolin [LFC: 6.10], and kaempferol [LFC: 5.88]), and the TCA cycle (thiamine pyrophosphate [LFC: 5.86] and oxalosuccinic acid [LFC: 5.53]).

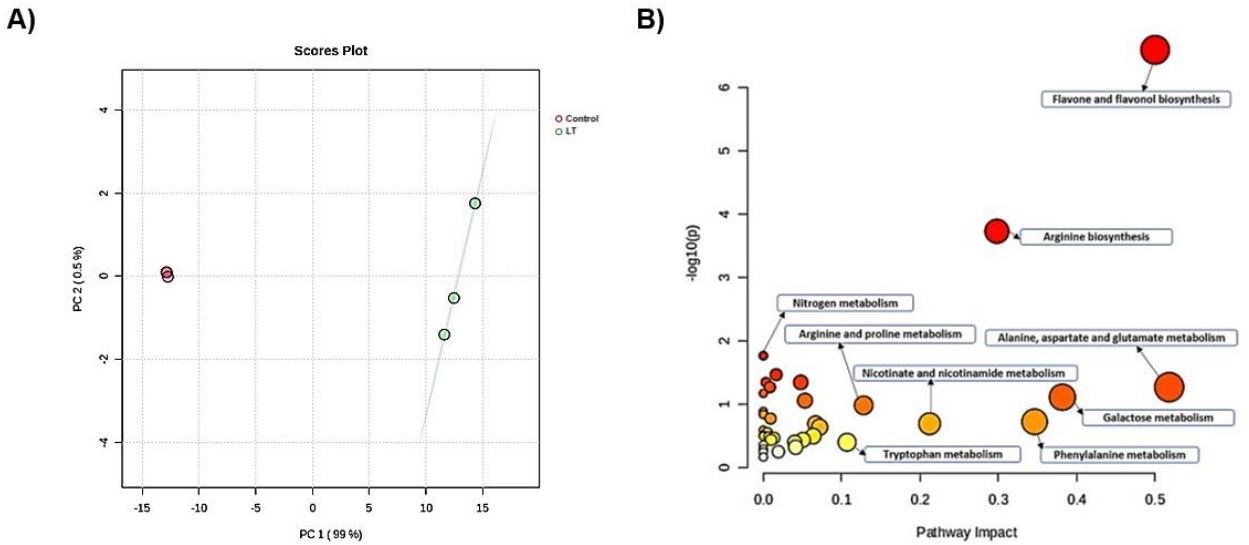

Figure 2. Untargeted Metabolomic Analysis of Pepper Stems in Response to Cold Stress. (A) PCA of three biological replicates for the control (light red) and cold treatment (green). (B) Overview of the metabolic pathways identified in response to cold stress using the pathway analysis module of MetaboAnalyst (https://www.metaboanalyst.ca, accessed January 8, 2024) and differentially accumulated metabolites (DAMs). Circles represent metabolic pathways, with yellow circles indicating lower p-values and red circles indicating higher p-values. The size of the circle represents the impact of the pathway.

Targeted Metabolomic Analysis

Based on the data obtained from the untargeted metabolomic analysis, a targeted analysis focused on phenolic compounds was performed to confirm the previously mentioned results. A total of 16 compounds from various chemical phenolic subcategories were identified and quantified in the *C. annuum* stem samples (Table 1). In the low-temperature treated samples, compounds such as chlorogenic acid, 3-coumaric acid, salicylic acid, quercetin-3-glucoside, and quercitrin were found in higher content than the control samples. Conversely, concentrations of compounds such as phenylalanine, 4-hydroxybenzoic acid, vanillic acid, 4-coumaric acid, vanillin, luteolin, luteolin-7-O-glucoside, rutin, and penta-O-galloyl-β-D-glucose were higher in the control samples compared to the treated ones. However, compounds such as ferulic acid and protocatechuic acid were present in approximately equal proportions in both sample types.

Table 1. Phenolic compound profiling was performed using metabolomics analyses in stem bell pepper under Cold stress.

Compound	Samples		
	Control	LT	LFC
Phenylalanine	101.75±2.39	98.73±8.57	-0.043468
Protocatechuic acid	0.31±0.01*	0.37±0.02*	0.25526
4-Hydroxybenzoic acid	1±0.05	0.55±0.04	-0.8625
Vanillic acid	1.04±0.01	0.71±0.06	-0.55069
Chlorogenic acid	22.89±0.20	25.85±0.23	0.17545
4-Coumaric acid	0.26±0.01*	0.17±0.01*	-0.61298
3-Coumaric acid	3.61±0.03	3.95±0.11	0.12985
Ferulic acid	0.05±0.01*[a]	0.02±0.01*[b]	-1.3219
Salicylic acid	2.31±0.14[a]	9.48±0.09[b]	2.037
Vanillin	1.44±0.06	1.13±0.05	-0.34975
Luteolin	0.66±0.01*	0.59±0.04*	-0.16175
Luteolin-7-O-glucoside	82.86±2.17	64.93±4.79	-0.35179
Quercetin-3-glucoside	4.97±0.12	5.22±0.09	0.070804
Quercitrin	3.97±0.05	4.84±0.09	0.28587
Rutin	9.17±0.33	8.00±0.24	-0.19692
Penta-O-galloyl-B-D-glucose	1.47±0.42*[a]	0.58±0.30*[b]	-1.3417

Concentration is defined in μg/g dry matter. Values show mean ± standard deviation (n=3). *Data below the limit of quantification. Different letters indicate marked changes in LFC between treatment and control samples for each compound.

Integration of Omics Data

An omics data integration analysis was performed using both proteomic and metabolomic data to investigate the impact of protein and metabolite accumulation on metabolic pathways. The analysis revealed that the pathways most significantly affected by LT stress were flavone flavone and flavonol biosynthesis, phenylpropanoid biosynthesis, arginine biosynthesis, pyruvate oxidation, and citrate cycle (TCA cycle), as illustrated in Figure 3 and Table S5.

The flavone and flavonol biosynthesis pathway were the most significantly altered, showing positive accumulation of metabolites such as apigenin, luteolin, kaempferol, kaempferin

(kaempferol 3-rhamnoside), quercetin 3-O-rhamnoside 7-O-glucoside, quercitrin, and isoquercitrin (quercetin-3-glucoside). Conversely, a decrease was observed in luteolin 7-O-glucoside (luteoloside) and rutin. No altered proteins were detected in this metabolic pathway.

In the phenylpropanoid biosynthesis pathway, enzymes such as caffeic acid 3-O-methyltransferase (OMT1; PHT87609), caffeoyl-CoA O-methyltransferase 6 (CCoAMT-6; PHT62646), caffeoyl alcohol, p-hydroxyphenyl lignin, and chlorogenic acid (caffeoylquinic acid) showed over-accumulation.. In contrast, suberization-associated anionic peroxidase 2 (TMP2; PHT90648), ferulic acid, *p*-coumaric acid (4-coumaric acid), and phenylalanine exhibited reduced accumulation.

The Pyruvate Oxidation and TCA cycle pathways are two interconnected stages in cellular respiration that play crucial roles in energy production. Changes were observed in the accumulation of isocitrate dehydrogenase [NADP] (PHT73980), 2-oxoglutarate dehydrogenase E2 (PHT77307), thiamine pyrophosphate (ThPP), and oxalosuccinic acid (oxalosuccinate). Additionally, a decrease in the accumulation of dihydrolipoamide acetyltransferase, long form protein (PHT75188), and succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial (PHT80416) was noted.

Finally, in the arginine biosynthesis pathway, there was an over-accumulation of N-acetyl-gamma-glutamyl-phosphate reductase (PHT64376) and peptidase M20/M25/M40 family protein (PHT83311), as well as a significant increase in glutamine, glutamate, arginine, and N-acetyl-L-glutamate 5-semialdehyde.

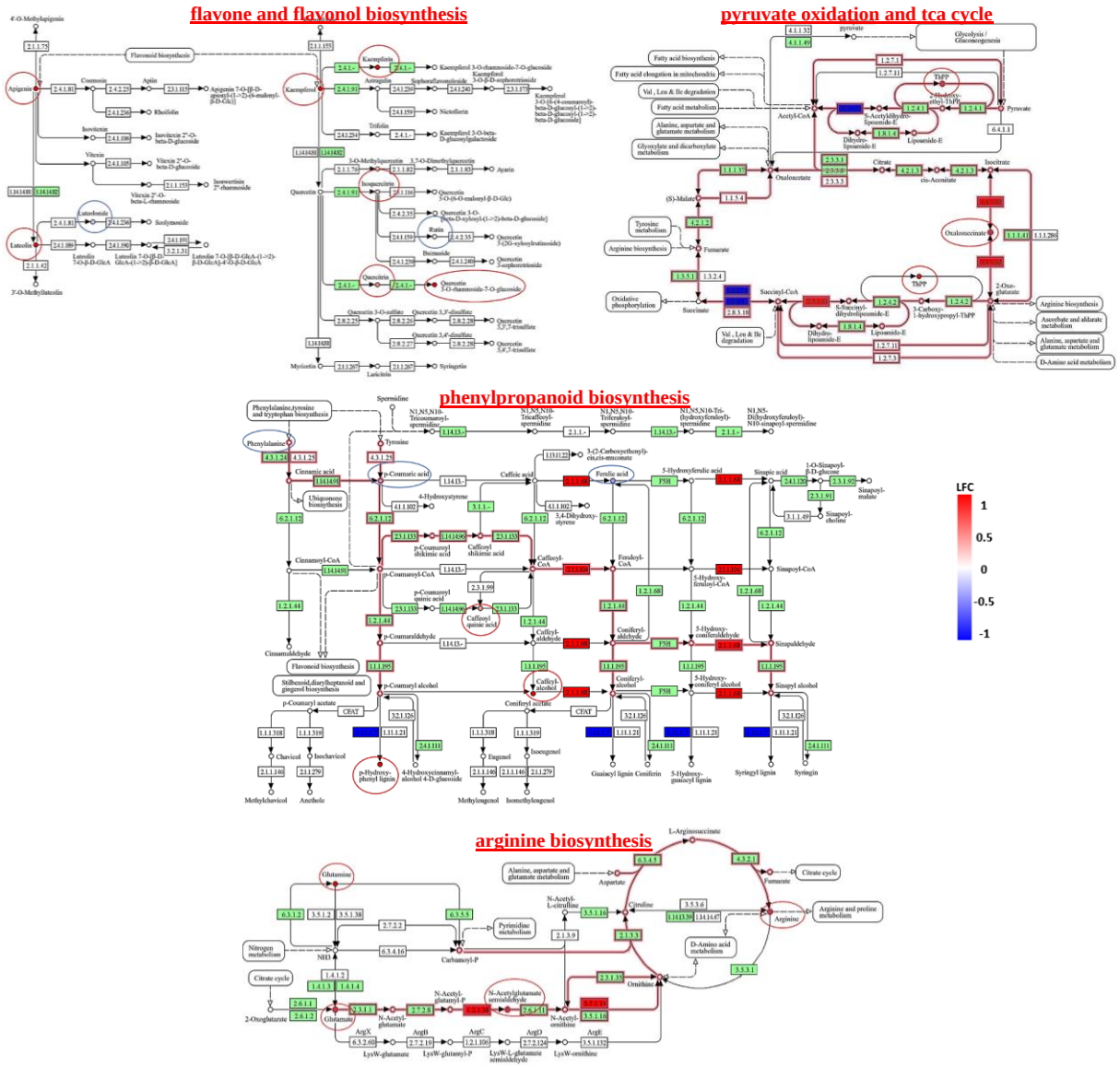

Figure 3. Plant Metabolic Pathways in Response to Cold Stress. phenylpropanoids biosynthesis, citrate cycle, and arginine biosynthesis (https://www.genome.jp/kegg/mapper/, accessed June 15, 2024). Omics data from proteomic and metabolomic analyses were used for mapping. The color scale represents log2-fold change values, with red hues indicating over-accumulation/upregulation and blue hues indicating under-accumulation/downregulation. Squares represent proteins, while circles represent metabolites. Light red shaded lines indicate the primary metabolic pathways, and green-marked squares indicate other important but unidentified proteins in the metabolic pathway.

Discussion

This study employed an integrated multi omics approach combining proteomic and metabolomic analyses to enhance the understanding of central metabolic processes in response to LT stress in pepper stems.

LT Induces Changes in Phenylpropanoid and Flavonoid Biosynthesis Pathways

Phenylpropanoids and flavonoids are two important groups of phytochemical compounds in plants that play crucial roles in abiotic stress responses. These compounds act through various mechanisms, including modifying cellular structures, neutralizing reactive oxygen species (ROS), modulating metabolic pathways, and participating in cellular signaling (Schulz et al. 2016; Sun et al. 2021; Morales-Merida et al. 2024). In the present study, we identified alterations in the pathways leading to phenylpropanoid and flavonoid biosynthesis. Several studies have reported changes in these metabolic pathways in response to LT stress in various plant types (Xu et al. 2020; Hu et al. 2022; Zhan et al. 2022; Yang et al. 2023; Gao et al. 2024).

In bell peppers, alterations in these pathways were identified, and an increase in the expression of *MYB, F3H, F3'5'H,* and *DFR* genes, as well as an increase in the accumulation of apigenin-7-O-glucoside in plants exposed to LT, was observed (León-Chan et al. 2017; León-Chang et al. 2020). using KEGG analysis with transcriptomic data Grimaldi-Olivas et al. (2023) found that the flavonoid and phenylpropanoid biosynthesis pathways were significantly enriched under LT conditions. Similar findings were reported when analyzing pepper plants exposed to combined stress (LT + UV-B) (Morales-Merida et al. 2021).

In this study, we observed an over-accumulation of the enzymes Caffeic acid 3-O-methyltransferase (OMT1; PHT87609) and Caffeoyl-CoA O-methyltransferase 6 (CCoAMT-6; PHT62646), which have been shown to play a fundamental role in the biosynthesis of lignin, flavonoids, and sinapoyl malate in *Arabidopsis* (Do et al. 2007; Fellenberg et al. 2012).

The over-accumulation of metabolites in the phenylpropanoid biosynthesis pathway included caffeoyl alcohol, a precursor in lignin biosynthesis, and *p*-hydroxyphenyl lignin, a type of lignin and one of the three primary monolignols (along with guaiacol and syringyl lignins) that compose the lignin polymer. Lignin is a complex polymer that provides structural support and protection to plants. Several studies have demonstrated that plants can alter lignin biosynthesis during low-temperature stress to enhance rigidity and resist stress-induced damage (Khaledian et al. 2015; Yadav and Chattopadhyay 2023). Similarly, it has been shown that the biosynthesis of *p*-hydroxyphenyl lignin can be upregulated in response to environmental stress, including LT, to strengthen cell walls and improve plant resilience (Xie et al. 2018). Finally, chlorogenic acid (caffeoylquinic acid), a phenolic compound known for its antioxidant properties, was also over-

accumulated. This metabolite is effective in neutralizing ROS produced under stress conditions, such as LT thus protecting plant cells from oxidative damage and enhancing stress tolerance (Wang et al. 2020).

According to this information, lignin and chlorogenic acid play crucial roles in response to low-temperature stress in bell pepper by improving cell wall rigidity and providing antioxidant activity to counteract the effects of this stress.

In the flavonoid biosynthesis pathway, we observed that five of the identified flavonoids were flavonoid glycosides. Our results align with those reported by Morales-Meridaet al. (2024), who identified flavonoid glycosides in a combined proteome and metabolome analysis of pepper stems in response to combined stress (LT + UV-B). This highlights the role of flavonoid glycosides in response to low-temperature stress, as the presence of a sugar molecule enhances their solubility and structural stability, facilitating their transport and storage to better address such stress (Zhao et al. 2019; Bozzo and Unterlander 2021; Morales-Merida et al. 2024).

LT-Induced Alterations in Arginine Biosynthesis

LT induces alterations in the arginine biosynthesis pathway. Numerous studies have demonstrated that the amino acid metabolism is essential for plant growth, development, and resistance to stresses such as LT (Heinemann and Hildebrandt 2021; Zuo et al. 2022; Kumari et al. 2023).

In this study, we observed that LT stress disrupts the arginine biosynthesis pathway, affecting both arginine and its precursor amino acids. Arginine has been shown to play various regulatory roles in plants, including nitrogen storage and stress resistance (Shi and Chan 2013; Hussein et al. 2022; Sun et al. 2023). Arginine biosynthesis involves two processes: a cyclic or linear pathway for ornithine formation from glutamate, followed by arginine synthesis from ornithine (Winter et al. 2015). We observed an accumulation of glutamine, a precursor for glutamate synthesis, which was also over-accumulated. We also found two enzymes, N-acetyl-gamma-glutamyl-phosphate reductase (PHT64376) and peptidase M20/M25/M40 family protein (PHT83311), involved in the linear pathway of ornithine biosynthesis. The enzyme N-acetyl-gamma-glutamyl-phosphate reductase facilitates the synthesis of N-acetyl-L-glutamate 5-semialdehyde from N-acetyl-glutamyl-P, which was also over-accumulated along with the enzyme catalyzing its reaction.

In wheat, it has been shown that stresses involving water loss, such as LT, salinity, and drought, lead to alterations in the expression of genes that encode essential enzymes for the synthesis and metabolism of arginine (Anwar et al. 2020; Li et al. 2024b). Similarly, exogenous administration of arginine has been shown to enhance germination and cold tolerance in wheat cultivars that were subjected to arginine priming (Liet al. 2024b).

Calhoun et al. (2021), evaluating *Scenedesmus sp.* in response to low-temperature stress, observed increased abundance of metabolites involved in arginine biosynthesis, such as ornithine, citrulline, and N-acetyl-L-glutamate, in cold-stress samples. This finding aligns with our study and the results reported by Cheng et al. (2023), who demonstrated that arginine activates specific cold-regulation pathways in tea plants, enhancing cold tolerance. Arginine is also involved in the biosynthesis of molecules crucial for plant stress regulation, such as proline and polyamines (Mega et al. 2023; Li et al. 2024a; Liet al. 2024b). Extensive data suggest a positive correlation between proline accumulation and stress in plants. Proline, an amino acid, plays a critical role in plant adaptation to various stressful conditions. Beyond its role as an efficient osmolyte, proline has three main functions during stress: metal chelation, antioxidant defense, and signaling (Hayat et al. 2012). Similarly, polyamines play a fundamental role in mitigating cold-induced damage across different plant species and, like proline, have been identified as cytoprotectors due to their role in protecting plants against abiotic and biotic stresses (Khan et al. 2010; Baier et al. 2019; Ahouvi et al. 2022).

In this study, we could not identify proline or polyamines; however, it is hypothesized that these metabolites might be detected during a prolonged period of low-temperature stress, as arginine, which was over-accumulated, and could serve as a precursor for their biosynthesis. Additionally, our metabolic pathway impact analysis included both arginine and proline metabolism pathways (Table S4), although these did not present significant values. Based on this information, the exogenous application of arginine could serve as an effective biostimulant for enhancing cold stress responses in pepper plants, given its potential to trigger a defensive response to adverse conditions.

LT induces changes in the Pyruvate Oxidation and TCA cycle pathways

Pyruvate oxidation and the citrate cycle (TCA cycle) are fundamental stages of cellular respiration, facilitating the conversion of glucose into usable metabolic energy (Zhang and Fernie

2023). These processes are highly temperature-dependent; thus, LT could significantly impact their efficiency. In this study, we identified an accumulation of thiamine diphosphate (ThPP), an essential intermediate of the pyruvate dehydrogenase complex, previously reported to accumulate in pepper fruit under low-temperature stress (Xuet al. 2023). Additionally, we observed the accumulation of enzymes involved in the TCA cycle, such as isocitrate dehydrogenase [NADP] (PHT73980) and 2-oxoglutarate dehydrogenase E2 (PHT77307), as well as an accumulation of oxalosuccinic acid (oxalosuccinate). Oxalosuccinic acid plays a crucial role as it decarboxylates to form alpha-ketoglutarate, a critical step in the continuation of the TCA cycle catalyzed by isocitrate dehydrogenase (identified in this metabolic pathway). Conversely, the succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial (PHT80416), exhibited downr-accumulation. Despite this, succinyl-CoA ligase, like the enzymes above, plays a crucial role in cellular respiration and energy production. Our results align with Xuet al. (2023), who reported increased TCA cycle enzymes, including isocitrate dehydrogenase (IDH), 2-oxoglutarate dehydrogenase (OGDH), succinyl-CoA ligase (SCOA), malate dehydrogenase (MDH), citrate synthase (CS), and aconitase, in green pepper fruits exposed to low-temperature stress.

Isocitrate dehydrogenase [NADP] is responsible for the production of alpha-ketoglutaric acid (α-ketoglutarate), which is not only an intermediary in the TCA cycle but also plays a role in enzyme regulation favoring the biosynthesis of amino acids, glucosinolates, flavonoids, alkaloids, and gibberellins, which may be involved in the low-temperature stress response (Araújo et al. 2014)

There is evidence that 2-oxoglutarate dehydrogenase, which converts 2-oxoglutarate to succinyl-CoA, is a key regulatory component of the TCA cycle, serving as a limiting factor during mitochondrial respiration and playing a central role in carbon-nitrogen interactions (Araújo et al. 2012a; Araújo et al. 2012b; Araújo et al. 2013), This suggests that this enzyme is crucial in the low-temperature stress response, aiding in the production of sufficient energy to maintain cellular homeostasis.

Conclusion

A comprehensive profile of proteins and metabolites was generated in *C. annuum* seedlings exposed to low-temperature stress (41 h). The integration of multi-omic data revealed alterations in metabolic pathways, including flavone and flavonol biosynthesis, phenylpropanoid biosynthesis, arginine biosynthesis, and pyruvate oxidation and the citric acid cycle (TCA cycle). These analyses

suggest that phenylpropanoids and flavonoids play an antioxidant role against oxidative stress, while lignin may provide stability and protection to cell walls against low-temperature damage. Arginine biosynthesis showed significant alterations, which induce protective osmolytes against cold stress. Cold stress strongly activated the enzymes 2-oxoglutarate dehydrogenase and isocitrate dehydrogenase (NADP). This demonstrated how important they are in the TCA cycle as well as in generating precursors of amino acids, glucosinolates, flavonoids, alkaloids, and gibberellins. Therefore, we propose that these enzymes play a critical role in pepper's cold stress response. The generated data could be of great support in developing LT hybrids or bio-stimulants that allow for early mitigation of such stress.

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Authors' contributions: JLF, BH and LLR, who designed and coordinated the study. JCGO performed the experimental analysis. BEMM, ACM, JLF, MLM, EBV, JLMV, CV and JCGO analyzed the results. Contribution of reagents/materials/analytical tools: RLC, JLF, LLR, ERM, JAGA, JLMV and BH. CV, MLM, and ACM edited the manuscript's English grammar. All authors drafted, read, and approved the manuscript.

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Data availability: Data presented in this study are available on fair request to the corresponding author.

Declarations

Conflict of interest: All other authors declare no conflicts of interest.

Ethical approval: Not applicable.

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4. CONCLUSIONES GENERALES

- El análisis RNA-seq de tallos de *C. annuum* expuestos a baja temperatura permitió identificar genes expresados diferencialmente relacionados con las vías de biosíntesis de flavonoides y regulados al alza en el ritmo circadiano de la planta y a la baja en genes implicados en la transducción de hormonas vegetales y la vía de señalización MAKK en todos los puntos tiempos de exposición demostrando que a 41h de exposición a baja temperatura ocasionó una mayor expresión de genes diferenciales.
- La integración de datos ómicos, del proteoma y metabolóma de *C. annuum* permitió la identificación de las vías metabólicas de biosíntesis de flavonas y flavonoles, biosíntesis de fenilpropanoides, biosíntesis de arginina, oxidación de piruvato y ciclo del ácido cítrico (ciclo TCA). Este análisis sugiere que los fenilpropanoides y los flavonoides ejercen un papel antioxidante contra el estrés oxidativo, y que la lignina podría estar brindando estabilidad y protección a las paredes celulares contra el daño generado por la baja temperatura.
- La arginina podría jugar un papel importante en la respuesta a baja temperatura ya que es un intermediario en para la biosíntesis de poliaminas y prolina, mimos que se han demostrado que actúan como osmolitos protectores ante diversos estreses abióticos.
- Las enzimas 2-oxoglutarato deshidrogenasa e isocitrato deshidrogenasa [NADP] mostraron cambios importantes en la vía del ciclo del ácido cítrico (ciclo TCA). La 2-oxoglutarato deshidrogenasa se ha relacionado como un punto clave en la regulación de esta ruta, mientras que la isocitrato deshidrogenasa [NADP] está encargada de la generación de 2-oxoglutárico, un metabolito importante en el TCA y precursor de aminoácidos, glucosinolatos, flavonoides, alcaloides y giberelinas. Por este motivo, proponemos que estas enzimas desempeñan un papel clave en la respuesta al estrés por bajas temperaturas en el pimiento morrón.