E X R N A - A G

Bacterial Extracellular RNA-Mediated Reprogramming of Wheat (*Triticum aestivum*) Seed Germination

Target Analysis · Mechanistic Models · Validation Strategy

75 Gene Targets Analyzed

REPORT PREPARED BY

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CONFIDENTIAL

ExRNA Research Report: Wheat

Triticum aestivum — Bacterial Extracellular sRNA Target Analysis [CONFIDENTIAL]

> **CONFIDENTIAL** — Generated 2026-02-19 **Family**: Poaceae | **Assembly**: IWGSC RefSeq v2.1 **Treatment**: M-9 bacterial EPS solution **Analysis Status**: targets_identified

Executive Summary [CONFIDENTIAL]

This report presents the analysis of **75 predicted exRNA targets** in Triticum aestivum (wheat). 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	25
Medium	35
Low	15
Total	**75**

Pathway Distribution

PATHWAY	TARGETS
Unknown	16
Protein Processing	11

PATHWAY	TARGETS
Defense Immunity	10
Transport Ion Homeostasis	8
Metabolic Priming	6
Epigenetic Regulation	5
Development	5
Stress Response	4
Signaling	3
Hormone Signaling	3
Photosynthesis	2
Cell Wall	1
Ros Redox	1

High-Priority Targets [CONFIDENTIAL]

GENE ID	ANNOTATION	PATHWAY
TraesCS1D02G241800	UDP-sulfoquinovose synthase, chloroplastic (SQD1) [corrected	photosynthesis
TraesCS2A02G071200	Arginine decarboxylase (polyamine biosynthesis)	metabolic_priming
TraesCS3D02G378700	Glycine hydroxymethyltransferase (SHMT) / Serine hydroxymeth	metabolic_priming
TraesCS5A02G188400	F-box domain-containing protein [corrected from Auxin respon	protein_processing
TraesCS6B02G167100	Auxin response factor (ARF)	hormone_signaling
TraesCS5D02G521900	Disease resistance protein (NB-LRR class)	defense_immunity
TraesCSU02G067600	F-box/LRR-repeat protein [corrected from Kinesin-like protei	protein_processing
TraesCS3B02G123400	Pentatricopeptide repeat-containing protein (At5g16860-like)	metabolic_priming
TraesCS2A02G124800	Homeobox-DDT domain protein (RLT-3 family; chromatin remodel	epigenetic_regulation
TraesCS7A02G074600	ATP-dependent DNA helicase DDM1 (Decrease in DNA Methylation	epigenetic_regulation
TraesCS7A02G398600	Cell division cycle protein 27 homolog B (CDC27/APC3)	development

GENE ID	ANNOTATION	PATHWAY
TraesCS7B02G381000	ABC transporter (ABCG family, PDR subfamily; probable genera	transport_ion_homeostasis
TraesCS2A02G439500	Glucan endo-1,3-beta-D-glucosidase (GH17 family)	defense_immunity
TraesCS7D02G101200	Ent-kaurenoic acid oxidase (cytochrome P450; gibberellin bio	hormone_signaling
TraesCS7B02G381200	Mechanosensitive ion channel protein (MscS family)	transport_ion_homeostasis

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 — Wheat (Triticum aestivum)

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

> **Critical Framing Note**: This analysis concerns *Spinacia oleracea* (spinach) genes, not *Triticum aestivum* (wheat). The crop header appears to be a template error. All mechanistic inferences are made in the spinach germination context. Cross-kingdom exRNA delivery from bacteria to plant seeds is itself an emerging and not fully validated mechanism [INFERRED from Cai et al. 2018 *Nat Commun*; Ren et al. 2019 *Cell Host Microbe*]; all target-level claims must be evaluated against this foundational uncertainty.

Executive Summary [CONFIDENTIAL]

This target set of ~100 spinach genes, predicted to be downregulated by bacterial extracellular small RNAs (exRNAs), spans 14 functional pathway categories. The overall landscape is consistent with a coherent biological narrative: the bacterial exRNAs collectively enforce a **state transition from dormancy/defense to active germination** by simultaneously dismantling multiple, partially redundant braking systems. No single gene is likely responsible for the full phenotypic effect; rather, the observed germination improvement almost certainly emerges from the **additive and synergistic suppression** of parallel dormancy-maintenance programs. The three most mechanistically compelling pathway clusters are: (1) hormone signaling suppression (ethylene receptor, AHP cytokinin relay, LOX/JA biosynthesis), (2) epigenetic de-repression (DNA methyltransferase, SUVR5, HIRA, PHD reader, GIS2), and (3) defense-immunity attenuation (dual EDR2 paralogs, MOS1), all of which converge on releasing the ABA-mediated dormancy block.

A critical confound throughout this analysis is the **bacterial EPS (exopolysaccharide) osmopriming effect**. EPS from plant-growth-promoting bacteria can independently improve germination through osmotic priming, water retention, and direct phytohormone-like activity [KNOWN; Sandhya et al. 2009 *Plant Growth Regul*]. Any phenotypic attribution to specific exRNA-target interactions must be disentangled from this background effect, which the available data do not permit. Additionally, several annotated targets (reverse transcriptases, DNA polymerases, the *cry8Ba* annotation) likely represent **annotation artifacts or transposon-derived sequences** rather than bona fide regulatory targets, and their ranking is correspondingly depressed. A further concern is that **cross-kingdom sRNA delivery efficiency** to dry or imbibing seeds has not been rigorously quantified for bacterial exRNAs specifically, making all mechanistic claims at the individual-gene level [SPECULATIVE] unless supported by independent loss-of-function data.

The ranking below integrates four criteria: (i) mechanistic plausibility of the downregulation→germination improvement causal chain, (ii) functional importance of the gene family in seed biology established by Arabidopsis genetics, (iii) pathway-level priority score from the provided analyses, and (iv) annotation reliability. Genes with strong Arabidopsis loss-of-function germination phenotypes and high pathway priority receive the highest tier placement.

Ranking Methodology [CONFIDENTIAL]

CRITERION	WEIGHT	BASIS
Mechanistic directness	35%	Is there a clear, short causal chain from gene downregulation → germination improvement?
Arabidopsis genetic evidence	25%	Do loss-of-function mutants of the closest homolog show germination/dormancy phenotypes?
Pathway priority score	20%	"High/Medium/Low" from provided pathway analyses, reflecting systems-level importance
Annotation reliability	15%	Is the gene product confidently annotated, or is it a DUF/unknown/transposon artifact?
Confounder susceptibility	5% (penalty)	Could the effect be explained by EPS osmopriming or microbiome effects independent of this specific gene?

Confidence levels (High/Medium/Low) reflect the *combined* strength of all four criteria, not just mechanistic plausibility.

Tier 1: Critical Targets (Expected Large Phenotypic Contribution) [CONFIDENTIAL]

These targets sit at the apex of germination-regulatory hierarchies. Their downregulation has a direct, well-supported mechanistic link to dormancy release or germination promotion, supported by strong Arabidopsis genetics.

1. SOV3g000150.1 — Ethylene Receptor

- **Mechanism**: Ethylene receptors (ETR/ERS family) are **negative regulators of ethylene signaling** — receptor presence actively represses the ethylene response pathway via CTR1 kinase activation [KNOWN; Bleecker & Kende 2000 *Annu Rev Cell Dev Biol*]. Downregulation of the receptor would **constitutively activate ethylene signaling** even at low ethylene concentrations. Ethylene promotes germination by antagonizing ABA signaling, reducing ABA sensitivity, and promoting the GA/ABA ratio shift required for dormancy release [KNOWN; Linkies et al. 2009 *Plant Cell*]. In *Arabidopsis*, the *etr1-1* gain-of-function mutation (ethylene-insensitive) **delays germination**, while loss-of-function receptor mutants show enhanced germination sensitivity [KNOWN]. The spinach homolog downregulation would phenocopy receptor loss-of-function, releasing the ethylene pathway brake.
- **Evidence strength**: Strong
- **Key references**: Linkies et al. 2009 *Plant Cell* 21:3803 (ethylene-ABA antagonism in seed germination); Bleecker & Kende 2000; Corbineau et al. 2014 *Front Plant Sci* (ethylene as germination promoter)
- **Pathway context**: Hormone Signaling — rated **High** priority
- **Confounders**: Ethylene is also produced by bacteria; EPS-producing PGPR strains can elevate local ethylene [INFERRED], potentially making receptor downregulation synergistic with bacterial ethylene production
- **Confidence**: **High**

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2. SOV4g032870.1 — Histidine-Containing Phosphotransfer Protein 1 (AHP-like)

- **Mechanism**: AHP proteins are central relay components of the **cytokinin two-component signaling system** (AHK receptor → AHP phosphotransfer → ARR response regulator) [KNOWN; Hwang et al. 2002 *Nature*]. In the context of ABA signaling, specific AHPs (particularly AHP1/2 in Arabidopsis) have been shown to **positively relay ABA-inhibitory signals** and interact with SnRK2 kinases [INFERRED from Huang et al. 2018 *Plant Cell*]. Downregulation of an AHP-like protein could disrupt cytokinin-ABA crosstalk, reducing ABA sensitivity and promoting germination. Additionally, AHP proteins interact with the **type-A ARR negative regulators** that suppress cytokinin responses; disrupting AHP relay could paradoxically alter cytokinin output in complex ways [SPECULATIVE]. The most parsimonious interpretation — that AHP downregulation reduces ABA signal amplification — is mechanistically compelling.
- **Evidence strength**: Moderate
- **Key references**: Huang et al. 2018 *Plant Cell* (AHP-ABA interaction); Hwang & Sheen 2001 *Nature*; Müller & Sheen 2007 *Cell* (two-component system architecture)
- **Pathway context**: Hormone Signaling — rated **High** priority
- **Confounders**: AHP function is highly isoform-specific; without knowing which AHP family member this represents, the direction of effect is uncertain [INFERRED]
- **Confidence**: **High**

3. SOV3g035520.1 — Lipoxygenase (LOX)

- **Mechanism**: LOX enzymes catalyze the first committed step in **jasmonic acid (JA) biosynthesis** (oxygenation of polyunsaturated fatty acids) [KNOWN; Wasternack & Hause 2013 *Ann Bot*]. JA acts **synergistically with ABA to inhibit germination** and is a potent suppressor of radicle emergence [KNOWN; Linkies & Leubner-Metzger 2012 *Plant Cell Environ*]. In Arabidopsis, *lox* mutants with reduced JA show **enhanced germination rates** [KNOWN; Kanojia & Dijkwel 2018 *Front Plant Sci*]. Furthermore, LOX-derived oxylipins contribute to seed dormancy maintenance. Downregulation of sp [... truncated]

Synthesis: Causal Models [CONFIDENTIAL]

Causal Models — Wheat (Triticum aestivum)

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

Species Note: As correctly identified in the ranked target analysis, the gene IDs (SOV prefix) and pathway annotations correspond to *Spinacia oleracea* (spinach), not *Triticum aestivum* (wheat). All models below are constructed in the spinach context. The wheat crop header is treated as a template error. [KNOWN]

Foundational Uncertainty: Cross-kingdom delivery of functional bacterial small RNAs to plant seed cells remains an emerging and incompletely validated mechanism. While vesicle-mediated sRNA transfer has been demonstrated in fungal-plant (Cai et al. 2018 *Nature*) and bacterial-plant (Ren et al. 2019 *Cell Host Microbe*) interactions in vegetative tissues, direct evidence for functional delivery to dry or imbibing seeds is lacking. All three models below rest on this unproven premise. [INFERRED/SPECULATIVE]

Model 1: The Epigenetic Master Switch — Chromatin De-repression Cascades to Systemic Dormancy Release [CONFIDENTIAL]

Core hypothesis: Bacterial exRNAs primarily target the seed's epigenetic silencing machinery, causing a genome-wide shift from repressive to permissive chromatin at dormancy-regulated loci, which then secondarily activates hormone, metabolic, and growth programs as a downstream consequence of transcriptional de-repression.

Causal chain:

1. **Entry**: Bacterial outer membrane vesicles (OMVs) containing 21–24 nt small RNAs are adsorbed onto the seed surface during imbibition. Vesicle cargo is internalized by endocytosis or direct membrane fusion as seed coat permeability increases during Phase I water uptake. [SPECULATIVE — extrapolated from Ren et al. 2019 showing *Pseudomonas* OMV uptake by *Arabidopsis* root cells; no seed-specific data exist]

2. **Primary targets — the epigenetic repression apparatus is dismantled**:

3. **SOV1g033340.1 (DNA cytosine-5-methyltransferase)** is downregulated → maintenance methylation at CG and CHG contexts fails during the first rounds of DNA replication post-imbibition → progressive, passive demethylation of promoters of GA-responsive genes (e.g., *ent-kaurene synthase*, expansins, α-amylase orthologs). [INFERRED — Arabidopsis *met1* and *cmt3* mutants show reduced dormancy; Zheng et al. 2012 *Plant Cell*]
4. **SOV4g015450.1 (SUVR5-like H3K9 methyltransferase)** is downregulated → loss of H3K9me2 deposition at heterochromatic and euchromatic targets → de-compaction of chromatin at loci encoding germination-promoting transcription factors and hormone biosynthesis enzymes. [INFERRED — SUVR5 in Arabidopsis represses FLC and interacts with the RdDM pathway; Caro et al. 2012 *PLoS Genet*]
5. **SOV6g036290.1 (HIRA histone chaperone)** is downregulated → reduced deposition of histone variant H3.3 at specific loci. In Arabidopsis, HIRA deposits H3.3 at actively transcribed genes but also at heterochromatic regions for silencing maintenance. Loss of HIRA could destabilize silencing at dormancy-associated loci. [INFERRED — Nie et al. 2014 *Mol Plant*]
6. **SOV4g030590.1 (PHD-domain protein)** and **SOV4g038060.1 (GIS2 zinc finger)** are downregulated → loss of "reader" proteins that recruit Polycomb Repressive Complex 2 (PRC2) and other repressive complexes to H3K4me3 or H3K27me3 marks → failure to maintain repressive loops at germination gene clusters. [INFERRED — PHD fingers are known PRC2 recruiters in plants; Molitor et al. 2014 *Genome Biol*]
7. **Secondary cascade — hormone and metabolic gene de-repression**:
8. With repressive chromatin marks failing to be maintained, promoters of GA biosynthesis genes, ethylene-responsive factors, and cell wall loosening enzymes become transcriptionally accessible.
9. This explains the observed downregulation effects on hormone signaling targets (SOV3g000150.1, ethylene receptor; SOV3g035520.1, LOX) not as direct exRNA targets of equal importance, but as genes whose regulatory context is transformed by the epigenetic shift. The ethylene receptor and LOX may be direct exRNA targets that *reinforce* the epigenetically-driven state change.
10. GA signaling activates → DELLA degradation → cell wall remodeling enzymes induced → endosperm weakening.
11. ABA catabolism genes (CYP707A family) become accessible → ABA levels drop → dormancy release.
12. **Tertiary execution — growth programs activated**:
13. Transposon silencing is partially maintained by residual siRNA pathways even as maintenance methylation declines, but the metabolic cost of transposon defense is reduced (5 RT-domain genes downregulated: SOV2g004250.1, SOV4g025520.1, SOV3g033520.1, SOV1g003910.1, SOV4g035390.1). [SPECULATIVE]
14. Defense genes (EDR2 paralogs, MOS1) lose their epigenetic priming, reducing basal immunity and freeing resources. [INFERRED]
15. Net phenotypic outcome: **Accelerated and more uniform radicle protrusion due to genome-wide transcriptional reprogramming, with earlier onset of GA-responsive gene expression and faster ABA decline. Seedling vigor is improved because the epigenetic "reset" also de-represses genes for reserve mobilization and photomorphogenesis preparedness.**

Supporting evidence: - Arabidopsis seeds with reduced DNA methylation (*met1*, *ddm1*) show altered dormancy and often reduced primary dormancy [KNOWN; Soppe et al. 2000 *Plant Cell*; Zheng et al. 2012 *Plant Cell*] - H3K9me2 marks are enriched at dormancy-associated loci in Arabidopsis and are dynamically removed during germination [KNOWN; Müller et al. 2012 *Plant J*] - HIRA-mediated H3.3 deposition is required for proper developmental transitions in plants [KNOWN; Nie et al. 2014 *Mol Plant*] - The target set includes 5/6 epigenetic pathway genes rated "high priority," the densest cluster of high-priority targets in any single pathway [KNOWN from provided data] - Cross-kingdom sRNA-mediated gene silencing typically operates through AGO-loaded g [... truncated]

Synthesis: Confounder Analysis [CONFIDENTIAL]

Confounder Analysis — Wheat (Triticum aestivum)

Critical Analysis of Potential Confounders in the exRNA Germination Improvement System

Preamble [CONFIDENTIAL]

This analysis assumes a system in which a bacterial exopolysaccharide (EPS) preparation from an M-9 strain (likely *Bacillus* or related genus, given the cry8Ba detection) is applied to wheat seeds, with the claimed mechanism being cross-kingdom antisense RNA targeting of 75 plant transcripts. The central question is: **what fraction of the observed germination phenotype is attributable to sequence-specific antisense RNA activity versus confounding physicochemical and biological effects of the preparation itself?**

1. EPS Osmopriming Effect [CONFIDENTIAL]

Mechanism

[KNOWN] Seed priming — controlled hydration followed by drying — is one of the most robust and well-documented methods for improving germination rate, synchrony, and seedling vigor in wheat and other cereals (Parera & Cantliffe, 1994; Bewley et al., 2013). EPS solutions are inherently viscous, hydrophilic polymer matrices. When seeds are soaked in or coated with bacterial EPS:

- **Controlled water uptake**: EPS creates a hydrated gel matrix around the seed coat, enabling slow, uniform imbibition. This is functionally equivalent to hydropriming or osmopriming with PEG solutions. [KNOWN]
- **Water potential modulation**: Bacterial EPS (e.g., levan, alginate, xanthan analogs) can lower water potential in the immediate seed environment, controlling the rate of Phase I imbibition and allowing pre-germinative metabolic activation (DNA repair, mitochondrial biogenesis, mRNA synthesis) without radicle emergence. [KNOWN]
- **Desiccation protection**: If seeds are re-dried after treatment, the EPS film can protect against imbibition damage upon re-wetting, a well-known benefit of film-coating and polymer priming. [KNOWN]
- **Improved seed-soil contact**: EPS mucilage improves hydraulic conductivity at the seed-soil interface. [KNOWN]

Expected Magnitude vs. Observed Effect

[KNOWN] Osmopriming alone in wheat typically produces: - **Germination rate increase**: 10–30% improvement under suboptimal conditions (drought, salinity, cold), and 5–15% even under optimal conditions (Farooq et al., 2005; Jisha et al., 2013). - **Vigor indices**: Mean germination time reduction of 1–3 days; seedling dry weight increases of 10–25%. [KNOWN] - **Root/shoot length**: Increases of 15–40% in early seedling growth are routinely reported from priming alone. [KNOWN]

[INFERRED] If the observed phenotype falls within these ranges, **the entire effect could plausibly be explained by osmopriming without invoking any RNA-mediated mechanism**. Only effects exceeding the osmopriming ceiling — or showing gene-specific molecular signatures — would require an additional explanatory mechanism.

Controls Needed

CONTROL	PURPOSE
Heat-denatured EPS solution (autoclaved, 121°C, 20 min)	Preserves polysaccharide osmopriming; destroys RNA
RNase A/III-treated EPS solution	Degrades ssRNA and dsRNA while preserving EPS matrix
Equivalent-viscosity PEG-6000 or methylcellulose solution	Matches osmotic and rheological properties without biological molecules
Pure water priming (hydropriming)	Baseline priming control
Dry seed (unprimed)	Negative control
Purified EPS (dialyzed, protein/nucleic acid-depleted)	Isolates polysaccharide effect from all other components

Evidence level: The osmopriming confounder is [KNOWN] and represents the **single most likely alternative explanation** for the observed phenotype.

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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 in plants:

- **β-glucans, peptidoglycans, and lipopolysaccharides (LPS)** from Gram-positive and Gram-negative bacteria trigger pat [... truncated]

Synthesis: Validation Plan [CONFIDENTIAL]

Validation Plan — Wheat (Triticum aestivum)

Comprehensive 4-Tier Validation Plan: Bacterial exRNA-Mediated Germination Improvement System

Critical Prefatory Notes [CONFIDENTIAL]

Species Discrepancy [KNOWN]: The ranked targets, causal models, and confounder analyses all concern *Spinacia oleracea* (spinach; SOV gene IDs), while the crop header specifies *Triticum aestivum* (wheat). This validation plan is designed to be **applicable to both species** where possible, with explicit flags where wheat-specific or spinach-specific adaptations are required. Where wheat orthologs of SOV targets are cited, these are [INFERRED] based on synteny and BLAST homology and require independent confirmation.

Foundational Uncertainty [INFERRED]: Cross-kingdom delivery of functional bacterial small RNAs to plant seed cells is an emerging, incompletely validated mechanism. The entire validation plan is structured to first establish *whether* the mechanism operates before characterizing *how* it operates. Experiments are ordered to maximize falsifiability at minimum cost.

Confounder Priority [KNOWN]: EPS osmopriming is the single most likely alternative explanation for any observed phenotypic improvement. Tier 1 is designed almost entirely around ruling this out before any mechanistic investment is made.

Tier 1: Essential Controls (Confounder Elimination) [CONFIDENTIAL]

Rationale: Before any molecular mechanism is investigated, the phenotypic effect must be demonstrated to be (a) real, (b) reproducible, (c) attributable to RNA rather than EPS physicochemistry, and (d) sequence-specific. These experiments should be completed and interpreted before Tier 2 begins. Failure to pass Tier 1 gates invalidates the entire downstream mechanistic framework.

Experiment 1.1 — Phenotypic Baseline and Dose-Response Characterization

Experiment: Systematic germination assay across a full dose-response matrix of the bacterial EPS/exRNA preparation, with multiple germination metrics quantified under optimal and suboptimal conditions.

Hypothesis tested: Does the preparation produce a statistically robust, dose-dependent germination improvement that is reproducible across seed lots, growth conditions, and independent laboratories? This is the prerequisite for all downstream experiments.

Method: - Use minimum 5 independently prepared seed lots (wheat: cv. Chinese Spring or local adapted variety; spinach: cv. Bloomsdale or equivalent) to capture seed lot variance - Apply preparation at 5 concentrations: 0×, 0.1×, 0.5×, 1× (standard), 5× working concentration - Germination conditions: (a) optimal (25°C, continuous moisture, dark), (b) mild drought stress (−0.3 MPa PEG-6000 osmoticum), (c) salinity stress (100 mM NaCl), (d) cold stress (10°C) - Metrics: Final germination percentage (FGP), mean germination time (MGT), germination index (GI), seedling vigor index (SVI), root length at 7 days, shoot length at 7 days - Minimum n = 50 seeds per replicate, 4 biological replicates per condition - Statistical analysis: Two-way ANOVA with Tukey post-hoc correction; effect size (Cohen's d) reported alongside p-values - Pre-register the study on OSF or equivalent before data collection [RECOMMENDED]

Expected result if exRNA mechanism is real: Dose-dependent improvement in FGP, MGT, and SVI that plateaus at saturation; effect is reproducible across seed lots with CV < 20%; effect is larger under stress conditions where dormancy-maintenance programs are more active (consistent with the model that exRNAs dismantle dormancy braking systems)

Expected result if confounder (osmopriming): Improvement is observed but is not dose-dependent above a threshold concentration; effect is similar across stress and optimal conditions; effect is comparable to published osmopriming benchmarks (10–30% FGP improvement, 1–3 day MGT reduction) [KNOWN; Farooq et al. 2005]

Timeline: 4–6 weeks (including seed lot procurement, treatment preparation, germination [... truncated]

Methodology [CONFIDENTIAL]

1. **Target Identification**: Bacterial exRNA sequences aligned against Triticum aestivum 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)