



Protein networks mediating embryogenic callus induction in *Carica papaya* L. respond to dose-dependent 2,4-dichlorophenoxyacetic acid treatment

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Abstract

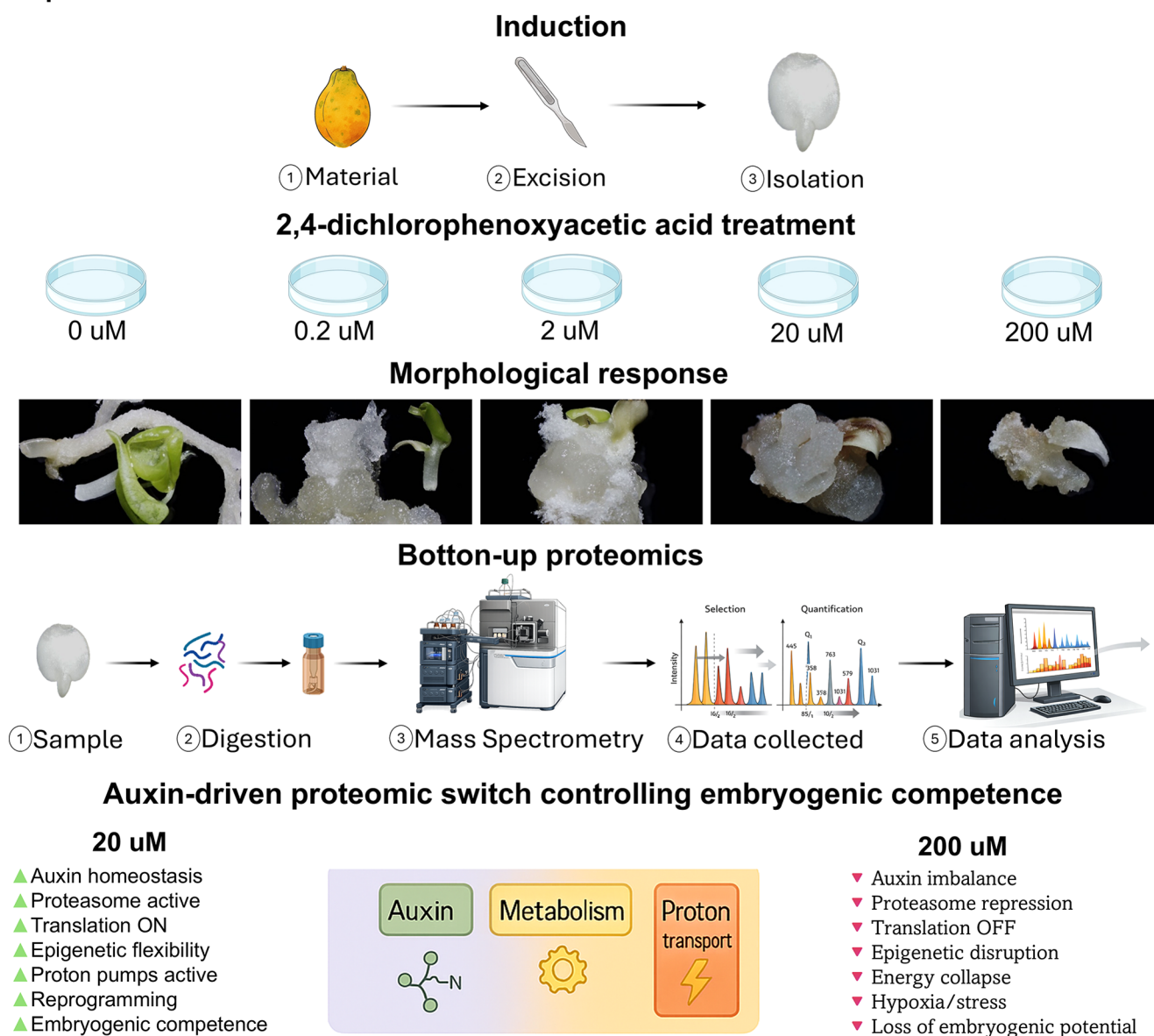
2,4-Dichlorophenoxyacetic acid (2,4-D) is widely used to induce somatic embryogenesis (SE), yet the concentration-dependent molecular mechanisms governing this process remain poorly resolved. Here, we investigated the morphological responses and proteomic changes in *Carica papaya* zygotic embryos exposed to a range of 2,4-D concentrations (0–200 μ M) to identify regulatory pathways underlying embryogenic competence. Morphological assays showed that 20 μ M 2,4-D was the only treatment capable of inducing embryogenic callus by day 14, whereas supra-optimal doses delayed or impaired SE. A label-free quantitative proteomic analysis performed at seven days after induction identified 1168 proteins, of which 726 were differentially accumulated at the onset of callus induction. Clustering revealed six dose-dependent accumulation patterns, with high 2,4-D concentrations broadly repressing proteins associated with auxin signaling, proteasome-mediated turnover, methylation-related metabolism, proton transport and chromatin remodeling. In contrast, moderate 2,4-D levels preserved proton pump activity, maintained SCF-AUX/IAA signaling, supported GH3-mediated auxin conjugation, sustained proteasomal components and promoted SAM/SAHH-dependent methylation, collectively creating a molecular environment permissive to cellular reprogramming. At 200 μ M 2,4-D, the proteomic profile showed a coordinated repression of biosynthetic, redox and developmental pathways, as demonstrated by the under-enrichment of metabolic and energy-associated GO terms. Functional enrichment also revealed only limited activation of stress-associated categories, with hypoxia emerging as the sole enriched BP term. Together, GO and KEGG analyses indicate that supra-optimal auxin simultaneously suppresses core metabolic pathways and fails to activate broader adaptive stress programs, disrupting the interconnected functional modules required for embryogenic reprogramming. These findings demonstrate that SE in *C. papaya* is governed by a narrow auxin window that balances hormonal signaling, proteostasis, metabolic activity and chromatin regulation, while excessive auxin shifts the proteome toward a metabolically repressed state. This work establishes a mechanistic framework for optimizing auxin-based SE protocols in *C. papaya*.

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Graphical abstract



Key message

Optimal 2,4-D concentration promotes embryogenic callus formation by coordinating metabolic, proteomic, and epigenetic networks, while higher doses trigger stress and inhibit somatic embryogenesis.

Keywords Somatic embryogenesis · *Carica papaya* · Quantitative proteomics · Auxin signaling · Epigenetic regulation

Introduction

Somatic embryogenesis is a morphogenetic process analogous to zygotic embryogenesis, in which a single cell or a small group of somatic cells acts as precursors of embryos, capable of germinating and regenerating complete plants (Loyola-Vargas and Ochoa-Alejo 2016). Somatic embryos

can originate either directly from the explant, without an intermediate callus phase (direct somatic embryogenesis), or indirectly, following callus formation (indirect somatic embryogenesis). Developed under controlled and sterile in vitro conditions, somatic embryogenesis holds significant potential for application in various plant species (Fehér 2015; Gladfelter et al. 2021), although the optimization and deeper understanding of its underlying mechanisms remain

largely unexplored. Indirect somatic embryogenesis is regulated by a complex network of interactions between genes and proteins, making it a promising approach for efficient micropropagation and biotechnological applications, such as genetic engineering and genome editing (Heringer et al. 2018; Salaün et al. 2021). Among the compounds used to induce this process, 2,4-Dichlorophenoxyacetic acid (2,4-D) is an auxinic compound widely used in plant tissue culture to induce callus formation during somatic embryogenesis (Raghavan 2004; Peterson et al. 2016). 2,4-D can activate auxin signaling pathways, including the TIR1/AFB receptor-mediated pathway, which leads to the degradation of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors and the activation of auxin-responsive genes (Song 2014; Leyser 2018).

Beyond its positive role in callus induction and the promotion of embryogenic competence at moderate concentrations, high concentrations of 2,4-D are associated with genetic, epigenetic, and physiological disruptions, as well as morphological abnormalities in somatic embryos, often resulting in malformed structures (de Moraes Oliveira et al. 2023). Even at later developmental stages, residual 2,4-D during the maturation phase can negatively affect somatic embryo differentiation disturbing endogenous auxin balance and distribution, delaying the accumulation of storage reserves, and altering the abundance of key proteins involved in stress responses, hormone signaling, and reserve metabolism (Reis et al. 2021). These findings underscore the importance of optimizing 2,4-D concentrations to improve somatic embryogenesis efficiency while preserving proper embryo differentiation and development.

Micropropagation protocols, particularly those involving somatic embryogenesis, often rely on 2,4-D to establish embryogenic cell lines. Here, we investigated how different concentrations of 2,4-D are associated with distinct proteomic profiles during the early stages of somatic embryogenesis in *Carica papaya* L. To address this question, we performed a 2,4-D dose-dependent experiment and carried out a proteomic analysis after seven days of induction, evaluating molecular responses to each treatment. Our findings reveal that varying concentrations of 2,4-D are associated with differential accumulation of proteins linked to somatic embryogenesis, particularly those involved in hormonal signaling and epigenetic regulation, supporting their role in the acquisition of embryogenic competence. These insights provide valuable information for the optimization of in vitro propagation protocols for this species.

Materials and methods

Plant material and callus induction

Mature fruits of *C. papaya* cv. 'Golden' were washed under running water and surface-sterilized under aseptic conditions by immersion in 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 1 min, followed by 50% (v/v) commercial bleach (Qboa®, Anhembi SA, Osasco, Brazil) containing 1.25% sodium hypochlorite for 30 min. After three rinses with type 2, deionized, and autoclaved water, fruits were halved, and mature zygotic embryos were dissected and used as explants. For the induction experiment, embryos were cultured in Petri dishes containing 20 mL of Murashige and Skoog culture medium (Phytotechnology Lab, Overland Park, USA) (Murashige and Skoog 1962) supplemented with 30 g L⁻¹ sucrose (Labsynth, Diadema, Brazil) and 2,4-D (Sigma-Aldrich) (0, 0.2, 2, 20, or 200 µM), solidified with 2 g L⁻¹ Phytigel® (Sigma-Aldrich). The pH of the medium was adjusted to 5.8±0.1 and autoclaved at 121 °C for 15 min. Zygotic embryos were cultured on induction medium supplemented with the different 2,4-D treatments and incubated at 25±1 °C in darkness. For proteomic analysis, a subset of explants was collected at seven days after culture initiation to capture early molecular events associated with embryogenic reprogramming prior to visible morphological differentiation. Three biological replicates were prepared for each treatment, each consisting of pooled material from fifteen independent explants. The remaining explants were maintained in culture and monitored morphologically every seven days over a 42-day period.

Protein extraction and digestion

For proteomic analysis, explants corresponding to zygotic embryos cultured under the different 2,4-D treatments were collected at seven days after induction. Three biological replicates were prepared, each consisting of a combined sample of fifteen independent explants. The explants were macerated in 1.5 mL microtubes until fully homogenized. Subsequently, 300 µL of extraction buffer, comprising 7 M urea (Cytiva, PlusOne™, Sweden), 2 M thiourea (Cytiva), 2% Triton X-100 (Sigma-Aldrich), 1% dithiothreitol (Bio-Rad, Hercules CA, USA), and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich), was added to the powdered samples. Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) (Bradford 1976), with bovine serum albumin (BSA) as the standard, following procedures adapted for plant tissues. A blank containing only the extraction buffer was included to account for possible interference. Absorbance was measured at

595 nm using a microplate spectrophotometer, and protein concentration was calculated based on the standard curve.

A total of 100 µg of protein from each sample was used for digestion. Protein precipitation was performed using the methanol/chloroform method to remove interfering compounds and detergent contaminants, according to Nanjo et al. (2012). The resulting protein pellets were resuspended in a denaturing buffer containing 7 M urea and 2 M thio-urea. Tryptic digestion was carried out following the filter-aided sample preparation (FASP) protocol described by Wiśniewski et al. (2009), with modifications introduced by Xavier et al. (2025). Briefly, protein samples were double-desalted on Microcon-30 kDa centrifugal filters (Merck Millipore, Darmstadt, Germany) using 50 mM ammonium bicarbonate (Sigma-Aldrich), pH 8.5. Reduction was performed by incubating the proteins at 60 °C for 20 min in 100 µL of 50 mM dithiothreitol prepared in 8 M urea and 50 mM ammonium bicarbonate. After reduction and cooling, 200 µL of 8 M urea in 50 mM ammonium bicarbonate were added, and proteins were alkylated for 20 min at 25 °C in the dark with 100 µL of 50 mM iodoacetamide (Sigma-Aldrich) prepared in the same buffer. The filters were then washed twice with 8 M urea and 50 mM ammonium bicarbonate, followed by three washes with 50 mM ammonium bicarbonate to remove residual reagents. Protein digestion was performed overnight (18 h) at 37 °C using sequencing-grade trypsin (V5111, Promega, Madison, WI, USA) at a 1:100 enzyme-to-protein ratio, in the presence of 0.2% RapiGest (Waters, Manchester, UK). To inactivate trypsin and precipitate RapiGest, 5 µL of 15% trifluoroacetic acid (Sigma-Aldrich) was added to reach a final concentration of 0.5%, followed by incubation for 30 min at 37 °C. The resulting peptides were vacuum-dried and resuspended at a final concentration of 0.7–1 µg µL⁻¹ in 50 µL of a solution containing 5% (v/v) LC-MS-grade acetonitrile (Thermo Fisher Scientific, Waltham, MA, USA) and 0.1% (v/v) formic acid (Sigma-Aldrich) in LC-MS-grade water. Peptide concentrations were determined by measuring absorbance at A205 nm using a NanoDrop One^c spectrophotometer (Thermo Fisher Scientific), and samples were directly injected into the mass spectrometer for LC-MS/MS analysis.

Mass spectrometry analysis

Mass spectrometry analysis was performed based on the parameters described by Passamani et al. (2018). A nano-Acquity UPLC M-class (Waters, Manchester, UK) coupled to a Synapt G2-Si mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI)-MS/MS source was used. A total of 2 µg of peptides was loaded onto a ACQUITY UPLC M-Class Symmetry C18 Trap (100 Å, 5 µm, 180 µm × 20 mm, 2D; Waters) at 5

µL min⁻¹ for 3 min, and then onto an ACQUITY UPLC M-Class HSS T3 analytical reversed-phase column (100 Å, 1.8 µm, 75 µm × 150 mm; Waters) at 400 nL min⁻¹ heated to 45 °C. Peptide separation was performed using a binary gradient elution system. Mobile phase A consisted of MS-grade water with 0.1% formic acid, while mobile phase B contained MS-grade acetonitrile with 0.1% formic acid. The gradient began at 5% B and was linearly increased to 40% B over 92 min. From 92 to 96 min, the concentration of phase B was ramped up to 99% and held constant until 100 min. It was then reduced back to 5% by 102 min and maintained at this composition until the end of the run at 118 min. The mass spectrometer was operated in positive ion mode, with resolution set to 35,000 FWHM (V-mode) and data-independent acquisition (DIA) enabled via ion mobility separation (HDMSE). The ion mobility wave velocity was programmed from 800 to 500 m s⁻¹. Collision energy in high-energy mode was ramped from 25 to 55 V. The cone voltage was set to 40 V, and the capillary voltage to 2.8 kV. Additional source parameters included a nano-flow gas pressure of 0.5 bar, a purge gas flow rate of 150 L h⁻¹, and a source temperature of 80 °C. For the time-of-flight (TOF) acquisition, the scan time was set at 0.6 s in continuum mode, covering an m/z range from 50 to 2000 Da. External calibration was performed using human [Glu1]-fibrinopeptide B at 100 fmol µL⁻¹, with lock mass correction applied every 30 s. Data acquisition was carried out using MassLynx version 4.1 (Waters).

Proteomics data analysis

The MS/MS spectra processing and database search were performed using ProteinLynx Global SERVER (PLGS) software v.3.02 (Waters) against the *C. papaya* ASGPBv0.4 protein bank from Phytozome (<https://phytozome.jgi.doe.gov>, accessed on 30 August 2024). The raw data processing settings included a low-energy threshold of 150 counts, a high-energy threshold of 50 counts, and an intensity threshold of 750 counts. Additionally, the analysis was performed with the following parameters: two missed cleavages, at least three fragment ions per peptide, a minimum of seven fragment ions per protein, a minimum of two peptides per protein, fixed carbamidomethyl (C) modifications, and variable methionine oxidation and phosphoryl (STY) modifications. The false discovery rate (FDR) for peptide and protein identification was set to a maximum of 1%, with a minimum peptide length of six amino acids and automatic peptide and fragment mass tolerance. Label-free quantification analyses were performed using the TOP3 quantification approach, followed by the multidimensional normalization process implemented within ISOQuant software v.1.7 (Distler et al. 2014). The mass spectrometry proteomics

data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2025) with the dataset identifier PXD072983. To ensure data reliability, only proteins consistently present in all three biological replicates were considered for differential accumulation analysis. Proteins were classified as differentially accumulated proteins (DAPs) if they showed a statistically significant difference in abundance based on Student's t-test (two-tailed, $p < 0.05$) and a $\log_2$ fold change (FC) greater than 0.58 (upregulated) or less than -0.58 (downregulated). In addition, proteins that were exclusively detected in one condition (i.e., consistently present in all replicates of one treatment and completely absent in the other) were also considered differentially accumulated, as they reflect

condition-specific expression. All pairwise comparisons among the five 2,4-D concentrations (0, 0.2, 2, 20, and 200 μM) were performed, totaling ten differential analyses.

Following protein identification, their ontology and biological functions were verified using UniProt and TAIR (last accessed on 10 September 2024). *Arabidopsis thaliana* orthologs of DAPs were used for enrichment analysis ($p\text{-value} < 0.05$) of biological processes (BP), cellular components (CC), molecular functions (MF), and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways via the g: Profiler tool (Kolberg et al. 2023). These orthologs were also employed to predict protein-protein interaction networks using STRING v12 database (Szklarczyk et al. 2023), and to identify kinases, transcription factors, and transcriptional regulators with iTAK (Zheng et al. 2016). The Mfuzz package (Kumar and Futschik 2007) was utilized for clustering analysis of DAPs detected in at least one comparison among the studied induction treatments.

Protein-protein interaction (PPI) analysis was performed as described by Pacheco et al. (2023). In brief, the Cytoscape 3.10.4 (Shannon et al. 2003) StringApp plugin was used to identify associations among differentially expressed proteins, employing a confidence score cutoff of 0.700 to visualize the PPI network. The MCODE (Molecular Complex Detection) application was used to extract highly connected protein clusters (modules) from the network, applying a degree cutoff ≥ 3 while keeping other parameters at default settings (node score cutoff ≥ 2 , K-Core ≥ 2 , and maximum depth = 100). Finally, the CytoHubba plugin was employed to extract the top 10 hub proteins from the PPI network using the Maximum Clique Centrality (MCC) ranking method, which calculates the degree of each protein node (Bader and Hogue 2003).

Results

Morphological responses of *C. papaya* explants induced by 2,4-D

The auxinic herbicide 2,4-D is widely used to induce somatic embryogenesis in plants with well-documented dose-dependent effects. To expand this understanding, we evaluated the impact of varying concentrations of 2,4-D on the early morphological responses of zygotic embryo explants. The morphological characterization during the initial stages of in vitro culture, prior to visible callus formation, is shown in Fig. 1. At time zero, explants exhibited intact cotyledons and a well-formed embryonic axis (Fig. 1a). After seven days, explants without 2,4-D showed normal germination with root hair development (Fig. 1b), while explants treated with 0.2 and 2 μM 2,4-D exhibited partial inhibition of

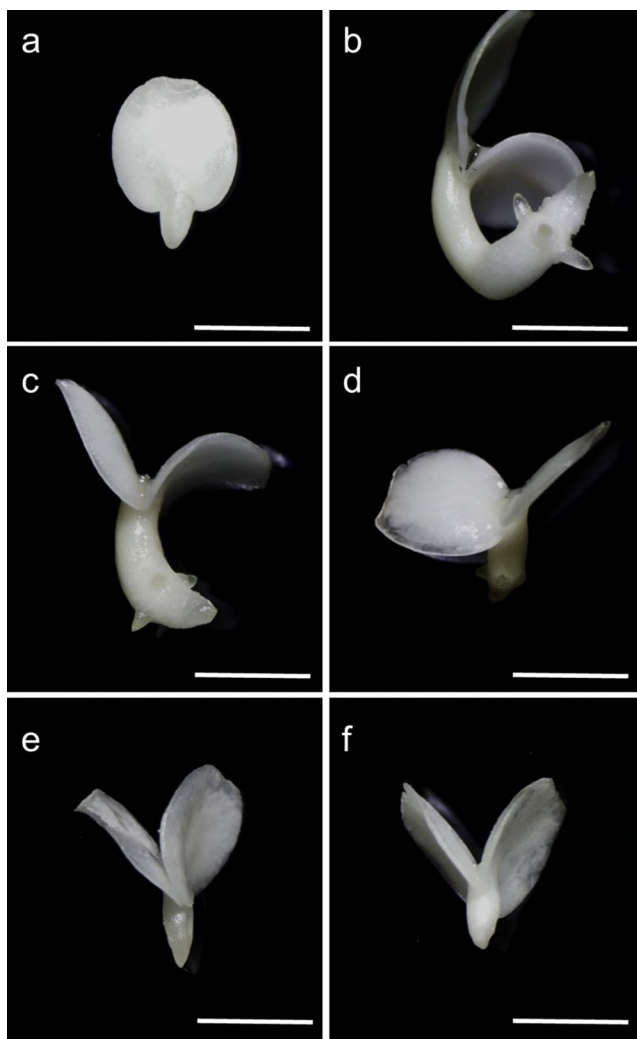


Fig. 1 Morphological changes in *C. papaya* explants during somatic embryogenesis induction under different 2,4-D concentrations. (a) Zygotic embryo immediately after dissection from the seed (initial state, time 0); (b) seven days of induction without 2,4-D (control); (c) seven days of induction with 0.2 μM 2,4-D; (d) seven days of induction with 2 μM 2,4-D; (e) seven days of induction with 20 μM 2,4-D; (f) seven days of induction with 200 μM 2,4-D. Scale bars: 4 mm

germination (Fig. 1c-d, respectively). Explants exposed to 20 μ M 2,4-D began to display early signs of developmental redirection, characterized by cotyledon opening and inhibition of radicle protrusion, indicative of a deviation from germinative growth (Fig. 1e). Although no macroscopically visible callus had formed at this stage, explants treated with 20 and 200 μ M 2,4-D exhibited a distinct morphological profile suggestive of early cellular reprogramming (Fig. 1e-f, respectively). Cotyledon opening and the absence of root development were observed in both 20 and 200 μ M 2,4-D treatments at day seven (Fig. 1e-f). Visible callus formation was confirmed only in 20 μ M 2,4-D treatment around day 14, whereas no such formation was detected under 200 μ M 2,4-D at the same time point (Supplementary Fig. S1). Explants exposed to 200 μ M 2,4-D exhibited a morphological profile similar to that of the 20 μ M 2,4-D treatment at day seven (Fig. 1e) but failed to initiate visible callus formation by day 14 (Supplementary Fig. S1). By day 14, callus formation was restricted to explants treated with 20 μ M 2,4-D, whereas explants under 200 μ M 2,4-D showed no evidence of callogenesis despite presenting a similar early morphology. This divergence illustrates a differential response associated with auxin concentration during the induction period.

Without 2,4-D, embryo germination and seedling development proceeded normally, with consistent growth throughout the period. Under low concentrations of 2,4-D (0.2 and 2 μ M), germination occurred but was progressively impaired over time. This led to stunted seedling development and no apparent callus formation, reflecting insufficient hormonal stimulation by these suboptimal auxin doses (Supplementary Fig. S1). In the 20 μ M 2,4-D treatment, initial signs of callus formation were observed around day 14, when the explants progressively developed compact, brownish structures characteristic of embryogenic callus. By day 21, the explants had fully transitioned into callus masses, with no residual morphological features of their zygotic embryo origin. These tissues continued to grow and mature until day 42, maintaining a dense and organized appearance typical of embryogenic callus morphology.

At the highest 2,4-D concentration tested (200 μ M), explants began forming callus-like structures only around day 21. These structures were translucent and whitish, contrasting with the compact, brownish calli formed under the 20 μ M 2,4-D treatment. Moreover, remnants of the original zygotic embryo, such as persistent cotyledons, remained clearly visible, indicating incomplete cellular reprogramming. By day 42, explants treated with 200 μ M 2,4-D had fully transformed into callus, yet these tissues continued to lack the structural integrity and morphological features observed in 20 μ M 2,4-D treatment at the same time point. The callus structures formed under 200 μ M 2,4-D remained translucent and disorganized, retaining visible remnants of

the original zygotic tissue, in contrast to the compact, embryogenic calli with an opaque brown coloration observed under 20 μ M 2,4-D (Supplementary Fig. S1). Taken together, the morphological analyses presented in Fig. 1 and Supplementary Fig. S1 indicate that explants treated with 20 μ M 2,4-D initiate visible embryogenic callus formation around day 14. Therefore, to investigate the molecular changes that precede visible callogenesis and are likely associated with the acquisition of embryogenic competence, day seven after induction was selected for proteomic analysis, representing an early stage of cellular reprogramming prior to detectable morphological differentiation.

The explant proteome is modulated by 2,4-D in a dose-dependent manner

A bottom-up proteomic analysis was conducted at seven days after induction using explants collected from all tested 2,4-D concentrations (0, 0.2, 2, 20, and 200 μ M) to examine proteomic responses associated with auxin dose variation at the explant level. This approach revealed dose-dependent alterations in the proteome, leading to the identification and quantification of 1168 *C. papaya* proteins (Supplementary Fig. S2a; Supplementary Table S1). After statistical and fold-change analysis, 726 proteins were classified as DAPs in at least one of the ten possible pairwise comparisons among the 2,4-D treatments, reflecting extensive proteomic reprogramming at the explant level under different auxinic conditions. Principal component analysis (PCA) showed a clear separation of samples according to 2,4-D concentration, indicating that each treatment induced a distinct proteomic profile in the explants (Supplementary Fig. S2b). All DAPs were further annotated via BLASTp against *A. thaliana* proteins in TAIR and grouped according to their Mfuzz expression clusters (Supplementary Table S2).

Among the DAPs, the main biological processes (BP) regulated by 2,4-D included protein folding, amide biosynthetic process, translation, amide and peptide metabolic process (Supplementary Fig. S3a). Among the cellular components (CC), DAPs were mainly regulated in ribosomes and cytosol (Supplementary Fig. S3b). The molecular functions (MF) enriched among DAPs comprised unfolded protein binding, structural molecule activity, copper binding, ATP-dependent chaperone activity, and GTPase activity (Supplementary Fig. S3c). It was also observed that 2,4-D mainly regulated the metabolic pathways of carbon metabolism, amino acid and pyruvate biosynthesis, translation, and proteasome activity (Supplementary Fig. S3d). Together, these enriched pathways suggest that 2,4-D triggers broad metabolic and translational reprogramming in the explants during the early stages of embryogenic induction.

Enhanced translation-related activity supports proteome reprogramming during early embryogenic induction

Among the upregulated DAPs identified under 20 μ M 2,4-D relative to the 0 μ M control, enrichment analysis revealed the predominance of translation-related processes and ribosome biogenesis (Fig. 2). Within the BP category, two terms reached significance, namely translation and response to heat (Fig. 2a). Both exhibited low gene ratio values. In the CC category (Fig. 2b), enriched terms were mainly associated with ribosome-related compartments, including ribosome, cytosolic large ribosomal subunit, and nucleolus. The MF analysis (Fig. 2c) showed enrichment for mRNA binding, GTP binding, and structural constituent of ribosome, functions typically associated with translational activity. Additionally, nutrient reservoir activity was identified among the enriched terms. KEGG pathway analysis (Fig. 2d) revealed a single significantly enriched pathway, Ribosome, with a

gene ratio of ≈ 0.041 , confirming the predominance of translation-related proteins among the upregulated group.

Repression of biosynthetic and developmental pathways under supra-optimal auxin concentration

GO and KEGG enrichment analyses were performed for proteins downregulated under 200 μ M 2,4-D relative to 20 μ M (Fig. 3). In the BP ontology (Fig. 3a), significantly depleted terms included nucleoside phosphate metabolism (gene ratio ≈ 0.057), carboxylic acid biosynthetic process (≈ 0.05), serine family amino acid metabolism (≈ 0.15), and sulfur compound metabolism (≈ 0.057). Embryo development (≈ 0.05), cytoplasmic translation initiation (≈ 0.20), and response to cold (≈ 0.05) were also among the significantly downregulated processes. In the CC category (Fig. 3b), downregulated proteins were associated mainly with the cytoplasm (≈ 0.025), cytosol (≈ 0.060), and intracellular anatomical structures (≈ 0.020). Terms related to

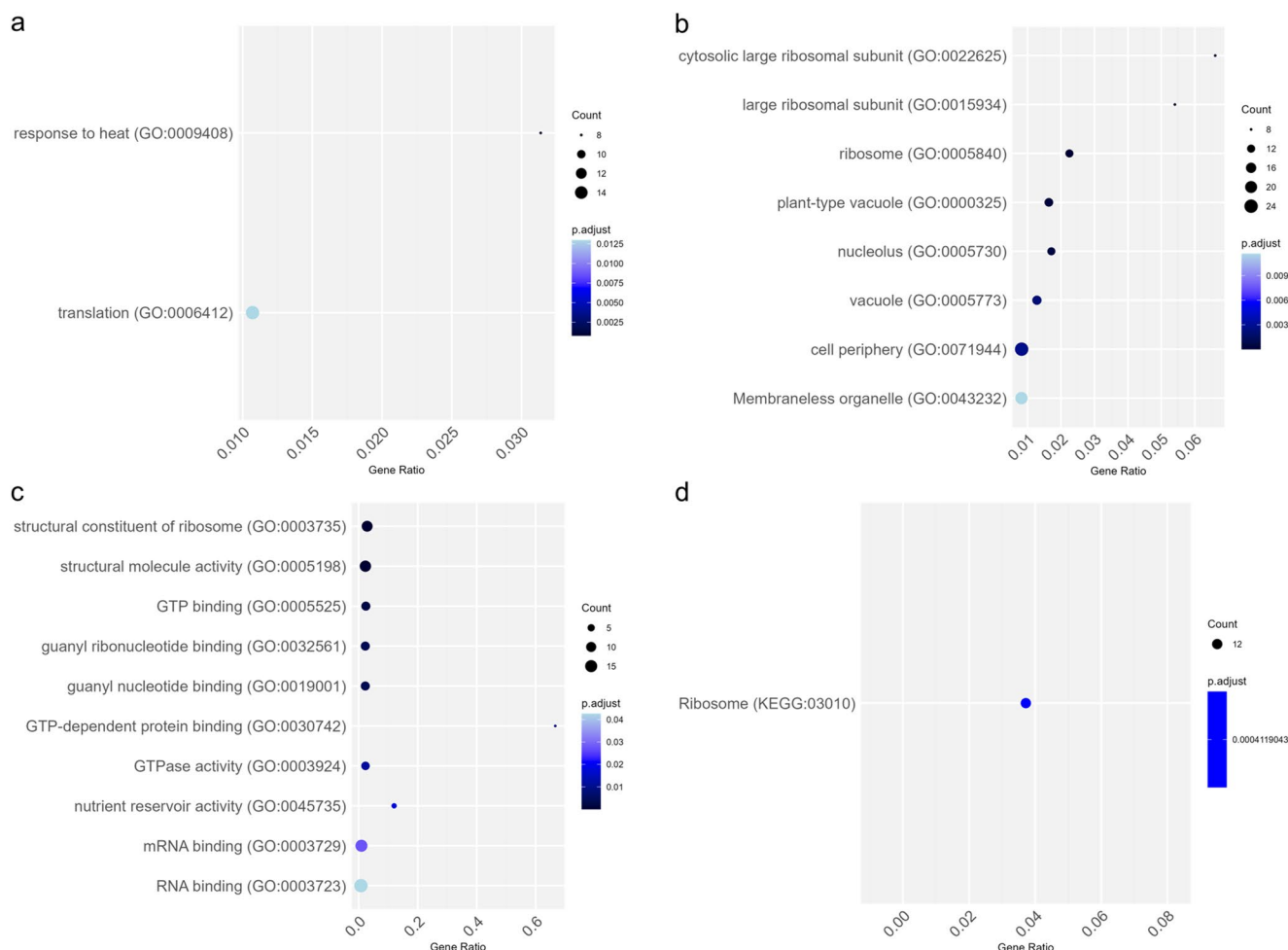


Fig. 2 Functional enrichment of upregulated proteins in the 20/0 μ M 2,4-D comparison. GO and KEGG enrichment analysis of proteins with increased accumulation under 20 μ M 2,4-D relative to the control

(0 μ M). Bubble plots display the top enriched terms from each ontology: **(a)** BP, **(b)** CC, **(c)** MF, and **(d)** KEGG. Bubble size and color reflect enrichment statistics

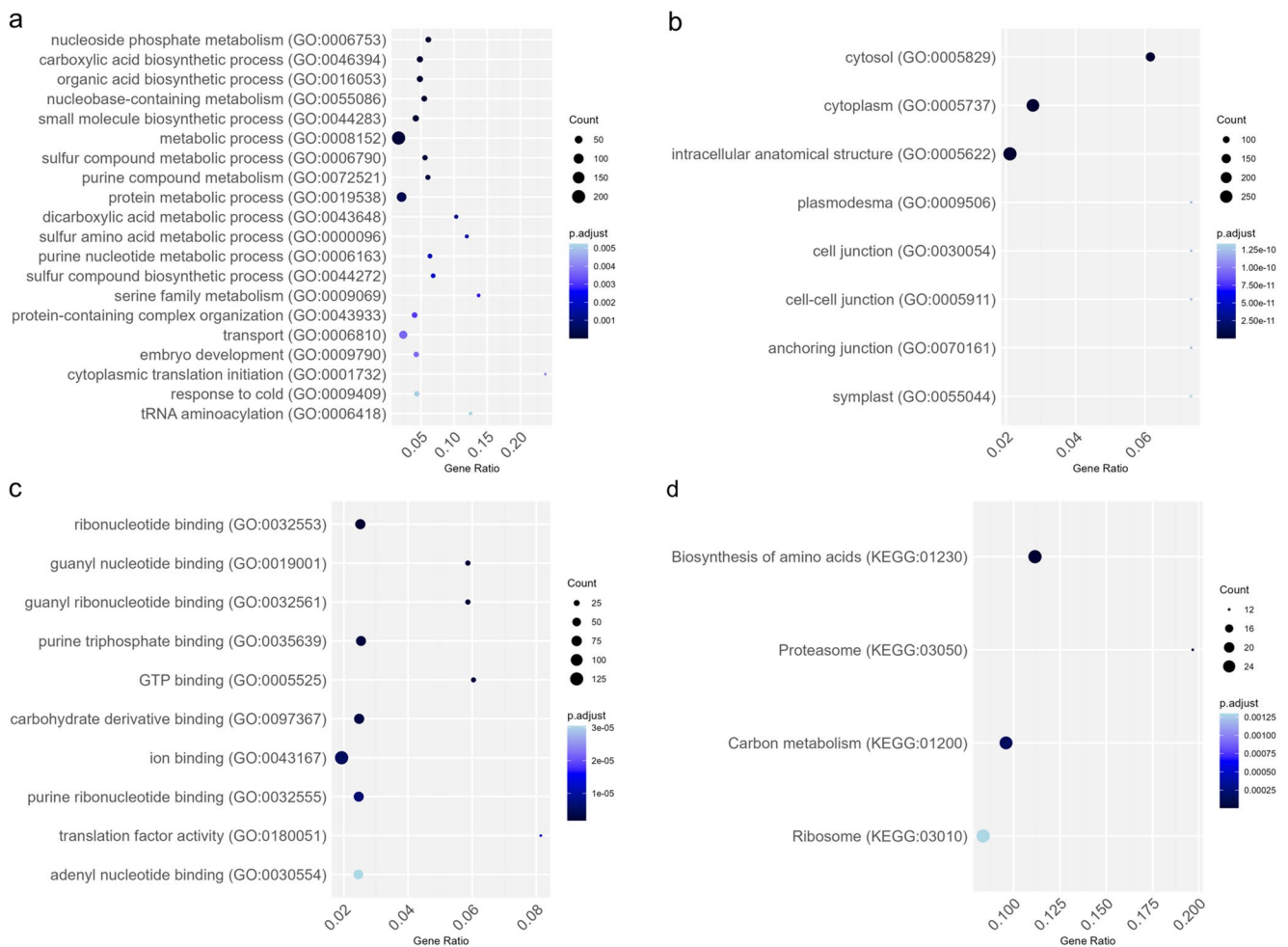


Fig. 3 Functional enrichment of downregulated proteins in the 200/20 μM 2,4-D comparison. GO and KEGG enrichment analysis of proteins with reduced accumulation under the supra-optimal 2,4-D concentra-

tion (200 μM) relative to the optimal treatment (20 μM). Bubble plots display the top enriched terms from each ontology: **(a)** BP, **(b)** CC, **(c)** MF, and **(d)** KEGG. Bubble size and color reflect enrichment statistics

plasmodesma, symplast, and cell junction (all >0.065) were also depleted. Within the MF ontology (Fig. 3c), reduced representation was observed for ribonucleotide binding (≈ 0.023), GTP binding (≈ 0.060), translation factor activity (≈ 0.080), and ion binding (≈ 0.020). KEGG pathway analysis (Fig. 3d) identified significant depletion in biosynthesis of amino acids (≈ 0.114), carbon metabolism (≈ 0.100), and proteasome (≈ 0.200). The ribosome pathway also appeared downregulated.

Limited activation of hypoxia-responsive proteins at supra-optimal auxin concentration

The analysis of upregulated proteins in the 200/20 comparison revealed a restricted functional profile, with enrichment confined to the BP ontology (Fig. 4). No significant terms were identified in the MF, CC, or KEGG categories. Within the BP ontology, enriched terms were primarily associated with oxygen deprivation responses, including cellular

response to hypoxia and cellular response to decreased oxygen levels (≈ 0.0150), cellular response to oxygen levels and response to oxygen levels (≈ 0.0065), and response to hypoxia (≈ 0.0151). These terms indicate that the upregulated protein set at supra-optimal 2,4-D concentration is mainly associated with hypoxia-related processes. A complete list of all significant GO and KEGG terms identified across the three contrasts, including full BP, CC, MF and pathway categories, is provided in Supplementary Table S3.

Clustering analysis reveals six dose-dependent protein accumulation patterns induced by 2,4-D

Clustering analysis using the MFUZZ algorithm revealed six distinct protein accumulation patterns in response to varying 2,4-D concentrations applied to *C. papaya* explants derived from zygotic embryos (Fig. 5). These clusters represent dose-sensitive regulation profiles, highlighting the proteomic reprogramming triggered by auxin during somatic

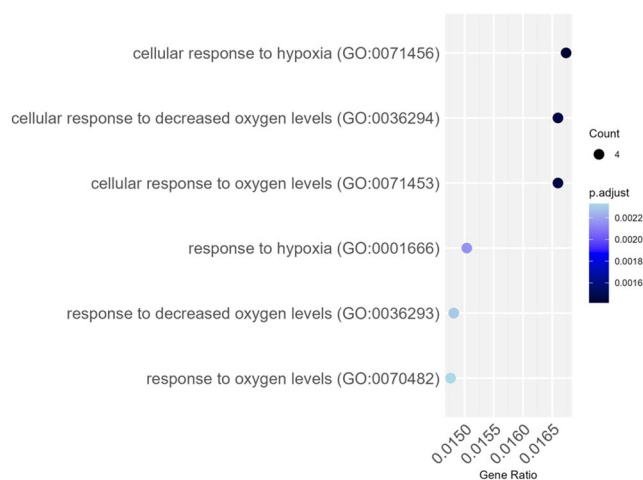


Fig. 4 Functional enrichment of upregulated proteins in the 200/20 μM 2,4-D comparison. Only BP terms reached significance, whereas no enrichment was detected for CC, MF, or KEGG. Bubble size and color reflect enrichment metrics. The enriched BP terms indicate an oxygen-deprivation-related response under supra-optimal auxin concentration

embryogenesis induction. Cluster 1, which included 68 proteins, showed low accumulation in the absence of 2,4-D, followed by a sustained increase that was maintained across all 2,4-D concentrations. Cluster 2, comprising 44 proteins, displayed a fluctuating expression pattern, with variable abundance across treatments and a pronounced peak under the highest auxin concentration. Cluster 3 grouped 204 proteins that maintained high abundance in the control and at lower auxin concentrations (0.2 and 2 μM), remained stable at 20 μM , but showed a marked decline at 200 μM . Cluster 4, with 110 proteins, exhibited a progressive and continuous decrease in abundance as auxin concentration increased from 0 to 200 μM . Cluster 5, containing 107 proteins, presented a bell-shaped pattern, characterized by low accumulation without 2,4-D, increased abundance at 0.2, 2, and 20 μM , and strong repression at 200 μM . Finally, cluster 6, which included 193 proteins, showed high abundance in the absence of 2,4-D but progressively decreased as the concentration increased, culminating in strong repression at 200 μM . Overall, clusters 3, 4, 5, and 6, which represented the majority of DAPs, were characterized by repression at 200 μM 2,4-D, whereas clusters 1 and 5 highlighted specific protein accumulation under intermediate auxin concentrations, particularly at 20 μM . These distinct accumulation profiles underscore the dose-dependent modulation of the papaya explant proteome by 2,4-D.

Regulatory network analysis of transcription factors and differentially accumulated proteins in response to 2,4-D

The effects of 2,4-D are closely associated with the modulation of regulatory proteins, such as transcription factors and chromatin remodelers. Eight regulatory proteins were identified in the proteomic dataset (Table 1). Three of these proteins exhibited differential accumulation under at least one 2,4-D treatment, as indicated by their cluster assignments (Fig. 5). These included the histone deacetylase (HDT3; *evm.model.supercontig_5.85*) (cluster 3) and two transcription factors putatively corresponding to Cold shock domain-containing protein (CSP3; *evm.model.supercontig_6.261* and CSP4; *evm.model.supercontig_37.170*) and Zinc finger CCCH domain-containing protein 52 (MPH15.13; *evm.model.supercontig_235.5*) (cluster 5). Functional analysis of DAPs regulated by 2,4-D treatments, specifically in the comparison between 20 μM (optimal for embryogenic callus induction) and 200 μM (embryogenic competence inhibition) 2,4-D, enabled the identification of additional proteins potentially associated with 2,4-D-mediated embryogenic callus induction (Table 2). These candidates likely contribute to transcriptional regulation and signaling cascades in response to auxin concentration gradients.

Protein-protein interactions and predicted hub genes associated with embryogenic callus induction

Given the broad impact of 2,4-D treatments on the explant proteome, we searched for predicted protein-protein interactions (PPIs) among the DAPs. This analysis resulted in a network comprising 630 nodes and 3605 edges, enabling the investigation of protein interaction modules responsive to 2,4-D (Supplementary Fig. S4). In addition, we integrated the clustering analysis data into the predicted interaction network to visualize biological processes potentially regulated by proteins co-accumulated at specific 2,4-D concentrations. After constructing the predicted interaction network among all DAPs, the MCODE algorithm was applied to identify highly interconnected nodes that may represent molecular complexes regulated in a dose-dependent manner by 2,4-D. As a result, 19 complexes were identified, each comprising DAP groups enriched in distinct biological processes (Supplementary Fig. S5). Each of these modules exhibited predicted interactions between proteins associated with specific pathways modulated by 2,4-D, with the largest complexes being linked to gene expression, proteasome-mediated protein catabolism, energy metabolism, protein folding, and small-molecule metabolic processes. Furthermore, these proteins formed interconnected networks bridging effectors of hormonal responses, transcriptional regulation, protein

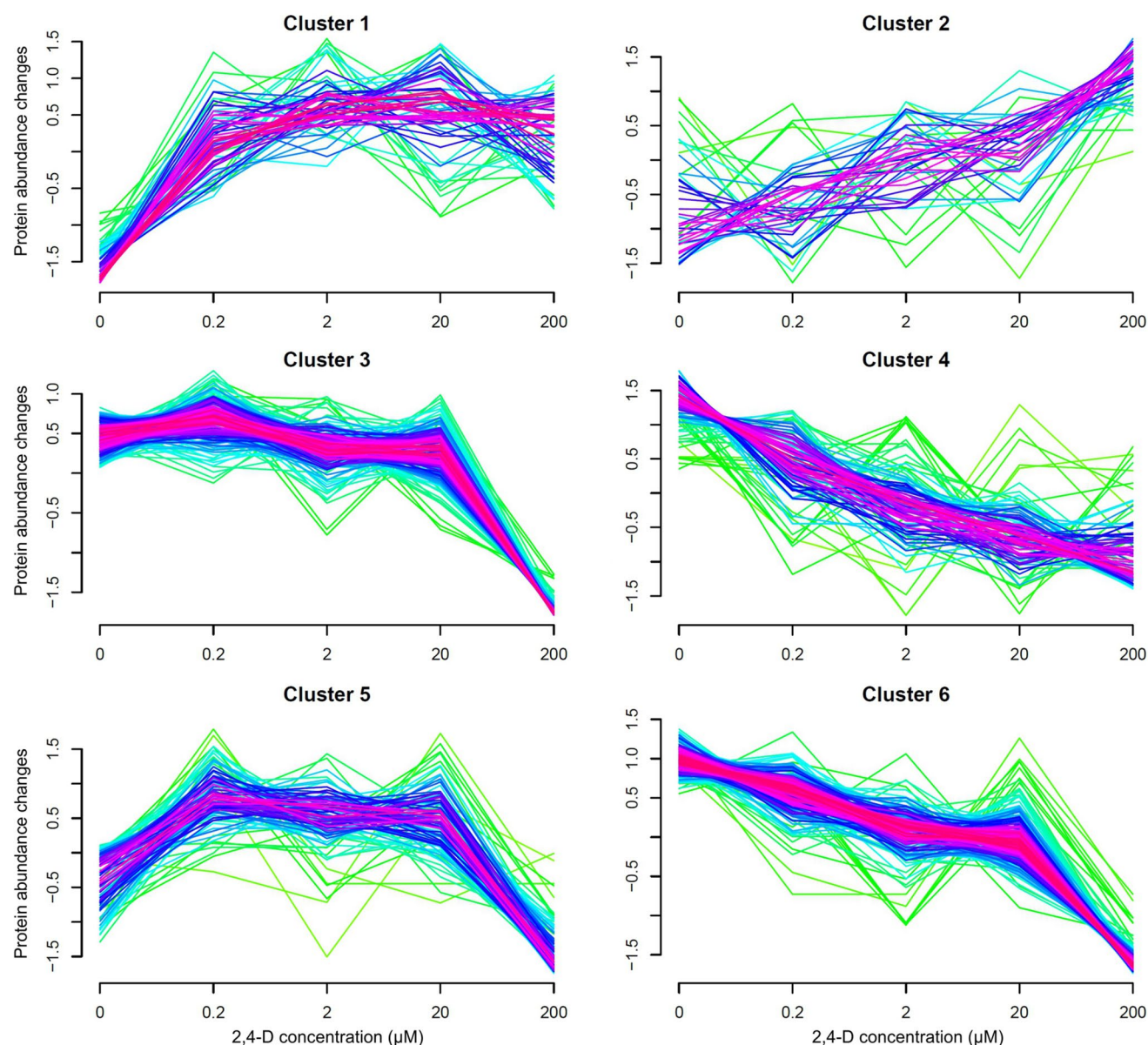


Fig. 5 Dose-dependent proteomic response to 2,4-D in *C. papaya* explants. Cluster analysis of DAPs revealed distinct response patterns across the 2,4-D concentration gradient. Clusters 1 and 2 show

increased protein accumulation, whereas clusters 3, 4, and 6 exhibit decreased accumulation. Cluster 5 displays a unique response at intermediate doses

Table 1 List of transcription regulators identified in proteomic analysis

Protein ID	BLASTp Annotation	Class	Type	Cluster
evm.model.supercontig_5.85	Histone Deacetylase 3	C2H2	TF	3
evm.model.supercontig_235.5	Zinc finger CCCH domain-containing protein 52	C3H	TF	5
evm.model.supercontig_37.170	Cold shock domain-containing protein 4	CSD	TF	5
evm.model.supercontig_72.55	Ribosomal protein L30/L7 family protein	CDK	Kinase	5
evm.model.supercontig_235.4	Zinc finger CCCH domain-containing protein 36	C3H	TF	-
evm.model.supercontig_30.148	Coatomeer subunit delta	MADS-MIKC	TF	-
evm.model.supercontig_6.261	Cold shock domain-containing protein 3	CSD	TF	-
evm.model.supercontig_77.11	Multiprotein-bridging factor 1b	MBF1	TR	-

The column with the cluster information is associated with the classification in Fig. 5

Table 2 List of differentially accumulated proteins regulated by 2,4-D associated with the embryogenic callus induction in *C. papaya*

Protein ID	BLASTp Annotation	Gene symbol (<i>A. thaliana</i>)	Cluster
evm.model.supercontig_70.83	V-type proton ATPase subunit C	VHA-C	6
evm.model.supercontig_122.22	V-type proton ATPase subunit E1	VHA-E1	5
evm.model.supercontig_666.2	V-type proton ATPase subunit G1	VHA-G1	6
evm.model.supercontig_43.106	V-type proton ATPase subunit H	VHA-H	4
evm.model.supercontig_26.289	SKP1-like protein 1A	SKP1A	4
evm.model.supercontig_20.89	SKP1-like protein 1B	SKP1B	6
evm.model.supercontig_6.295	SKP1-like protein 1B	SKP1B	4
evm.model.supercontig_173.13	Cullin-associated NEDD8-dissociated protein 1	CAND1	3
evm.model.supercontig_36.169	14-3-3-like protein GF14 omega	GRF2	6
evm.model.supercontig_309.6	14-3-3-like protein GF14 kappa	GRF8	6
evm.model.supercontig_1.115	14-3-3-like protein GF14 iota	GRF12	6
evm.model.supercontig_179.17	14-3-3-like protein GF14 psi	GRF3	3
evm.model.supercontig_36.140	14-3-3-like protein GF14 omicron	GRF11	1
evm.model.supercontig_13.7	Serine/threonine-protein phosphatase 2 A 65 kDa regulatory subunit A alpha isoform	PP2AA1	3
evm.model.supercontig_85.31	Serine/threonine-protein phosphatase PP2A-4 catalytic subunit	PP2A4	3
evm.model.supercontig_1.195	Serine/threonine-protein phosphatase 2 A 65 kDa regulatory subunit A beta isoform	PP2AA2	3
evm.model.supercontig_13.7	Serine/threonine-protein phosphatase 2 A 65 kDa regulatory subunit A alpha isoform	PP2AA1	3
evm.model.supercontig_6.74	Indole-3-acetic acid-amido synthetase GH3.3	GH3.3	1
evm.model.supercontig_292.1	Probable indole-3-acetic acid-amido synthetase GH3.1	GH3.1	1
evm.model.supercontig_146.36	S-adenosylmethionine synthase 4	METK4	6
evm.model.supercontig_67.71	S-adenosylmethionine synthase	SAM2	4
evm.model.supercontig_102.23	S-adenosylmethionine synthase 3	METK3	1
evm.model.supercontig_157.46	Adenosylhomocysteinase 1	SAHH1	3
evm.model.supercontig_5.85	Histone deacetylase HDT3	HDT3	3
evm.model.supercontig_599.1	Histone deacetylase HDT3	HDT3	3

translation, and energy metabolism through hub proteins (Supplementary Fig. S6). A representative high-confidence PPI subnetwork highlighting these hubs and their associated functional groups is shown in Fig. 6.

Within this context, the ten hub proteins predicted in the complete interaction network were ribosomal proteins, including RPS6B (evm.model.supercontig_209.28), RPS28C (evm.model.supercontig_1.384), RPS3AA (evm.model.supercontig_58.59), RPS5A (evm.model.supercontig_103.75), RPS7A (evm.model.supercontig_3.500), RPS8B (evm.model.supercontig_12.207), RPS10C (evm.model.supercontig_107.113), RPS7B (evm.model.supercontig_2.174), and RPL4A (evm.model.supercontig_10.79). Following functional annotation of the DAPs, prediction of transcriptional regulators, and network integration, we established a list of DAPs associated with embryogenic callus induction in response to 2,4-D (Table 2). These DAPs participate in diverse biological processes, with predicted PPI involving epigenetic modulation (SAHH1; evm.model.supercontig_157.46), hormone response (EBP1; evm.model.supercontig_120.60), stress response (HSP70-4; evm.model.supercontig_1.398), and proteasome-mediated degradation (RPN10; evm.model.supercontig_37.159, and SKP1A; evm.model.supercontig_26.289) (Fig. 6). Additionally, predicted interactions were identified among proteins

regulated by 2,4-D that are involved in S-adenosylmethionine (SAM) metabolism (METK3; evm.model.supercontig_102.23, METK4; evm.model.supercontig_146.36, SAM2; evm.model.supercontig_67.71, and SAHH1; evm.model.supercontig_157.46), cell division and proteasome function (CDC48D; evm.model.supercontig_65.33, RPN10; evm.model.supercontig_37.159 and evm.model.supercontig_6.273, RPN6; evm.model.supercontig_83.90, RPN8A; evm.model.supercontig_163.7, and RPT3-2; evm.model.supercontig_2.273), and auxin-associated chaperone (P23-1; evm.model.supercontig_9.223).

Discussion

As expected, zygotic embryos without 2,4-D exhibited normal germination and radicle protrusion (Fig. 1b), whereas intermediate doses (0.2 and 2 μ M) partially inhibited these processes without inducing visible callus formation (Fig. 1c-d; Supplementary Fig. S1). In contrast, 20 μ M 2,4-D fully suppressed radicle elongation and germination, resulting in the emergence of embryogenic calli by day 14 (Fig. 1e; Supplementary Fig. S1). At the highest 2,4-D concentration (200 μ M), callus formation was delayed until day 21, and the resulting calli lacked typical embryogenic

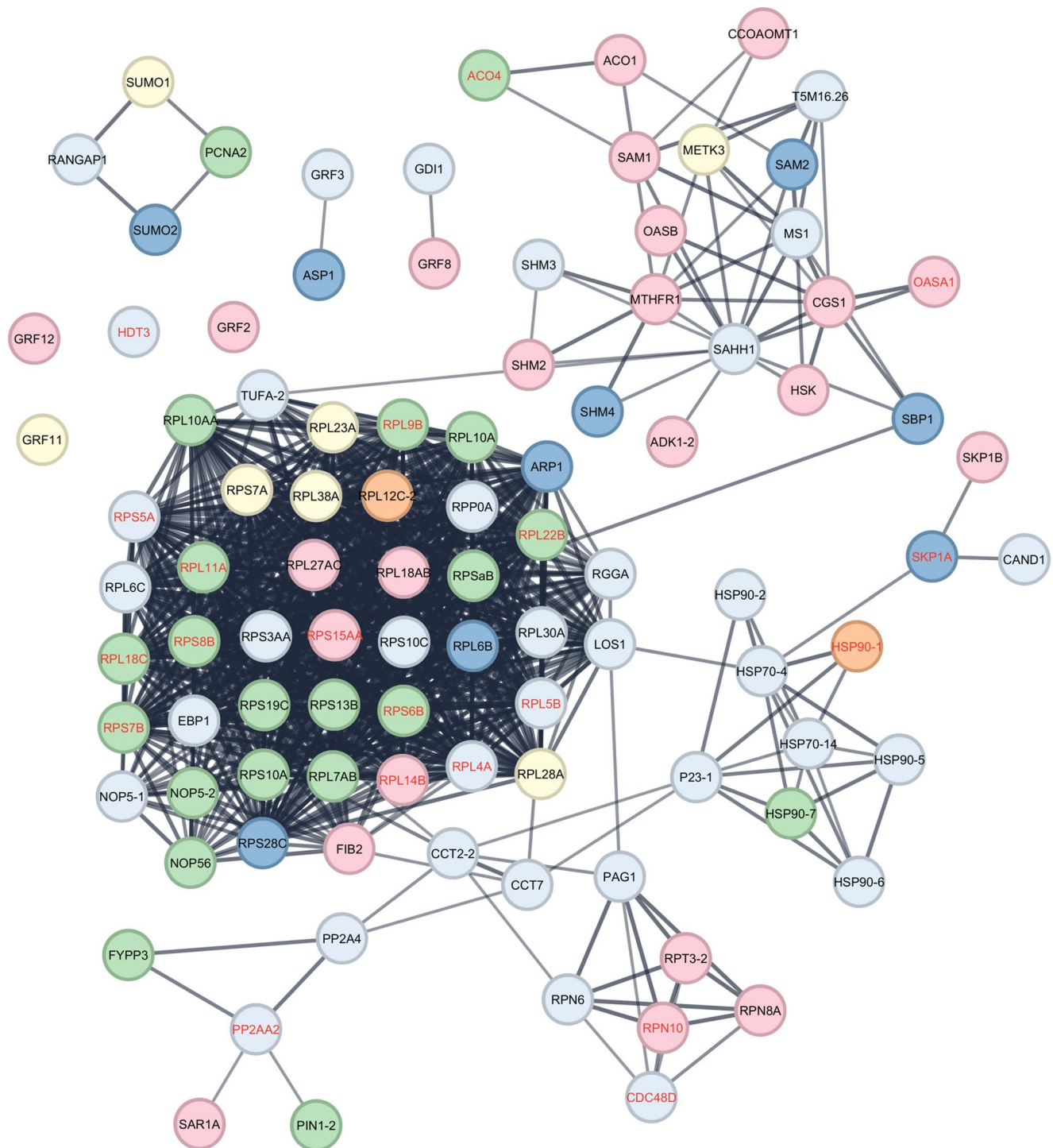


Fig. 6 Protein-protein interaction network of proteins associated with embryogenic callus induction mediated by 2,4-D in *C. papaya*. Network generated in Cytoscape via stringApp, with confidence cut-off=0.7. Edges are shown in confidence view, edge thickness and opacity scale with the STRING combined score. Names indicate Arabidopsis proteins of orthologues identified in *C. papaya* by STRING analysis. The network was constructed using DAPs listed in Table 2

and the first neighbors extracted from the complete PPI network (Supplementary Figure S4). Node color represent the ortholog classification on clustering analysis (mfuzz): Yellow (cluster 1), orange (cluster 2), light blue (cluster 3), blue (cluster 4), green (cluster 5) and pink (cluster 6). Nodes with red names represents duplicated orthologs of Arabidopsis identified within *C. papaya* DAPs that could be present in more than one cluster

features, indicating compromised developmental potential (Fig. 1f; Supplementary Fig. S1). Collectively, these findings support the concept that acquisition of embryogenic competence requires an optimal auxin window, whereas supra-optimal doses induce stress, disrupt hormonal homeostasis, and impair the transcriptional programs underlying SE (Karami and Saidi 2010; Méndez-Hernández et al. 2021). Excessive auxin levels have been shown to activate oxidative and osmotic stress pathways, perturb cell cycle progression and favor disorganized callogenesis instead of coordinated embryogenic development (Fehér 2015; Pasternak et al. 2005).

The proteomic analysis of *C. papaya* explants exposed to increasing concentrations of 2,4-D revealed dose-dependent changes in proteins associated with proton transport, particularly within the V-H⁺-ATPase and P-H⁺-ATPase families. Among the DAPs (Table 2), we identified the vacuolar H⁺-ATPase subunits VHA-C (evm.model.supercontig_70.83) and VHA-E1 (evm.model.supercontig_122.22), both showing maximal accumulation at intermediate auxin concentrations followed by a marked decline at supra-optimal levels, whereas VHA-G1 (evm.model.supercontig_666.2) and VHA-H (evm.model.supercontig_43.106) remained relatively stable. The activation of plasma-membrane H⁺-ATPases by auxin has been consistently associated with cell elongation processes during embryonic and root development (Rober-Kleber et al. 2003). Mechanistically, this activation involves phosphorylation of the pumps and their interaction with 14-3-3 proteins, as demonstrated in *Cucumis sativus* (Li et al. 2020) and *Oryza sativa* (Wang et al. 2021). Supporting the functional role of 14-3-3 proteins under stress, overexpression of MdGRF6 (a 14-3-3 gene) in *Malus domestica* callus led to increased sensitivity to salinity stress, suggesting that specific 14-3-3 isoforms can negatively modulate stress tolerance through their interaction with membrane proteins such as H⁺-ATPases (Zhu et al. 2023). Hormonal crosstalk plays a crucial role in modulating H⁺-ATPase activity, as auxin promotes its activation whereas abscisic acid (ABA) inhibits it by preventing the binding of 14-3-3 proteins, as observed in *A. thaliana* (Xue et al. 2018). Given the central role of pH regulation in auxin-mediated cell wall loosening and signal propagation (Spartz et al. 2014), the reduced abundance of these ATPases under 200 μ M 2,4-D may reflect alterations in cellular processes associated with auxin signaling and pH regulation during embryogenic induction. Accordingly, the supra-optimal auxin dose fully inhibited explant growth and repressed key proteins involved in energy metabolism, stress adaptation and embryogenesis. Collectively, these findings indicate that excessive 2,4-D is associated with alterations in proteins related to proton pump activity and cellular energy metabolism, which may interfere with the

molecular pathways required for embryogenic competence. Consistent with these observations, proteins upregulated under the optimal 20 μ M 2,4-D treatment were strongly enriched for translation, ribosome biogenesis, mRNA binding, and GTP-binding functions (Fig. 2). These enriched categories reinforce the interpretation that moderate auxin levels stimulate translational capacity and initiate metabolic adjustments required for early cellular reprogramming. The predominance of ribosome-related terms suggests that the 20 μ M dose activates the protein synthesis machinery necessary to sustain the initial steps of somatic embryogenesis. This reinforces the notion that SE induction in *C. papaya* is highly auxin-sensitive and depends on a finely balanced interplay between growth-promoting and stress-mitigating signals.

In addition to modulating proton pumps, the proteomic analysis further revealed changes in protein kinases and phosphatases, as well as components of the ubiquitin-proteasome system, in a dose-dependent manner. Among them, SKP1-like protein 1A (SKP1A; evm.model.supercontig_26.289), grouped in cluster 4, exhibited a progressive and continuous reduction in abundance as auxin concentration increased, and SKP1-like protein 1B (SKP1B; evm.model.supercontig_20.89, cluster 6, and evm.model.supercontig_6.295, cluster 4), suggests a key role in auxin-responsive signal transduction pathways that orchestrate early cellular reprogramming. Notably, proteins in cluster 4 tend to decrease in abundance with increasing 2,4-D, while those in cluster 6 exhibit transient accumulation at intermediate concentrations followed by repression at 200 μ M (Fig. 5). This biphasic pattern suggests a dose-sensitive regulation of auxin perception and signaling machinery, which may reflect the tight temporal control required for embryogenic competence acquisition (Radoeva and Weijers 2014). The SKP1B remained unchanged in the 200/20 μ M 2,4-D comparison, showing a profile characterized by relative stability at lower to intermediate auxin doses and a pronounced decrease only at the highest dose. Also, SKP1-like protein 1A remained unchanged in this comparison, but displayed a progressive, continuous reduction in abundance as auxin increased, and appeared downregulated whenever detected as differentially accumulated in other comparisons (Supplementary Table S1). Cullin-associated NEDD8-dissociated protein 1 (CAND1; evm.model.supercontig_173.13) was significantly downregulated and followed the same progressive decline with rising auxin. Moreover, the proteins SKP1A, SKP1B, and CAND1 have predicted interactions in the PPI analysis (Fig. 6). Together, these patterns point to attenuation of SCF-complex components under specific auxin regimes, potentially limiting AUX/IAA turnover and modulating auxin signal output at supra-optimal doses. These trends suggest that moderate levels of 2,4-D favor the

proper assembly or activation of the SCF complex, whereas supra-optimal concentrations may disrupt this balance. This aligns with previous studies indicating that auxin homeostasis and signal propagation must be tightly controlled to enable the acquisition of embryogenic competence (Staswick et al. 2005; Méndez-Hernández et al. 2019). Supporting this, the SCF complex is known to interact with the ubiquitin-proteasome system and other post-translational regulators, forming a dynamic proteostasis network responsive to hormone fluctuations (Zhang and Zeng 2020). In this respect, the search for hub proteins in the complete PPI of DAPs showed that ribosomal proteins play an important role in the changes promoted by 2,4-D (Supplementary Fig. S6), highlighting the genetic reprogramming associated with callus induction. We propose that the optimal 2,4-D dose fine-tunes the SCF-mediated degradation of AUX/IAA repressors, while excess auxin may impair this process, leading to aberrant auxin responses and reduced embryogenic potential. The global repression observed at 200 μ M 2,4-D is further supported by the significant depletion of proteins involved in amino acid biosynthesis, carbon metabolism, translation initiation and proteasome activity (Fig. 3). These enriched downregulated categories highlight the collapse of essential biosynthetic and developmental pathways under supra-optimal auxin conditions, corroborating the inhibitory metabolic environment observed in the proteomic profile. This metabolic and developmental collapse is consistent with previous studies showing that supra-optimal auxin disrupts polarity and patterning mechanisms mediated by ROP GTPases, which are essential for embryo development and directional auxin transport (Chen et al. 2014). Elevated 2,4-D levels have also been shown to exacerbate oxidative stress and impair somatic embryogenesis in other species, such as *Medicago truncatula* (Orłowska and Kępczyńska 2020), reinforcing the idea that excessive auxin shifts ROS homeostasis toward a damaging state. Because ROS operate in a dual fashion, promoting differentiation at controlled levels yet impairing developmental competence when excessively accumulated (Prudente et al. 2020), the pronounced repression of biosynthetic, translational and proteasome-related pathways detected in our dataset (Fig. 3) likely reflects a shift toward a stress-dominated cellular state that is incompatible with embryogenic reprogramming. Conversely, the set of proteins upregulated at 200 μ M revealed a completely different functional signature (Fig. 4). Whereas the downregulated proteins at 200 μ M 2,4-D were mainly associated with biosynthetic and translational functions, the proteins upregulated under this condition showed enrichment exclusively for hypoxia- and oxygen-deprivation-related biological process terms. This narrow functional activation indicates that at supra-optimal auxin levels explants prioritize stress mitigation rather than

developmental reprogramming, reinforcing the view that excessive auxin shifts the cellular environment toward a stress-dominated state. This interpretation aligns with evidence that hypoxia signaling is tightly integrated with auxin, redox balance and energy metabolism (Renziehausen et al. 2025). Additionally, 2,4-D exposure has been shown to reduce oxygen consumption in non-plant systems (Barbieri 2009), supporting the hypothesis that supra-optimal auxin disrupts aerobic metabolism. Together with the downregulation of proteasome components, these alterations reinforce the conclusion that excessive 2,4-D compromises proteostasis, oxygen balance and cellular homeostasis, ultimately impairing the acquisition of embryogenic competence.

Auxin signaling fundamentally relies on proteolysis, classically through SCF-TIR1/AFB-mediated degradation of AUX/IAA repressors to activate transcription. Emerging evidence further demonstrates that auxin also promotes the turnover of additional signaling components, revealing a broader and more integrative role of the ubiquitin-proteasome system (UPS) in auxin-driven cellular reprogramming (De Roij et al. 2024). In our study, the proteomic profiles of explants exposed to increasing concentrations of 2,4-D revealed a clear dose-dependent modulation of proteins involved in proteostasis, particularly components of the 26S proteasome, in addition to several predicted interactions, forming a molecular complex (Supplementary Fig. 5b). Most displayed low basal abundance followed by progressive accumulation across increasing auxin concentrations, suggesting an early proteolytic activation phase consistent with auxin perception. This separation between high-dose-repressed subunits and context-dependent responders reinforces the notion that proteasome activity is actively maintained under moderate auxin conditions, supporting AUX/IAA turnover and transcriptional reprogramming, but is suppressed under supra-optimal doses, leading to proteome imbalances detrimental to embryogenic competence. This dose-sensitive behavior aligns with findings in *C. canephora*, where supra-optimal auxin levels disrupted proteasome integrity during somatic embryogenesis (Quintana-Escobar et al. 2023). Moreover, auxin promotes the interaction between SCFTIR1 and AUX/IAA repressors, triggering their ubiquitination and subsequent degradation, which is essential for auxin-induced gene activation (Dos Santos Maraschin et al. 2009). Accordingly, mutations in SCFTIR1 components stabilize AUX/IAA repressors and block auxin responses, confirming that proteasome-mediated degradation is central to auxin signaling (Dos Santos Maraschin et al. 2009). Taken together, these patterns indicate that moderate 2,4-D treatments are associated with proteasome activity patterns consistent with AUX/IAA turnover and transcriptional reprogramming during embryogenic induction, whereas high doses are associated

with reduced abundance of proteasome components, which may interfere with proteostasis and auxin-related regulatory processes during embryogenic induction.

During the acquisition of embryogenic competence, multiple members of the 14-3-3 (GF14) protein family were detected in our dataset, each displaying distinct abundance profiles across the auxin treatments. In the 200/20 μM 2,4-D comparison, several isoforms showed a repressed profile, including General Regulatory Factor 8 (GRF8; evm.model.supercontig_309.6) and General Regulatory Factor 2 (GRF2; evm.model.supercontig_36.169), displayed high basal levels followed by progressive downregulation, indicating sensitivity to elevated auxin concentrations. In contrast, General Regulatory Factor 12 (GRF12; evm.model.supercontig_1.1152) and General Regulatory Factor 3 (GRF3; evm.model.supercontig_179.17) remained unchanged in this comparison but displayed reduced accumulation in other contrasts (Supplementary Table S1), suggesting broader modulation under different auxin regimes. Additionally, General Regulatory Factor 11 (GRF11; evm.model.supercontig_36.140) remained unchanged in this contrast but accumulated more strongly in another, indicating potential participation at earlier or intermediate stages of cellular reprogramming. Predicted interactions among several of these isoforms were also identified (Fig. 6). The modulation of these General Regulatory Factors is consistent with previous findings in *Coffea canephora*, where multiple 14-3-3 proteins accumulate during the induction phase of somatic embryogenesis alongside other regulators such as PP2A, SERK1, MADS-box transcription factors, and calreticulin (Quintana-Escobar et al. 2023). In agreement with this, serine/threonine-protein phosphatase 2A regulatory subunit A2 (PP2AA1; evm.model.supercontig_13.7), grouped in cluster 3, was significantly repressed in the 200/20 μM 2,4-D treatments comparison. Two other phosphatases from the same cluster, PP2A4 (evm.model.supercontig_85.31) and PP2AA2 (evm.model.supercontig_1.195), remained unchanged in this specific contrast but followed the characteristic cluster 3 profile, showing high abundance under lower auxin levels. Together, these findings indicate that General Regulatory Factors (14-3-3 proteins) and PP2A-related components may act as conserved regulators of hormonal and stress-associated signaling adjustments that support the acquisition of embryogenic competence.

During the early stages of somatic embryogenesis, auxin-related regulatory processes play an important role in determining cell fate. In our dataset, 2,4-D application led to a strong and consistent accumulation of the enzyme Probable indole-3-acetic acid-amido synthetase (GH3.1; evm.model.supercontig_292.1), which was upregulated in the 200/20 μM 2,4-D comparison and consistently accumulated across

other contrasts, suggesting a robust and sustained role in auxin conjugation throughout the dose-response curve. In contrast, GH3.3 (evm.model.supercontig_6.74) remained unchanged in the 200/20 μM treatment but was upregulated in other contrasts, indicating a more stable participation in auxin buffering under alternative auxin regimes. GH3 enzymes catalyze the conjugation of indole-3-acetic acid (IAA)-amido synthetases, conjugating IAA to amino acids and thereby reducing the pool of free auxin, effectively buffering its cellular effects (Staswick et al. 2005). This conjugation mechanism has been shown to negatively regulate auxin responses such as radicle elongation and shoot cell expansion (Nakazawa et al. 2001), consistent with our observation of inhibited root development in 2,4-D-treated explants (Fig. 1). In *C. papaya*, GH3 protein accumulation has also been associated with enhanced somatic embryo maturation under specific light conditions (Almeida et al. 2019) and with the progression of somatic embryogenesis in *C. canephora* (Méndez-Hernández et al. 2021; Quintana-Escobar et al. 2023). Together, these findings suggest that GH3-mediated auxin conjugation is associated with auxin-related regulatory adjustments during early embryogenic reprogramming under different 2,4-D treatments. The suppression of radicle growth and the modulation of auxin-responsive proteins suggest that auxin detoxification via conjugation is not only a homeostatic mechanism but a prerequisite for cellular reprogramming during somatic embryogenesis.

Likewise, proteins involved in S-adenosylmethionine (SAM) metabolism displayed pronounced dose-dependent changes. METK4 (evm.model.supercontig_146.36) exhibited a progressive and continuous decline in abundance as auxin concentration increased, with marked repression in the 200/20 comparison (Table 2). This pattern matches the functional profile of cluster 4, where methyl donor biosynthesis is progressively suppressed under supra-optimal auxin. Although SAM2 (evm.model.supercontig_67.71) and METK3 (evm.model.supercontig_102.23) remained unchanged in this specific comparison, both displayed consistent downregulation in other contrasts, while SAM2 (evm.model.supercontig_48.212) maintained stable abundance across all treatments, suggesting a constitutive role in sustaining basal SAM levels during somatic embryogenesis (Supplementary Table S1). This repression of SAM biosynthesis at high auxin concentrations is further compounded by changes in Adenosylhomocysteinase 1 (SAHH1; evm.model.supercontig_157.46), assigned to cluster 3, which maintained high abundance under lower auxin levels but showed a sharp decline only at 200 μM 2,4-D. The SAM metabolism forms a group of proteins with predicted interactions, where SAHH1 represents a hub (Fig. 6). In support of this functional link, predicted interactions between

SAHH and SAM synthetases have been reported during sugarcane somatic embryo maturation (Xavier et al. 2022), where SAHH, orthologous to *A. thaliana* HOG1, was shown to associate with SAM1 and SAM2 in a methylation-related protein network. In *A. thaliana*, HOG1 is required for DNA methylation-dependent gene silencing (Rocha et al. 2005) and encodes a SAH hydrolase that ensures methyl group availability through the hydrolysis of SAH, which is generated by SAM synthetases (Ouyang et al. 2012). Although SAM biosynthetic enzymes showed contrasting auxin-dependent profiles, their interpretation must consider the broader epigenetic context of somatic embryogenesis. Several studies demonstrate that reduced methylation capacity can facilitate cellular dedifferentiation and acquisition of embryogenic competence, often through the relaxation of chromatin and the reactivation of developmental regulators (Fraga et al. 2012; Nic-Can et al. 2013; Xu and Huang 2014; Fehér 2015). Conversely, other systems rely on the maintenance of methyl group availability to stabilize newly established embryogenic programs (Neves et al. 2021). Together, these findings indicate that the SAM/SAH cycle functions as a dynamic regulatory node whose impact on somatic embryogenesis is species, context and stage dependent, integrating hormonal inputs such as 2,4-D with epigenetic mechanisms that modulate developmental plasticity.

Histone modifications emerged as an additional regulatory layer. Histone deacetylase 3 (HDT3; evm.model.supercontig_5.85) was strongly repressed in the 200/20 μM comparison, while another HDT3 paralog (evm.model.supercontig_599.1) showed consistent downregulation in contrasts involving 200 μM 2,4-D. HDACs contribute to dedifferentiation and the acquisition of embryogenic competence by repressing differentiation-related genes (Hollender and Liu 2008; De-la-Peña et al. 2015; Sena et al. 2024). In *A. thaliana*, HDA6 and HDA19 restrict embryonic programs after germination, illustrating that HDACs can also limit regeneration or embryogenic potential under specific contexts (Tanaka et al. 2008). Conversely, increased histone acetylation, mediated by histone acetyltransferases or HDAC inhibition, has been associated with enhanced reprogramming capacity (Grzybkowska et al. 2018; Kim et al. 2018). Broader epigenetic analyses have shown that auxin-induced somatic embryogenesis involves extensive chromatin remodeling, including histone acetylation, DNA methylation and miRNA-mediated regulation (Wójcik et al. 2020; Wójcikowska et al. 2020). DNA methylation pathways also play a critical and complex role in somatic embryogenesis, with both maintenance and de novo methylation contributing to the regulation of somatic embryogenesis-related transcription factors, as evidenced by the altered expression of LEC1, LEC2 and

BBM in DNA-methylase mutants and 5-AzaC-treated cultures (Grzybkowska et al. 2018). Collectively, this evidence supports the interpretation that HDAC repression under supra-optimal auxin integrates hormonal and chromatin-based mechanisms, potentially weakening the epigenetic control required for effective somatic embryo induction.

Conclusions

This study demonstrates that proteomic responses associated with 2,4-D treatments during the induction of somatic embryogenesis in *C. papaya* are strongly dose-dependent and modulate multiple regulatory layers related to embryogenic reprogramming. Moderate auxin concentrations, particularly 20 μM 2,4-D, activated a coordinated network involving auxin perception and conjugation, proteasome-mediated turnover, methylation-related metabolism and histone deacetylation, collectively establishing a molecular environment permissive to embryogenic competence. In contrast, supra-optimal auxin (200 μM) repressed proton pumping, destabilized proteostasis, disrupted auxin homeostasis and weakened epigenetic regulation, resulting in a stress-induced failure to reprogram cells toward embryogenesis. The differential accumulation of kinases, phosphatases and 14-3-3 proteins further highlight the role of post-translational control in modulating auxin-dependent developmental transitions. Together, these findings reveal that somatic embryo induction relies on a narrow auxin window that preserves the balance between signaling, proteostasis and chromatin regulation. This work provides a mechanistic framework that can guide the refinement of auxin-based protocols for callus induction in *C. papaya* and potentially in other plant systems where somatic embryogenesis is employed.

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Author contributions DDRP, LRX, and VS designed the research. DDRP and LRX developed the methodology, performed data acquisition and analysis, and drafted the initial version of the manuscript. CEAdS contributed to the experimental work. RPDp and VS provided technical support and assisted in the partial interpretation of the results. VS and CS-C critically reviewed the manuscript and provided suggestions to improve clarity and scientific discussion. All authors

read and approved the final version of the manuscript.

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Data availability The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2025) with the dataset identifier PXD072983. All other data generated or analyzed during this study are included in this published article and its Supplementary Information files.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. Work with plants complies with relevant institutional, national and international guidelines and legislation.

Consent to participate Not applicable.

Consent for publication All authors provide their consent for publication of this manuscript.

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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