E X R N A - A G

Bacterial Extracellular RNA-Mediated Reprogramming of Spinach (*Spinacia oleracea*) Seed Germination

Target Analysis · Mechanistic Models · Validation Strategy

109 Gene Targets Analyzed

REPORT PREPARED BY

Sarthak Tiwary

ExRNA-Ag

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CONFIDENTIAL

ExRNA Research Report: Spinach

Spinacia oleracea — Bacterial Extracellular sRNA Target Analysis [CONFIDENTIAL]

> **CONFIDENTIAL** — Generated 2026-02-19 **Family**: Amaranthaceae | **Assembly**: SOV_r1.1 **Treatment**: M-9 bacterial EPS solution **Analysis Status**: completed

Executive Summary [CONFIDENTIAL]

This report presents the analysis of **109 predicted exRNA targets** in Spinacia oleracea (spinach). These transcripts were identified as potential targets of bacterial extracellular small RNAs (exRNAs) that may improve seed germination and seedling vigor when seeds are treated with M-9 bacterial EPS solution.

Target Distribution

PRIORITY	COUNT
High	21
Medium	49
Low	39
Total	**109**

Pathway Distribution

PATHWAY	TARGETS
Transport Ion Homeostasis	18
Metabolic	15

PATHWAY	TARGETS
Protein Turnover	11
Signaling	11
Rna Processing	10
Unknown	7
Epigenetic Regulation	6
Organelle Biogenesis	6
Dna Repair Replication	6
Defense Immunity	5
Transposon Related	5
Ros Redox	3
Cell Wall Remodeling	3
Hormone Signaling	3

High-Priority Targets [CONFIDENTIAL]

GENE ID	ANNOTATION	PATHWAY
SOV3g040200.1	Glutathione S-transferase L3-like	ros_redox
SOV6g036290.1	Protein HIRA	epigenetic_regulation
SOV1g021960.1	Cation-chloride cotransporter 1-like	transport_ion_homeostasis
SOV2g025380.1	Cation-chloride cotransporter 1-like	transport_ion_homeostasis
SOV3g043450.1	ENHANCED DISEASE RESISTANCE 2 (EDR2)	defense_immunity
SOV6g048760.1	ENHANCED DISEASE RESISTANCE 2 (EDR2)	defense_immunity
SOV1g033340.1	DNA (cytosine-5)-methyltransferase	epigenetic_regulation
SOV4g015450.1	Histone-lysine N-methyltransferase SUVR5 (putative)	epigenetic_regulation
SOV4g032870.1	Histidine-containing phosphotransfer protein 1 (AHP-like)	hormone_signaling
SOV5g005530.1	Modifier of SNC1 1 (MOS1-like / immune regulator)	defense_immunity

GENE ID	ANNOTATION	PATHWAY
SOV3g038840.1	Peroxidase	ros_redox
SOV6g029280.1	6-phosphogluconate dehydrogenase (PPP / NADPH)	metabolic
SOV1g020340.1	MYB transcription factor	signaling
SOV1g018480.1	Cyclic nucleotide-gated channel (CNGC)	transport_ion_homeostasis
SOV2g014810.1	NAC domain-containing protein	signaling

Pathway Analysis Summary [CONFIDENTIAL]

Pathway Analysis Index

TL;DR: Gene targets grouped by 14 biological pathways. Key pathways include hormone signaling, defense/immunity, epigenetics, ROS/redox, transport, and metabolic priming. Last Updated: 2026-02-18

Pathways [CONFIDENTIAL]

PATHWAY	FILE	TARGETS	SUMMARY
Cell Wall Remodeling	cell_wall_remodeling.md	3	Of course. As a plant systems biologist, I will analyze the coordinated downregu
Defense Immunity	defense_immunity.md	5	Of course. As a plant systems biologist, I will analyze this "Defense Immunity"
Dna Repair Replication	dna_repair_replication.md	6	Of course. As a plant systems biologist, I will analyze this pathway by integrat
Epigenetic Regulation	epigenetic_regulation.md	6	Of course. As a plant systems biologist, I will analyze this set of epigenetical
Hormone Signaling	hormone_signaling.md	3	Of course. As a plant systems biologist, I will analyze the coordinated function
Metabolic	metabolic_priming.md	15	Of course. As a plant systems biologist, I will analyze this set of downregulate
Organelle Biogenesis	organelle_biogenesis.md	6	Of course. As a plant systems biologist, I will analyze this "Organelle Biogenes
Protein Turnover	protein_turnover.md	11	Of course. As a plant systems biologist, I will analyze the coordinated downregu
Rna Processing	rna_processing.md	10	Of course. As a plant systems biologist, here is a pathway-level analysis of the
Ros Redox	ros_redox.md	3	Of course. As a plant systems biologist, here is a detailed pathway-level analys
Signaling	signaling.md	11	Of course. As a plant systems biologist, here is a pathway-level analysis of the
Transport Ion Homeostasis	transport_ion_homeostasis.md	18	Of course. As a plant systems biologist, I will analyze the "Transport Ion Homeo

PATHWAY	FILE	TARGETS	SUMMARY
Transposon Related	transposon_related.md	5	Of course. As a plant systems biologist, here is a detailed pathway-level analys
Unknown	unknown_function.md	7	Of course. As a plant systems biologist, I will analyze this gene set. The key c

Cross-Pathway Analysis [CONFIDENTIAL]

See cross_pathway_interactions.md

Theme Analysis Summary [CONFIDENTIAL]

Themes Index

> TL;DR: 6 cross-cutting biological themes extracted from target analysis. Last Updated: 2026-02-18

Themes [CONFIDENTIAL]

THEME	FILE	SUMMARY
Defense Downshift	defense_downshift.md	Theme analysis for defense downshift
Epigenetic Remodeling	epigenetic_remodeling.md	Theme analysis for epigenetic remodeling
ROS Optimization	ros_optimization.md	Theme analysis for ros optimization
Hormone Nodes	hormone_nodes.md	Theme analysis for hormone nodes
Transport / Ion Homeostasis	transport_ion_homeostasis.md	Theme analysis for transport / ion homeostasis
Metabolic Priming	metabolic_priming.md	Theme analysis for metabolic priming

Theme Interactions [CONFIDENTIAL]

See individual theme files for cross-theme analysis.

Synthesis: Ranked Targets [CONFIDENTIAL]

Ranked Target Analysis — Spinach (Spinacia oleracea)

Definitive Ranked Target Analysis: Bacterial exRNA-Mediated Germination Improvement in *Spinacia oleracea*

Executive Summary [CONFIDENTIAL]

This analysis evaluates 109 predicted gene targets across 14 functional pathways, all putatively downregulated by bacterial extracellular small RNAs (exRNAs) to improve spinach seed germination and early seedling vigor. The target landscape reveals a coherent, multi-layered biological strategy rather than a collection of independent hits: the bacterial exRNAs appear to simultaneously dismantle dormancy-maintenance programs (epigenetic silencing, ABA signaling, immune activation), reduce energetic costs of non-essential processes (transposon mobilization, de novo organelle biogenesis, secondary metabolism), and directly facilitate the physical act of germination (cell wall loosening, ion homeostasis, turgor generation). The convergence of multiple independent pathways on the ABA/GA hormonal axis and the growth-defense tradeoff is the most compelling systems-level feature of this dataset.

Critical caveats must be acknowledged before accepting any mechanistic interpretation. The entire framework rests on unverified assumptions: (1) that bacterial exRNAs can traverse the seed coat and enter embryonic cells in sufficient quantities to silence specific mRNAs—a process demonstrated in limited plant-fungal and plant-bacterial systems but not yet rigorously established for this specific bacterium-spinach interaction; (2) that the annotated gene functions are correct, as spinach genomics remains less mature than Arabidopsis; and (3) that the observed germination improvement is causally attributable to exRNA-mediated silencing rather than to confounding effects of bacterial exopolysaccharides (EPS) acting as osmopriming agents, polysaccharide elicitors of plant immunity, or microbiome-mediated soil conditioning. Several targets (reverse transcriptases, DNA polymerases, unknown proteins) have annotations too generic or uncertain to support confident mechanistic claims.

The highest-confidence targets are those where: (a) the gene family has well-established, experimentally validated roles in seed dormancy or germination in Arabidopsis or closely related species; (b) the direction of effect (downregulation → germination improvement) is mechanistically coherent with known biology; and (c) the target occupies a high-connectivity node in the regulatory network (i.e., its modulation would have cascading downstream effects). By these criteria, the ethylene receptor, DNA methyltransferase, HIRA histone chaperone, trehalose-phosphate synthase, cation-chloride cotransporters, and the EDR2/MOS1 immune regulators emerge as the most compelling candidates for driving the observed phenotype.

Ranking Methodology [CONFIDENTIAL]

Targets were scored on six criteria, each weighted by biological relevance to germination:

CRITERION	WEIGHT	RATIONALE
Mechanistic coherence: Is the direction of effect (downregulation → improved germination) supported by established biology?	30%	Primary filter; incoherent mechanisms disqualify high ranking
Homolog evidence: Is there direct experimental evidence from Arabidopsis or related species for this gene family's role in seed dormancy/germination?	25%	Strongest available proxy for spinach function
Network centrality: Does the gene occupy a hub position (TF, kinase, epigenetic regulator) with broad downstream effects?	20%	Hub genes have larger phenotypic footprints
Annotation confidence: Is the gene family annotation reliable, or is it generic/uncertain?	15%	Penalizes reverse transcriptases, unknowns, DUF proteins
Pathway priority assignment: Was the gene assigned "high" priority within its pathway analysis?	5%	Internal consistency check
Confounder susceptibility: Could the effect be explained by EPS osmopriming, elicitor effects, or microbiome changes rather than specific mRNA silencing?	5% (penalty)	Reduces confidence in non-specific targets

Targets with annotation flags (contamination, misannotation) were automatically demoted to Tier 3 or excluded from mechanistic ranking.

Tier 1: Critical Targets (Expected Large Phenotypic Effect)

[CONFIDENTIAL]

These targets occupy regulatory hubs with direct, well-supported connections to the ABA/GA dormancy-germination switch, epigenetic reprogramming, or the physical mechanics of radicle emergence.

1. SOV3g000150.1 — Ethylene Receptor

- **Mechanism**: Ethylene receptors (ETR/EIN4 family) are constitutive repressors of ethylene signaling; receptor protein presence suppresses ethylene responses. Downregulation of the receptor by exRNA would derepress ethylene signaling, promoting germination. Ethylene is well-established as an antagonist of ABA-mediated dormancy and acts synergistically with GA to promote radicle emergence. In Arabidopsis, *etr1* loss-of-function

mutants show enhanced germination under ABA stress. [KNOWN for receptor family; INFERRED for this specific spinach gene]

- **Evidence strength**: Strong
- **Key references**: Bleecker & Kende (2000) *Annu Rev Cell Dev Biol*; Linkies et al. (2009) *Plant Cell* — demonstrated ethylene promotes endosperm cap weakening in *Lepidium sativum* (close Brassicales relative); Arc et al. (2013) *Plant Cell Environ* — ethylene-ABA antagonism in seed germination
- **Spinach context**: Spinach seeds are notoriously difficult to germinate uniformly; ethylene signaling enhancement is a plausible mechanism for breaking thermodormancy [INFERRED]
- **Confounders**: EPS could independently trigger ethylene production via PAMP-triggered immunity, making attribution to specific receptor silencing difficult [SPECULATIVE]
- **Confidence**: **High**

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2. SOV1g033340.1 — DNA (Cytosine-5)-Methyltransferase

- **Mechanism**: DNA methyltransferases (CMT3, MET1, DRM2 classes in Arabidopsis) maintain repressive methylation marks at dormancy-associated loci including ABA-responsive genes and transposon-adjacent regulatory regions. Downregulation during imbibition would reduce maintenance methylation, allowing de-repression of germination-promoting genes. In Arabidopsis, *met1* mutants show reduced seed dormancy. [KNOWN for gene family; INFERRED for this spinach paralog]
- **Evidence strength**: Strong
- **Key references**: Footitt et al. (2015) *Plant J* — DNA methylation changes during Arabidopsis seed after-ripening; Narsai et al. (2017) *Plant Physiol* — methylome dynamics during rice germination; Sano et al. (2012) *Plant Cell Physiol* — CMT3 role in transposon silencing during germination
- **Network centrality**: Extremely high — DNA methylation is upstream of virtually all transcriptional programs [KNOWN]
- **Caveat**: The specific methyltransferase class (MET1 vs. CMT3 vs. DRM2) determines which genomic contexts are affected; annotation does not specify class, reducing mechanistic precision [INFERRED]
- **Confidence**: **High**

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3. SOV6g036290.1 — Protein HIRA (Histone Chaperone)

- **Mechanism**: HIRA is a replication-independent histone H3.3 chaperone that deposits H3.3 at actively transcribed loci and participates in chromatin reprogramming during developmental transitions. In seeds, HIRA activity is associated with maintaining repressive chromatin states during dormancy. Downregulation would facilitate chromatin opening at germination-promoting loci. In Arabidopsis, HIRA interacts with the Polycomb

pathway and is required for proper seed development and germination timing. [KNOWN for gene family; INFERRED for germination-specific role]

- **Evidence strength**: Moderate-Strong
- **Key references**: Duc et al. (2015) *Plant Cell* — AtHIRA function in chromatin assembly; Jiang & Berger (2017) *Curr Opin Plant Biol* — histone variant dynamics in plant development
- **Network centrality**: High — chromatin remodeling affects entire transcriptional pr [... truncated]

Synthesis: Causal Models [CONFIDENTIAL]

Causal Models — Spinach (Spinacia oleracea)

Alternative Causal Models: Bacterial exRNA-Mediated Germination Improvement in *Spinacia oleracea*

Preamble: Critical Assumptions and Caveats [CONFIDENTIAL]

Before constructing these models, several foundational uncertainties must be stated explicitly:

1. **exRNA delivery to embryonic cells is unproven in this system.** Cross-kingdom RNA interference has been demonstrated in select plant–fungal (e.g., *Botrytis cinerea–Arabidopsis*; Weiberg et al., 2013, *Science*) and plant–parasitic plant systems, but the physical barriers of the spinach seed coat (testa) and endosperm are formidable. Whether bacterial small RNAs can penetrate these layers in biologically relevant quantities during imbibition is unknown. [KNOWN: cross-kingdom RNAi exists; SPECULATIVE: it operates here]

2. **Confounders are substantial.** Bacterial exopolysaccharides (EPS) can act as osmopriming agents, altering water potential gradients. Bacterial lipopolysaccharides and peptidoglycan fragments can act as microbe-associated molecular patterns (MAMPs), triggering or suppressing plant immune responses. Live bacteria alter the spermosphere microbiome. Any or all of these could explain improved germination independently of mRNA silencing.

3. **Spinach genome annotation is incomplete.** Many gene assignments are based on homology to *Arabidopsis thaliana* or *Beta vulgaris* and may not reflect true function in *S. oleracea*.

All three models below assume, for the sake of hypothesis construction, that bacterial exRNAs do reach embryonic cells and engage the plant's RNAi machinery (likely loading into AGO1 or AGO-family proteins to direct target cleavage or translational repression). The models differ in which subset of targets they prioritize as the primary causal drivers and in the temporal logic of the causal chain.

Model 1: The Epigenetic Master Switch Model [CONFIDENTIAL]

Core hypothesis: Bacterial exRNAs primarily target the seed's epigenetic silencing machinery, causing a genome-wide de-repression of pro-germination gene programs that were locked in a dormancy-associated chromatin state; all other observed pathway effects are downstream consequences of this transcriptional liberation.

Causal chain:

1. **Bacterial exRNA enters seed cells during early imbibition** via symplastic uptake through hydrated cell walls of the micropylar endosperm, potentially facilitated by membrane destabilization during rehydration. Small RNAs (likely 20–24 nt) are loaded into spinach AGO proteins. [SPECULATIVE: delivery mechanism; INFERRED: AGO loading based on conserved plant RNAi machinery]
2. **Three core epigenetic regulators are simultaneously downregulated**:
3. **SOV1g033340.1 (DNA cytosine-5-methyltransferase)** → Reduced maintenance methylation at CG and CHG contexts during the first rounds of DNA replication post-imbibition. This passively demethylates promoters of germination-associated genes (e.g., GA biosynthesis genes *GA3ox*, *GA20ox*; cell wall loosening enzymes; aquaporins). [KNOWN: DNA methylation maintains dormancy gene silencing in Arabidopsis; Nee et al., 2017, *Plant Cell*; INFERRED: analogous in spinach]
4. **SOV4g015450.1 (SUVR5-like histone methyltransferase)** → Reduced deposition of H3K9me2 repressive marks at heterochromatic loci, including dormancy-maintenance genes and transposable element-adjacent regulatory regions. [KNOWN: SUVR5 deposits H3K9me2 in Arabidopsis; Caro et al., 2012; INFERRED: functional conservation]
5. **SOV6g036290.1 (HIRA histone chaperone)** → Disrupted replication-independent deposition of histone variant H3.3 into transcriptionally active loci. Paradoxically, HIRA loss may prevent the re-establishment of specific chromatin states that maintain dormancy gene expression during imbibition. [KNOWN: HIRA deposits H3.3 in Arabidopsis; Nie et al., 2014; INFERRED: dormancy-specific role]
6. **Chromatin readers and transcriptional repressors lose their substrates and targets**:
7. **SOV4g030590.1 (PHD domain protein)** downregulation removes a "reader" of histone methylation marks, breaking the positive feedback loop where repressive marks recruit more silencing machinery. [INFERRED]
8. **SOV4g038060.1 (GIS2 zinc finger)** downregulation removes a transcriptional repressor that, in Arabidopsis, promotes trichome development and represses flowering; its spinach homolog may repress germination-associated transcription factor cascades. [KNOWN: GIS2 function in Arabidopsis; SPECULATIVE: spinach germination role]
9. **Cascading de-repression activates multiple downstream programs simultaneously**:
10. **Hormone signaling genes become transcriptionally accessible**: GA biosynthesis and signaling genes are expressed → DELLA repressors are degraded → germination program initiates. ABA catabolism genes (*CYP707A* family) are de-repressed → ABA levels decline. [KNOWN: epigenetic control of ABA/GA genes; Liu et al., 2013]
11. **Cell wall remodeling enzymes are expressed**: Expansins, endo-β-mannanases, and xyloglucan endotransglucosylase/hydrolases (XTHs) are transcribed from newly accessible chromatin → micropylar endosperm weakening proceeds. [KNOWN: these genes are epigenetically regulated during germination]
12. **Defense genes are collaterally suppressed**: The broad chromatin opening paradoxically reduces expression of some defense genes that were maintained by specific active chromatin states (H3.3-dependent), while the

simultaneous exRNA-mediated knockdown of EDR2 and MOS1 (SOV3g043450.1, SOV6g048760.1, SOV5g005530.1) ensures immune suppression is robust. [INFERRED]

13. **Transposon silencing is partially released**: The 5 reverse transcriptase/gag targets (SOV2g004250.1, SOV4g025520.1, SOV3g033520.1, SOV1g003910.1, SOV4g035390.1) may represent a side effect—their transcripts increase due to chromatin opening but are simultaneously targeted by the bacterial exRNAs, creating a fail-safe against genomic instability. [SPECULATIVE] [... truncated]

Synthesis: Confounder Analysis [CONFIDENTIAL]

Confounder Analysis — Spinach (Spinacia oleracea)

Critical Confounder Analysis: Bacterial exRNA Germination Improvement System

Preamble [CONFIDENTIAL]

This analysis assumes a system in which *Spinacia oleracea* seeds are treated with an EPS (exopolysaccharide) solution derived from an M-9 bacterial strain, with the claimed mechanism being antisense RNA-mediated silencing of 109 plant target transcripts. The goal here is rigorous: to determine what fraction of the observed germination phenotype could be explained by mechanisms **other than** sequence-specific antisense RNA targeting.

1. EPS Osmopriming Effect [CONFIDENTIAL]

Mechanism

[KNOWN] Osmopriming — the controlled hydration of seeds in osmotic solutions — is one of the most well-established seed enhancement technologies. EPS solutions are inherently viscous, high-molecular-weight polysaccharide matrices that generate significant osmotic potential.

- **Controlled imbibition**: EPS solutions restrict water availability (lowered water potential, ψ), allowing seeds to progress through Phase I and early Phase II of imbibition without completing germination. This permits pre-germinative metabolic activation — DNA repair, mitochondrial biogenesis, mRNA synthesis, and antioxidant system priming — without radicle emergence. [KNOWN — reviewed in Paparella et al., 2015, *Plant Cell Rep*; Lutts et al., 2016]
- **Membrane reorganization**: Slow hydration allows orderly membrane phase transitions from gel to liquid-crystalline state, reducing solute leakage and improving seedling vigor. [KNOWN]
- **ROS priming**: Controlled hydration generates a mild oxidative signal (H_2O_2 accumulation) that activates antioxidant defense systems (APX, CAT, SOD, GR), creating a "primed" redox state. [KNOWN — Wojtyla et al., 2016, *Front Plant Sci*]
- **Hormone rebalancing**: Osmopriming is known to reduce ABA/GA ratio, promote GA biosynthesis, and modulate ethylene signaling — all of which directly overlap with the claimed exRNA targets in hormone signaling pathways. [KNOWN — Chen & Arora, 2013]

Expected Magnitude

[KNOWN] Osmopriming with PEG or other osmolytes routinely produces: - 15–40% improvement in germination rate under suboptimal conditions - 20–50% improvement in germination speed (T50 reduction) - Significant improvement in seedling vigor indices

These magnitudes are **comparable to or exceed** typical reported improvements in bacterial treatment germination studies. Without knowing the exact magnitude of the observed phenotype in this system, this is a **major confounder** because EPS osmopriming alone could plausibly account for the entire phenotype.

Critical Overlap with Claimed Targets

The claimed exRNA target pathways — hormone signaling, ROS/redox, defense priming, metabolic activation — are **precisely the pathways activated by osmopriming itself**. This represents a profound confound: even if these pathways change in expression, it is impossible to attribute the change to antisense RNA versus osmotic priming without proper controls.

Controls Needed

1. **EPS-only control (RNA-depleted)**: Treat seeds with EPS solution subjected to RNase A/T1 cocktail + RNase III treatment, then verify RNA removal by qPCR/Bioanalyzer
2. **Osmotic equivalent control**: PEG 6000 or PEG 8000 at matched water potential (ψ) to the EPS solution
3. **Viscosity-matched control**: Methylcellulose or carboxymethylcellulose at matched viscosity but different composition
4. **Water potential measurement**: Characterize ψ of the EPS solution precisely using a vapor pressure osmometer

Evidence Level: [KNOWN] — This is the single most likely confounder and the most difficult to separate from the exRNA effect.

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2. Polysaccharide Elicitor Effects [CONFIDENTIAL]

Known Defense/Growth Priming by Bacterial Polysaccharides

[KNOWN] Bacterial EPS and related polysaccharides are well-established microbe-associated molecular patterns (MAMPs) and elicitors:

- **Direct receptor engagement**: Plants possess pattern [... truncated]

Synthesis: Validation Plan [CONFIDENTIAL]

Validation Plan — Spinach (Spinacia oleracea)

Comprehensive 4-Tier Validation Plan: Bacterial exRNA-Mediated Germination Improvement in *Spinacia oleracea*

Preamble: Validation Philosophy [CONFIDENTIAL]

This plan is designed around a **falsification-first** logic. The most important experiments are those capable of *disproving* the exRNA mechanism, not merely confirming it. Given the substantial confounders identified (EPS osmopriming, polysaccharide elicitor effects, microbiome conditioning), the burden of proof requires demonstrating that:

1. Sequence-specific RNA silencing occurs in embryonic cells [not just osmotic priming]
2. Specific target mRNAs are reduced in abundance in treated seeds [not just transcriptome-wide stress responses]
3. Target reduction is causally linked to germination improvement [not merely correlated]
4. The effect is reproducible across environmental conditions and seed lots [not an artifact of a single experiment]

Label conventions throughout: [KNOWN], [INFERRED], [SPECULATIVE] applied to all mechanistic claims. All experimental predictions are falsifiable hypotheses, not guaranteed outcomes.

Tier 1: Essential Controls — Confounder Elimination

[CONFIDENTIAL]

These experiments must be completed and interpreted before any mechanistic work begins. A positive result from Tier 1 (i.e., the exRNA mechanism survives confounder testing) is the prerequisite gate for all subsequent tiers.

Experiment T1.1: RNA-Depleted EPS Control

Experiment: Enzymatic RNA depletion from bacterial EPS preparation to isolate EPS osmopriming effects from exRNA effects.

Hypothesis tested: Is the observed germination improvement attributable to the RNA component of the bacterial preparation, or is it fully explained by the EPS matrix acting as an osmopriming agent?

Method: 1. Prepare bacterial EPS solution at standard treatment concentration using established protocol 2. Split preparation into four aliquots: - **Treatment A**: Intact EPS preparation (positive control, standard treatment) - **Treatment B**: EPS + RNase A (100 µg/mL, 37°C, 2h) + RNase III (1 U/µL, 37°C, 1h) + RNase T1 (10 U/µL, 37°C, 1h) — degrades ssRNA, dsRNA, and ssRNA at G residues respectively; heat-inactivate at 95°C for 10 min - **Treatment C**: EPS + heat-inactivated RNase cocktail (boiled 15 min before addition) — protein denaturation control to verify RNase treatment does not introduce confounding proteins - **Treatment D**: Water control (no EPS, no RNA) 3. Verify RNA depletion in Treatment B by: (a) Bioanalyzer RNA 6000 Pico chip — confirm absence of RNA peaks; (b) RT-qPCR with universal bacterial 16S primers on the preparation — confirm no amplifiable RNA; (c) Fluorometric RNA quantification (RiboGreen assay) — confirm >99% RNA removal 4. Measure osmotic potential (ψ) of Treatments A and B using vapor pressure osmometer (Wescor VAPRO or equivalent) — confirm ψ is not significantly altered by RNA removal 5. Apply all four treatments to spinach seeds (minimum n=4 biological replicates, 50 seeds per replicate per treatment) under controlled germination conditions (20°C, 12h light/dark, filter paper, Petri dish) 6. Record: germination percentage at 24h, 48h, 72h, 96h, 120h; T50 (time to 50% germination); radicle length at 120h; seedling fresh weight at 7 days

Expected result if exRNA mechanism is real: Treatment A (intact) significantly outperforms Treatment B (RNA-depleted) in germination rate and/or percentage. Treatment B performs similarly to Treatment D (water) or to the osmotic equivalent control (see T1.2). The difference between A and B is attributable to the RNA component. [INFERRED from exRNA hypothesis]

Expected result if EPS osmopriming is the primary confounder: Treatment A and Treatment B perform equivalently (no significant difference in germination metrics). Both outperform Treatment D. The EPS matrix, not the RNA, drives the phenotype. [KNOWN: osmopriming mechanism is well-established; Papa [... truncated]

Methodology [CONFIDENTIAL]

1. **Target Identification**: Bacterial exRNA sequences aligned against Spinacia oleracea transcriptome
2. **Gene Analysis (Stage 1)**: Individual gene function analysis via Gemini 2.5 Flash
3. **Pathway Mapping (Stage 2)**: Pathway-level grouping and interaction analysis via Gemini 2.5 Pro
4. **Literature Dive (Stage 3)**: Homolog research and deep literature review
5. **Theme Extraction (Stage 4)**: Cross-cutting biological theme identification
6. **Synthesis (Stage 5)**: Claude-powered ranking, causal modeling, and validation design

Generated by ExRNA Autonomous Research Platform Gemini (bulk research) + Claude (synthesis & critical review)