E X R N A - A G

Bacterial Extracellular RNA-Mediated Reprogramming of Broccoli (*Brassica oleracea var. italica*) Seed Germination

Target Analysis · Mechanistic Models · Validation Strategy

89 Gene Targets Analyzed

REPORT PREPARED BY

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CONFIDENTIAL

ExRNA Research Report: Broccoli

Brassica oleracea var. italica — Bacterial Extracellular sRNA Target Analysis [CONFIDENTIAL]

> **CONFIDENTIAL** — Generated 2026-02-19 **Family**: Brassicaceae | **Assembly**: None **Treatment**: M-9 bacterial EPS solution **Analysis Status**: targets_identified

Executive Summary [CONFIDENTIAL]

This report presents the analysis of **89 predicted exRNA targets** in Brassica oleracea var. italica (broccoli). 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	36
Medium	18
Low	35
Total	**89**

Pathway Distribution

PATHWAY	TARGETS
Unknown	35
Signaling	10

PATHWAY	TARGETS
Transport Ion Homeostasis	9
Development	7
Epigenetic Regulation	6
Protein Processing	5
Metabolic Priming	4
Defense Immunity	4
Cell Wall	3
Hormone Signaling	3
Stress Response	2
Ros Redox	1

High-Priority Targets [CONFIDENTIAL]

GENE ID	ANNOTATION	PATHWAY
Bo1g016960.1	CDK-Activating Kinase 1 (CAK1AT / CDKD;1-like)	signaling
Bo9g173970.1	Chaperone DnaJ-domain superfamily protein (DNAJD5-like)	protein_processing
Bo8g090760.1	tRNA (guanine-N2)-dimethyltransferase (TRM1-like)	epigenetic_regulation
Bo2g05500.1	RING finger (C3HC4-type) / BRCT domain protein	protein_processing
Bo6g067490.1	PDR9 / ABCG37 pleiotropic drug resistance transporter	transport_ion_homeostasis
Bo1g087970.1	Patellin 1 (PATL1) - SEC14/GOLD domain protein	transport_ion_homeostasis
Bo9g173960.1	Rab GDP Dissociation Inhibitor (GDI)	transport_ion_homeostasis
Bo1g079000.1	General Transcription Factor IIIC subunit 5 (TFIIIC5)	epigenetic_regulation
Bo4g076880.1	Pentatricopeptide Repeat (PPR) superfamily protein	metabolic_priming
Bo7g119590.1	CRK26 - Cysteine-Rich Receptor-Like Kinase 26	defense_immunity
Bo2g047480.1	DNA Primase large subunit / PrimPol	development
Bo4g061330.1	THIC - Thiamine (Vitamin B1) Biosynthesis Protein	metabolic_priming

GENE ID	ANNOTATION	PATHWAY
Bo9g126160.1	Glycosyl Hydrolase Family 17 (GH17) beta-1,3-glucanase	defense_immunity
Bo8g068060.1	MADS-box transcription factor (Type II MIKC-type) [corrected	development
Bo7g107400.1	CRK29 - Cysteine-Rich Receptor-Like Kinase 29	defense_immunity

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 — Broccoli (Brassica oleracea var. italica)

Definitive Ranked Target Analysis: Bacterial exRNA-Mediated Germination Improvement in *Spinacia oleracea* (Applied Context: *Brassica oleracea* var. *italica*)

Critical Prefatory Note: All gene IDs are from *Spinacia oleracea* (spinach). The stated crop is *Brassica oleracea* var. *italica* (broccoli). Cross-species extrapolation of exRNA targeting efficacy is [SPECULATIVE] unless direct evidence of conserved target site complementarity is demonstrated. Brassicaceae and Caryophyllales diverged ~100 Mya; ortholog conservation varies substantially by gene family. All mechanistic claims below are assessed in the spinach context unless otherwise stated.

Executive Summary [CONFIDENTIAL]

This target set of ~110 spinach genes, predicted to be downregulated by bacterial extracellular small RNAs (exRNAs), represents a remarkably coherent systems-level intervention in seed dormancy-to-germination transition. The targets are not randomly distributed across cellular functions; they cluster into 14 functional modules that collectively dismantle the molecular architecture of dormancy while simultaneously releasing brakes on growth-promoting pathways. The highest-confidence targets are those with (a) well-characterized roles in ABA/GA hormonal balance, (b) direct mechanistic links to cell wall loosening at the micropylar endosperm cap, or (c) epigenetic repressor functions with clear Arabidopsis precedent. These three functional clusters likely account for the majority of the observed germination/vigor phenotype.

A critical analytical challenge is separating genuine exRNA-mediated target suppression from confounding effects of the bacterial extracellular polysaccharide (EPS) matrix used as the delivery vehicle. EPS itself is a potent elicitor of plant immune responses and osmotic adjustment, and osmopriming effects alone can improve germination rate by 15–40% in Brassicaceae [KNOWN, based on established osmopriming literature]. Furthermore, several annotated targets (reverse transcriptases, retrotransposon Gag proteins, the putative cry8Ba protein) are either genomic "noise" targets with negligible germination relevance or likely misannotations, and their apparent downregulation may reflect off-target exRNA activity or annotation artifacts rather than functionally meaningful suppression.

The overall mechanistic model supported by this target set is a **multi-layer dormancy dissolution program**: bacterial exRNAs simultaneously (1) suppress ABA-pathway amplifiers and cytokinin relay components in hormone signaling, (2) dismantle epigenetic repressor complexes that lock dormancy-associated chromatin states, (3) reduce cell wall reinforcement enzyme activity to lower the mechanical resistance against radicle protrusion, (4) modulate ROS homeostasis to permit the pro-germination oxidative burst, and (5) suppress immune/defense pathway activity to redirect metabolic resources from defense to growth. The convergence of multiple independent pathways on a common phenotypic outcome substantially increases confidence that the observed germination improvement is mechanistically real, though the relative contribution of each module remains to be experimentally dissected.

Ranking Methodology [CONFIDENTIAL]

Targets were ranked using a weighted multi-criteria scoring system:

CRITERION	WEIGHT	RATIONALE
Mechanistic directness — how proximal is the target to the rate-limiting step of germination (radicle protrusion)?	30%	Proximal targets have larger expected effect sizes
Arabidopsis/Brassica homolog functional evidence — is the ortholog's role in germination experimentally demonstrated?	25%	Reduces inference distance
Pathway priority score — as assigned in the provided pathway analyses (high/medium/low)	20%	Reflects expert curation of functional importance
Pathway-level redundancy — does the target operate in a module with multiple co-targeted genes, increasing robustness?	15%	Pathway-level effects are more phenotypically stable than single-gene effects
Annotation confidence — is the gene annotation reliable, or is it a DUF/unknown/potentially misannotated?	10%	Uncertain annotations reduce confidence in mechanistic claims

Confounders assessed: EPS osmopriming effect (reduces attributable effect of specific targets), microbiome remodeling by bacterial treatment (could alter seed surface microbiome independently of exRNA), polysaccharide elicitor effects on immune priming (could confound defense pathway interpretations).

Tier 1: Critical Targets (Expected Large Phenotypic Effect)

[CONFIDENTIAL]

These targets are predicted to individually or collectively account for the majority of the germination/vigor improvement. Downregulation of any single Tier 1 target is expected to produce a measurable phenotypic shift; their combined suppression is predicted to be synergistic.

1. SOV3g000150.1 — Ethylene Receptor

- **Mechanism**: Ethylene receptors (ETR/ERS family) are **negative regulators** of ethylene signaling — receptor presence represses the ethylene response pathway [KNOWN]. In Arabidopsis, *etr1* loss-of-function mutants show enhanced germination and reduced dormancy (Bleecker et al. 1988; Corbineau et al. 2014). Ethylene promotes germination partly by antagonizing ABA signaling and partly by directly promoting endosperm weakening. Downregulating the receptor therefore **de-represses ethylene signaling**, mimicking an ethylene-rich environment and accelerating the ABA→GA transition. This is the most direct, best-characterized single-gene mechanism in the entire target set.
- **Evidence strength**: Strong
- **Key references**: AtETR1 (AT1G66340); *etr1-1* mutants (Bleecker et al. 1988 *Science* 241:1086); ethylene-ABA antagonism in germination (Linkies & Leubner-Metzger 2012 *Plant Cell Environ* 35:1727); endosperm cap weakening by ethylene (Linkies et al. 2009 *Plant Cell* 21:3803)
- **Confidence**: **High**
- **Confounders**: EPS may independently elicit ethylene production via PAMP recognition, partially masking the receptor-specific effect [INFERRED]

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

- **Mechanism**: AHPs are central relay components of the **two-component cytokinin signaling system** [KNOWN]. In Arabidopsis, AHPs transfer phosphoryl groups from receptor histidine kinases (AHK) to type-B ARR transcription factors, activating cytokinin-responsive gene expression. Critically, cytokinin signaling intersects with ABA pathways: cytokinin generally antagonizes ABA-mediated dormancy, but specific AHP isoforms also relay signals that can maintain dormancy-associated transcriptional programs [INFERRED]. Downregulation of this AHP-like protein is predicted to disrupt cytokinin signal relay, potentially reducing ABA-pathway amplification. The pathway analysis correctly identifies this as a high-priority target within hormone signaling. The net effect depends critically on which downstream ARR targets this specific AHP activates — if it primarily relays to type-A ARRs (negative regulators of cytokinin signaling), downregulation would paradoxically increase cytokinin sensitivity [SPECULATIVE].

- **Evidence strength**: Moderate
- **Key references**: AtAHP1-5 (Hutchison et al. 2006 *Plant Cell* 18:3073); AHP role in ABA-cytokinin crosstalk (Tran et al. 2007 *Plant Cell* 19:3847); cytokinin promotion of germination (Riefler et al. 2006 *Plant Cell* 18:40)
- **Confidence**: **Medium-High**
- **Confounders**: AHP family has 5 members in Arabidopsis with partially redundant functions; single-member knockdown may be buffered by paralogs [KNOWN]

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

- **Mechanism**: DNA methyltransferases (CMT/MET/DRM families) maintain cytosine methylation, a core epigenetic mark that silences dormancy-associated genes and [... truncated]

Synthesis: Causal Models [CONFIDENTIAL]

Causal Models — Broccoli (Brassica oleracea var. italica)

Alternative Causal Models: Bacterial exRNA-Mediated Germination Improvement in *Brassica oleracea* var. *italica* (Broccoli)

> **Cross-Species Caveat**: All target gene IDs derive from *Spinacia oleracea*. Application to *Brassica oleracea* var. *italica* requires conserved target-site complementarity at the sRNA binding loci, which is [SPECULATIVE] given ~100 My divergence between Caryophyllales and Brassicaceae. Ortholog-level functional conservation is more plausible for deeply conserved pathways (epigenetic regulation, hormone signaling, cell wall remodeling) than for rapidly evolving gene families (NLR immune receptors, transposable elements). All models below assume sufficient conservation for the core mechanistic logic to apply.

Model 1: The Epigenetic Derepression Cascade ("Unlock the Blueprint") [CONFIDENTIAL]

Core hypothesis: Bacterial exRNAs primarily dismantle the multi-layered epigenetic repressor architecture that locks dormancy-associated chromatin states, and the resulting transcriptional derepression of pro-germination gene programs is the dominant driver of improved germination speed and seedling vigor.

Causal chain:

1. **Entry & Loading**: Bacterial exRNAs (likely 20–25 nt small RNAs) are delivered to the seed surface within the bacterial EPS matrix during priming treatment. Upon imbibition, exRNAs enter seed cells — most likely the metabolically active aleurone/endosperm cap and outer embryonic cell layers — via endocytosis of EPS-associated vesicles or through transient membrane permeabilization during rehydration [SPECULATIVE]. Once intracellular, exRNAs are loaded into host AGO proteins (most likely AGO1 or AGO4 orthologs), forming functional RISC complexes [INFERRED from cross-kingdom RNAi precedent in *Botrytis cinerea–Arabidopsis* and *C. elegans*–bacteria systems; Weiberg et al., 2013, Science; Cai et al., 2018, Science].
2. **Epigenetic repressor suppression** → Immediate chromatin decompaction:
3. **DNA (cytosine-5)-methyltransferase (SOV1g033340.1)** is downregulated → maintenance methylation at CG and CHG contexts fails during the first rounds of DNA replication post-imbibition → passive demethylation of

promoters of GA-responsive genes (e.g., *EXPANSIN*, *α-amylase*, *endo-β-mannanase* orthologs) [INFERRED from Arabidopsis *met1*/*cmt3* mutant phenotypes showing reduced dormancy; Zheng et al., 2012, PNAS].

4. **SUVR5-like histone methyltransferase (SOV4g015450.1)** is downregulated → reduced deposition of H3K9me2 at heterochromatic loci → decompaction of pericentromeric and intergenic regions harboring dormancy-regulated genes [INFERRED from Arabidopsis *suvh4/kyp* mutants].
5. **HIRA histone chaperone (SOV6g036290.1)** is downregulated → altered H3.3 variant deposition dynamics → destabilization of nucleosome occupancy at transcriptional start sites of dormancy-maintaining genes, increasing accessibility to GA-responsive transcription factors [INFERRED from Arabidopsis HIRA functional studies; Nie et al., 2014].
6. **PHD-domain protein (SOV4g030590.1)** is downregulated → loss of a "reader" that recruits PRC2 or other repressive complexes to H3K4me0 marks → failure to reinforce Polycomb-mediated silencing of growth genes [INFERRED].
7. **GIS2 zinc-finger protein (SOV4g038060.1)** is downregulated → loss of a transcriptional repressor that integrates stress signals to suppress growth; in Arabidopsis, GIS family members regulate trichome/epidermal differentiation and interact with GA/cytokinin pathways [KNOWN for Arabidopsis GIS; Sun et al., 2015].
8. **Chromatin decompaction enables downstream transcriptional activation**:
9. With repressive DNA methylation, H3K9me2, and PRC2-mediated H3K27me3 all simultaneously reduced, the chromatin landscape shifts globally toward a euchromatic, transcriptionally permissive state [INFERRED].
10. GA-responsive promoters become accessible → endogenous GA (even at basal levels) can now drive expression of cell wall loosening enzymes, storage mobilization enzymes, and cell cycle re-entry genes [INFERRED].
11. This creates a feed-forward loop: derepressed GA biosynthesis genes produce more GA → further activation of GA-responsive targets [SPECULATIVE but consistent with GA-epigenetic crosstalk models].
12. **Downstream amplification through secondary pathway effects**:
13. The epigenetic "unlock" potentiates the effects of all other targeted pathways. Specifically:
    - Hormone signaling targets (AHP, LOX, ethylene receptor) are more effectively suppressed because their regulatory regions are now in an open chromatin context accessible to exRNA-guided RISC [SPECULATIVE].
    - Cell wall remodeling genes that *promote* germination (non-targeted expansins, mannanases) are transcriptionally derepressed [INFERRED].
    - Defense gene loci that were epigenetically maintained in a "poised" state lose their repressive marks, but because the defense pathway activators (EDR2, MOS1, RLKs) are simultaneously targeted by exRNAs, the net effect is defense suppression rather than activation [INFERRED].
14. **Net phenotypic outcome**: Faster, more synchronous radicle protrusion (reduced T50) due to earlier activation of the germination transcriptional program; improved seedling vigor due to earlier mobilization of storage reserves and earlier onset of cell division in the embryonic axis. Predicted effect: 20–40% reduction in mean germination time, with improved uniformity (reduced T90–T10 spread).

Supporting evidence: - Arabidopsis seeds with reduced DNA methylation (*met1*, *ddm1* mutants) show decreased primary dormancy [KNOWN; Zheng et al., 2012, PNAS; Footitt et al., 2015] - SUVH4/KYP loss-of-function reduces seed dormancy in Arabidopsis [KNOWN; Zheng et al., 2012] - Histone deacetylase inhibitors (trichostatin A) can break dormancy in multiple species [KNOWN; reviewed in Nonogaki, 2014, Plant Science] - Cross-kingdom RNAi via small RNAs [... truncated]

Synthesis: Confounder Analysis [CONFIDENTIAL]

Confounder Analysis — Broccoli (Brassica oleracea var. italica)

Critical Analysis of Potential Confounders in the exRNA Germination Improvement System

Preamble [CONFIDENTIAL]

This analysis assumes a system in which a bacterial extracellular polymeric substance (EPS) solution from an M-9 bacterial strain is applied to *Brassica oleracea* var. *italica* seeds, with the claim that 89 antisense small RNA targets mediate germination and vigor improvements. The central question is: **what fraction of the observed phenotype can be confidently attributed to sequence-specific antisense RNA activity versus alternative mechanisms inherent to the treatment matrix?**

1. EPS Osmopriming Effect [CONFIDENTIAL]

Mechanism

[KNOWN] Osmopriming is one of the oldest and most robust seed invigoration techniques. Seeds imbibed in osmotic solutions (PEG, mannitol, or any high-molecular-weight solute) undergo controlled hydration that allows pre-germinative metabolic activation — DNA repair, mitochondrial biogenesis, mRNA accumulation — without radicle protrusion (Paparella et al., 2015, *Plant Cell Reports*; Bewley et al., 2013, *Seeds: Physiology of Development, Germination and Dormancy*).

[KNOWN] Bacterial EPS is a complex mixture of high-molecular-weight polysaccharides (typically 10^5–10^6 Da), including polyglucans, polymannans, alginate-like polymers, and others depending on the species. These macromolecules are inherently osmotically active and viscous. An EPS solution will reduce water potential (Ψ) at the seed surface, creating a classic osmopriming environment.

[KNOWN] The magnitude of osmopriming effects on Brassicaceae germination is well-documented: - Germination rate increases of 10–30% under suboptimal conditions are routine with PEG priming in *Brassica* species (Zheng et al., 2016, *Scientia Horticulturae*). - Germination speed (T_{50} reduction) of 12–48 hours is commonly reported. - Seedling vigor indices (hypocotyl length × germination%) can increase 20–50%.

[INFERRED] Unless the EPS solution is formulated at an osmotic potential equivalent to pure water (which is physically impossible given the dissolved EPS), **every seed treated with the EPS solution is being osmoprimed to some degree**. The viscosity of EPS solutions also modulates the rate of water uptake, which itself constitutes a form of hydropriming control.

Expected Magnitude vs. Observed Effect

[INFERRED] If the observed germination improvement falls within the range of 10–30% rate increase and moderate vigor enhancement, **the entire phenotype could plausibly be explained by osmopriming alone** without invoking any RNA-mediated mechanism. Only effects that exceed the osmopriming ceiling — or that are qualitatively different (e.g., specific gene expression changes not seen with osmotic priming) — would require an additional explanation.

Controls Needed

CONTROL	PURPOSE
Heat-denatured EPS solution (autoclaved, 121°C/20 min)	Destroys RNA but preserves polysaccharide osmopriming capacity
RNase A/III–treated EPS solution	Degrades ssRNA and dsRNA; preserves EPS matrix
Size-matched PEG solution (equivalent Ψ)	Matched osmotic priming without any biological molecules
Purified EPS without RNA (ultrafiltration, <100 kDa cutoff to remove RNA-protein complexes, then reconstituted)	Separates polysaccharide from RNA cargo
Water control at equivalent volume	Baseline

Evidence level: [KNOWN] for osmopriming mechanism; [INFERRED] for its contribution to this specific system

2. Polysaccharide Elicitor Effects [CONFIDENTIAL]

Known Defense/Growth Priming by Bacterial Polysaccharides

[KNOWN] Bacterial exopolysaccharides are well-established microbe-associated molecular patterns (MAMPs) and can also function as damage-associated signals when partially degraded. Key examples:

- **Lipopolysaccharides (LPS):** If the M-9 strain is Gram-negative, LPS contamination of EPS preparations is essentially guaranteed. LPS triggers defen [... truncated]

Synthesis: Validation Plan [CONFIDENTIAL]

Validation Plan — Broccoli (Brassica oleracea var. italica)

Comprehensive 4-Tier Validation Plan: Bacterial exRNA-Mediated Germination Improvement in *Brassica oleracea* var. *italica* (Broccoli)

Prefatory Note on Cross-Species Translation: All ranked targets derive from *Spinacia oleracea* gene models. This validation plan explicitly incorporates experiments to determine whether orthologous *B. oleracea* var. *italica* genes are (a) present, (b) expressed during imbibition, and (c) accessible to exRNA-mediated suppression. Cross-species efficacy is [SPECULATIVE] at the outset and must be empirically established before mechanistic interpretation is valid. The plan is structured so that Tier 1 experiments generate the go/no-go decision before significant resources are committed to mechanistic dissection.

Strategic Framework [CONFIDENTIAL]

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OVERARCHING VALIDATION LOGIC:

Observed phenotype (germination improvement)
         ↓
Is it real and reproducible? ——————————— [Tier 1, Expts 1.1–1.3]
         ↓ YES
Is it RNA-dependent (not EPS/osmopriming)? — [Tier 1, Expts 1.4–1.6]
         ↓ YES
Which specific targets are suppressed? ———— [Tier 2, Expts 2.1–2.6]
         ↓
Do target suppressions cause the phenotype? — [Tier 3, Expts 3.1–3.5]
         ↓
Is it deployable and scalable? ———————————  [Tier 4, Expts 4.1–4.4]
```

Epistemic standards applied throughout: - [KNOWN]: Established in peer-reviewed literature with direct experimental evidence - [INFERRED]: Logically derived from known mechanisms; not directly demonstrated in this system - [SPECULATIVE]: Plausible hypothesis requiring experimental support

Tier 1: Essential Controls [CONFIDENTIAL]

Purpose: Establish phenotypic reality, reproducibility, and RNA-dependence before any mechanistic investment. These experiments must be completed and interpreted before Tier 2 begins. Estimated duration: 3–4 months.

Experiment 1.1: Multi-Environment Phenotypic Baseline

Experiment: Rigorous germination phenotyping across controlled environments with full statistical power calculation prior to data collection.

Hypothesis tested: The germination improvement phenotype attributed to bacterial exRNA treatment is real, reproducible across environments, and of sufficient effect size to warrant mechanistic investigation. This rules out batch effects, seed lot variation, and laboratory-specific artifacts.

Method: - Obtain minimum 3 independent seed lots of *B. oleracea* var. *italica* (e.g., cv. Marathon, Calabrese, and one open-pollinated variety) from different suppliers to capture genetic and physiological variation - Conduct germination assays in: (a) growth chamber at 20°C constant, (b) 15/25°C alternating temperature (simulates field diurnal variation), (c) suboptimal temperature 10°C (stress condition), (d) salt stress (50 mM NaCl), (e) osmotic stress (−0.3 MPa PEG-8000) - Treatments: Full EPS treatment, water control, positive control (established osmopriming: −0.8 MPa PEG-8000 for 24h at 15°C [KNOWN effective in Brassicaceae; Zheng et al., 2016]) - Sample size: n = 4 replicates × 50 seeds per replicate per treatment per condition = 200 seeds/treatment/condition; power calculation targeting 80% power to detect 15% germination rate difference at $\alpha = 0.05$ - Measure: Final germination percentage (FGP), mean germination time (MGT), germination index (GI), $T_{10}/T_{50}/T_{90}$ (time to 10/50/90% germination), seedling vigor index (SVI = radicle length × FGP), uniformity coefficient - Conduct across minimum 3 independent experimental runs separated by ≥2 weeks - Pre-register the statistical analysis plan (primary endpoint: T_{50} at 20°C; secondary endpoints: all others) on OSF or equivalent before data collection

Expected result if exRNA mechanism is real: Consistent, statistically significant improvement in T_{50} (predicted: 10–30% reduction) and SVI across ≥2 of 3 seed lots and ≥3 of 5 environmental conditions, with effect size exceeding the established osmopriming [... truncated]

Methodology [CONFIDENTIAL]

1. **Target Identification**: Bacterial exRNA sequences aligned against Brassica oleracea var. italica 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

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