E X R N A - A G

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

Target Analysis, Mechanistic Models, and Validation Strategy

REPORT PREPARED BY

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CONFIDENTIAL

ExRNA-Mediated Reprogramming of Spinach Seed Germination

Sarthak Tiwary | ExRNA-Ag | February 2026 **Evidence:** [KNOWN] = peer-reviewed; [INFERRED] = deduced from homolog data; [SPECULATIVE] = untested hypothesis

Bottom Line Up Front [CONFIDENTIAL]

M-9 (bacterial EPS seed treatment) improves spinach germination. sRNA sequencing identified ~109 spinach gene targets with complementarity to bacterial exRNAs. We asked: **Are these real targets? What's the mechanism? Can we prove it?**

Answers:

1. **21 high-priority targets** hit three regulatory levers: epigenetic brakes, ABA/GA hormone balance, and defense cost [1, 2, 3]. The target list is coherent -- not random.
2. **The mechanism is testable.** Two causal models make opposite predictions distinguishable by time-course qRT-PCR [4, 5].
3. **Six confounders threaten the hypothesis.** The simplest explanation -- osmopriming -- requires no novel biology [6, 7]. One target (cry8Ba) is bacterial contamination, not a spinach gene.
4. **One experiment decides everything.** RNase treatment of M-9: 2 weeks, <$500. If the phenotype survives RNase, RNA is irrelevant. If it doesn't, proceed.
5. **Minimum viable validation:** 5 experiments, 10-12 weeks, ~$3,600.

Caveat: "Zhu et al. (2022, *Nature Plants*)" -- cited in source documents as precedent for bacteria-to-plant sRNA transfer during imbibition -- **could not be verified** in PubMed, Google Scholar, or Nature Plants. Cross-kingdom RNAi is established in fungal-plant systems [8, 9, 10, 11, 12], but this specific bacterial-seed precedent may not exist. If confirmed, our finding would be genuinely novel.

1. Top 21 Targets [CONFIDENTIAL]

Scored by annotation quality, *Arabidopsis* homolog relevance [13], pathway membership, and mechanistic plausibility of downregulation promoting germination.

RANK	GENE ID	ANNOTATION	THEME	SCORE
1	SOV3g000150.1	Ethylene receptor	Hormone	10
2	SOV1g033340.1	DNA methyltransferase	Epigenetic	10
3	SOV3g043450.1	EDR2	Defense	9
4	SOV6g048760.1	EDR2 (paralog)	Defense	9
5	SOV4g015450.1	SUVR5 histone methyltransferase	Epigenetic	9
6	SOV3g035520.1	Lipoxygenase (LOX)	Hormone	9
7	SOV6g036290.1	HIRA histone chaperone	Epigenetic	8
8	SOV5g005530.1	MOS1-like (NLR regulator)	Defense	8
9	SOV4g032870.1	AHP (cytokinin relay)	Hormone	8
10	SOV1g020340.1	MYB transcription factor	Signaling	8
11	SOV2g014810.1	NAC domain protein	Signaling	8
12	SOV3g040200.1	Glutathione S-transferase	ROS	7
13	SOV3g038840.1	Peroxidase	ROS	7
14	SOV6g029280.1	6-PGDH (NADPH source)	Metabolic	7
15	SOV4g038060.1	GIS2 zinc finger	Epigenetic	7
16	SOV3g033920.1	PP2A regulatory subunit	Signaling	7
17	SOV1g018480.1	CNGC (Ca2+ channel)	Transport	7
18	SOV1g021960.1	Cation-Cl cotransporter	Transport	7
19	SOV2g025380.1	Cation-Cl cotransporter	Transport	6
20	SOV2g009230.1	Trehalose-P synthase	Metabolic	5
21	SOV4g030590.1	PHD-domain protein	Epigenetic	6

+49 medium-priority, +39 low-priority (Appendix A).

Why These Matter

- **Ethylene receptor (#1):** Negative regulator. *etr1* mutants show reduced dormancy [1, 14]. Downregulation = ethylene hypersensitivity = pro-germination.
- **DNA methyltransferase (#2):** Maintains dormancy via promoter silencing. *met1* mutants show altered dormancy [15]. Downregulation = passive demethylation of GA biosynthesis genes.

- **EDR2 (#3-4, two paralogs):** SA-defense regulator [16]. Dual knockdown = defense cost eliminated.
- **SUVR5 (#5):** Writes repressive H3K9me2/3. Downregulation = chromatin opening at germination loci.
- **LOX (#6):** First step of JA biosynthesis [17]. Downregulation = less JA + less ABA cross-talk + less lipid peroxidation. Triple benefit from one target.

Red Flags

- **cry8Ba (SOV2g038830.1):** *B. thuringiensis* crystal protein. **Bacterial contamination, not a spinach gene.** Remove from all analyses.
- **RT-domain proteins (4 targets):** Transposon alignment artifacts. Already low-priority.

2. Mechanism: Six Themes, Two Models [CONFIDENTIAL]

The 109 targets cluster into six functional themes that reinforce each other:

THEME	KEY TARGETS	WHAT DOWNREGULATION DOES
Defense downshift	EDR2 x2, MOS1, RLKs	Dismantles costly immunity [18, 19, 16]. Frees ATP/NADPH/carbon for growth.
Epigenetic remodeling	DNA-MTase, SUVR5, HIRA [20], GIS2	Removes repressive marks on pro-germination promoters [15, 21]. Opens GA3ox, CYP707A loci.
ROS optimization	Peroxidase, GST, 6-PGDH	Reduces antioxidant capacity --> controlled ROS burst into the "oxidative window" [22, 23]. Breaks ABA-ROS dormancy loop [22, 24].
Hormone rebalancing	Ethylene receptor, LOX, AHP, MYB, NAC	Targets regulatory nodes, not enzymes. Shifts ABA/GA ratio [1, 4, 5]. LOX + ethylene receptor = "brakes off + accelerator on."
Transport	CNGC [25], Cl- cotransporters, ABC	Blunts defense Ca2+ signaling. Adjusts osmotic potential for radicle emergence.
Metabolic priming	TPS [26], aspartokinase, CTP synthase	[SPECULATIVE] TPS down --> SnRK1 activated --> reserve mobilization. Contradicts simple model -- needs validation.

Two Testable Models

Model 1: Defense-Epigenetic Reprogramming - Primary targets: DNA-MTase, SUVR5, EDR2, MOS1 - Pathway: Epigenetic derepression --> GA gene activation --> ABA/GA shift - Test: Paclobutrazol (GA inhibitor) blocks the effect - Temporal: Epigenetic/defense targets down first (3-6h), ROS changes follow (12-24h)

Model 2: Metabolic-Hormonal Priming - Primary targets: Peroxidase, LOX, GST, CNGC, TPS - Pathway: ROS attenuation --> ABA-ROS loop broken --> passive hormone shift - Test: Ascorbic acid partially mimics the effect in untreated seeds - Temporal: ROS/metabolic targets down first (1-3h), epigenetic changes follow

Time-course qRT-PCR distinguishes them.

Minimal Effective Cocktail (6 genes from 109)

If mechanism validates, these 6 genes are the synthetic exRNA design targets: 1. DNA methyltransferase + SUVR5 (epigenetic) 2. Ethylene receptor + LOX (hormone) 3. EDR2 + MOS1 (defense)

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3. Confounders: What Could Kill This Hypothesis [CONFIDENTIAL]

#	CONFOUNDER	THREAT	CONTROL	KILL CONDITION
1	**EPS osmopriming**	CRITICAL	Iso-osmotic PEG 8000 vs M-9 [6]	PEG replicates phenotype = RNA irrelevant
2	**Polysaccharide MAMP elicitation**	HIGH	RNase treatment of M-9 [19, 27]	RNase has no effect = RNA irrelevant
3	**Live bacteria (PGPR)**	CONDITIONAL	Was M-9 filter-sterilized (0.22um)? [28, 29]	If no, serious threat
4	**Contamination / misannotation**	CRITICAL	Re-map to bacterial genome; BLAST all targets	Filtered list is empty = all artifacts
5	**RNA instability**	HIGH	Spike synthetic RNA, measure half-life [30, 31]	Degrades in minutes = mechanism implausible
6	**Non-specific RNA (PAMP)**	MEDIUM	Scrambled RNA control (same length/GC) [27]	Scrambled works = not sequence-specific

The Killer Experiment

RNase A/T1 treatment of M-9 + heat-inactivated RNase control.

This one experiment addresses confounders #1, #2, and #3 simultaneously because the treated solution retains identical EPS, osmolality, and polysaccharides -- only RNA is destroyed.

- **RNase kills phenotype:** Confounders #1-3 ruled out. exRNA hypothesis lives. Proceed.
- **RNase has no effect:** exRNA hypothesis is dead. Redirect to EPS/polysaccharide mechanism.

Cost: <$500. Time: 2 weeks. Do this first.

4. Validation Plan [CONFIDENTIAL]

Gate 1: Go/No-Go (Weeks 1-3, ~$400)

EXP	WHAT	COST	DECIDES
1.1	RNase treatment	$200	Is RNA the active molecule?
1.4	PEG iso-osmotic control	$200	Is it just osmopriming?
1.5	Bioinformatic cleanup	$0	Does target list survive filtering?

If any fails: STOP. Redirect program.

Gate 2: Target Validation (Weeks 4-8, ~$3,200)

EXP	WHAT	COST	DECIDES
1.2	EPS fractionation	$700	RNA fraction vs polysaccharide fraction?
1.3	Dose-response	$150	Quantitative RNA-phenotype link?
2.1	qRT-PCR time-course (15 genes)	$2,500	Are targets actually silenced?

qRT-PCR panel: 10 high-priority + 3 secondary + 2 negative controls (RT-domain artifact + cry8Ba). Timepoints: 0, 4, 8, 12, 24, 48h. Reference genes validated by geNorm [32] / NormFinder [33].

GO: >=6/10 targets downregulated (FC <0.5, p<0.05) at 8-24h. Controls unchanged. **NO-GO:** <3 targets downregulated.

Gate 3+: Mechanistic & Publication-Grade (If Gates 1-2 pass)

EXP	WHAT	COST	TIMELINE
3.1	ROS assays	$1,000	3-4 weeks
3.2	sRNA uptake imaging	$4,000	8-12 weeks
4.1	Degradome/PARE-seq [34, 35]	$6,500	12-16 weeks
4.2	Synthetic RNA mimics	$4,000	8-10 weeks
4.3	*Arabidopsis* mutant validation	$750	6-8 weeks

Timeline

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Week:  1   2   3   4   5   6   7   8   9  10  11  12
       |---|---|---|---|---|---|---|---|---|---|---|---|
RNase + PEG        [===]
Bioinformatics      [==]
       GATE 1 ---------*
Dose-Response       [===]
EPS Fractionation       [=======]
       GATE 2 -----------------*
qRT-PCR (15 genes)            [===============]
Hormone Markers                         [======]
       DATA COMPLETE --------------------------*
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Risks

RISK	IMPACT	MITIGATION
RNase doesn't kill phenotype	Program-ending	Redirect to polysaccharide/osmotic mechanism
PEG replicates phenotype	Program-ending	Accept: it's osmopriming, not RNAi
Unstable reference genes	Invalidates qRT-PCR	Validate 3 references with geNorm [32]
Poor seed RNA quality	Delays Tier 2	TRIzol + PVP-40 protocol

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5. Recommendation [CONFIDENTIAL]

Spend $400 and 2 weeks on Gate 1 (RNase + PEG + bioinformatics). This decides everything.

- **Pass:** High-value, high-novelty territory. One of the first bacteria-to-plant sRNA demonstrations during seed imbibition. Commercially and scientifically significant.
- **Fail:** Stop immediately. Save $5,000-20,000 on downstream experiments. The EPS itself may still be commercially valuable as a priming agent.

What we don't know yet: - Whether sRNAs enter spinach embryo cells - Whether any target is actually downregulated - Whether the phenotype depends on RNA at all - Whether the cited "Zhu et al. 2022" precedent is a real paper

The confounders analysis is a strength. It protects against investing in an uncontrolled hypothesis.

Appendix A: Medium Priority Targets (49) [CONFIDENTIAL]

GENE ID	ANNOTATION	PATHWAY	SCORE
SOV4g000330.1	Phytoene synthase	Metabolic	6
SOV1g021670.1	Disease resistance protein	Defense	5
SOV3g021300.1	Stress response NST1	Defense	5
SOV1g027650.1	Receptor-like kinase	Signaling	5
SOV4g000660.1	Ser/Thr kinase (RLK)	Signaling	5
SOV1g043000.1	RING E3 ubiquitin transferase	Turnover	5
SOV1g002960.1	F-box protein	Turnover	5
SOV4g010600.1	Glycosyltransferase	Cell wall	5
SOV1g032780.1	ABC transporter	Transport	5
SOV4g055600.1	Cytochrome P450	Metabolic	5
SOV5g006110.1	F-box protein-like	Turnover	4
SOV2g038280.1	F-box protein	Turnover	4
SOV2g028550.1	E3 ubiquitin ligase	Turnover	4
SOV2g021870.1	RING-type domain	Turnover	4
SOV1g033840.1	Glyco_transf_64	Cell wall	4
SOV4g051070.1	Beta-galactosidase	Cell wall	4
SOV4g041000.1	ABC transporter	Transport	4
SOV5g008400.1	Cation/H+ antiporter	Transport	4
SOV2g038560.1	DETOXIFICATION protein	Transport	4
SOV5g032210.1	NRT1/PTR transporter	Transport	4
SOV6g014710.1	Cd resistance-like	Transport	4
SOV3g000640.1	G3P transporter	Transport	4
SOV1g004930.1	GDSL esterase/lipase	Metabolic	4
SOV4g008190.1	GDSL esterase/lipase	Metabolic	4
SOV6g042250.1	GDSL esterase/lipase	Metabolic	4

GENE ID	ANNOTATION	PATHWAY	SCORE
SOV1g048270.1	Aspartokinase	Metabolic	4
SOV5g001320.1	CTP synthase	Metabolic	4
SOV6g037220.1	PPR protein	RNA proc.	4
SOV6g035270.1	PPR protein	RNA proc.	4
SOV5g000510.1	RNA helicase	RNA proc.	4
SOV1g048290.1	Glutamate receptor	Signaling	4
SOV2g039720.1	Ca-binding protein	Signaling	4
SOV5g030510.1	Protein kinase	Signaling	4
SOV1g019270.1	DNA topoisomerase 2	DNA repair	4
SOV4g051610.1	ATR kinase	DNA repair	4
SOV1g034720.1	Mito. morphology 35	Organelle	4
SOV2g013310.1	Folate transporter	Transport	3
SOV4g006140.1	Choline phosphotransferase	Metabolic	3
SOV6g042110.1	Glyoxylate reductase	Metabolic	3
SOV4g005210.1	PPR protein	RNA proc.	3
SOV4g023530.1	LUC7 (splicing)	RNA proc.	3
SOV4g046320.1	Ser/Thr kinase	Signaling	3
SOV6g037890.1	Patellin-6	Signaling	3
SOV4g011580.1	DNA polymerase	DNA repair	3
SOV5g013920.1	CRM factor CFM3	Organelle	3
SOV2g025780.1	TIM50-like import	Organelle	3
SOV5g034290.1	Cyt c biogenesis	Organelle	3
SOV3g020770.1	TIC214 (chloroplast)	Organelle	3
SOV4g054740.1	RETICULATA	Organelle	3

Low-priority (39 targets): chaperones, unknowns, transposon elements, housekeeping, contamination artifacts. Full list available in knowledge base.

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