E X R N A - A G

Bacterial Extracellular RNA-Mediated Reprogramming of Maize (*Zea mays*) Seed Germination

Target Analysis · Mechanistic Models · Validation Strategy

20 Gene Targets Analyzed

REPORT PREPARED BY

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CONFIDENTIAL

ExRNA Research Report: Maize

Zea mays — Bacterial Extracellular sRNA Target Analysis

[CONFIDENTIAL]

> **CONFIDENTIAL** — Generated 2026-02-19 **Family**: Poaceae | **Assembly**: Zm-B73-REFERENCE-NAM-5.0
> **Treatment**: M-9 bacterial EPS solution **Analysis Status**: targets_identified

Executive Summary [CONFIDENTIAL]

This report presents the analysis of **20 predicted exRNA targets** in Zea mays (maize). 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	11
Medium	5
Low	4
Total	**20**

Pathway Distribution

PATHWAY	TARGETS
Unknown	4
Hormone Signaling	3

PATHWAY	TARGETS
Protein Processing	3
Metabolic Priming	2
Epigenetic Regulation	2
Cell Wall	2
Ros Redox	1
Transport Ion Homeostasis	1
Stress Response	1
Signaling	1

High-Priority Targets [CONFIDENTIAL]

GENE ID	ANNOTATION	PATHWAY
Zm00001eb197370_T001	ABI40 - ABA-Insensitive 4-like / ABI4-related transcription	hormone_signaling
Zm00001eb154520_T001	HEX6 - Hexokinase 6 (dual-function glucose sensor and glycol	metabolic_priming
Zm00001eb333290_T001	PRX91 - Class III peroxidase 91 (secretory/apoplastic; ROS g	ros_redox
Zm00001eb385450_T002	NPF15 - NRT1/PTR Family 6.3-like transporter (nitrate/peptid	transport_ion_homeostasis
Zm00001eb065740_T001	AHL9 - AT-hook motif nuclear-localized protein 9 (chromatin	epigenetic_regulation
Zm00001eb044800_T001	RING63 - RING-type E3 ubiquitin ligase (C3H2C3/RING-H2 type;	protein_processing
Zm00001eb194870_T002	RING265 - RING-type E3 ubiquitin ligase (C3HC4 type; selecti	protein_processing
Zm00001eb303410_T002	ppr377 - Pentatricopeptide repeat protein 377 (P-type; organ	metabolic_priming
Zm00001eb159250_T002	CYP10 - Cytochrome P450 family member (CYP71/CYP72 clade; po	hormone_signaling
Zm00001eb187270_T001	MYBR64 - MYB-related transcription factor 64 (ABA response;	hormone_signaling
Zm00001eb397700_T001	IBP1 - Bowman-Birk type trypsin inhibitor / stress-responsiv	stress_response

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 — Maize (Zea mays)

Definitive Ranked Analysis of Bacterial exRNA Target Genes in *Spinacia oleracea*: Contribution to Germination/Vigor Phenotype

> **Note on Scope**: All gene IDs are *Spinacia oleracea* (spinach) targets identified in a cross-kingdom exRNA experiment. The requested crop is *Zea mays*, but all targets are spinach genes. This analysis ranks spinach targets by their inferred contribution to the observed germination/vigor phenotype. Where maize orthologs are relevant for comparative context, they are noted explicitly.

Executive Summary [CONFIDENTIAL]

This target landscape represents a remarkably broad, multi-pathway reprogramming of the spinach seed transcriptome by bacterial extracellular small RNAs (exRNAs), likely delivered in the context of an exopolysaccharide (EPS)-primed bacterial inoculant. The 110+ predicted targets span 14 functional pathway categories, ranging from hormone signaling and epigenetic regulation to transposon silencing and unknown proteins. [KNOWN] The sheer breadth of targeting is consistent with the known promiscuity of small RNA-mediated silencing and the documented ability of bacterial sRNAs to enter plant cells via extracellular vesicles or direct uptake. [INFERRED] However, this breadth also introduces significant interpretive challenges: not all targets will contribute equally to the phenotype, and some may represent off-target effects or bystander downregulation with negligible functional consequence.

The dominant mechanistic theme emerging from pathway-level integration is the **suppression of the dormancy-defense-stress tradeoff**. The seed's default state—characterized by ABA dominance, epigenetic silencing of growth genes, active immune priming, and metabolic quiescence—is systematically dismantled across multiple independent regulatory nodes simultaneously. [INFERRED] This multi-node attack is likely more effective than targeting any single pathway, as it prevents compensatory buffering through redundant pathways. The three highest-impact pathway clusters are: (1) **Hormone Signaling** (ABA/ethylene/cytokinin suppression releasing the GA-growth axis), (2) **Epigenetic Regulation** (dismantling transcriptional repression of germination genes), and (3) **Defense/Immunity suppression** (resolving the growth-defense tradeoff). These three clusters are deeply interconnected and their simultaneous modulation likely produces a synergistic, non-additive effect on germination rate and vigor.

A critical caveat must be stated at the outset: [SPECULATIVE] the causal attribution of the germination phenotype to any specific target or subset of targets has not been experimentally validated through individual gene knockdown/knockout in spinach. The ranking below is based on (a) the known biology of homologous genes in *Arabidopsis thaliana* and other model systems, (b) the pathway-level priority assigned in the input data, (c) the mechanistic directness of the predicted effect on germination, and (d) the degree of cross-pathway convergence. Rankings should be treated as testable hypotheses, not established facts.

Ranking Methodology [CONFIDENTIAL]

Targets were scored across five weighted criteria:

CRITERION	WEIGHT	RATIONALE
Mechanistic Directness	30%	Does downregulation of this gene have a direct, well-characterized effect on germination rate or seed vigor in *Arabidopsis* or other species?
Pathway Priority Score	25%	Was the gene assigned "high" priority within its pathway? High = 3, Medium = 2, Low = 1
Cross-Pathway Convergence	20%	Does this gene sit at a node where multiple pathways intersect (e.g., ABA signaling, ROS, epigenetics)?
Evidence Quality	15%	Is the function based on [KNOWN] biochemistry, [INFERRED] homology, or [SPECULATIVE] domain prediction?
Confound Risk	10%	Could the effect be explained by EPS osmopriming, polysaccharide elicitor effects, or microbiome remodeling rather than specific exRNA targeting?

Targets with identical scores were separated by mechanistic specificity (a gene with a single, well-defined germination-relevant function ranks above one with broad, pleiotropic roles).

Confounders explicitly considered: - EPS (exopolysaccharide) osmopriming can independently improve germination by lowering water potential and improving imbibition kinetics [KNOWN] - Polysaccharide elicitors can trigger pattern-triggered immunity (PTI) and hormonal changes independent of exRNA [KNOWN] - Microbiome remodeling by the inoculant may alter the rhizosphere environment [INFERRED] - Annotation errors (notably SOV2g038830.1 "cry8Ba") reduce confidence in some targets [KNOWN concern]

Tier 1: Critical Targets (Expected Large Phenotypic Effect)

[CONFIDENTIAL]

These targets have strong mechanistic connections to core germination regulatory nodes, high pathway priority, and robust homolog data from model systems.

T1.1 — SOV3g000150.1 — Ethylene Receptor

- **Mechanism**: Ethylene receptors (ETR1/ERS family) are **negative regulators** of ethylene signaling—their presence suppresses ethylene responses. [KNOWN] Downregulation of the receptor would therefore **derepress ethylene signaling**, promoting germination. In *Arabidopsis*, ethylene promotes germination by antagonizing ABA signaling; *etr1* loss-of-function mutants show enhanced ethylene sensitivity and faster germination under stress. [KNOWN] The *Arabidopsis* homolog AT1G66340 (*ETR1*) is one of the best-characterized hormone receptors in plant biology. Downregulation of the spinach ortholog would shift the ABA/ethylene balance decisively toward germination. [INFERRED]
- **Evidence strength**: Strong
- **Key references**: Bleecker & Kende (2000) *Annu Rev Cell Dev Biol*; Linkies et al. (2009) *Plant Cell* (ethylene promotes endosperm cap weakening in *Lepidium sativum*); Arc et al. (2013) *Front Plant Sci* (ethylene-ABA antagonism in seeds)
- **Cross-pathway convergence**: Directly intersects with ABA signaling, ROS redox, and cell wall remodeling (endosperm cap weakening)
- **Confound risk**: Low — the mechanism is specific and directional
- **Confidence**: **High**

T1.2 — SOV4g032870.1 — Histidine-containing Phosphotransfer Protein 1 (AHP-like)

- **Mechanism**: AHP proteins are central relay components of the **two-component cytokinin signaling pathway** (AHK receptor → AHP → ARR response regulators). [KNOWN] In seeds, cytokinin signaling via type-A ARRs promotes ABA catabolism and reduces ABA sensitivity, thereby promoting germination. [KNOWN] However, AHP proteins also relay signals that can activate type-B ARRs, which repress germination by maintaining ABA-responsive gene expression. [INFERRED] Downregulation of a specific AHP isoform could disrupt this relay, potentially reducing ABA-mediated dormancy maintenance. The *Arabidopsis* homologs *AHP1-5* (AT3G21510, AT3G29350, AT5G39340, AT3G16360, AT1G03430) have well-characterized roles in cytokinin signal

transduction. [KNOWN] The pathway analysis assigns this gene "high" priority within hormone signaling, and it sits at a critical junction between cytokinin, ABA, and GA cross-talk.

- **Evidence strength**: Moderate-Strong
- **Key references**: Hwang et al. (2012) *Annu Rev Plant Biol*; Kushwah & Laxmi (2014) *Plant J* (AHP role in seed germination); Müller & Sheen (2007) *Plant Cell*
- **Cross-pathway convergence**: Hormone signaling × general signaling × metabolic priming
- **Confound risk**: Moderate — cytokinin effects on germination are context-dependent and isoform-specific
- **Confidence**: **High**

T1.3 — SOV1g033340.1 — DNA (Cytosine-5)-Methyltransferase

- **Mechanism**: DNA methyltransferases (CMT/MET/DRM family) establish and maintain cytosine methylation, a key epigenetic mark for transcriptional silencing of dormancy-associated genes and transposons. [KNOWN] In *Arabidopsis*, the *met1* mutant shows reduced seed dormancy and faster germ [... truncated]

Synthesis: Causal Models [CONFIDENTIAL]

Causal Models — Maize (Zea mays)

Alternative Causal Models: Bacterial exRNA-Mediated Improvement of Maize Germination and Seedling Vigor

Critical Framing Note: The target gene IDs are from *Spinacia oleracea* (spinach). For application to *Zea mays* (maize), these models assume functional conservation of orthologous pathways. Maize orthologs are referenced where identifiable (e.g., *ZmAHP*, *ZmLOX*, *ZmETR*). All models must be considered in light of confounders: EPS osmopriming, polysaccharide elicitor effects, and microbiome remodeling may contribute independently to the phenotype. The causal attribution to specific exRNA-target interactions remains **[SPECULATIVE]** until validated by individual gene perturbation experiments in maize.

Model 1: The Epigenetic Gatekeeper Model — "Unlock First, Then Grow" [CONFIDENTIAL]

Core hypothesis: Bacterial exRNAs primarily target the seed's epigenetic silencing machinery, causing a global chromatin de-repression event that is the necessary and rate-limiting precondition for all downstream hormonal, metabolic, and growth responses; without this epigenetic "unlock," the other targeted pathways would remain transcriptionally inaccessible.

Causal chain:

1. **Bacterial exRNA enters seed cells via extracellular vesicles (EVs) or direct uptake during imbibition.** [INFERRED] Bacterial outer membrane vesicles (OMVs) containing small RNAs are taken up by plant cells, as demonstrated in *Arabidopsis* with *Pseudomonas*-derived vesicles (Cai et al., 2018, *Mol Plant*). During imbibition, the hydrating seed coat and endosperm become permeable, and EPS from the bacterial inoculant may enhance vesicle stability and adhesion to cell surfaces. In maize, the pericarp and aleurone layer represent the primary uptake barriers; the coleorhiza and scutellar epithelium are likely initial uptake sites. [SPECULATIVE]
2. **Epigenetic repressor genes are downregulated → immediate de-repression of dormancy-silenced chromatin.**

3. **SOV1g033340.1 (DNA cytosine-5-methyltransferase)** ortholog in maize: likely *ZmMET1* or *ZmCMT3*. Downregulation prevents maintenance methylation at CG and CHG sites during the first round of DNA replication post-imbibition. [KNOWN: MET1 loss in *Arabidopsis* causes genome-wide demethylation and de-repression of silenced loci; Saze et al., 2003]
4. **SOV4g015450.1 (SUVR5-like H3K9 methyltransferase)** ortholog in maize: likely *ZmSUVH4/KYP* family. Downregulation reduces deposition of H3K9me2, the hallmark heterochromatic mark that reinforces DNA methylation through the self-reinforcing loop between CMT3 and KYP. [KNOWN: SUVH4/KYP mutants show reduced H3K9me2 and transposon de-repression in *Arabidopsis*; Jackson et al., 2002]
5. **SOV6g036290.1 (HIRA histone chaperone)** ortholog in maize: *ZmHIRA*. HIRA deposits the replication-independent histone variant H3.3 into chromatin. In dormant seeds, HIRA activity may maintain specific repressive chromatin configurations at stress-responsive loci. Downregulation disrupts this maintenance, allowing replication-coupled histone exchange to dilute repressive marks. [INFERRED from *Arabidopsis* HIRA function; Nie et al., 2014]
6. **SOV4g038060.1 (GIS2 zinc finger)** ortholog in maize: *ZmGIS2-like*. In *Arabidopsis*, GIS family members regulate trichome and epidermal development via epigenetic pathways; in the seed context, GIS2 likely acts as a transcriptional repressor of growth-promoting genes under ABA control. [INFERRED]
7. **SOV4g030590.1 (PHD domain protein)** — PHD fingers read H3K4me3 (active) or unmodified H3 (recruiting repressors). Downregulation of a repressive PHD reader releases PRC2-associated silencing at target loci. [INFERRED from structural homology]
8. **Chromatin de-repression cascades into three downstream domains:**

3a. Hormone-responsive promoters become accessible → ABA/GA balance shifts. - With repressive DNA methylation and H3K9me2 reduced at ABA-responsive element (ABRE)-containing promoters and GA-responsive promoters, the hormone signaling targets (SOV3g000150.1/ethylene receptor, SOV4g032870.1/AHP, SOV3g035520.1/LOX) become more susceptible to exRNA-mediated silencing because their transcripts are now being actively produced and thus targetable by RISC. [INFERRED] In maize, the *VP1/ABI3* locus and *GA20ox* loci are known to be epigenetically regulated during seed development (Hoecker et al., 1995). The ethylene receptor ortholog *ZmETR2* acts as a negative regulator of ethylene signaling; its downregulation would constitutively activate ethylene responses, which antagonize ABA. [KNOWN: *etr1* loss-of-function in *Arabidopsis* constitutively activates ethylene signaling; Chang et al., 1993] - AHP downregulation (ortholog: *ZmHP2*) attenuates cytokinin → ABA crosstalk via the two-component phosphorelay, reducing ABA sensitivity. [INFERRED from *Arabidopsis* AHP function in ABA signaling; Nishimura et al., 2004] - LOX downregulation (ortholog: *ZmLOX* family) reduces jasmonate (JA) biosynthesis, removing JA-ABA synergistic inhibition of germination. [KNOWN: LOX is rate-limiting for JA biosynthesis; Wasternack & Hause, 2013]

3b. Defense gene promoters become accessible but their products are simultaneously silenced → resource reallocation. - EDR2 (SOV3g043450.1, SOV6g048760.1), MOS1 (SOV5g005530.1), and R-gene (SOV1g021670.1) transcripts are produced from de-repressed chromatin but are immediately targeted by exRNAs, creating a futile cycle that consumes these defense transcripts. The net effect is that the transcriptional machinery is redirected toward germination genes while defense mRNAs are degraded. [SPECULATIVE] - In maize, defense genes including

ZmNLR family members and *ZmMYB* stress-responsive transcription factors (ortholog of SOV1g020340.1) are known to be metabolically costly; their suppression during early germination would free carbon and nitrogen. [INFERRED]

**3c. Transposon loci are de-re [... truncated]

Synthesis: Confounder Analysis [CONFIDENTIAL]

Confounder Analysis — Maize (Zea mays)

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 a related species, given the cry8Ba detection) is applied to *Zea mays* seeds, with the proposed mechanism being cross-kingdom antisense RNA silencing of ~20 plant target transcripts. The observed phenotype — improved germination rate, vigor, and early seedling growth — is multifactorial by nature, and the burden of proof for an exRNA-specific mechanism is high. Below, I systematically evaluate each class of confounder.

1. EPS Osmopriming Effect [CONFIDENTIAL]

Mechanism

[KNOWN] Seed priming with osmotic solutions (hydropriming, osmopriming) is one of the most well-established techniques in seed technology. Bacterial EPS is a high-molecular-weight polysaccharide matrix that:

- **Controls water uptake kinetics**: EPS solutions are viscous and have defined water potential (ψ). Controlled imbibition allows seeds to progress through Phase I and Phase II of germination (metabolic activation, DNA repair, mRNA synthesis) without completing Phase III (radicle emergence), effectively "priming" the seed. [KNOWN — reviewed in Paparella et al., 2015, *Plant Cell Reports*]
- **Maintains hydration envelope**: EPS forms a hydrogel around the seed coat, buffering against desiccation stress during early imbibition. [KNOWN — demonstrated for *Pseudomonas putida* and *Azospirillum* EPS; Sandhya et al., 2009]
- **Modulates seed coat permeability**: Polysaccharide solutions can alter the rate of solute exchange across the seed coat. [INFERRED]

Expected Magnitude vs. Observed Effect

- **[KNOWN]** Osmopriming alone (e.g., with PEG-6000 at -1.0 to -1.5 MPa) routinely improves maize germination rate by **10–30%** under stress conditions and **5–15%** under optimal conditions, with concomitant increases in seedling vigor indices (Hussain et al., 2015, *J. Agron. Crop Sci.*).
- **[KNOWN]** Bacterial EPS-based priming (e.g., from *Pseudomonas*, *Bacillus*) has been shown to improve germination by **8–25%** in cereals, with effects on root length and shoot length comparable to PEG osmopriming (Naseem et al., 2018, *Arch. Microbiol.*).
- **[INFERRED]** If the observed germination improvement falls within this 10–25% range, the **entire phenotype could plausibly be explained by osmopriming alone**, without invoking any RNA-based mechanism.

Controls Needed

1. **Water-only control** (hydropriming at equivalent volume/duration)
2. **Osmotically matched control**: PEG or methylcellulose solution matched to the water potential (ψ) of the EPS preparation
3. **Heat-denatured EPS control**: Autoclaved EPS solution (degrades RNA but preserves polysaccharide osmotic properties)
4. **RNase-treated EPS control**: EPS + RNase A/RNase III treatment (degrades ssRNA and dsRNA, preserves polysaccharide)
5. **Purified EPS control**: Polysaccharide fraction only, after nucleic acid removal (proteinase K + RNase + DNase treatment followed by dialysis)

Evidence Level

The osmopriming confounder is **HIGH LIKELIHOOD, HIGH IMPACT**. Without the above controls, it is not possible to attribute any portion of the phenotype to exRNA. **[KNOWN]**

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2. Polysaccharide Elicitor Effects [CONFIDENTIAL]

Known Defense/Growth Priming by Bacterial Polysaccharides

[KNOWN] Bacterial EPS and lipopolysaccharides (LPS) are well-characterized microbe-associated molecular patterns (MAMPs) / damage-associated molecular patterns that trigger plant innate immune signaling:

- **β-glucans, peptidoglycan fragments, and EPS oligosaccharides** are recognized by plant pattern recognition receptors (PRRs). In maize, relevant PRRs include members of the LysM-RLK family (e.g., *ZmLYK* genes,

homologs of *Arabidopsis* CERK1/LYK5) and leucine-rich repeat receptor-like kinases. [KNOWN — reviewed in Zipfel, 2014, *Curr. Opin. [... truncated]

Synthesis: Validation Plan [CONFIDENTIAL]

Validation Plan — Maize (Zea mays)

Comprehensive 4-Tier Validation Plan: Bacterial exRNA-Mediated Germination Improvement in *Zea mays*

Scope and Epistemic Framing: This validation plan addresses a system in which bacterial extracellular small RNAs (exRNAs), delivered in an EPS-containing inoculant, are proposed to improve maize germination and seedling vigor by cross-kingdom silencing of orthologous targets identified in spinach (*Spinacia oleracea*). All causal attributions to specific exRNA-target interactions are **[SPECULATIVE]** until validated. The plan is designed to systematically eliminate confounders before claiming mechanistic specificity, following the principle that extraordinary claims (cross-kingdom RNA silencing as the primary driver of an agronomic phenotype) require extraordinary evidence. Maize-specific biology (pericarp impermeability, aleurone signaling, endosperm-embryo communication) introduces additional barriers and considerations not present in spinach that are explicitly addressed throughout.

Tier 1: Essential Controls — Confounder Elimination

[CONFIDENTIAL]

These experiments must be completed and interpreted before any mechanistic claims are made. Failure to pass Tier 1 controls invalidates the exRNA hypothesis as currently framed.

T1-E1: Osmotic Equivalence Control

Experiment: Matched-osmolarity germination assay comparing full EPS inoculant against osmotically equivalent synthetic solutions.

Hypothesis tested: Rules out EPS osmopriming as the sole driver of improved germination rate and vigor. If PEG-matched controls produce identical phenotypes, the exRNA hypothesis is not supported and the effect is attributable to controlled imbibition kinetics alone.

Method: 1. Measure water potential (ψ) of the bacterial EPS inoculant preparation using a vapor pressure osmometer (Wescor VAPRO or equivalent) or psychrometer. [KNOWN: EPS solutions from *Bacillus* strains typically range from -0.3 to -1.2 MPa depending on concentration] 2. Prepare five treatment groups (n = 4 replicates × 50 seeds per replicate = 200 seeds per treatment): - **T1**: Full EPS inoculant (positive treatment) - **T2**: PEG-6000 solution matched to ψ of T1 (±0.05 MPa tolerance) - **T3**: Methylcellulose solution matched to ψ of T1 (non-ionic, non-elicitor polymer control) - **T4**: Distilled water (hydropriming control) - **T5**: Dry seed, no treatment (absolute baseline) 3. Imbibe seeds on germination paper (Anchor Paper #1 germination blotter) at 25°C in darkness for the duration matching the EPS treatment protocol (typically 6–24 hours), then transfer to standard germination conditions (25°C, 12h light/12h dark, 95% RH). 4. Score: Germination rate (GR = seeds germinated/total × 100 at 48h, 72h, 96h), Mean Germination Time (MGT), Seedling Vigor Index (SVI = germination% × mean shoot length at 7 days), root length, shoot length, and fresh weight at 7 days post-imbibition. 5. Statistical analysis: One-way ANOVA with Tukey HSD post-hoc; significance threshold $p < 0.05$. Effect sizes (Cohen's d) reported for all pairwise comparisons with T1.

Expected result if exRNA mechanism is real: T1 (full EPS inoculant) significantly outperforms T2 and T3 on at least germination rate, MGT, and SVI ($p < 0.05$, Cohen's $d > 0.5$). T2 and T3 may show modest improvement over T4 and T5 due to osmopriming, but T1 should show a statistically distinct, superior phenotype.

Expected result if confounder (osmopriming): T1, T2, and T3 show statistically indistinguishable germination rates, MGTs, and vigor indices. All three outperform T4 and T5 by 10–25% [KNOWN range for osmopriming in maize]. No significant difference between T1 and T2 would indicate the phenotype is fully explained by osmotic priming.

Critical decision point: If T1 ≈ T2 ≈ T3, **stop and reformulate**. The exRNA hypothesis cannot be the primary driver. If T1 >> T2 and T1 >> T3, proceed to T1-E2.

Timeline: 3–4 weeks (seed p [... truncated]

Methodology [CONFIDENTIAL]

1. **Target Identification**: Bacterial exRNA sequences aligned against Zea mays 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)