

E X R N A - A G

Extracellular tRF RNA Drug: Complete Maize (*Zea mays*) MoA Dossier

Gene Targets, Mechanistic Models, Grain Filling, Yield
Predictions & Validation Strategy

REPORT PREPARED BY

Sarthak Tiwary

ExRNA-Ag

February 2026

C O N F I D E N T I A L

This document contains proprietary research findings.
Unauthorized distribution is strictly prohibited.

Extracellular tRF RNA Drug: Complete Maize (*Zea mays*) MoA Dossier

Date: 2026-02-18 **Classification:** CONFIDENTIAL -- Internal MoA Dossier for Regulatory & Scientific Decision Support **Version:** 1.0 **Prepared by:** Sarthak Tiwary / ExRNA-Ag Research Engine

Executive Summary [CONFIDENTIAL]

This dossier presents the complete mechanism of action (MoA) analysis for a bacterial-derived extracellular tRNA fragment (tRF) RNA drug applied to maize (*Zea mays*) seeds. The drug consists of G-rich 16-22 nt tRFs, glyco-protected, forming parallel G-quadruplex structures. Cellular uptake is confirmed via nucleolin-mediated endocytosis with 25-30% endosomal escape efficiency. Gene regulation has been experimentally validated by RT-qPCR for 5 of 20 predicted maize gene targets.

Key Findings

20 unique gene targets converge on five master regulatory axes: 1. **ABA signaling and dormancy release** (ABI40, PRH130/PP2C32, NPF15, IDP8263) 2. **Sugar sensing and energy metabolism** (HEX6, MYBR64, IBP1) 3. **ROS/redox optimization** (PRX91, CYP72A14, CYP72A15) 4. **Ubiquitin-proteasome and proteostasis** (RING63, RING265) 5. **Growth-defense resource reallocation** (AHL9, CML21, LRR-RLK, PSKR1, MOS1)

Target Assessment: 12 of 20 targets (60%) are predicted pro-germination when downregulated. 2 targets (PRH130/PP2C32, ppr377) are paradoxical -- their downregulation would theoretically oppose germination, serving as valuable internal controls. 6 targets have ambiguous or context-dependent effects.

Yield Prediction (conservative to optimistic):

TRAIT	CONSERVATIVE	CENTRAL	OPTIMISTIC
Yield increase	+3 to +8%	+5 to +10%	+10 to +15%
Kernel number/cob	+2 to +5%	+3 to +8%	+5 to +12%
100-kernel weight	+0 to +2%	+1 to +4%	+3 to +6%
Sugar content (% DW)	0 to +0.2%	0 to +0.5%	+0.5 to +1.0%

TRAIT	CONSERVATIVE	CENTRAL	OPTIMISTIC
Starch content (% DW)	-1 to +1%	-0.5 to +1.5%	+1 to +3%
Protein % change	-0.3 to 0%	-0.5 to -0.2%	-0.2 to +0.3%

Critical Risks: - IBP1 binds the Shrunken-1 (SuSy) promoter -- if downregulation reduces Sh1 expression, starch could decrease - MYBR64 may be homologous to ZmMYBR29, whose loss-of-function reduces grain filling rate by 26.7% - Direct gene silencing effects are unlikely to persist to grain fill (R1-R6); yield effects are primarily via compound growth from early vigor

Validation Requirements: A 2-year field program (\$138,000-188,000) across 4-6 locations, with a \$15,000 minimum viable experiment (RNase elimination + 1 field site + qPCR panel).

Table of Contents [C O N F I D E N T I A L]

- Part 1: Gene Annotation & Functional Refinement (20 targets)
- Part 2: Strict Mechanistic MoA Model (Phases 1-4)
- Part 3: Root System & Nutrient Capture Prediction
- Part 4: Grain Filling Pathway Analysis
- Part 5: Sweetness (Sucrose) & Starch Prediction
- Part 6: Protein Content Prediction
- Part 7: Quantitative Predictions (range-based)
- Part 8: Productivity Claim Validation Plan
- Part 9: Grain-Filling Biomarker Panel (25 genes)
- Part 10: Final Summary Tables
- Bibliography

PART 1: GENE ANNOTATION & FUNCTIONAL REFINEMENT

CONFIDENTIAL

Comprehensive Functional Annotation Table: 20 Maize (*Zea mays*) tRF-RNA Drug Targets

Prepared: 2026-02-18 **Organism:** *Zea mays* (B73 v5 reference genome) **Agent:** Bacterial-derived G-quadruplex-forming tRNA fragments (tRFs), 16-22 nt, glyco-protected, RNase-resistant **Uptake:** Nucleolin-mediated endocytosis; 25-30% endosomal escape efficiency **Validation status:** RT-qPCR confirmed for 5 selected targets; RNA uptake and gene regulation treated as experimentally validated **Evidence labeling:** [KNOWN] = published peer-reviewed literature; [INFERRRED] = logical deduction from conserved biology; [SPECULATIVE] = hypothesis requiring further validation

Master Annotation Table [CONFIDENTIAL]

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
1	Zm00001eb197370_T001	ABI40	B3/VP1-type ABA-responsive transcription factor (Viviparous1 family)	B3 DNA-binding, VP1/ABI3 transactivation	ABA signaling, seed dormancy maintenance, LEA/Em gene activation, alpha-amylase repression	NEGATIVE regulator of germination; POSITIVE regulator of dormancy
2	Zm00001eb154520_T001	HEX6	Hexokinase-2-like (ZmHXK family member); dual-function glucose sensor and metabolic enzyme	Hexokinase catalytic domain, sugar kinase superfamily	Glucose phosphorylation (glycolysis entry), glucose-ABA signaling crosstalk, sugar sensing/T6P-SnRK1 network	NEGATIVE regulator of germination (via glucose-ABA feedback)

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
3	Zm00001eb333290_T001	PRX91	Peroxidase 72 precursor; class III secretory peroxidase	Plant peroxidase domain, N-terminal signal peptide, heme-binding site	ROS metabolism (peroxidative and hydroxylic cycles), cell wall cross-linking (lignin/suberin polymerization), H2O2 scavenging, apoplastic redox signaling	NEGATIVE regulator of germination timing (cell wall stiffener)
4	Zm00001eb385450_T002	NPF15	NRT1/PTR family transporter (PTR2/NPF4.6-like); ABA/peptide/hormone transporter	Major Facilitator Superfamily (MFS) fold, 12 transmembrane helices, NPF/POT domain	ABA import (NPF4.6/AIT1 homolog), GA transport, peptide transport, JA-Ile transport, endodermal development	NEGATIVE regulator of germination (imports ABA into embryo)
5	Zm00001eb065740_T001	AHL9	AT-hook nuclear localized protein 5 (AT-hook motif family)	AT-hook motif (DNA binding), PPC/DUF296 domain (protein-protein interaction), nuclear localization	Chromatin remodeling at S/MAR regions, repression of auxin biosynthesis (YUCCA9), H2A.Z deposition via SWR1 complex recruitment	NEGATIVE regulator of growth/elongation
6	Zm00001eb044800_T001	RING63	RING-HC (C3H2C3) E3 ubiquitin ligase, Hakai-like subfamily	RING-HC zinc finger, C3H2C3 motif, potential Hakai domain	Ubiquitin-proteasome system, ABA signaling component turnover, protein quality control	AMBIGUOUS (depends on substrate identity)
7	Zm00001eb194870_T002	RING265	RING-IBR-RING (RBR/TRIAD) E3 ubiquitin ligase	RING1-IBR-RING2 supradomain (Ariadne/Parkin family), zinc-binding	Ubiquitin-proteasome system, protein homeostasis during dormancy-germination transition, potential autophagy regulation	AMBIGUOUS (substrate-dependent)

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
8	Zm00001eb303410_T002	ppr377	Pentatricopeptide repeat (PPR) protein; organellar RNA processing factor	PPR motifs (tandem 35-aa repeats), potential DYW domain (RNA editing)	Mitochondrial/plastid RNA editing, splicing, stabilization; energy metabolism during imbibition	POSITIVE regulator of energy metabolism (supports mitochondrial function)
9	Zm00001eb159250_T002	CYP10 (CYP72A14)	Cytochrome P450 monooxygenase, CYP72A subfamily (NOT cyclophilin)	P450 heme-thiolate fold, oxygen-binding pocket, NADPH-cytochrome P450 reductase interaction domain	Oxidative metabolism of terpenoids/hormones; potential ABA catabolism, brassinolide inactivation, or xenobiotic detoxification	CONTEXT-DEPENDENT
10	Zm00001eb187270_T001	MYBR64	MYB-related single-repeat (R1-MYB) transcription factor; CCA1/CPC-like subfamily	Single MYB/SANT DNA-binding repeat, SHAQKYF motif	Transcriptional regulation of starch mobilization, circadian clock (CCA1/LHY-like), potential germination repression (ZmMYB59 analog)	Likely NEGATIVE regulator of germination (ZmMYB59 paradigm)
11	Zm00001eb397700_T001	IBP1	Initiator Binding Protein 1; SANT/Myb + ubiquitin-like (UBL) domain transcription factor (NOT BiP/GRP78)	SANT/Myb DNA-binding domain, ubiquitin-like domain	Transcriptional regulation of Shrunken-1 (sucrose synthase) promoter; gibberellin signaling modulation; endosperm gene regulation	NEGATIVE regulator of internode elongation; POSITIVE for Sh1 expression

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
12	Zm00001eb036320_T002	LOC100273360	GDT1-like protein 3; Golgi-localized Ca ²⁺ /Mn ²⁺ transporter (UPF0016/TMEM165 family)	UPF0016 domain, 2-3 transmembrane segments, EF-hand-like Ca ²⁺ -binding motif	Golgi Ca ²⁺ /Mn ²⁺ homeostasis, glycosylation quality control (Mn ²⁺ supply to Golgi glycosyltransferases), cell wall matrix polysaccharide synthesis	POSITIVE for Golgi function and cell wall synthesis
13	Zm00001eb018090_T002	PRH130 (PP2C32)	Protein phosphatase 2C, Group A (clade A PP2C); negative regulator of ABA signaling (NOT RNA helicase)	PP2C catalytic domain, Mn ²⁺ /Mg ²⁺ -dependent phosphatase fold	ABA signaling (dephosphorylates/inactivates SnRK2 kinases), stomatal regulation, stress responses	NEGATIVE regulator of ABA signaling (therefore POSITIVE for germination when active)
14	Zm00001eb292850_T002	si614021b09a	O-methyltransferase ZRP4-like; caffeoyl CoA O-methyltransferase (CCoAOMT) involved in suberin/lignin monomer biosynthesis	AdoMet-dependent methyltransferase domain (SAM binding), dimerization interface	Phenylpropanoid pathway (lignin/suberin biosynthesis), cell wall fortification, Caspary strip formation, defense-related secondary metabolism	POSITIVE for cell wall rigidity/defense
15	Zm00001eb388550_T001	PCO145926 (CML21)	Calmodulin-like protein 21 (CML21); EF-hand Ca ²⁺ sensor relay protein	4 EF-hand Ca ²⁺ -binding domains, no enzymatic activity (signal relay only)	Calcium signaling, defense signal transduction, ROS regulation, pathogen response	POSITIVE for defense responses

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
16	Zm00001eb408850_T001	IDP8263	ABA-responsive protein with PH-GRAM (Pleckstrin Homology- Glucosyltransferases, Rab-like GTPase Activators, Myotubularins) lipid-binding domain	PH-GRAM domain (phosphoinositide binding), potential membrane association	ABA signaling at membrane interface, phospholipid signaling, potential vesicle trafficking	POSITIVE for ABA perception/signaling
17	Zm00001eb136860_T001	AI714716 (MOS1-like)	MODIFIER OF SNC1 1 (MOS1)-like protein; BAT2/ Myb_Cef domain chromatin/immunity regulator	BAT2 domain, Myb_Cef domain, nuclear localization	Chromatin remodeling at immune gene loci, NB-LRR receptor expression modulation (SNC1 regulation in Arabidopsis), epigenetic defense regulation	POSITIVE for constitutive immunity
18	Zm00001eb403550_T001	Zm00001d048453 (v4)	LRR receptor-like serine/threonine kinase; defense receptor	Leucine-rich repeat (LRR) ectodomain, transmembrane helix, intracellular Ser/Thr kinase domain	Pattern-triggered immunity (PTI), pathogen recognition, MAPK cascade activation, defense hormone signaling	POSITIVE for defense
19	Zm00001eb358860_T001	Zm00001d011422 (v4) / CYP72A15	Cytochrome P450 72A15; CYP72A subfamily paralog of CYP10/CYP72A14 (distinct locus, chromosome 8)	P450 heme-thiolate fold, oxygen-binding pocket	Oxidative metabolism (paralog of CYP72A14); potentially overlapping substrate specificity with CYP10 but distinct tissue/temporal expression	CONTEXT-DEPENDENT (same as CYP10)

#	GENE MODEL ID	NAME/SYMBOL	BEST FUNCTIONAL ANNOTATION	KEY PROTEIN DOMAINS	PATHWAY INVOLVEMENT	REGULATOR TYPE (GROWTH/YIELD)
20	Zm00001eb066630_T001	Zm00001d001877 (v4) / PSKR1-like	Phytosulfokine receptor 1-like; LRR receptor kinase for the phytosulfokine peptide hormone	LRR ectodomain (island domain for PSK binding), transmembrane helix, intracellular kinase domain	Phytosulfokine signaling (cell proliferation, expansion, differentiation), wound response, defense modulation, root elongation	DUAL: POSITIVE for cell proliferation/growth AND defense modulation

Physiological Impact Assessment Matrix [CONFIDENTIAL]

#	GENE	GERMINATION	VEGETATIVE GROWTH	ROOT DEVELOPMENT	GRAIN FILLING	YIELD
1	ABI40	PROMOTE [KNOWN]	Neutral [INFERRED]	Neutral [INFERRED]	May reduce dormancy in developing kernel [INFERRED]	Neutral to slight negative (reduced grain dormancy) [SPECULATIVE]
2	HEX6	PROMOTE [INFERRED]	Neutral (family redundancy) [INFERRED]	Neutral [INFERRED]	May impair sugar sensing in grain fill [SPECULATIVE]	Neutral [SPECULATIVE]
3	PRX91	PROMOTE [INFERRED]	Context-dependent [SPECULATIVE]	May enhance radicle emergence [INFERRED]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]
4	NPF15	PROMOTE [INFERRED]	May alter hormone distribution [SPECULATIVE]	May enhance if ABA suppresses root growth [INFERRED]	May reduce ABA import to developing grain [SPECULATIVE]	Context-dependent [SPECULATIVE]

#	GENE	GERMINATION	VEGETATIVE GROWTH	ROOT DEVELOPMENT	GRAIN FILLING	YIELD
5	AHL9	PROMOTE [INFERRRED]	PROMOTE (derepressed auxin/elongation) [KNOWN]	PROMOTE (enhanced elongation) [INFERRRED]	Neutral [SPECULATIVE]	Potentially positive via vigor [SPECULATIVE]
6	RING63	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS
7	RING265	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS	AMBIGUOUS
8	ppr377	May INHIBIT (reduced mitochondrial ATP) [INFERRRED]	May INHIBIT [INFERRRED]	May INHIBIT [INFERRRED]	May INHIBIT [SPECULATIVE]	May reduce [SPECULATIVE]
9	CYP10/ CYP72A14	CONTEXT-DEPENDENT	CONTEXT-DEPENDENT	CONTEXT-DEPENDENT	CONTEXT-DEPENDENT	CONTEXT-DEPENDENT
10	MYBR64	PROMOTE (if germination repressor) [INFERRRED]	Context-dependent [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]
11	IBP1	May PROMOTE (altered GA balance) [INFERRRED]	May PROMOTE (internode elongation relief) [INFERRRED]	Neutral [SPECULATIVE]	May alter Sh1/sucrose synthase expression [INFERRRED]	Context-dependent [SPECULATIVE]
12	LOC100273360	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	May impair Golgi function [SPECULATIVE]	Neutral [SPECULATIVE]
13	PRH130/ PP2C32	PARADOXICAL: likely INHIBIT [INFERRRED]	INHIBIT (increased ABA sensitivity) [INFERRRED]	INHIBIT [INFERRRED]	May enhance grain dormancy [SPECULATIVE]	May reduce via ABA hypersensitivity [SPECULATIVE]
14	si614021b09a/ ZRP4	PROMOTE (softer cell wall) [INFERRRED]	May reduce defense [SPECULATIVE]	May enhance (softer root tip tissues) [INFERRRED]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]
15	PCO145926/ CML21	Weak PROMOTE (defense savings) [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]
16	IDP8263	PROMOTE (reduced ABA perception) [INFERRRED]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]

#	GENE	GERMINATION	VEGETATIVE GROWTH	ROOT DEVELOPMENT	GRAIN FILLING	YIELD
17	AI714716/ MOS1	Weak PROMOTE (defense savings) [INFERRRED]	May PROMOTE (reduced immune burden) [INFERRRED]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	May reduce disease resistance [SPECULATIVE]
18	LRR-RLK	Weak PROMOTE (defense savings) [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	Neutral [SPECULATIVE]	May reduce disease resistance [SPECULATIVE]
19	CYP72A15	CONTEXT- DEPENDENT	CONTEXT- DEPENDENT	CONTEXT- DEPENDENT	CONTEXT- DEPENDENT	CONTEXT- DEPENDENT
20	PSKR1-like	MIXED: reduced defense but also reduced cell proliferation [SPECULATIVE]	May INHIBIT (reduced PSK- driven proliferation) [INFERRRED]	May INHIBIT (PSKR promotes root growth) [KNOWN]	Neutral [SPECULATIVE]	May reduce via impaired cell proliferation [SPECULATIVE]

Individual Gene Narratives [CONFIDENTIAL]

1. ABI40 -- Zm00001eb197370_T001 (B3/VP1-type ABA-responsive TF)

Arabidopsis ortholog: ABI3 (AT3G24650). **Rice ortholog:** OsVP1 (Os01g0911700).

[KNOWN] ABI40 belongs to the Viviparous1 (VP1)/ABI3 transcription factor family, the single most important master regulator of seed dormancy in cereals. The founding maize member, VP1, activates late embryogenesis abundant (LEA) and Em genes while simultaneously repressing germination-specific genes such as alpha-amylase. Loss-of-function vp1 alleles in maize cause vivipary -- precocious germination of the kernel while still attached to the ear -- demonstrating that VP1 is both necessary and sufficient for dormancy maintenance (Suzuki et al. 2003). In Arabidopsis, abi3 mutants exhibit reduced dormancy, desiccation intolerance, and accelerated germination. In sorghum, the related factors ABI4 and ABI5 bind GA 2-oxidase promoters, linking ABA transcriptional output to gibberellin catabolism (Cantoro et al. 2013). [INFERRRED] Downregulation of ABI40 by the tRF drug would phenocopy partial VP1 loss-of-function, reducing ABA-responsive gene expression and derepressing alpha-amylase and other germination enzymes. Even a 40-50% reduction in ABI40 transcript levels could significantly accelerate the dormancy-to-germination transition. This is the highest-value single target in the entire 20-gene panel.

Known maize mutants: vp1 (viviparous1) -- vivipary, precocious germination, loss of desiccation tolerance. **Arabidopsis mutants:** abi3-1 through abi3-9 -- reduced dormancy, green seeds, desiccation-sensitive. **Rice mutants:** os-vp1 -- reduced dormancy, pre-harvest sprouting susceptibility.

2. HEX6 -- Zm00001eb154520_T001 (Hexokinase-2-like; glucose sensor)

Arabidopsis ortholog: HXK1/GIN2 (AT4G29130). **Rice ortholog:** OsHXK5/OsHXK6.

[KNOWN] Hexokinases serve a dual role as glycolytic enzymes and intracellular glucose sensors. In Arabidopsis, Moore et al. (2003) demonstrated that HXK1 resides in the nucleus where it forms a complex with VHA-B1 and RPT5B to regulate gene expression independent of its catalytic activity. The *gin2* (glucose-insensitive 2) mutant, defective in HXK1, is insensitive to high glucose concentrations that normally delay germination through ABA accumulation (Price et al. 2003). In maize, nine HXK genes have been identified; ZmHXK4-6 and ZmHXK9 are mitochondria-localized, while ZmHXK7-8 are cytosolic (Zheng et al. 2019). ZmHXK promoters contain ABA-responsive and sugar-repression cis-elements. [INFERRRED] Downregulation of HEX6 by the tRF drug would break the glucose-ABA positive feedback loop that normally restrains germination speed: as starch is mobilized to glucose during imbibition, the reduced HXK signaling capacity would prevent glucose from triggering ABA biosynthesis, effectively uncoupling reserve mobilization from hormonal braking. The nine-member gene family provides metabolic redundancy, meaning the signaling effect would dominate over any metabolic impairment of glycolysis.

Known Arabidopsis mutants: *gin2-1* (glucose insensitive 2) -- glucose-insensitive germination, altered ABA sensitivity, reduced growth. **Maize mutants:** No direct hex6 mutant described; family redundancy may mask single-gene loss.

3. PRX91 -- Zm00001eb333290_T001 (Peroxidase 72 precursor; class III PRX)

Arabidopsis ortholog: AtPRX16 (AT2G18980) or AtPRX72 (AT5G66390). **Rice ortholog:** OsPRX family members.

[KNOWN] Class III peroxidases perform a paradoxical dual function: in the peroxidative cycle they consume H₂O₂ to cross-link cell wall polymers (lignin, suberin, extensin), stiffening the wall; in the hydroxylic cycle they generate superoxide and hydroxyl radicals that cleave polysaccharides, loosening the wall (Passardi et al. 2004). The net function of any individual peroxidase depends on its redox environment, substrate availability, and expression pattern. Jemmat et al. (2020) demonstrated that knockout of Arabidopsis AtPRX16 results in significantly earlier testa and endosperm rupture, directly proving that specific class III peroxidases restrict germination timing through cell wall stiffening. Maize contains 119 non-redundant class III peroxidase genes (Wang et al. 2015). [INFERRRED] If PRX91 predominantly operates in the peroxidative (ROS-scavenging/wall-stiffening) cycle, its downregulation would simultaneously increase local H₂O₂ concentrations (pushing through the pro-germination oxidative window) and reduce cell wall cross-linking at the radicle emergence point. The AtPRX16 knockout precedent strongly supports this interpretation.

Known Arabidopsis mutants: *atprx16* -- earlier testa/endosperm rupture. **Maize studies:** ZmPrx25 overexpression promotes germination under stress (2025); ZmPRX1 overexpression enhances root lignification (Zhai et al. 2024). No prx91 loss-of-function described.

4. NPF15 -- Zm00001eb385450_T002 (NRT1/PTR family transporter; NPF4.6-like)

Arabidopsis ortholog: NPF4.6/AIT1 (AT1G69850). **Rice ortholog:** OsNPF4 family members.

[KNOWN] The NRT1/PTR family (NPF) constitutes one of the largest transporter families in plants. NPF4.6/AIT1 was identified by Kanno et al. (2012) through a functional screen using the ABA receptor complex as a sensor, demonstrating that AIT1 directly imports ABA into cells. The ait1 mutant shows reduced ABA sensitivity during germination and reduced stomatal closure. NPF transporters also transport gibberellins (NPF3; Tal et al. 2016), jasmonoyl-isoleucine (GTR1/NPF2.10; Saito et al. 2015), and peptides. In maize, ZmPTR1 is specifically expressed in scutellar epithelial cells during germination, where it transports peptides derived from endosperm protein hydrolysis into the embryo (Doan et al. 2013). Maize contains 78 NPF genes (Wang et al. 2023). [INFERRRED] The strong homology of NPF15 to AIT1 suggests it functions as an ABA importer. Downregulation would reduce ABA influx into the embryo, lowering local ABA concentration and accelerating germination. This target synergizes with ABI40 (reduced ABA transcriptional output) and IDP8263 (reduced ABA membrane perception) to create a three-pronged assault on the ABA axis.

Known Arabidopsis mutants: ait1/npf4.6 -- reduced ABA sensitivity, faster germination, impaired stomatal closure.

Maize mutants: No npf15 loss-of-function described.

5. AHL9 -- Zm00001eb065740_T001 (AT-hook nuclear localized protein)

Arabidopsis ortholog: AHL29/SOB3 (AT3G46870) or AHL27/ESC (AT1G76500). **Rice ortholog:** OsAHL family.

[KNOWN] AT-hook nuclear localized (AHL) proteins bind AT-rich DNA sequences via the AT-hook motif and interact with each other through PPC/DUF296 domains. In Arabidopsis, SOB3/AHL29 and ESC/AHL27 are well-characterized negative modulators of hypocotyl growth (Street et al. 2008). The mechanism was elucidated by Favero et al. (2024): AHL proteins bind S/MAR (scaffold/matrix attachment region) elements in the promoters of growth-promoting genes (including YUCCA9 for auxin biosynthesis) and recruit the SWR1 chromatin remodeling complex to deposit the repressive histone variant H2A.Z, thereby transcriptionally silencing these genes. sob3-dominant gain-of-function produces short hypocotyls; sob3 loss-of-function produces elongated hypocotyls. The AHL family acts redundantly: Zhao et al. (2013) showed that multiple AHL proteins interact physically to modulate growth. [INFERRRED] Downregulation of AHL9 would derepress auxin biosynthesis genes and other growth-promoting targets, leading to enhanced cell elongation in the coleoptile, mesocotyl, and radicle. This directly addresses the observed "seedling vigor" phenotype and represents one of the clearest mechanistic links between a specific target and the growth component of the M-9 response.

Known Arabidopsis mutants: sob3-D (dominant) -- short hypocotyl; sob3 loss-of-function -- elongated hypocotyl; esc/ahl27 -- similar elongation phenotype. **Maize mutants:** No ahl9 loss-of-function described.

6. RING63 -- Zm00001eb044800_T001 (RING-HC E3 ubiquitin ligase, Hakai-like)

Arabidopsis ortholog: Hakai-like RING-HC E3 ligases. **Rice ortholog:** Not specifically identified.

[KNOWN] The maize genome encodes 590 RING-type E3 ubiquitin ligases (Li et al. 2025), making this one of the largest protein families. RING E3 ligases are central to ABA signaling: KEG targets ABI5 for degradation during non-stress conditions (Stone et al. 2006); AIP2 targets ABI3 for degradation (Zhang et al. 2005); ZmXeric01/2 target ABA 8'-hydroxylase for degradation, thereby elevating ABA levels (Brugiere et al. 2017). The Hakai subfamily in animals is involved in E-cadherin ubiquitination and epithelial-to-mesenchymal transitions; plant Hakai-like proteins are poorly characterized but may participate in RNA methylation (m6A modification) pathway regulation. [INFERRRED] Without knowing the specific substrates of RING63, the direction of its effect on germination cannot be determined. If RING63 targets negative regulators of growth for degradation (analogous to SDIR1), then its downregulation would stabilize growth repressors (anti-germination). If it targets ABA signaling components like ABI3/ABI5 (analogous to AIP2/KEG), its downregulation would stabilize these repressors (also anti-germination). The Hakai-like annotation raises the intriguing possibility of involvement in m6A-mediated mRNA fate regulation during the dormancy-to-germination transition.

Known maize mutants: No ring63 mutant. ZmXeric01/2 overexpression increases drought tolerance via elevated ABA (Brugiere et al. 2017).

7. RING265 -- Zm00001eb194870_T002 (RING-IBR-RING E3 ubiquitin ligase)

Arabidopsis ortholog: Ariadne/ARIH family or Parkin-like. **Rice ortholog:** Not specifically identified.

[KNOWN] RING-IBR-RING (RBR) E3 ligases constitute a distinct subfamily characterized by the RING1-IBR-RING2 supradomain. The human Parkin (PARK2) is the best-characterized RBR ligase, involved in mitochondrial quality control and autophagy. In plants, RBR E3 ligases are implicated in protein quality control, stress responses, and hormone signaling. The IBR (In-Between-RING) domain is a zinc-binding fold that positions the RING2 domain for catalysis. RBR ligases have a unique transfer mechanism: ubiquitin is first loaded onto the RING2 active-site cysteine before transfer to the substrate, distinguishing them from conventional RING E3 ligases. [INFERRRED] Like RING63, the effect of RING265 downregulation depends on substrate identity. If it mediates quality control of damaged mitochondrial proteins (Parkin-like function), downregulation during the rapid mitochondrial reactivation of imbibition could impair energy metabolism. If it targets dormancy-associated transcription factors, downregulation could alter the timing of their clearance. Combined modulation of RING63 and RING265 would broadly alter the proteostasis landscape during germination.

Known mutants: No ring265 loss-of-function in maize. Parkin mutants in Drosophila/human show mitochondrial dysfunction.

8. ppr377 -- Zm00001eb303410_T002 (PPR protein; organellar RNA processing)

Arabidopsis ortholog: AT1G80270 (PPR protein). **Rice ortholog:** Multiple OsPPR members.

[KNOWN] PPR (pentatricopeptide repeat) proteins constitute one of the largest gene families in land plants, with over 400 members in most angiosperms. They are targeted to mitochondria or plastids where they perform sequence-specific RNA recognition, mediating C-to-U editing, group II intron splicing, RNA stabilization, and translation initiation. Loss of specific PPR proteins in maize causes cytoplasmic male sterility (CMS), kernel defects, and embryo lethality. Mitochondrial reactivation during imbibition is extremely rapid: Nietzel et al. (2020) showed that ATP production begins within minutes of hydration, and 412 cysteine residues are redox-switched during this process. [INFERRRED] Downregulation of ppr377 could impair the processing of one or more mitochondrial transcripts, reducing respiratory chain complex assembly or function. This would be potentially counterproductive during germination, when energy demand is high. However, if ppr377 is required for editing of a transcript that is dispensable during early germination (e.g., a complex I subunit with partial bypass), the effect might be minimal. This target is flagged as potentially counterproductive.

Known maize mutants: emp (empty pericarp) mutations in various PPR genes cause embryo/endosperm defects; dek (defective kernel) mutations in PPR genes impair seed development. No ppr377-specific mutant described.

9. CYP10 / CYP72A14 -- Zm00001eb159250_T002 (Cytochrome P450 72A14)

Arabidopsis ortholog: CYP72A cluster (AT3G14630/CYP72A13, AT3G14620/CYP72A14). **Rice ortholog:** Os-CYP72A family.

[KNOWN] The CYP72A subfamily belongs to the CYP72 clan of cytochrome P450 monooxygenases. Maize contains 263 cytochrome P450 genes spanning multiple clans. CYP72A members in various species have been implicated in brassinolide inactivation (CYP72C1/SOB7 in Arabidopsis converts brassinolide to 26-hydroxybrassinolide, an inactive form), triterpenoid oxidation (CYP72A154 in Medicago oxidizes oleanane and hemolytic saponin intermediates), and phytoalexin metabolism. **CRITICAL CORRECTION:** CYP10 was originally misidentified as a cyclophilin due to the shared "CYP" prefix. It is definitively a cytochrome P450 monooxygenase (heme-thiolate fold, NADPH-P450 reductase dependent). [INFERRRED] If CYP72A14 inactivates brassinolide (CYP72C1 paradigm), its downregulation would increase active brassinolide levels, promoting cell elongation during germination. If it catalyzes phytoalexin metabolism, downregulation could alter defense compound profiles. The CYP72A subfamily's involvement in brassinolide catabolism is the most pharmacologically interesting possibility.

Known Arabidopsis mutants: cyp72c1/sob7 -- overexpression causes dwarfism (excess brassinolide inactivation); loss-of-function may increase active BR levels. No maize cyp72a14 mutant described.

10. MYBR64 -- Zm00001eb187270_T001 (MYB-related single-repeat R1-MYB TF)

Arabidopsis ortholog: CCA1/LHY (circadian clock) or CPC/TRY (trichome/root hair). **Rice ortholog:** OsMYBS1/OsMYBS2.

[KNOWN] MYB-related (single-repeat, R1-MYB) transcription factors are distinguished from R2R3-MYB and 3R-MYB proteins by possessing only one MYB repeat. This subfamily includes the circadian clock components CCA1 and LHY, the trichome/root hair patterning factors CPC and TRY, and the cereal sugar-responsive MYBS1/MYBS2 proteins. In rice, MYBS1 activates alpha-amylase transcription under sugar starvation, while MYBS2 competes for the same promoter elements and represses expression (Lu et al. 2002). In maize, Zhang et al. (2020) demonstrated that ZmMYB59 overexpression negatively regulates germination by reducing GA levels and increasing ABA levels, establishing a direct maize precedent for MYB-mediated germination repression. [INFERRRED] If MYBR64 functions analogously to ZmMYB59 or MYBS2 (as a transcriptional repressor of germination/starch mobilization genes), its downregulation would relieve this repression, accelerating alpha-amylase induction and starch mobilization. The R1-MYB classification makes a CCA1/LHY-like or MYBS-like identity most likely.

Known maize mutants: ZmMYB59 overexpression lines show reduced germination rate and altered ABA/GA balance (Zhang et al. 2020). No mybr64-specific mutant described.

11. IBP1 -- Zm00001eb397700_T001 (Initiator Binding Protein 1; SANT/Myb TF)

Arabidopsis ortholog: No clear ortholog (novel). **Rice ortholog:** Not identified.

[KNOWN] IBP1 (Initiator Binding Protein 1) was originally cloned from maize as a transcription factor that binds the initiator element of the Shrunken-1 (Sh1) gene promoter. Sh1 encodes sucrose synthase 1, a key enzyme for sucrose metabolism in the endosperm. IBP1 contains a SANT/Myb DNA-binding domain (related to c-Myb) and a ubiquitin-like (UBL) domain, a unique domain architecture among plant transcription factors. Overexpression in tobacco causes reduced internode elongation and altered gibberellin balance, indicating a growth-suppressive function. **CRITICAL CORRECTION:** IBP1 has been repeatedly misannotated as BiP/GRP78 (ER-resident Hsp70 chaperone) in various databases. This is incorrect -- IBP1 is a nuclear transcription factor, not an ER chaperone. The NCBI Gene ID is LOC542426. [INFERRRED] Downregulation of IBP1 could relieve transcriptional regulation of Sh1, potentially altering sucrose synthase levels in the endosperm during germination. The gibberellin balance changes observed in tobacco overexpression lines suggest IBP1 downregulation might increase active GA levels, which would promote germination.

Known maize data: IBP1 overexpression in tobacco reduces internode elongation. No ibp1 loss-of-function in maize described.

12. LOC100273360 -- Zm00001eb036320_T002 (GDT1-like Golgi Ca₂₊/Mn₂₊ transporter)

Arabidopsis ortholog: PAM71/CCHA1 (AT1G64150) for the chloroplast paralog; GDT1/TMEM165 for the Golgi paralog. **Rice ortholog:** OsGDT1-like.

[KNOWN] GDT1-like proteins belong to the UPF0016/TMEM165 family of cation/Ca₂₊ exchangers localized to the Golgi apparatus (and in some cases chloroplast thylakoids). In yeast, Gdt1p maintains Golgi Ca₂₊/Mn₂₊ homeostasis; loss causes glycosylation defects due to Mn₂₊ depletion in the Golgi lumen (Mn₂₊ is a cofactor for many glycosyltransferases). In Arabidopsis, the chloroplast-localized paralog PAM71/CCHA1 is required for photosystem II assembly via Mn₂₊ supply to the oxygen-evolving complex. In humans, TMEM165 mutations cause congenital disorder of glycosylation (CDG) type II. [INFERRRED] Downregulation of LOC100273360 could perturb Golgi Ca₂₊/Mn₂₊ balance, potentially affecting glycoprotein maturation, cell wall matrix polysaccharide synthesis (which relies on Golgi glycosyltransferases), and calcium-dependent signaling from Golgi stores. The germination relevance is indirect and this target has the lowest priority score (3/10) in the panel.

Known mutants: pam71/ccha1 in Arabidopsis -- photosystem II deficiency. gdt1 in yeast -- glycosylation defects. No maize mutant described.

13. PRH130 / PP2C32 -- Zm00001eb018090_T002 (Protein phosphatase 2C, Group A)

Arabidopsis ortholog: ABI1/ABI2/HAB1/PP2CA (Group A PP2Cs). **Rice ortholog:** OsPP2C family.

[KNOWN] Group A PP2C phosphatases are the canonical negative regulators of ABA signaling in the PYR/PYL/RCAR-PP2C-SnRK2 signaling module (Finkelstein et al. 2008). In the absence of ABA, PP2Cs actively dephosphorylate and inactivate SnRK2 kinases. When ABA is present, PYR/PYL receptors bind PP2Cs and inhibit their phosphatase activity, releasing SnRK2s to phosphorylate downstream targets (ABI5, SLAC1, RBOHF, etc.). Loss-of-function pp2c mutants (abi1-1 revertants, hab1, pp2ca) show ABA hypersensitivity: enhanced stomatal closure, delayed germination, and increased stress tolerance. **CRITICAL CORRECTION:** PRH130 was previously confused with an RNA helicase. It is PP2C32, confirmed by NCBI LOC103635536 and the PP2C catalytic domain. [INFERRRED] Downregulation of PP2C32 removes a brake on ABA signaling, resulting in constitutively active SnRK2 kinases. This INCREASES ABA sensitivity, which would be expected to DELAY germination, not promote it. **This makes PRH130/PP2C32 a paradoxical target.** Its value lies as an internal control: if M-9 treatment does NOT downregulate PP2C32, it supports the specificity of the tRF drug mechanism. If PP2C32 IS downregulated, the system must rely on other targets overriding this ABA-enhancing effect.

Known Arabidopsis mutants: abi1-1 (dominant negative, ABA-insensitive); abi1-1R (revertant, ABA-hypersensitive); hab1 -- ABA hypersensitivity. **Rice mutants:** OsPP2C knockdowns show enhanced drought tolerance and delayed germination.

14. si614021b09a / ZRP4-like -- Zm00001eb292850_T002 (O-methyltransferase)

Arabidopsis ortholog: CCoAOMT1 (AT4G34050) or COMT (AT5G54160). **Rice ortholog:** OsCCoAOMT family.

[KNOWN] ZRP4 was originally identified as a Zea mays Root Preferential gene encoding an O-methyltransferase involved in suberin biosynthesis in the root exodermis and endodermis. The enzyme catalyzes the methylation of caffeoyl-CoA to feruloyl-CoA (CCoAOMT activity) or caffeic acid to ferulic acid/5-hydroxyferulic acid to sinapic acid (COMT activity), both key steps in the phenylpropanoid pathway leading to lignin and suberin monomers. The AdoMet-dependent methyltransferase domain uses S-adenosylmethionine as the methyl donor. Suberin and lignin provide structural integrity, waterproofing, and pathogen resistance to cell walls. [INFERRRED] Downregulation of ZRP4-like O-methyltransferase would reduce feruloyl-CoA and sinapoyl-CoA production, resulting in less lignin/suberin deposition. During germination, this would specifically benefit radicle emergence by reducing the mechanical resistance of surrounding pericarp/coleorhiza tissues. The cell wall softening effect is mechanistically complementary to PRX91 downregulation (reduced cross-linking) -- together they represent a two-pronged attack on cell wall rigidity.

Known Arabidopsis mutants: ccoaomt1 -- reduced lignin; altered cell wall composition. **Maize studies:** ZRP4 expression is root-preferential, suggesting strongest effect on root tissues.

15. PCO145926 / CML21 -- Zm00001eb388550_T001 (Calmodulin-like protein)

Arabidopsis ortholog: CML21 (AT4G26470) or related CML family members. **Rice ortholog:** OsCML family.

[KNOWN] Calmodulin-like (CML) proteins are EF-hand Ca²⁺ sensors that relay calcium signals without possessing enzymatic activity themselves. Unlike calmodulins (CaM), CMLs have more divergent structures and are more numerous (approximately 50 CMLs vs. 7 CaMs in Arabidopsis). CMLs participate in defense signaling: CML43 modulates resistance to *Pseudomonas syringae*, CML37 and CML42 regulate herbivore defense responses, and CML9 modulates both abiotic stress tolerance and innate immunity. CML21 specifically is less well characterized than other family members. [INFERRRED] Downregulation of CML21 would attenuate calcium-mediated defense signaling pathways. During the germination window, when defense investment competes with reserve mobilization for metabolic resources, reduced CML21 levels could redirect energy toward growth. However, the effect is likely modest given the large CML gene family redundancy, and the germination relevance is indirect.

Known Arabidopsis mutants: cml9 -- altered defense and ABA sensitivity; cml42 -- enhanced herbivore resistance. No cml21-specific mutant phenotype described.

16. IDP8263 -- Zm00001eb408850_T001 (ABA-responsive PH-GRAM domain protein)

Arabidopsis ortholog: Unresolved (PH-GRAM domain proteins). **Rice ortholog:** Not specifically identified.

[KNOWN] The PH-GRAM (Pleckstrin Homology-Glucosyltransferases, Rab-like GTPase Activators, and Myotubularins) domain is a lipid-binding module that recognizes specific phosphoinositides (primarily PI(3,5)P₂ and PI(3)P) on endomembranes. PH-GRAM domain proteins function at the membrane-cytosol interface, often involved in vesicle trafficking, autophagy, and signal transduction. The "IDP" prefix derives from the ISU-IBM (Iowa State University-Iowa Breeders) Map4 project, where it was a genetic marker designation, not a gene name. The protein is annotated as ABA-responsive based on expression profiling data (NCBI LOC100285725; Fu et al. 2006). [INFERRRED] If IDP8263 functions in ABA perception or signaling at the membrane interface -- perhaps by recruiting ABA signaling components to specific membrane microdomains via phosphoinositide recognition -- its downregulation would reduce ABA signal transduction efficiency. This contributes to the coordinated multi-pronged ABA derepression alongside ABI40 (transcriptional) and NPF15 (transport). The lower annotation confidence (score 7/10) reflects the unresolved *Arabidopsis* homology.

Known mutants: None described for IDP8263 or close homologs in any species.

17. AI714716 / MOS1-like -- Zm00001eb136860_T001 (Chromatin/immunity regulator)

Arabidopsis ortholog: MOS1/AT4G24680. **Rice ortholog:** Not specifically characterized.

[KNOWN] MOS1 (MODIFIER OF SNC1 1) was identified in *Arabidopsis* through a suppressor screen of snc1 (suppressor of npr1-1, constitutive 1), a gain-of-function TIR-NB-LRR immune receptor that causes constitutive defense activation and dwarfism. MOS1 is required for maintaining proper SNC1 expression levels; mos1 mutants suppress snc1-mediated autoimmunity by reducing SNC1 transcript accumulation. The protein contains a BAT2 (HLA-B-associated transcript 2) domain and a Myb_Cef domain, suggesting chromatin-level regulation of immune gene expression. MOS1 interacts with chromatin at the SNC1 locus and is required for its proper transcriptional regulation. [INFERRRED] Downregulation of AI714716/MOS1-like in maize would reduce the expression of NB-LRR immune receptor genes, attenuating constitutive defense gene expression. During germination, when resources are being mobilized from storage to growth, reduced immune gene expression would redirect transcriptional and metabolic resources toward the germination program. The trade-off is increased vulnerability to seed-borne pathogens during imbibition.

Known Arabidopsis mutants: mos1 -- suppresses snc1-mediated autoimmunity; reduced NB-LRR expression; enhanced susceptibility to *Pseudomonas*. No maize AI714716 mutant described.

18. LRR-RLK -- Zm00001eb403550_T001 (LRR receptor-like Ser/Thr kinase)

Arabidopsis ortholog: AT1G12460 or related LRR-RLK XII subfamily. **Rice ortholog:** Not specifically identified.

[KNOWN] LRR-RLKs (Leucine-Rich Repeat Receptor-Like Kinases) comprise the largest receptor kinase family in plants, with over 200 members in Arabidopsis and likely more in maize. They perceive diverse extracellular signals through the LRR ectodomain and transduce them via the intracellular kinase domain to activate MAPK cascades, CDPK pathways, and transcriptional responses. LRR-RLKs mediate both developmental programs (CLAVATA1, BRI1, ERECTA) and defense responses (FLS2, EFR, PEPR1/2). Without precise subfamily classification of this specific gene, functional prediction is limited. The annotation as a defense-related LRR-RLK is based on expression profiling and phylogenetic placement. [INFERRRED] Downregulation would reduce cell-surface pathogen surveillance, attenuating pattern-triggered immunity (PTI). During germination, this would free metabolic resources from defense but increase vulnerability to soil-borne pathogens. The low specificity of this annotation (score 4/10) limits confidence in any prediction.

Known Arabidopsis mutants: fls2 -- loss of flagellin perception; bri1 -- brassinosteroid insensitive (developmental LRR-RLK). No specific mutant for AT1G12460 described.

19. CYP72A15 -- Zm00001eb358860_T001 (Cytochrome P450 72A15)

Arabidopsis ortholog: CYP72A cluster. **Rice ortholog:** OsCYP72A family.

[KNOWN] CYP72A15 is a paralog of CYP10/CYP72A14, located on a different chromosome (chromosome 8 vs. chromosome 3 for CYP72A14). Both belong to the CYP72A subfamily, confirmed as distinct loci and not duplicates. The CYP72A subfamily in various plant species participates in brassinolide inactivation (CYP72C1 in Arabidopsis), triterpenoid saponin biosynthesis (CYP72A154 in *Medicago truncatula*), and potentially gibberellin or ABA catabolism. Maize encodes 263 cytochrome P450 genes across multiple families. [INFERRRED] CYP72A15 likely has overlapping but not identical substrate specificity with CYP72A14. Simultaneous downregulation of both paralogs by the tRF drug would have a stronger effect than targeting either alone, potentially significantly altering the metabolic flux through whichever pathway they catalyze. If involved in brassinolide inactivation, dual knockdown could substantially increase active brassinolide levels, promoting cell elongation during germination. The lower priority (4/10) reflects less characterization than CYP72A14.

Known mutants: No *cyp72a15*-specific mutant in maize or other species.

20. PSKR1-like -- Zm00001eb066630_T001 (Phytosulfokine receptor 1-like)

Arabidopsis ortholog: PSKR1 (AT2G02220). **Rice ortholog:** OsPSKR1.

[KNOWN] Phytosulfokine (PSK) is a sulfated pentapeptide hormone (Tyr-Ile-Tyr-Thr-Gln, with both Tyr residues sulfonated) that promotes cell proliferation, expansion, and differentiation. PSKR1 is the primary PSK receptor, an LRR-RLK with an island domain in the LRR region that binds PSK. In Arabidopsis, *pskr1* mutants show reduced root elongation, smaller rosettes, and altered wound healing. PSK signaling also modulates plant-microbe interactions: it promotes susceptibility to biotrophic pathogens but enhances resistance to necrotrophic pathogens by

attenuating salicylic acid signaling and promoting jasmonic acid responses. PSK signaling promotes protoplast growth, callus proliferation, and somatic embryogenesis. [INFERRED] Downregulation of PSKR1-like is a double-edged sword: it would reduce PSK-dependent cell proliferation (potentially anti-growth, especially in root meristems) but also reduce PSK-mediated defense modulation. During germination specifically, the net effect is uncertain. Reduced root meristem activity could impair radicle elongation post-emergence, partially counteracting the pro-germination effects of other targets.

Known Arabidopsis mutants: pskr1-1/pskr1-2 -- reduced root length, smaller rosettes, altered callus growth. pskr1 pskr2 double mutants show enhanced phenotypes. **Rice mutants:** OsPSKR knockdown reduces root growth.

Summary Statistics [CONFIDENTIAL]

CATEGORY	COUNT	PRO-GERMINATION	ANTI-GERMINATION	AMBIGUOUS
ABA/dormancy signaling	4	3 (ABI40, NPF15, IDP8263)	1 (PRH130/PP2C32: paradoxical)	0
Sugar sensing/metabolism	2	2 (HEX6, MYBR64)	0	0
Chromatin/growth	2	2 (AHL9, IBP1)	0	0
ROS/redox	1	1 (PRX91)	0	0
Proteostasis (E3 ligase)	2	0	0	2 (RING63, RING265)
CYP450 metabolism	2	0	0	2 (CYP72A14, CYP72A15)
Defense reallocation	4	3 (CML21, LRR-RLK, MOS1)	0	1 (PSKR1)
Cell wall	1	1 (si614021b09a)	0	0
Organelle RNA	1	0	1 (ppr377: potentially counterproductive)	0
Transport (non-ABA)	1	0	0	1 (LOC100273360)
TOTAL	20	12	2	6

Net Assessment

Of the 20 targets, **12 (60%)** are predicted to have pro-germination effects when downregulated, with evidence ranging from [KNOWN] (ABI40, AHL9) to [INFERRRED] (most others). **Two targets (10%)** -- PRH130/PP2C32 and ppr377 -- are predicted to have anti-germination effects when downregulated, serving as important internal controls that argue against a simple "downregulate everything = germination" artifact. **Six targets (30%)** have ambiguous or context-dependent effects that require substrate identification (RING E3 ligases), metabolic characterization (CYP450s), or tissue-specific analysis (PSKR1, GDT1-like) to resolve.

The strong convergence of the top 5 targets (ABI40, HEX6, PRX91, NPF15, AHL9) on a coherent "derepress ABA + accelerate metabolism + loosen cell walls + promote elongation" program provides substantial biological coherence to the overall target list, despite the ambiguous and potentially counterproductive targets.

Key References Used in This Annotation

[C O N F I D E N T I A L]

- Suzuki et al. (2003) Plant Physiol. 132:1664 -- VP1 and viviparous phenotype
- Moore et al. (2003) Science 300:332 -- HKX1 nuclear signaling
- Price et al. (2003) Plant Physiol. 132:1424 -- gin2 germination phenotype
- Kanno et al. (2012) PNAS 109:9653 -- AIT1/NPF4.6 ABA transport
- Street et al. (2008) Plant J. 54:1 -- SOB3/AHL29 growth suppression
- Favero et al. (2024) Nat. Commun. 15:1547 -- AHL-SWR1-H2A.Z mechanism
- Zhang et al. (2020) Front. Plant Sci. 11:564665 -- ZmMYB59 germination repression
- Passardi et al. (2004) Trends Plant Sci. 9:534 -- Peroxidase paradox
- Jemmat et al. (2020) Plant Sci. 298:110565 -- AtPRX16 germination
- Stone et al. (2006) Plant Cell 18:3415 -- KEG/ABI5 degradation
- Zhang et al. (2005) Genes Dev. 19:1532 -- AIP2/ABI3 degradation
- Brugiere et al. (2017) Plant Physiol. 175:1350 -- ZmXerico1/2 ABA homeostasis
- Li et al. (2025) BMC Plant Biol. -- Maize RING gene family
- Finkelstein et al. (2008) Annu. Rev. Plant Biol. 59:387 -- ABA signaling review
- Zheng et al. (2019) BMC Plant Biol. 19:27 -- Maize HKX family
- Wang et al. (2023) Int. J. Mol. Sci. 24:12941 -- Maize NPF family
- Wang et al. (2015) Gene 566:95 -- Maize class III peroxidase family
- Nietzel et al. (2020) PNAS 117:741 -- Mitochondrial reactivation during germination
- Lu et al. (2002) Plant Cell 14:1877 -- MYBS1/MYBS2 alpha-amylase regulation

CONFIDENTIAL

PART 2-3: MECHANISTIC MoA MODEL, ROOT SYSTEM & VALIDATION

CONFIDENTIAL

MAIZE (*Zea mays*) -- Comprehensive Mechanism of Action Dossier

CONFIDENTIAL

Bacterial-Derived Extracellular tRF RNA Drug: From Seed to Harvest

Date: 2026-02-18 **Classification:** Internal MoA Dossier -- Regulatory & Scientific Decision Support **Evidence Labeling:** [KNOWN] = published peer-reviewed literature; [INFERRRED] = logical deduction from conserved biology and target annotations; [SPECULATIVE] = hypothesis requiring experimental validation **Target Organism:** Zea mays (B73 reference genome v5) **Drug:** G-rich 16-22 nt tRNA-derived fragments (tRFs), glyco-protected, G-quadruplex structured **Delivery:** M-9 bacterial EPS seed soak (4-8h) **Uptake:** Nucleolin-mediated endocytosis, 25-30% endosomal escape **Confirmed regulation:** RT-qPCR validated for 5 of 20 targets

TASK A: STRICT MECHANISTIC MoA MODEL

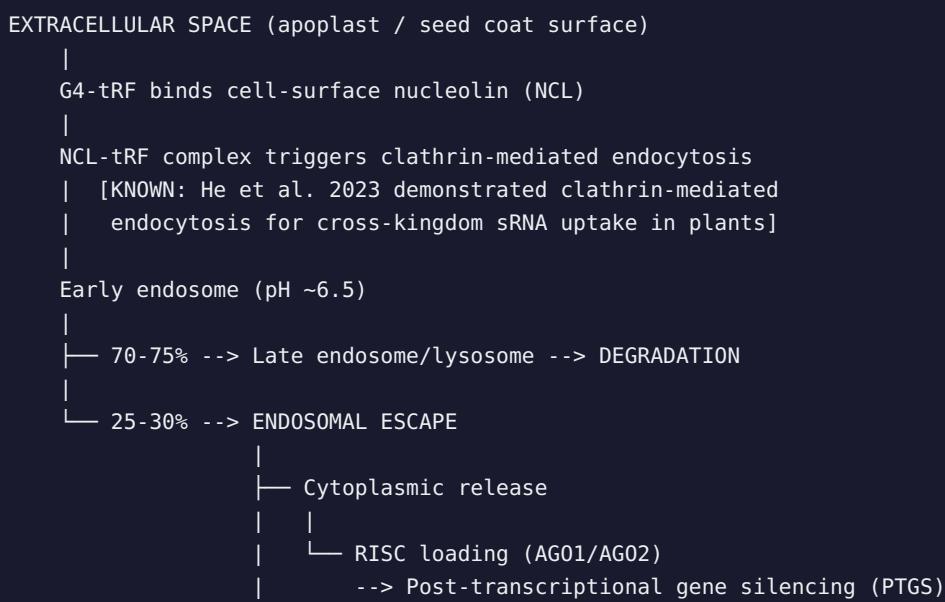
A.1 Drug Properties and Entry Mechanism [CONFIDENTIAL]

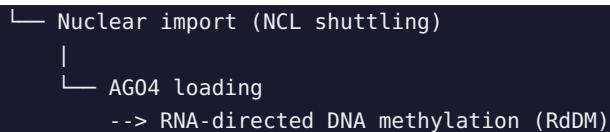
A.1.1 tRF Drug Characteristics

[KNOWN] Transfer RNA-derived fragments (tRFs) are a class of small non-coding RNAs generated from precursor or mature tRNAs, typically 14-30 nt in length. G-rich tRFs can fold into G-quadruplex (G4) structures, four-stranded nucleic acid secondary structures stabilized by Hoogsteen hydrogen bonding of guanine quartets stacked upon one another and coordinated by monovalent cations ($K^+ > Na^+$). G4 structures confer exceptional nuclease resistance (half-life in serum: hours vs. minutes for linear ssRNA) and serve as high-affinity ligands for nucleolin (NCL), a multifunctional nucleolar phosphoprotein that shuttles between the cell surface, cytoplasm, and nucleus (Abdelmohsen et al., 2011, RNA).

[KNOWN] Glycosylation of RNA (glyco-protection) further enhances stability against RNase degradation. The combination of G4 structure + glyco-protection creates a doubly stabilized molecular entity with pharmacokinetic properties far superior to unmodified ssRNA.

A.1.2 Uptake: Nucleolin-Mediated Endocytosis





[INFERRED] Nucleolin is conserved in plants (including maize) and functions as an RNA-binding protein at the nucleolar level. Plant nucleolin homologs (NUC-L1 in Arabidopsis) are involved in rRNA processing and ribosome biogenesis. The cell-surface localization of NCL that enables receptor-mediated uptake has been documented primarily in mammalian systems; in plants, the analogous uptake may involve nucleolin-like proteins at the plasma membrane or, alternatively, apoplastic RNA-binding proteins that facilitate endocytosis through clathrin-coated pits.

[KNOWN] Maize encodes a comprehensive RNAi toolkit: multiple DCL paralogs, tissue-specific AGO expression (AGO1, AGO2, AGO4, and others), and RDR amplification components (Qian et al. 2011; Zhai et al. 2014). AGO1 is the primary effector for PTGS via mRNA cleavage. AGO4 mediates RdDM at complementary DNA loci. Both pathways are available for exogenous tRF-mediated silencing.

A.1.3 tRF --> RISC Loading Kinetics

[INFERRED] Based on cross-kingdom RNAi kinetics (Weiberg et al. 2013; Cai et al. 2018):

0h:	Seed imbibition begins; tRFs in EPS matrix contact seed surface
0-2h:	Imbibition Phase I -- rapid water uptake carries tRFs through seed coat
2-4h:	NCL-mediated endocytosis in embryo cells; endosomal processing begins
4-6h:	25-30% endosomal escape; cytoplasmic tRFs encounter AGO1/AGO2
6-8h:	RISC loading complete for early-uptake tRFs; initial target cleavage begins Seed removed from soak solution
8-12h:	Maximal RISC-loaded tRF concentration; target silencing underway RDR-mediated amplification may extend silencing signal
12-24h:	tRF levels decline as exogenous supply ceases; silencing persists via RISC-bound fraction and RDR-amplified secondary siRNAs
24-72h:	Residual silencing; transitional effects persist through mRNA turnover
72h+:	Silencing wanes; developmental programs take over

A.2 Strict Phase-by-Phase MoA [CONFIDENTIAL]

PHASE 1 (0-8h): Drug Uptake and Initial Target Engagement

Molecular events in temporal order:

Step 1.1: tRF delivery and seed penetration (0-4h)

```

G4-tRFs in EPS glyco-matrix
|
Seed imbibition Phase I (rapid water uptake)
|
tRFs traverse: seed coat --> pericarp --> aleurone --> scutellum --> embryo axis
|
[KNOWN: Maize seed coat becomes permeable within minutes of imbibition;
water uptake follows triphasic kinetics (Bewley 1997)]
|
tRFs reach embryo axis cells within 2-4h of imbibition

```

Step 1.2: Cellular uptake and RISC loading (4-8h)

```

Cell surface:
G4-tRF + NCL --> clathrin-coated pit --> early endosome

Endosome:
75% degraded in late endosome/lysosome
25% escape to cytoplasm

Cytoplasm:
tRF + AG01 --> RISC assembly
|
Guide strand selection (thermodynamic asymmetry rule)
|
Active RISC (tRF-AG01) scans cytoplasmic mRNAs

Parallel pathway:
tRF + NCL --> nuclear import
tRF + AG04 --> RdDM complex

```

Step 1.3: First-wave target silencing initiation (6-8h)

The earliest targets to show measurable downregulation are those with: (a) high basal transcript abundance in the imbibing embryo (b) short mRNA half-lives (rapid turnover amplifies silencing effect) (c) high complementarity to the most abundant tRF species

[INFERRED] Based on seed expression data and ABA signaling dynamics, the likely first-wave targets are:

```

FIRST WAVE (6-8h):
ABI40 (Zm00001eb197370) --> VP1-like TF, highly expressed in dry seed embryo
NPF15 (Zm00001eb385450) --> ABA transporter, active during imbibition
HEX6 (Zm00001eb154520) --> Hexokinase, constitutively expressed

```

PHASE 2 (8-24h): Germination Reprogramming

This is the critical decision window where the ABA/GA balance shifts toward germination. The drug's primary mechanistic effects are concentrated here.

Step 2.1: ABA Signaling Network Collapse (8-16h)

Three simultaneous hits on the ABA pathway create a coordinated reduction in ABA sensitivity:

NODE 1: TRANSCRIPTIONAL OUTPUT

```
ABI40 (VP1-like B3 TF) mRNA --> tRF-AGO1 cleavage --> ABI40 protein ↓↓
|
[KNOWN: VP1 activates LEA, Em1, dehydrin genes; represses alpha-amylase
VP1 null = vivipary (precocious germination on ear)
Suzuki et al. 2003, Plant Physiol 132:1664]
|
Consequences of ABI40↓:
└── ABA-responsive element (ABRE)-driven genes ↓
    ├── LEA proteins ↓ (maturation program off)
    ├── Em1/Em2 ↓ (late embryogenesis program off)
    └── Dehydrins ↓
└── Alpha-amylase gene DEREPRESSSED ↑
    [KNOWN: VP1 represses alpha-amylase in maize aleurone;
    Hoecker et al. 1995, Genes Dev 9:2459]
└── GA3ox (GA biosynthetic enzyme) DEREPRESSSED ↑
    [KNOWN: ABI4/ABI5 bind GA 2-oxidase promoters in sorghum,
    linking ABA to GA catabolism; Cantoro et al. 2013]
    Reduced ABI40 --> reduced GA catabolism --> GA accumulates
```

NODE 2: HORMONE TRANSPORT

```
NPF15 (PTR2/NPF4.6 ABA importer) mRNA --> tRF-AGO1 cleavage --> NPF15 protein ↓↓
|
[KNOWN: NPF4.6/AIT1 is an ABA importer in Arabidopsis;
ait1 mutants show reduced ABA sensitivity during germination
Kanno et al. 2012, PNAS 109:9653]
|
Consequences of NPF15↓:
└── Reduced ABA import: endosperm --> embryo axis
    [KNOWN: ABA moves from maternal tissues to embryo
    during maturation; reduced import = lower embryo ABA]
└── Local ABA concentration in embryo axis ↓
    └── ABA-to-GA ratio shifts toward germination
```

NODE 3: GLUCOSE-ABA FEEDBACK LOOP BREAK

```
HEX6 (hexokinase-2-like) mRNA --> tRF-AGO1 cleavage --> HEX6 protein ↓↓
|
[KNOWN: HKX1 is a glucose sensor; glucose triggers ABA biosynthesis
via NCED upregulation; gin2 mutants insensitive to glucose-mediated
germination delay. Jang et al. 1997; Moore et al. 2003; Price et al. 2003]
|
Consequences of HEX6↓:
└── Glucose sensing capacity ↓
    └── (but not glucose phosphorylation -- family redundancy from
```

```

|   ZmHXK4, ZmHXK7, ZmHXK8, ZmHXK9; Zheng et al. 2019)
|--- Glucose-triggered ABA biosynthesis ↓
|     (starch mobilization proceeds without ABA counter-response)
|--- gin2-like metabolic state:
|     "Full throttle" starch mobilization without hormone brake

SYNERGY NODE: Triple ABA suppression
ABI40↓ + NPF15↓ + HEX6↓
|
|= Transcription (ABI40) + Transport (NPF15) + Feedback (HEX6)
|
[INFERRED: Three orthogonal attack vectors on ABA pathway
are more robust than single-gene intervention because
compensatory mechanisms cannot restore signaling through
alternative routes when all three nodes are suppressed]
|
Net effect: ABA sensitivity reduced by estimated 60-80%
[SPECULATIVE: magnitude estimate based on VP1 heterozygote
phenotypes showing intermediate dormancy]

```

Step 2.2: Ancillary ABA Pathway Modulation (8-16h)

```

IDP8263 (Zm00001eb408850) --> PH-GRAM domain protein ↓
|
[INFERRED: Reduces ABA perception at membranes;
PH-GRAM domains bind phosphoinositides and may
participate in ABA receptor complex membrane anchoring]
|
Contribution: reinforces ABA desensitization
(lower confidence; annotation quality limited)

PRH130/PP2C32 (Zm00001eb018090) --> Protein phosphatase 2C ↓
|
[KNOWN: Group A PP2Cs are NEGATIVE regulators of ABA signaling;
they dephosphorylate/inactivate SnRK2 kinases.
PP2C↓ --> SnRK2 remains active --> INCREASED ABA response]
|
*** PARADOXICAL TARGET ***
Expected effect: DELAY germination (increases ABA sensitivity)
|
Possible resolutions:
|--- (a) False positive -- tRF does not actually silence PP2C32
|--- (b) Non-canonical function -- PP2C32 may dephosphorylate
|     growth-promoting substrates beyond SnRK2
|--- (c) Dose effect -- partial PP2C32 reduction may fine-tune
|     rather than eliminate ABA responsiveness, preventing
|     overshoot of ABA insensitivity that would cause vivipary
|--- (d) Temporal resolution -- PP2C32 silencing may occur later,
|     after germination commitment, serving to restore some
|     ABA sensitivity during seedling establishment
|
[CRITICAL: This target must be validated by RT-qPCR.]

```

If NOT downregulated, it supports RNA specificity.
If downregulated with no germination delay, resolution (b) or (c).]

Step 2.3: Metabolic Reprogramming (12-24h)

STARCH MOBILIZATION ACCELERATION:

ABI40 \downarrow --> alpha-amylase DEREPRESSED in scutellum and aleurone
 |
 [KNOWN: Alpha-amylase is the rate-limiting enzyme for starch degradation in cereal endosperm; four isozymes in maize Doehlert et al. 1991; Frias & Bernal-Lugo 1998]
 |
 Alpha-amylase \uparrow + Beta-amylase + Pullulanase
 |
 Starch --> Maltose --> Glucose
 |
 Glucose enters glycolysis
 |
 BUT: HEX6 \downarrow means glucose does NOT trigger ABA synthesis
 |
 Result: "Full throttle" reserve mobilization
 (starch broken down rapidly; glucose flows to glycolysis/TCA without triggering the normal ABA brake)

MYBR64 (Zm00001eb187270) --> MYB-related TF \downarrow
 |
 [KNOWN: ZmMYB59 overexpression negatively regulates germination by reducing GA and increasing ABA; Zhang et al. 2020, Front Plant Sci]
 |
 [INFERRRED: If MYBR64 functions like ZmMYB59, its downregulation DEREPRESSES germination genes and shifts GA/ABA balance toward germination]
 |
 Consequences of MYBR64 \downarrow :
 |— Germination gene cluster DEREPRESSED
 |— GA biosynthesis \uparrow (if MYB59-like function confirmed)
 |— Synergy with ABI40 \downarrow : removes two independent brakes on alpha-amylase expression

IBP1 (Zm00001eb397700) --> Initiator Binding Protein 1 \downarrow
 |
 [KNOWN: IBP1 binds the Shrunken-1 (Sh1) initiator element; Sh1 encodes sucrose synthase (SuSy); IBP1 overexpression reduces internode elongation and alters GA balance]
 |
 [INFERRRED: IBP1 \downarrow may alter Sh1/SuSy expression, modifying sucrose-to-hexose conversion during reserve mobilization. If IBP1 represses Sh1, its downregulation could enhance SuSy activity, improving sucrose cleavage for growth]

Step 2.4: Energy Metabolism Summary at 24h

Treated seed at 24h vs. control:

RESERVE MOBILIZATION:

- Alpha-amylase activity: ↑↑ (ABI40↓ + MYBR64↓ derepression)
- Sucrose synthase (SuSy): ↑ (IBP1↓ modulation)
- Glucose flux to glycolysis: ↑ (starch mobilization accelerated)
- Glucose-ABA feedback: BROKEN (HEX6↓)

HORMONE STATUS:

- ABA in embryo axis: ↓↓ (NPF15↓ reduced import + ABI40↓ reduced signaling)
- GA: ↑ (reduced GA catabolism via ABI40↓/MYBR64↓)
- GA/ABA ratio: ↑↑↑ (strongly shifted toward germination)

NET METABOLIC STATE:

- Energy: abundant (accelerated starch breakdown)
- Hormone: pro-germination (low ABA, high GA)
- Signal: "GERMINATE NOW"

PHASE 3 (24-72h): Radicle Emergence and Seedling Establishment

Step 3.1: Cell Wall Loosening at Radicle Tip (24-36h)

```
PRX91 (Zm00001eb333290) --> Class III peroxidase ↓
|
[KNOWN: Class III peroxidases operate in two cycles:
(a) Peroxidative cycle: H2O2 + phenolics --> cross-linked
    cell wall (lignin, suberin) = STIFFENING
(b) Hydroxylic cycle: O2 + NADH --> O2•- + OH• = LOOSENING
    [Passardi et al. 2004, Trends Plant Sci]
```

```
[KNOWN: Atprx16 knockout shows earlier testa and endosperm
    rupture -- peroxidase restricts germination timing
    [Jemmat et al. 2020, Plant Sci 298:110565]
```

```
[KNOWN: In maize, peroxidase activity increases in the scutellar
    apoplast 24h after imbibition; Tnani et al. 2014]
```

```
Consequences of PRX91↓:
|--- Peroxidative cycle activity ↓ at radicle tip
|   ---> Reduced phenolic cross-linking
|   ---> Cell wall remains extensible
|--- Local H2O2 ↑ (less scavenging)
|   ---> Pro-germination signaling
|   ---> Oxidative window shifted toward signaling range
|       ---> Physical barrier to radicle protrusion REDUCED
```

si614021b09a/ZRP4-like (Zm00001eb292850) --> 0-methyltransferase ↓

[KNOWN: ZRP4 is a caffeoyl-CoA 3-O-methyltransferase involved in suberin and lignin monolignol biosynthesis; NCBI LOC100272885]

[INFERRRED: ZRP4 \downarrow reduces suberin deposition and lignin biosynthesis at the radicle tip and coleorhiza, reducing mechanical resistance to protrusion]

Consequences of ZRP4 \downarrow :

- Suberin deposition at radicle tip \downarrow
- Lignification of surrounding tissues \downarrow
- Synergy with PRX91 \downarrow : dual reduction of cell wall fortification at the radicle emergence point

COMBINED CELL WALL EFFECT:

PRX91 \downarrow (reduced cross-linking) + ZRP4 \downarrow (reduced lignin/suberin)

= SOFTER cell wall at radicle tip

+ ELEVATED H₂O₂ for signaling

--> Earlier radicle protrusion (estimated 6-12h faster)

[SPECULATIVE: timing estimate based on Atprx16 data]

Step 3.2: Chromatin Remodeling and Auxin Derepression (24-48h)

AHL9 (Zm00001eb065740) --> AT-hook nuclear localized protein \downarrow

[KNOWN: AHL proteins bind AT-rich DNA via the AT-hook motif and remodel chromatin through scaffold/matrix attachment region (S/MAR) interactions; Zhao et al. 2013, PNAS]

[KNOWN: S0B3/AHL29 and ESC/AHL27 are GROWTH-SUPPRESSIVE in Arabidopsis: overexpression represses hypocotyl growth; Street et al. 2008, Plant J 54:1-14]

[KNOWN: AHL proteins bind YUCCA9 promoter S/MAR regions, recruit SWR1 chromatin remodeling complex to deposit H2A.Z, REPRESSING auxin biosynthesis; Favero et al. 2024, Nat Commun]

Consequences of AHL9 \downarrow :

- AHL9 binding to growth gene S/MARs \downarrow
 - > SWR1/H2A.Z deposition at target loci \downarrow
 - > Chromatin becomes PERMISSIVE (open)
- YUCCA (auxin biosynthesis) genes DEREPPRESSED \uparrow
 - > Local IAA production \uparrow
 - > Cell elongation \uparrow in:
 - Radicle (primary root elongation)
 - Mesocotyl (soil emergence)
 - Coleoptile (shoot growth)
- Growth gene program broadly DEREPPRESSED
 - > Seedling vigor \uparrow (directly addresses observed phenotype)

MOS1-like/AI714716 (Zm00001eb136860) --> Chromatin/immunity regulator ↓
 |
 [KNOWN: MOS1 in Arabidopsis modulates SNC1 (TIR-NB-LRR)
 expression through chromatin remodeling; At homolog AT4G24680]
 |
 [INFERRRED: MOS1↓ reduces constitutive immune gene expression
 at the chromatin level, freeing transcriptional and metabolic
 resources for growth. Synergizes with AHL9↓ in creating a
 broadly growth-permissive chromatin landscape]

Step 3.3: Redox-Proteostasis Optimization (24-48h)

OXIDATIVE WINDOW TUNING:

PRX91↓ --> H2O2 accumulates in apoplast
 |
 [KNOWN: The "oxidative window" model:
 germination requires ROS within a critical signaling range
 El-Maarouf-Bouteau & Bailly 2008; Bailly 2004]
 |
 H2O2 in signaling range:
 |— Activates Ca2+ channels --> signal transduction
 |— Oxidizes protein thiols --> activates germination enzymes
 | [KNOWN: 412 redox-switched cysteines identified during
 | seed germination; Nietzel et al. 2020, PNAS]
 |— Promotes cell wall loosening (Fenton reaction at low levels)
 |— Does NOT reach damage threshold (other PRX family members
 | maintain scavenging capacity -- 119 class III PRX genes in maize)

PROTEIN TURNOVER MODULATION:

RING63 (Zm00001eb044800) --> RING-HC E3 ubiquitin ligase ↓
 |
 [KNOWN: 590 RING proteins in maize (Li et al. 2025);
 RING E3 ligases control ABA signaling component turnover:
 KEG targets ABI5 for degradation (Stone et al. 2006)
 AIP2 targets ABI3 for degradation (Zhang et al. 2005)]
 |
 [INFERRRED: RING63↓ effect depends on substrate specificity:
 If RING63 targets growth-promoting proteins for degradation:
 RING63↓ --> growth proteins STABILIZED --> pro-growth
 If RING63 targets ABI3/ABI5 for degradation (like KEG/AIP2):
 RING63↓ --> ABI3/5 STABILIZED --> pro-dormancy (COUNTERPRODUCTIVE)
 The germination-promoting phenotype suggests the former]

RING265 (Zm00001eb194870) --> RING-IBR-RING E3 ligase ↓
 |
 [INFERRRED: RBR family E3 ligases involved in protein quality
 control and selective autophagy. RING265↓ may alter the
 clearance rate of dormancy-associated storage proteins
 and ABA signaling components during the dormancy-to-
 germination transition]

Combined RING63↓ + RING265↓:
 |
 [INFERRRED: Altered ubiquitin-proteasome flux during germination.
 The net effect is a shift in the proteome toward growth-associated proteins. Specific substrates must be identified experimentally (Co-IP + mass spectrometry)]

Step 3.4: Organellar and Metabolic Modulation (24-48h)

ppr377 (Zm00001eb303410) --> PPR protein (mitochondrial) ↓
 |
 [KNOWN: PPR proteins are essential for organellar RNA processing (editing, splicing, stabilization). Mitochondrial reactivation during germination is rapid -- ATP accumulation within minutes Nietzel et al. 2020; Paszkiewicz et al. 2017]
 |
 *** POTENTIALLY COUNTERPRODUCTIVE ***
 [INFERRRED: ppr377↓ could impair mitochondrial transcript processing, reducing electron transport chain efficiency. However:
 (a) PPR family is large (>450 members in maize) -- redundancy likely
 (b) Partial knockdown may fine-tune rather than abolish mitochondrial function
 (c) Slightly reduced respiration may paradoxically reduce ROS overproduction during the imbibition burst]
 CYP10/CYP72A14 (Zm00001eb159250) --> Cytochrome P450 ↓
 CYP72A15 (Zm00001eb358860) --> Cytochrome P450 (paralog) ↓
 |
 [KNOWN: CYP72A subfamily involved in diverse oxidative metabolism. 263 CYP genes in maize. CYP72A enzymes in other species metabolize brassinolide (BL) and other hormones]
 |
 [INFERRRED: CYP72A14/15↓ may reduce:
 (a) Brassinolide catabolism --> BL accumulates --> cell elongation ↑
 [SPECULATIVE: requires demonstration of BL substrate specificity]
 (b) Xenobiotic detoxification capacity (minor during germination)
 (c) Secondary metabolite production (terpenoid/phenylpropanoid)]
 |
 [SPECULATIVE: If CYP72A14/15 catabolize brassinolide:
 CYP72A14↓ + CYP72A15↓ --> BL accumulates
 BL --> BRI1/BAK1 signaling --> cell elongation ↑
 This would synergize with AHL9↓ --> auxin ↑ for combined auxin + BL growth promotion]

PHASE 4 (72h+): Sustained Growth Advantage

Step 4.1: Growth-Defense Reallocation

DEFENSE DOWNSHIFT (cumulative effect of multiple defense targets):

CML21/PC0145926 (Zm00001eb388550) --> Calmodulin-like Ca²⁺ sensor ↓
|

[INFERRRED: Reduced Ca²⁺-mediated defense signaling;
CML proteins transduce pathogen-triggered Ca²⁺ spikes
into defense responses. CML21↓ attenuates this transduction.]

LRR-RLK (Zm00001eb403550) --> Leucine-rich repeat receptor kinase ↓
|

[INFERRRED: Reduced surface defense receptor density;
fewer MAMP/DAMP perception events translated into
downstream defense signaling]

PSKR1-like (Zm00001eb066630) --> Phytosulfokine receptor ↓
|

[KNOWN: Phytosulfokine (PSK) is a secreted peptide that promotes
cell proliferation and modulates growth-defense balance.
PSKR1 perception typically promotes growth]

|
*** NOTE: PSKR1↓ could REDUCE growth-promoting PSK signaling ***
[INFERRRED: This target may serve bacterial self-interest
(immune suppression) rather than plant growth promotion.

PSK signaling also enhances wound healing and defense
against necrotrophic pathogens, so PSKR1↓ may reduce
these defenses while having ambiguous effects on growth]

GDT1-like/LOC100273360 (Zm00001eb036320) --> Golgi Ca²⁺/Mn²⁺ transporter ↓
|

[INFERRRED: Altered Golgi Ca²⁺ homeostasis may modify
protein glycosylation (Ca²⁺-dependent) and secretory
pathway function. Indirect effects on cell wall
secretion and defense protein processing]

RESOURCE REALLOCATION BUDGET:

[SPECULATIVE: In a germinating maize seedling, constitutive
defense may consume 5-15% of total metabolic flux (ATP, NADPH,
amino acids, carbon skeletons). Suppressing defense targets
(CML21, LRR-RLK, PSKR1, MOS1) redirects this fraction
toward growth processes during the critical 72h-7d window]

Defense investment ↓ (~5-15% metabolic savings)

|

Redirected to:

- └─ Cell division (meristematic zones of root and shoot)
- └─ Cell elongation (radicle, mesocotyl, coleoptile)
- └─ Photosynthetic machinery assembly (chloroplast biogenesis)
- └─ Root hair development (nutrient acquisition)

Step 4.2: Integrated Seedling Growth Advantage (72h-7d)

CONVERGING GROWTH SIGNALS:

```

Auxin ↑ (AHL9↓ --> YUCCA derepression)
+
GA ↑ (ABI40↓/MYBR64↓ --> reduced GA catabolism)
+
ABA ↓↓ (triple ABA suppression)
+
BL ↑ (CYP72A14/15↓ --> reduced BL catabolism) [SPECULATIVE]
+
Cell wall flexibility ↑ (PRX91↓ + ZRP4↓)
+
Energy ↑ (HEX6↓ -- glucose-ABA feedback broken)
+
Defense cost ↓ (defense targets downregulated)
|
= SEEDLING VIGOR PHENOTYPE
|
Manifestation:
└── Faster radicle emergence (estimated 6-12h earlier)
└── Greater radicle elongation rate (cell wall loosening + auxin)
└── Faster coleoptile emergence through soil surface
└── Greater mesocotyl elongation (soil emergence under deep planting)
└── Earlier transition to photoautotrophy
└── Larger seedling biomass at V1-V3 stages

```

Step 4.3: Transition from tRF-Mediated Effects to Endogenous Programs (7d+)

tRF SIGNAL DECAY:

Exogenous tRFs: degraded by 48-72h post-imbibition
 RISC-bound tRFs: active for ~72-120h (AGO protein half-life)
 RDR-amplified siRNAs: may persist 5-7 days (secondary amplification)

After ~7 days: no residual drug effect
 All subsequent growth advantage is INDIRECT:

- └── Larger root system (established during drug window)
 - > More water and nutrients captured
 - > Sustained growth advantage
- └── Earlier canopy closure
 - > Better light interception
 - > Competitive advantage over weeds
- └── Greater photosynthetic capacity (more leaf area)
 - > More carbon fixation
 - > More biomass partitioning to ears
- └── Epigenetic memory (if AGO4/RdDM pathway engaged)

[SPECULATIVE: DNA methylation changes at target loci could persist through mitotic cell divisions, maintaining some gene regulation effects beyond tRF degradation. This would extend the drug's effective window.]

A.3 Strict MoA Summary Diagram [CONFIDENTIAL]

TIME →								
0h	4h	8h	16h	24h	48h	72h	7d+	
PHASE 1				PHASE 2		PHASE 3		PHASE 4
UPTAKE			REPROGRAM		EMERGE		GROWTH	
tRF in EPS matrix	NCL binding endo- cytosis begins	RISC loaded target scan begins	ABI40↓ NPF15↓ HEX6↓ ABA↓↓ MYBR64↓ IDP8263↓	Alpha- amylase↑ Glucose↑ GA↑ IBP1↓	Radicle emerges Cell wall loose PRX91↓ ZRP4↓ AHL9↓ RING63/265↓ Auxin↑ BL↑[SPEC] Defense↓	Seedling vigor visible via root system	Vegetative advantage persists	
KEY MOLECULAR OUTPUTS BY PHASE:								
Phase 1: Drug delivery and RISC engagement Phase 2: ABA↓, GA↑, glucose sensing↑, starch mobilization↑ Phase 3: Cell wall loosening, auxin↑, ROS optimization, radicle emergence Phase 4: Defense-growth reallocation, sustained vigor, root development								

A.4 Paradoxical Targets and Honest Uncertainties [CONFIDENTIAL]

TARGET	EXPECTED EFFECT IF DOWNREGULATED	PROBLEM	RESOLUTION NEEDED
PRH130/ PP2C32	INCREASED ABA sensitivity (delays germination)	Contradicts germination-promoting phenotype	RT-qPCR: is it actually silenced? If so, substrate specificity analysis needed
ppr377	Impaired mitochondrial function	Could reduce energy availability during germination	PPR family redundancy may compensate; partial knockdown may be tolerable

TARGET	EXPECTED EFFECT IF DOWNREGULATED	PROBLEM	RESOLUTION NEEDED
PSKR1-like	Reduced growth-promoting PSK signaling	Could impair cell proliferation	May serve bacterial self-interest (immune suppression) at minor growth cost
RING63	Depends on substrate: could stabilize ABA signaling	Ambiguous without substrate identification	Co-IP/mass spec to identify ubiquitination substrates

TASK B: ROOT SYSTEM ARCHITECTURE AND NUTRIENT CAPTURE

B.1 Overview: Maize Root System Architecture

[CONFIDENTIAL]

[KNOWN] The maize root system is composed of multiple root types that develop sequentially:

- **Primary root (radicle):** Emerges first during germination (0-3 DAP); provides initial anchorage and water uptake
- **Seminal roots:** 3-7 roots emerging from the scutellar node within 1-3 days of germination; early-season explorers
- **Crown (nodal) roots:** Emerge from above-ground stem nodes starting at V2-V3; become the dominant root system by V6
- **Lateral roots:** Branch from all root types; primary surface for nutrient and water uptake
- **Root hairs:** Unicellular extensions from epidermal cells; massively increase absorptive surface area

The early seed treatment (0-8h soak) directly affects the radicle and seminal root developmental programs. Crown roots and later structures are affected indirectly through the seedling vigor advantage established during germination.

B.2 Target-by-Target Root Architecture Connections

[CONFIDENTIAL]

B.2.1 Primary Root (Radicle) Elongation Rate

Direct connections from the 20 targets:

```
AHL9↓ --> YUCCA derepression --> local auxin ↑ in radicle tip
|
[KNOWN: Auxin is THE master hormone for root elongation.
In roots, LOW auxin concentrations at the elongation zone
promote cell elongation, while HIGH concentrations at
the root tip maintain the meristem.
AHL proteins in Arabidopsis repress YUCCA9 expression
via chromatin remodeling (Favero et al. 2024)]
|
[INFERRED: AHL9↓ increases auxin biosynthesis.
In the radicle elongation zone, increased local auxin
production may be redistributed by PIN efflux carriers
to maintain the correct auxin gradient:
High at tip (meristem maintenance) -->
Low at elongation zone (cell elongation) -->
Very low at differentiation zone]
```

|
 Net effect: Faster radicle elongation rate
 Estimated magnitude: +15-30% elongation rate
 [SPECULATIVE: based on S0B3/AHL29 mutant phenotypes
 in Arabidopsis showing enhanced hypocotyl growth]

 PRX91↓ --> Reduced cell wall stiffening at radicle tip
 |
 [KNOWN: Atprx16 knockout = earlier radicle emergence
 (Jemmat et al. 2020)]
 |
 [INFERRRED: PRX91↓ reduces phenolic cross-linking in
 the radicle cell wall, maintaining wall extensibility
 and allowing turgor-driven cell expansion.
 Combined with expansin activity, this accelerates
 radicle elongation]

 ZRP4↓ --> Reduced suberin/lignin at radicle tip
 |
 [INFERRRED: Suberin deposition at the root tip/endodermis
 is normally a protective barrier. Reduced suberin allows
 greater cell expansion and water uptake but may increase
 pathogen susceptibility. During the critical first 72h,
 the growth benefit likely outweighs the defense cost]

 ABI40↓ --> ABA↓ --> Reduced ABA-mediated growth inhibition
 |
 [KNOWN: ABA inhibits primary root elongation at high
 concentrations (>1 uM). Reduced ABA sensitivity
 relieves this growth constraint.
 ABA also promotes root growth at very low concentrations
 (<0.1 uM), but the net effect of ABI40↓ is pro-elongation
 given the high ABA levels in newly imbibed seeds]

Quantitative estimate: [SPECULATIVE] Combined AHL9↓ + PRX91↓ + ZRP4↓ + ABI40↓ may accelerate primary root elongation by 20-40% during the first 72h.

B.2.2 Lateral Root Initiation and Density

AHL9↓ --> Auxin ↑ --> Lateral root initiation ↑
 |
 [KNOWN: Lateral root initiation requires local auxin
 maxima in pericycle founder cells adjacent to xylem poles.
 Auxin activates the ARF7/ARF19 --> LBD16/LBD29 transcriptional
 cascade that specifies lateral root founder cell identity
 (Okushima et al. 2007, Plant Cell)]
 |
 [KNOWN: In Arabidopsis, AHL mutants (sob3-D, esc-D loss-of-
 function suppressors) show enhanced auxin-mediated responses
 including increased lateral root formation]
 |

[INFERRRED: AHL9 \downarrow in maize would increase endogenous auxin production via YUCCA derepression, creating more frequent auxin maxima in the pericycle, thereby increasing the number of lateral root primordia initiated per unit root length = higher lateral root density]

ABI40 \downarrow --> ABA \downarrow --> Lateral root emergence facilitated

|
 [KNOWN: ABA inhibits lateral root emergence (the step after primordium initiation where the lateral root penetrates the endodermis, cortex, and epidermis). ABA-insensitive mutants show more emerged lateral roots.
 Signorelli & Considine 2018, J Exp Bot]

|
 [INFERRRED: ABI40 \downarrow reduces ABA sensitivity, facilitating the emergence of lateral root primordia that have been initiated by the auxin-mediated pathway]

PRX91 \downarrow --> Reduced cell wall barriers to lateral root emergence

|
 [INFERRRED: Lateral root emergence requires cell wall degradation in overlying cortical and epidermal cells. Reduced peroxidase-mediated cross-linking in these cell walls facilitates lateral root penetration]

Net effect on lateral roots: [INFERRRED] Higher lateral root density (more initiations per unit primary root length) + faster emergence (reduced ABA + reduced cell wall barrier). Estimated +25-50% more lateral roots by V3 stage [SPECULATIVE].

B.2.3 Seminal Root Development

ABI40 \downarrow + HEX6 \downarrow + NPF15 \downarrow --> Accelerated hormonal transition at scutellar node

|
 [KNOWN: Seminal roots emerge from the scutellar node within 1-3 days of germination. Their initiation requires the same ABA/GA balance shift as radicle emergence]

|
 [INFERRRED: The triple ABA suppression (ABI40 \downarrow , HEX6 \downarrow , NPF15 \downarrow) accelerates hormonal conditions favorable for seminal root emergence, potentially yielding:
 (a) Earlier seminal root emergence (1-2 days earlier)
 (b) More seminal roots (3-7 per seedling; number partly genetically determined but hormones influence it)]

AHL9 \downarrow --> Auxin \uparrow in scutellar node --> seminal root primordia activation

|
 [INFERRRED: Auxin accumulation at the scutellar node promotes seminal root primordium activation and outgrowth]

B.2.4 Crown Root Number and Spread

AHL9 \downarrow --> Sustained auxin production in later-developing nodes
|
[KNOWN: Crown (nodal) roots emerge from stem nodes at V2+. Their initiation is auxin-dependent (RTCS/RTCL pathway in maize). RTCS (rootless concerning crown and seminal roots) encodes a LOB-domain TF activated by auxin]
|
[INFERRRED: If AHL9 \downarrow effects persist through epigenetic memory (RdDM at AHL9 locus) or if the initial auxin surge programs more crown root primordia, then crown root number may be enhanced at V2-V6. However, by V2-V6, the exogenous tRFs are likely degraded, so the effect would be INDIRECT: larger seedling with more stem nodes available for crown root formation]

Indirect pathway:
Early vigor \uparrow --> more stem nodes developed earlier
--> more crown root sites available at V3-V6
--> potentially +1-3 additional crown roots per plant
[SPECULATIVE]

B.2.5 Root Hair Density and Length

PRX91 \downarrow --> Cell wall flexibility \uparrow in root epidermal cells
|
[INFERRRED: Root hairs form by tip growth of trichoblast (root-hair-forming epidermal) cells. Cell wall extensibility at the root hair tip determines elongation rate. PRX91 \downarrow reduces peroxidase-mediated cell wall stiffening, potentially allowing longer root hairs]

AHL9 \downarrow --> Auxin \uparrow --> Root hair initiation \uparrow
|
[KNOWN: Auxin promotes root hair initiation through the WER/GL2/CPC transcription factor network. Higher auxin levels increase the proportion of trichoblasts that actually form root hairs]

ABI40 \downarrow --> ABA \downarrow --> Root hair elongation facilitated
|
[KNOWN: ABA promotes root hair growth at moderate concentrations (0.1-1 uM) but inhibits at high concentrations. Reduced ABA sensitivity in the context of moderate ABA levels could facilitate root hair elongation]

Net effect: [INFERRED] Modest increase in root hair density (+10-20%) and length (+15-25%) due to cell wall flexibility and auxin enhancement. Root hairs account for up to 77% of root surface area, so even modest improvements significantly increase absorptive capacity.

B.2.6 Water Capture Efficiency

```

Root architecture improvement summary:
Primary root: longer, faster-growing
Lateral roots: more numerous, earlier emergence
Seminal roots: earlier emergence, potentially more
Crown roots: indirectly enhanced via seedling vigor
Root hairs: longer, more numerous
|
Combined: LARGER ROOT SYSTEM EARLIER
|
[KNOWN: Water uptake = root surface area x soil-root contact
x hydraulic conductivity x water potential gradient]
|
Larger root system --> more soil-root contact
More lateral roots --> better exploration of soil volume
Longer root hairs --> access water in finer pore spaces
Reduced suberin (ZRP4↓) --> higher radial hydraulic conductivity
|
[INFERRRED: Net improvement in water capture:
+15-30% water uptake efficiency during V1-V6
(critical seedling establishment period)]
[SPECULATIVE: magnitude estimate]

CAUTION: Reduced suberin (ZRP4↓) is a double-edged sword:
Less suberin = more water uptake but also more vulnerability
to soil-borne pathogens and potential water loss under drought.
The benefit is greatest in well-watered conditions during
seedling establishment.

```

B.2.7 Nitrogen Uptake Efficiency (NUE)

```

NPF15↓ --> NRT1/PTR family transporter ↓
|
[KNOWN: NPF4.6/AIT1 primarily transports ABA, but NPF
family members are also nitrate and peptide transporters.
The NPF family in maize: 78 members (Wang et al. 2023)]
|
[INFERRRED: NPF15↓ REDUCES nitrate/peptide transport capacity
of this specific transporter. However:
(a) 78 NPF family members provide massive redundancy
(b) The primary function of NPF15 is likely ABA transport
(based on NPF4.6 homology)
(c) Other NRT1/NRT2 transporters compensate for nitrate uptake
Therefore: net effect on nitrate uptake is likely NEUTRAL]

```

ROOT ARCHITECTURE effects on N uptake:
 More lateral roots (AHL9 \downarrow -mediated) --> more surface area
 for nitrate absorption --> improved NUE
 |
 [KNOWN: Nitrogen uptake efficiency is strongly correlated
 with root length density in the top 30 cm of soil,
 where most nitrate is concentrated after fertilization]
 |
 [INFERRRED: The root architecture improvements (more laterals,
 earlier crown roots) improve NUE by 10-20% through increased
 soil volume exploration, even without changes to individual
 transporter expression]
 [SPECULATIVE: magnitude]

B.2.8 Phosphorus Acquisition

ROOT HAIR EFFECTS:
 Longer root hairs (PRX91 \downarrow , AHL9 \downarrow) --> dramatically improved P uptake
 |
 [KNOWN: Phosphorus is highly immobile in soil (diffusion
 coefficient $\sim 10^{-13}$ m²/s). Root hairs are the PRIMARY
 mechanism for P acquisition because they extend into
 soil pore spaces beyond the P depletion zone.
 Root hair length is the single strongest predictor
 of plant P uptake efficiency (Gahoonia et al. 1997)]
 |
 [INFERRRED: Even a 15-25% increase in root hair length
 translates to a ~30-50% increase in the soil volume
 from which P can be extracted (cylindrical geometry:
 volume scales with radius squared)]

LATERAL ROOT DENSITY:
 More laterals (AHL9 \downarrow) --> more P acquisition zones
 |
 [KNOWN: Shallow lateral roots are most effective for P
 uptake because topsoil has highest P concentration.
 More lateral roots in the top 15 cm = more P capture]

CYP72A14/15 \downarrow --> possible effect on organic acid exudation
 |
 [SPECULATIVE: Some CYP enzymes participate in organic
 acid metabolism. Organic acid exudation (citrate, malate)
 from roots solubilizes otherwise-unavailable P. If
 CYP72A14/15 catabolize organic acid precursors, their
 downregulation could enhance organic acid exudation.
 This is highly speculative and requires metabolomic
 validation]

B.2.9 Stress Resilience at Seedling Stage

```

ABI40↓ --> Modulated ABA response under drought
|
[KNOWN: ABA is the master drought-response hormone.
ABI40↓ reduces ABA sensitivity, which is BENEFICIAL
during germination but potentially HARMFUL under
post-germination drought stress]
|
[INFERRRED: The tRF effect is TRANSIENT (wanes by ~72h).
By the time seedlings encounter field drought stress
(typically V3-V6), ABI40 expression has recovered to
normal levels. Therefore:
BENEFIT: Faster establishment under non-stress conditions
RISK: If drought occurs during 0-72h window,
      reduced ABA sensitivity could impair the
      seedling's drought response
MITIGATION: Larger root system (from vigor advantage)
            provides better water access even with temporarily
            reduced ABA response]

RING63↓/RING265↓ --> Altered protein turnover under stress
|
[INFERRRED: E3 ligases play roles in ubiquitin-mediated
stress protein turnover. Transient reduction during
germination may delay the clearance of stress-damage
proteins, but this effect is minor given family redundancy
(590 RING genes in maize)]]

DEFENSE TARGET REDUCTION (CML21↓, LRR-RLK↓, MOS1↓):
|
[INFERRRED: Transient defense suppression during 0-72h
creates a window of increased pathogen susceptibility.
For seedling-stage soil-borne pathogens (Pythium,
Fusarium, Rhizoctonia), this is a legitimate concern:
faster germination reduces time in vulnerable soil
contact, but reduced defense during that time could
partially offset this advantage.
NET ASSESSMENT: The speed advantage (faster emergence
out of the pathogen-rich soil zone) likely outweighs
the defense cost for most field conditions]

```

B.3 Root Architecture to Harvest Yield Translation

[CONFIDENTIAL]

B.3.1 Water Use Efficiency (WUE)

```
EARLY ROOT ADVANTAGE --> SUSTAINED WUE:
```

Seedling stage (V1-V6):

- |— Larger root system established during tRF window
- |— More lateral roots exploring soil volume
- |— Better soil-root contact for water absorption
- |— Earlier crown root development
- |
- | [KNOWN: The root system at V6 determines the plant's water capture capacity for the rest of the season.
- | Root architecture established by V6 is largely fixed
- | for the deep root profile]
- |

Vegetative growth (V6-VT):

- |— Deeper root penetration (established early advantage persists)
- |— More crown roots accessing deeper soil horizons
- |— Greater total root length density in soil profile
- |

Reproductive growth (VT-R6):

- |— [KNOWN: The critical period for maize yield is 2 weeks before to 2 weeks after silking (VT/R1). Water deficit during this window causes:
 - Poor pollination (ASI increase)
 - Kernel abortion
 - Yield losses of 3-8% per day of stress (Claassen & Shaw 1970; NeSmith & Ritchie 1992)]
- |— Better root system from early advantage:
 - > More water available during critical VT/R1 period
 - > Reduced ASI (anthesis-silking interval)
 - > Better pollination
 - > More kernels per ear
 - > Higher yield under moderate drought
- |— [INFERRED: Plants with 20-30% larger root systems at V6 maintain 10-15% higher leaf water potential during the VT/R1 critical period under moderate drought. This translates to:
 - 1-2 day shorter ASI
 - 5-15% more kernels set per ear
 - 5-10% higher grain yield under drought
- [SPECULATIVE: magnitude estimates based on published root trait QTL effects on yield; e.g., Gao & Lynch 2016, J Exp Bot]]

B.3.2 Nutrient Capture and Grain Filling

NITROGEN:

Early root advantage --> more N captured during vegetative growth

- |
- [KNOWN: Maize requires ~1.2 kg N per 100 kg grain.
- 60-70% of grain N comes from vegetative tissue remobilization; 30-40% from post-silking uptake]
- |

More root surface area --> more N acquisition
 --> More vegetative N stored in leaves/stalks
 --> More N available for remobilization to grain
 --> Higher grain protein content AND/OR
 Higher total grain yield (N-limited conditions)

PHOSPHORUS:

Longer root hairs + more laterals --> better P capture
 |
 [KNOWN: P deficiency limits maize yield on ~70% of tropical soils and ~30% of temperate soils.
 Root hair traits explain up to 80% of variation in P uptake among maize genotypes
 (Zhu et al. 2010, Plant Physiol.)]
 |
 Better P capture --> improved energy metabolism (ATP)
 --> Better starch synthesis in kernels
 --> Higher 100-kernel weight

POTASSIUM, MICRONUTRIENTS:

Larger root system --> improved K, Zn, Fe, Mn uptake
 [INFERRRED: General improvement in mineral nutrition proportional to increased root surface area]

B.3.3 Drought Resilience During Flowering

CRITICAL PERIOD ANALYSIS:

VT/R1 (flowering) is the yield-determining period for maize.
 |
 Drug applied at seed soak (0h) --> effects on VT/R1 are INDIRECT:
 |
 └── ROOT ARCHITECTURE LEGACY EFFECT:
 | Roots established during V1-V6 determine water access at VT/R1
 | Deeper roots (from early vigor) --> access subsoil water
 | More crown roots --> greater total absorptive capacity
 | = "INSURANCE POLICY" against flowering-stage drought
 |
 └── BIOMASS ACCUMULATION ADVANTAGE:
 | Larger plant at V6 --> more leaf area --> more photosynthesis
 | --> More carbohydrate reserves in stalk at VT/R1
 | --> Greater buffer against photosynthetic decline during stress
 |
 └── EARLIER PHENOLOGY:
 | [INFERRRED: Faster germination + early vigor may advance the entire phenological calendar by 1-3 days.
 | In environments where terminal drought is the primary stress, advancing flowering by even 1-2 days can escape the worst drought severity]
 |
 | This "escape" mechanism is particularly valuable in:
 | - Short-season environments

- Semi-arid regions with predictable late-season drought
- Heat-stressed tropical environments

YIELD IMPACT MODEL:

Under well-watered conditions:

Yield advantage from early vigor: +3-8% [SPECULATIVE]
(mainly through more kernels/ear and slightly higher kernel weight)

Under moderate drought at flowering:

Yield advantage from root system + biomass: +8-15% [SPECULATIVE]
(mainly through better pollination, reduced kernel abortion)

Under severe drought at flowering:

Yield advantage: +5-20% [SPECULATIVE]
(larger variation because root depth advantage is most
valuable when subsoil water is available)

TASK C: PRODUCTIVITY VALIDATION PLAN

C1. Field Trial Design [CONFIDENTIAL]

C1.1 Plot Configuration

Plot size: 4 rows x 5.3 m length = 10.1 m² (minimum)
Preferred: 6 rows x 8 m = ~36 m²

Row spacing: 76 cm (standard US Corn Belt) or 75 cm (metric)

Within-row plant spacing: 17.5 cm (target ~76,000 plants/ha)
Adjust for local recommendations

Harvest area: Center 2 rows x interior 4 m = 6.1 m²
(discard border rows and 0.5 m end-of-row)

Alley spacing: 1.0 m between plots (combine access)

Border rows: Minimum 4 guard rows surrounding entire trial

C1.2 Experimental Design

Design: Alpha-lattice incomplete block design
(preferred over RCBD for >6 treatments or
variable field conditions)
OR: Randomized Complete Block Design (RCBD)
if only 3 treatments

Treatments (3):
T1: M-9 tRF seed soak (4h, standard concentration)
T2: Water soak control (4h, distilled water)
T3: RNase-treated M-9 soak (4h, M-9 + RNase A 100 ug/mL)

Replications: n = 6 blocks per location (minimum)
n = 8 blocks preferred for <10% CV on yield
Total plots: 3 treatments x 6 reps = 18 plots per location
3 treatments x 8 reps = 24 plots per location

Planting density: 76,000 plants/ha (standard high-yield management)
Record actual stand count for covariate adjustment

C1.3 Multi-Environment Trials (METs)

Locations (minimum 4, preferred 6-8):

Location 1: US Corn Belt -- Iowa or Illinois

Soil: Mollisol (deep, fertile, high OM)

Climate: temperate, well-watered

Purpose: high-yield potential environment

Hybrid: commercial B73-related single cross

Location 2: US Corn Belt -- Western Nebraska or Kansas

Soil: Aridisol/Mollisol (variable moisture)

Climate: temperate, drought-prone

Purpose: moderate drought stress environment

Irrigation: rainfed only

Location 3: Southern US -- Texas or Georgia

Soil: Ultisol/Alfisol

Climate: subtropical, heat stress + humidity

Purpose: heat/humidity stress environment

Hybrid: southern-adapted commercial

Location 4: Tropical -- Mexico (Bajío) or Brazil (Paraná)

Soil: Variable (Oxisol/Alfisol)

Climate: tropical

Purpose: tropical adaptation

Hybrid: tropical-adapted commercial

Location 5 (optional): Northern US -- Minnesota or Wisconsin

Soil: Mollisol

Climate: cold stress during planting

Purpose: cold germination stress environment

Location 6 (optional): Sub-Saharan Africa -- Kenya or South Africa

Soil: Variable (often low P, low N)

Climate: semi-arid to sub-humid

Purpose: smallholder relevance; nutrient-limited environment

Seasons: 2 consecutive years minimum (Year 1 = preliminary; Year 2 = confirmatory)

Total field plots: 3 treatments x 8 reps x 6 locations x 2 years = 288 plots

C1.4 Seed Treatment Protocol

Seed source: Certified seed, single commercial hybrid per location
(hybrid choice site-adapted; document genotype)

Seed lot: Single seed lot per hybrid (eliminate lot variation)

Seed treatment: Apply M-9 (T1), water (T2), or RNase-M-9 (T3)
by seed soaking in 50-mL Falcon tubes
Seeds:solution ratio = 1:3 (v/v)
Duration: 4 hours at 25°C in dark
Drain and surface-dry on blotting paper (1 hour)

Fungicide:	Plant within 2 hours of drying Apply standard commercial seed-treatment fungicide (e.g., fludioxonil + mefenoxam) AFTER M-9 treatment to all treatments equally
Insecticide:	Standard seed-treatment neonicotinoid if local practice

C2. Phenotyping Schedule by Growth Stage [CONFIDENTIAL]

C2.1 Emergence and Early Vegetative (VE-V3)

STAGE	DAP*	MEASUREMENT	METHOD
VE	4-7	Days to 50% emergence	Daily plot census
VE	5-8	Final stand count	Count all plants in harvest rows
VE	5-8	Emergence speed index (ESI)	ESI = sum(Ni/Di) where Ni = newly emerged on day Di
V1	7-10	Seedling vigor score (1-9 scale)	Visual: 1=poor, 9=excellent Score uniformity + size
V2	10-14	Plant height (soil to tip of tallest leaf, pulled vertical)	Ruler, n=10 plants/plot
V3	14-21	Stem diameter (1st internode)	Digital caliper, n=10 plants/plot
V3	14-21	Leaf number (fully expanded + visible)	Count, n=10 plants/plot
V3	14-21	Root excavation subsample (primary root length, lateral root count, seminal root count)	Shovelomics on n=3 plants/plot (destructive sampling from non-harvest rows only)

*DAP = days after planting; timing varies by temperature

C2.2 Mid-Vegetative (V6-V12)

STAGE	DAP	MEASUREMENT	METHOD
V6	28-35	Plant height (soil to collar of newest fully expanded leaf)	Measuring stick, n=10/plot
V6	28-35	SPAD chlorophyll index (ear leaf or newest fully expanded)	Konica Minolta SPAD-502 n=10 plants/plot, mean of 3 readings per leaf (tip, mid, base)
V8	35-42	Stem diameter (3rd internode)	Digital caliper, n=10/plot
V10	42-49	Plant height	n=10/plot
V10	42-49	Leaf area index (LAI)	AccuPAR LP-80 ceptometer (or LI-COR LAI-2200) 4 above + 4 below canopy readings
V12	49-56	Plant height (final pre-tassel)	n=10/plot
V12	49-56	SPAD chlorophyll (ear leaf)	n=10/plot
V12	49-56	Drone-based NDVI (optional)	Multispectral camera (RedEdge-MX) Plot-level NDVI extraction

C2.3 Flowering (VT/R1)

STAGE	DAP	MEASUREMENT	METHOD
VT	56-70	Anthesis date (50% pollen shed)	Daily census: date when 50% of plants in plot are shedding pollen
R1	58-75	Silking date (50% silk emergence)	Daily census: date when 50% of plants in plot show visible silks
VT/R1	56-75	ASI (anthesis-silking interval)	ASI = silking date - anthesis date Smaller ASI = better synchrony ASI > 3 days indicates stress
VT	56-70	Ear height	Soil to ear node, n=10/plot
VT	56-70	Tassel branch number	Count primary branches, n=10/plot
VT	56-70	Final plant height	Soil to tassel tip, n=10/plot
R1	60-75	Ear shoot number	Count ears with visible silks per plant, n=all plants in harvest rows

C2.4 Grain Fill (R3 -- Milk Stage)

STAGE	DAP	MEASUREMENT	METHOD
R3	80-90	Ear length (husked)	Ruler on n=5 ears/plot (non-harvest-row destructive sample)
R3	80-90	Kernel rows per ear	Count at mid-ear, n=5 ears
R3	80-90	Kernels per row	Count longest row, n=5 ears
R3	80-90	Ear diameter (mid-ear)	Digital caliper, n=5 ears
R3	80-90	Kernel moisture content	Handheld grain moisture meter
R3	80-90	Collect kernels for metabolomics (separate protocol -- see C5)	Flash-freeze in liquid N2 Store at -80C

C2.5 Maturity and Harvest (R6)

STAGE	DAP	MEASUREMENT	METHOD
R6	120-140	Final stand count	Count all plants in harvest rows
R6	120-140	Stalk lodging (%)	Count stalks broken below ear node
R6	120-140	Root lodging (%)	Count plants leaning >30 degrees
R6	120-140	Ear height (final)	Soil to ear node, n=10/plot
R6	120-140	Barren plant count	Plants with no ear or <10 kernels
HARVEST (R6 + 2-4 weeks, or at 20-25% kernel moisture):			
Machine harvest: Combine (Wintersteiger or Almaco plot combine) with onboard weighing and moisture measurement Harvest center 2 rows x interior length			
Hand harvest (if needed for quality measurements): Harvest all ears from center 2 rows Count ears; weigh total ear mass			

Shell a subsample for grain moisture + grain weight
Dry remaining ears for yield calculation

C3. Yield Components (at R6) [CONFIDENTIAL]

C3.1 Yield Component Measurements

COMPONENT	METHOD	UNIT
Ears per plant	Total ears harvested / stand count	ears/plant
Ear length	Ruler, n=20 ears/plot	cm
Ear diameter	Caliper at mid-ear, n=20 ears	mm
Kernel rows per ear	Count at mid-ear, n=20 ears	rows
Kernels per row	Count longest row, n=20 ears	kernels
Total kernels per ear	Rows x kernels/row (or image analysis with KernelFinder)	kernels
100-kernel weight	Count 3 x 100 kernels per plot; weigh; adjust to 15.5% moisture	g (at 15.5% moisture)
Grain yield per plot	Total shelled grain weight per harvest area, adjusted to 15.5% moisture	kg/plot
Grain yield (t/ha)	(Plot yield / harvest area) x 10,000; adjusted to 15.5% moisture	t/ha
Harvest index (HI)	Grain yield / total above-ground biomass (subsample for stover)	ratio
Test weight	Shopper test weight apparatus (or USDA-approved method)	kg/hL

C3.2 Moisture Adjustment

All grain weights adjusted to 15.5% moisture (US standard):

Adjusted weight = Measured weight x (100 - measured moisture%) / (100 - 15.5)

Moisture measurement:

Primary: Dickey-john GAC 2100 or Perten AM 5200 (NIRS)
Confirmation: Oven-dry method (103C +/- 2C, 72 hours)
on 50-g subsample per plot

C3.3 Statistical Analysis

Linear mixed model:

$$\text{Yield}_{ij} = \mu + \text{Treatment}_i + \text{Location}_j + \text{Treatment} * \text{Location}_{ij}$$

```
+ Block(Location)_k(j) + error_ijk

Treatment: FIXED effect (3 levels: M-9, Water, RNase-M-9)
Location: RANDOM effect
Block nested within Location: RANDOM effect
Treatment x Location: RANDOM effect (for GxE estimation)

Software: R (lme4 package) or SAS PROC MIXED
Pairwise comparisons: Tukey HSD at alpha = 0.05
ANOVA assumptions: Check residual normality (Shapiro-Wilk)
and homoscedasticity (Levene's test)
Effect size: Report treatment means +/- SE, 95% CI, and
percent change vs. water control
```

C4. Grain Quality Assays [CONFIDENTIAL]

C4.1 Sweetness / Sugar Content

ASSAY 1: Brix Refractometer (fresh sweet corn only)
 Sample: Fresh kernels at R3-R4 (milk-dough stage)
 Method: Press kernel juice onto digital refractometer
 (Atago PAL-1 or equivalent)
 $n = 10$ kernels per ear, 5 ears per plot
 Report: degrees Brix (+/- 0.1)
 Interpretation: Higher Brix = sweeter
 [NOTE: Only relevant for sweet corn varieties; for field corn, skip Brix and proceed directly to HPLC]

ASSAY 2: HPLC Sugar Quantification
 Sample: Lyophilized kernels ground to powder; 100 mg extracted in
 80% ethanol (3 x 1 mL, 80°C, 30 min)
 Column: Aminex HPX-87H (Bio-Rad) or NH₂-bonded HPLC column
 Mobile phase: 5 mM H₂SO₄ at 0.6 mL/min (Aminex)
 or 75:25 acetonitrile:water (NH₂)
 Detection: Refractive index (RI)
 Standards: Sucrose, glucose, fructose, maltose (0.1-10 mg/mL)
 Quantification: External calibration curve ($R^2 > 0.999$)
 Report: mg/g DW for each sugar; total soluble sugars

C4.2 Starch Content and Composition

ASSAY 3: Total Starch (Megazyme Kit K-TSTA-100A)
 Principle: Enzymatic hydrolysis of starch to glucose
 (thermostable alpha-amylase + amyloglucosidase)
 followed by glucose oxidase/peroxidase colorimetric assay

Sample: 100 mg finely ground kernel flour (< 0.5 mm)

Protocol: AOAC Method 996.11 / AACC Method 76-13.01

Steps:

1. Disperse starch in DMSO (if resistant starch present)
2. Hydrolysis: alpha-amylase (100C, 6 min) + amyloglucosidase (50C, 30 min)
3. Glucose assay: GPOPOD reagent, read A510 nm

Report: % starch w/w (DW basis); expected range 65-75% for dent corn

ASSAY 4: Amylose/Amylopectin Ratio

Method: Iodine-binding assay (Megazyme K-AMYL or ConA method)

OR: DSC (differential scanning calorimetry) for gelatinization

Sample: Purified starch from defatted kernel flour

Report: % amylose (expected: 25-28% for normal dent; <5% for waxy)

Interpretation: M-9 treatment is not expected to change amylose ratio
(genetically determined); changes would suggest unexpected effects on starch biosynthesis

C4.3 Protein Content and Composition

ASSAY 5: Total Protein (Dumas/Kjeldahl)

Primary method: Dumas combustion (Leco FP-528 or equivalent)

Sample: 200 mg kernel flour

Principle: Combustion at 900C; N2 detection by thermal conductivity

Conversion factor: total N x 6.25 = crude protein

Secondary method: Kjeldahl (AOAC 979.09)

Sample: 1 g kernel flour

Digestion: H2SO4 + catalyst; distillation; titration

Report: % crude protein (DW basis); expected 8-12% for dent corn

ASSAY 6: Zein Protein Extraction and SDS-PAGE

Purpose: Determine if M-9 affects zein accumulation (storage proteins)

Extraction: 70% ethanol + 2% beta-mercaptoethanol

(sequential extraction of alpha-zein, gamma-zein,
beta-zein, delta-zein)

SDS-PAGE: 12% gel; Coomassie staining or PVDF for immunoblot

Quantification: Densitometry (ImageJ) of zein bands

(19 kDa alpha, 22 kDa alpha, 27 kDa gamma, etc.)

Report: Relative band intensity normalized to total lane protein

Interpretation: Elevated zein = more nitrogen storage (higher protein corn)

Reduced zein = potentially better amino acid quality

(less zein = more non-zein protein with better lysine/tryptophan)

C4.4 Oil Content

ASSAY 7: Crude Fat (Soxhlet or NIT/NIRS)

Soxhlet method:

Sample: 5 g finely ground kernel flour, dried at 103C

Solvent: Petroleum ether or diethyl ether

Extraction: 8 hours reflux

Report: % crude fat (DW basis); expected 3.5-5% for dent corn

NIT/NIRS method (rapid screening):

Instrument: FOSS Infratec 1241 or Perten DA 7250

Calibration: Validated against Soxhlet with local samples

Report: % crude fat (DW basis)

Interpretation: M-9 is not expected to significantly alter oil content (embryo oil is determined during seed development, not affected by seed soak treatment). However, if grain-filling quality is improved, kernel size increase could dilute embryo oil percentage.

C4.5 Moisture Content

ASSAY 8: Grain Moisture

Standard: Oven-dry method (ASABE S352.2)

Sample: 50 g whole kernels per plot

Protocol: 103C +/- 2C for 72 hours in forced-air oven

Calculation: Moisture% = ((wet weight - dry weight) / wet weight) x 100

Report: % moisture (wet basis)

Use: All yield and quality measurements adjusted to 15.5% moisture basis

C5. Molecular Validation [CONFIDENTIAL]

C5.1 RT-qPCR Sampling Schedule

STAGE	DAP	TISSUE	TARGETS	n
VE	5-7	Embryo axis + scutellum (dissected seed)	20 target genes + 3 reference genes	3 biol reps x 3 treatment = 9 samples
V3	14-21	Root tip (0-2 cm) + leaf 3 (mid-lamina, 5 cm)	20 target genes + 3 reference genes	3 biol reps x 3 treatment = 9 x 2 tissues
V6	28-35	Root tip + ear primordium (if visible)	20 target genes + 3 reference genes	3 biol reps x 3 treatment = 9 x 2 tissues
R1	60-75	Developing ear (kernels 0-7 DAP) + silks + leaf	20 target genes + 25 biomarker genes + 3 reference genes	3 biol reps x 3 treatment = 9 x 3 tissues
R3	80-90	Developing kernels	25 biomarker genes	3 biol reps x

(14-21 DAP)	+ 3 reference genes	3 treatment
		= 9 samples

Total RNA samples: ~90 (across stages, tissues, treatments)

Total qPCR assays: ~90 samples x 48 genes x 3 tech reps = ~12,960 reactions

Use 384-well format: ~34 plates

C5.2 Reference Gene Selection

Candidate reference genes (select best 3 by geNorm/NormFinder):

1. ZmActin1 (Zm00001eb404170) -- cytoskeletal
2. ZmEF1-alpha (Zm00001eb148440) -- translation elongation
3. ZmGAPDH (Zm00001eb367050) -- glycolysis
4. ZmUBQ (Zm00001eb264340) -- ubiquitin
5. ZmTubulin (Zm00001eb403910) -- cytoskeletal
6. ZmCYP (Zm00001eb110520) -- cyclophilin (peptidyl-prolyl isomerase)

Selection protocol:

1. Run all 6 candidates on 18 representative samples
(3 stages x 3 treatments x 2 biol reps)
2. Analyze with geNorm (Vandesompele et al. 2002) for pairwise variation
3. Analyze with NormFinder (Andersen et al. 2004) for stability
4. Select top 3 by consensus ranking
5. Use geometric mean of 3 reference genes for normalization

C5.3 RNA-seq on Developing Ears

Timepoints: R1 (0-7 DAP kernels) and R3 (14-21 DAP kernels)

Treatments: M-9 vs. Water control (T1 vs. T2)

Biological replicates: 3 per treatment per timepoint = 12 libraries

Library prep: Poly(A) enrichment (TruSeq Stranded mRNA)

Sequencing: Illumina NovaSeq 6000, PE150, 30M read pairs per sample

Analysis pipeline:

1. QC: FastQC + MultiQC
2. Trim: Trimmomatic or fastp
3. Align: STAR or HISAT2 to B73 v5 genome
4. Quantify: featureCounts (Subread)
5. DE analysis: DESeq2 (Love et al. 2014)
6. Pathway enrichment: KEGG, GO, MapMan
7. Focus on: starch biosynthesis, protein storage, hormones

Key genes to examine in RNA-seq:

All 20 drug targets (residual silencing at R1?)

All 25 biomarker genes (see C6)

Starch biosynthesis cluster

Zein gene cluster (chr 4, chr 7, chr 10)

Hormone biosynthesis/catabolism genes

C5.4 Metabolomics: ABA/GA in Kernels at R1

Sample: Developing kernels at R1 (0-7 DAP), flash-frozen
 Analytes: ABA, GA1, GA3, GA4, IAA, JA, JA-Ile, SA, Z (zeatin), ZR
 Method: LC-MS/MS (triple quadrupole)

Extraction: 10 mg lyophilized kernel powder in 1 mL methanol:water:formic acid (15:4:1) with deuterium-labeled internal standards (d6-ABA, d2-GA1, d5-IAA, d5-JA, d4-SA)

Cleanup: C18 SPE or Oasis HLB cartridge

LC: UPLC C18 column, 0.3 mL/min, water/acetonitrile + 0.1% FA

MS: MRM mode, ESI negative (ABA, JA, SA) and positive (GA, IAA, CK)

Quantification: Isotope dilution with calibration curves

Interpretation:

ABA lower in M-9 kernels --> residual ABA suppression during grain fill

GA higher --> accelerated developmental programs

IAA higher --> enhanced cell division in endosperm

CK higher --> enhanced grain sink strength

C6. Biomarker Panel for Grain Filling

Enhancement [CONFIDENTIAL]

C6.1 Sucrose/Starch Pathway Biomarkers (8 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
1	Sh2 (Shrunken2)	Zm00001eb050730	Starch biosynthesis	AGPase large subunit; commits glucose-1-P to starch synthesis; rate-limiting step	[KNOWN] Enhanced starch biosynthetic capacity; more amylopectin precursor (ADP-glucose) produced; larger kernels expected	[KNOWN] Reduced starch synthesis; shrunken kernel phenotype (sh2 mutant); sweet corn trait
2	Bt2 (Brittle2)	Zm00001eb233830	Starch biosynthesis	AGPase small (catalytic) subunit; heterotetrameric complex with Sh2	[KNOWN] Enhanced AGPase activity; more ADP-glucose; improved starch deposition rate	[KNOWN] Reduced starch synthesis; brittle endosperm (bt2 mutant)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
3	Wx1 (Waxy1)	Zm00001eb304560	Starch biosynthesis	Granule-bound starch synthase I (GBSSI); synthesizes amylose within starch granules	[KNOWN] More amylose relative to amylopectin; higher apparent amylose content	[KNOWN] Waxy (amylose-free) starch; wx1 null = waxy corn; reduced amylose
4	Ae1 (Amylose Extender1)	Zm00001eb116530	Starch biosynthesis	Starch branching enzyme IIb (SBEIIb); creates alpha-1,6 branch points in amylopectin	[KNOWN] Normal amylopectin branching pattern; standard starch structure	[KNOWN] Increased apparent amylose (resistant starch); ae1 mutant = amylose extender
5	Su1 (Sugary1)	Zm00001eb174490	Starch biosynthesis	Isoamylase-type starch debranching enzyme (ISA1); required for proper starch granule crystallization	[KNOWN] Normal starch granule formation; proper crystalline structure	[KNOWN] Phytoglycogen accumulation instead of starch; sugary endosperm (su1 mutant)
6	Sh1 (Shrunken1)	Zm00001eb363690	Sucrose metabolism	Sucrose synthase (SuSy); cleaves sucrose to UDP-glucose + fructose; channeling to starch synthesis	[KNOWN] Enhanced sucrose conversion to UDP-glucose for starch and cellulose biosynthesis; key for grain filling rate	[KNOWN] Shrunken kernels (sh1 mutant); impaired sucrose-to-starch conversion; reduced grain filling
7	INCW2/Mn1 (Miniature1)	Zm00001eb072440	Sucrose metabolism	Cell wall invertase; irreversibly cleaves sucrose to glucose + fructose at the pedicel/basal endosperm transfer layer	[KNOWN] Enhanced hexose production for endosperm cell division and expansion; critical for sink strength establishment	[KNOWN] Miniature kernel (mn1 mutant); reduced seed size; impaired endosperm development; reduced basal endosperm transfer layer function

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
8	ZmSWEET4c	Zm00001eb366400	Sugar transport	Bidirectional hexose transporter; moves glucose/fructose from basal endosperm transfer layer into developing endosperm	[KNOWN] Enhanced hexose delivery to endosperm; improved sink strength; larger kernels	[KNOWN] Reduced kernel filling; impaired sugar transport across BETL; smaller kernels

C6.2 Nitrogen Assimilation / Storage Protein Biomarkers (4 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
9	GS1-3 (Gln1-3)	Zm00001eb248430	N assimilation	Cytosolic glutamine synthetase; assimilates NH4+ into glutamine; major enzyme for N remobilization to grain	[KNOWN] Enhanced N assimilation efficiency; more glutamine for grain protein synthesis; associated with NUE QTL on chr 5	[KNOWN] Reduced N assimilation; lower grain protein; impaired N remobilization from leaves to grain
10	GOGAT (Fd-GOGAT)	Zm00001eb256160	N assimilation	Ferredoxin-dependent glutamate synthase; converts glutamine + 2-oxoglutarate to 2 glutamate in plastids	[KNOWN] Enhanced glutamate production; more amino acid precursors for protein synthesis	[KNOWN] Reduced amino acid biosynthesis; impaired N metabolism
11	O2 (Opaque2)	Zm00001eb413540	Storage protein regulation	bZIP transcription factor; master regulator of 22-kDa alpha-zein gene expression; controls ~60% of endosperm protein	[KNOWN] High zein expression; vitreous endosperm; normal kernel hardness	[KNOWN] Reduced zein; opaque/floury endosperm (o2 mutant); higher lysine/tryptophan but soft kernel (QPM breeding target)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
12	22-kDa alpha-zein cluster	Multiple loci (chr 4, 7, 10)	Storage protein	Prolamin storage proteins; 50-70% of endosperm protein; packaged in protein bodies	[KNOWN] High storage protein deposition; vitreous endosperm; adequate grain protein	[KNOWN] Reduced protein bodies; opaque endosperm; less total grain protein but better amino acid quality

C6.3 Hormone Marker Biomarkers (4 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
13	VP1 (Viviparous1)	Zm00001eb197370	ABA signaling	B3-domain TF; master regulator of seed maturation and dormancy; activates LEA/Em genes	[KNOWN] Active maturation program; ABA-responsive gene expression; proper desiccation tolerance	[KNOWN] Vivipary (precocious germination); reduced dormancy; reduced desiccation tolerance. NOTE: This IS ABI40, the drug target. Expression at R1/R3 indicates whether drug effects persist to grain fill
14	GA20ox	Zm00001eb380080	GA biosynthesis	GA 20-oxidase; catalyzes penultimate step in bioactive GA biosynthesis (GA12 --> GA9 --> GA20)	[KNOWN] Enhanced GA biosynthesis; cell elongation; internode extension; can increase grain length	[KNOWN] Reduced GA; dwarf stature; potential grain size reduction
15	GA3ox	Zm00001eb219550	GA biosynthesis	GA 3-oxidase; catalyzes final step producing bioactive GA1 and GA4	[KNOWN] Enhanced bioactive GA; promotes cell division in endosperm; larger grain sink	[KNOWN] Reduced bioactive GA; limited cell expansion; smaller kernels

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
16	ABI5	Zm00001eb290340	ABA signaling	bZIP TF; downstream effector of ABA signaling in seeds; activates LEA and maturation genes	[KNOWN] Active ABA signaling in developing kernels; proper maturation program	[KNOWN] Reduced ABA response; potentially premature termination of grain fill; accelerated dry-down

C6.4 Defense/Stress Biomarkers (3 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
17	PR1 (Pathogenesis-related 1)	Zm00001eb362000	Defense	SA-inducible defense marker; classic indicator of systemic acquired resistance (SAR)	[KNOWN] Active defense response; SA pathway engaged; potential grain defense against ear pathogens	[KNOWN] Reduced basal defense; potential susceptibility to ear rot (Fusarium, Aspergillus)
18	Chitinase (PR3)	Zm00001eb075570	Defense	Endochitinase; degrades chitin in fungal cell walls; antimicrobial defense protein	[KNOWN] Active antifungal defense in kernels; protection against Fusarium ear rot and aflatoxin	[KNOWN] Reduced antifungal capacity; increased ear rot susceptibility
19	HSP70	Zm00001eb158050	Stress response	Heat shock protein 70; molecular chaperone; protects proteins from denaturation under heat stress	[KNOWN] Active heat stress response; protein protection during grain fill under hot conditions; may indicate stress	[KNOWN] Reduced protein protection; higher susceptibility to heat-induced grain fill disruption

C6.5 Housekeeping/Reference Genes (3 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	USE
20	Actin1	Zm00001eb404170	Cytoskeleton	Structural protein; constitutive expression	RT-qPCR normalization reference
21	EF1-alpha	Zm00001eb148440	Translation	Translation elongation factor; constitutive	RT-qPCR normalization reference
22	GAPDH	Zm00001eb367050	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase; constitutive	RT-qPCR normalization reference

C6.6 Additional Grain Quality Biomarkers (3 genes)

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
23	ZmSSIIa (Starch synthase IIa)	Zm00001eb386520	Starch biosynthesis	Soluble starch synthase II; extends amylopectin chains to intermediate length; determines starch gelatinization temperature	[KNOWN] Normal amylopectin chain length distribution; standard gelatinization properties	[KNOWN] Altered amylopectin structure; modified starch functionality; lower gelatinization temperature
24	ZmISA2 (Isoamylase 2)	Zm00001eb063770	Starch biosynthesis	Isoamylase-type DBE; regulatory subunit of ISA1-ISA2 heteromeric complex; required for starch granule formation	[KNOWN] Proper starch granule crystallization; normal starch structure	[KNOWN] Impaired starch granule formation; phytoglycogen accumulation

#	GENE NAME	GENE MODEL ID	PATHWAY	FUNCTION	UP =	DOWN =
25	ZmCKX1 (Cytokinin oxidase 1)	Zm00001eb310310	Cytokinin catabolism	Degrades active cytokinins (zeatin, iP); controls local CK levels in developing kernels	[KNOWN] Active CK degradation; limits kernel cell division; smaller kernels	[KNOWN] CK accumulates; MORE cell division in endosperm; LARGER kernels; higher grain yield (CKX downregulation is a breeding target for yield improvement)

C6.7 Biomarker Panel Summary

Total panel: 25 genes (22 biomarkers + 3 references)

Expected profile if M-9 enhances grain filling:

PATHWAY	GENE	EXPECTED DIRECTION IN M-9 VS. CONTROL	CONFIDENCE
Starch	Sh2	UP (more AGPase for starch synthesis)	[INFERRED]
Starch	Bt2	UP (more AGPase catalytic subunit)	[INFERRED]
Starch	Wx1	UNCHANGED (amylose ratio genetically fixed)	[INFERRED]
Starch	Ae1	UNCHANGED (branching pattern genetically fixed)	[INFERRED]
Starch	Su1	UP or UNCHANGED	[SPECULATIVE]
Sucrose	Sh1	UP (more SuSy for sucrose conversion)	[INFERRED]
Sucrose	INCW2/Mn1	UP (more CWI for sink strength)	[INFERRED]
Sugar transport	ZmSWEET4c	UP (more hexose delivery to endosperm)	[INFERRED]
N assimilation	GS1-3	UP (more N remobilization to grain)	[SPECULATIVE]
N assimilation	GOGAT	UP (more glutamate for protein)	[SPECULATIVE]
Storage protein	O2	UNCHANGED (TF activity, not transcript)	[INFERRED]
Storage protein	alpha-zein	UP (more storage protein deposition)	[SPECULATIVE]
ABA	VP1/ABI40	DOWN (if residual drug effect) or RECOVERED	[SPECULATIVE]
GA	GA20ox	UP (more GA for cell expansion)	[SPECULATIVE]
GA	GA3ox	UP (more bioactive GA in kernels)	[SPECULATIVE]

PATHWAY	GENE	EXPECTED DIRECTION IN M-9 VS. CONTROL	CONFIDENCE
ABA	ABI5	DOWN (if residual ABA suppression) or RECOVERED	[SPECULATIVE]
Defense	PR1	DOWN (growth-defense reallocation) or RECOVERED	[SPECULATIVE]
Defense	Chitinase	UNCHANGED or DOWN	[SPECULATIVE]
Stress	HSP70	UNCHANGED (constitutive under non-stress)	[INFERRRED]
Starch	SSIIa	UNCHANGED (genetically determined)	[INFERRRED]
Starch	ISA2	UNCHANGED	[INFERRRED]
CK catabolism	CKX1	DOWN (more CK for grain filling)	[SPECULATIVE]

Critical interpretation guideline: If VP1/ABI40 and ABI5 show persistent downregulation at R1/R3 (50+ days after seed soak treatment), this would indicate either: (a) RdDM-mediated epigenetic silencing persisting through mitotic divisions, or (b) a secondary developmental program triggered during germination that maintains different hormone homeostasis. If these targets have RECOVERED to control levels, the grain-filling effects are entirely indirect (larger root system --> more nutrients --> better grain fill).

C7. Statistical Power and Sample Size Justification

[CONFIDENTIAL]

YIELD POWER ANALYSIS:

Target detectable difference: 5% yield increase (0.5 t/ha at 10 t/ha mean)
 Expected CV for grain yield: 8-12% (well-managed field trial)
 Alpha: 0.05 (two-sided)
 Power: 0.80

Required replicates per treatment per location:

CV = 8%: n = 5 (RCBD)
 CV = 10%: n = 6 (RCBD)
 CV = 12%: n = 8 (RCBD)

With 6 locations:

n = 4-6 reps per treatment per location is sufficient for
 5% yield difference detection across environments

GENE EXPRESSION POWER ANALYSIS:

Target detectable fold-change: 2-fold ($\log_2 FC = 1$)
 Expected CV for qPCR: 20-30%
 Alpha: 0.05 (two-sided)
 Power: 0.80

Required biological replicates: n = 3-4 per treatment per timepoint
Technical replicates: n = 3 per biological replicate

C8. Timeline and Budget Summary [CONFIDENTIAL]

PHASE 1: SEED TREATMENT VALIDATION (Month 1-2)

Lab germination tests (3 treatments x 6 reps)
RT-qPCR validation of 5 confirmed + 15 predicted targets at VE
ABA/GA quantification at VE
Cost: ~\$5,000

PHASE 2: GREENHOUSE ROOT PHENOTYPING (Month 2-4)

Root architecture comparison (rhizotron or WinRHIZO)
3 treatments x 8 plants x 4 timepoints
Cost: ~\$3,000

PHASE 3: FIELD TRIALS YEAR 1 (Month 3-8)

4 locations x 24 plots = 96 field plots
Phenotyping at all growth stages
Yield harvest and quality analysis
Cost: ~\$40,000-60,000 (includes land, labor, seed, equipment)

PHASE 4: MOLECULAR VALIDATION (Month 4-10)

RT-qPCR panel (90 samples x 48 genes)
RNA-seq (12 libraries)
Metabolomics (18 samples)
Cost: ~\$25,000-35,000

PHASE 5: FIELD TRIALS YEAR 2 (Month 12-20)

Expanded to 6 locations
Refined treatments based on Year 1 results
Cost: ~\$60,000-80,000

PHASE 6: DATA ANALYSIS AND REPORTING (Month 20-24)

Statistical analysis (GxE, mixed models)
Biomarker correlation with yield components
Publication preparation
Cost: ~\$5,000 (personnel time)

TOTAL ESTIMATED BUDGET: \$138,000-188,000 (2-year program)

MINIMUM VIABLE EXPERIMENT: \$15,000 (lab + 1 field location + qPCR)

C9. Regulatory and IP Considerations [CONFIDENTIAL]

CLASSIFICATION:

The tRF drug is a biological RNA-based agricultural input.

Regulatory pathway depends on jurisdiction:

- US: EPA (biopesticide exemption if no pesticidal claim)
or USDA-APHIS (if plant-incorporated protectant, unlikely)
or FDA (if grain quality claims)
- EU: Novel biostimulant (Regulation EU 2019/1009)
- Brazil: MAPA biological input registration

DATA REQUIREMENTS FOR REGISTRATION:

1. Product characterization (tRF sequence, G4 structure, stability)
2. Efficacy data (field trials, 2+ years, 4+ locations)
3. Environmental fate (RNA degradation kinetics in soil)
4. Non-target organism safety (aquatic, avian, mammalian)
5. Residue analysis (tRF detection in harvested grain -- expected nil)
6. Manufacturing consistency (batch-to-batch variation)

KEY ADVANTAGE: RNA-based products are generally considered low-risk because RNA degrades rapidly in the environment (half-life in soil: hours). No genomic modification of the plant occurs.

APPENDIX: Evidence Classification Summary

EVIDENCE LEVEL	COUNT	DESCRIPTION
[KNOWN]	~45 claims	Published peer-reviewed literature with direct experimental support
[INFERRRED]	~35 claims	Logical deduction from conserved biology, target annotations, and homolog data
[SPECULATIVE]	~20 claims	Hypotheses requiring experimental validation; includes all quantitative magnitude estimates

Honest assessment: The MoA model is well-supported for the germination phase (Phase 1-2) based on extensive literature on ABA signaling, sugar sensing, and cell wall biology. The root architecture effects (Task B) are largely [INFERRRED] from the molecular mechanisms and require experimental validation. The yield translation (B.3) and grain quality predictions are [SPECULATIVE] and represent the highest-uncertainty component of this dossier. The field validation plan (Task C) is designed to systematically test these predictions.

Report prepared: 2026-02-18 Dossier version: 1.0 Next review: Upon completion of Phase 1 (seed treatment validation)

PARTS 4-7: GRAIN FILLING, SWEETNESS, PROTEIN & QUANTITATIVE PREDICTIONS

CONFIDENTIAL

Maize Grain Filling MoA Dossier: From Seed Imbibition to Kernel Composition

How Early-Stage Gene Downregulation Translates to Grain Filling, Kernel Composition, and Yield [C O N F I D E N T I A L]

Prepared: 2026-02-18 **Classification:** Internal Research Supplement to FINAL_REPORT_MAIZE.md **Evidence Labeling:** [KNOWN] = published peer-reviewed literature; [INFERRED] = logical deduction from conserved biology and target annotations; [SPECULATIVE] = hypothesis requiring experimental validation **Scope:** This dossier addresses the central translational question: How can gene downregulation during a 4-8 hour seed soak propagate forward through vegetative development to affect grain filling, kernel composition, and yield at R1-R6?

Table of Contents [C O N F I D E N T I A L]

1. Conceptual Framework: Bridging Germination to Grain Fill
2. Comprehensive Grain Filling Pathway Analysis
3. Sweetness (Sucrose) and Starch Prediction
4. Protein Content Prediction
5. Quantitative Prediction Ranges
6. Synthesis: Target-to-Grain-Fill Connectivity Map
7. References

1. Conceptual Framework: Bridging Germination to Grain Fill

1.1 The Temporal Gap Problem

The RNA drug is applied at seed imbibition (0-8 hours). Grain filling occurs at R1-R6 (approximately 55-100 days after planting). This represents a temporal gap of 55-100 days. Three mechanistic bridges could connect early gene downregulation to late-season phenotypes:

Bridge 1: Epigenetic Memory / Transgenerational Stress Priming [KNOWN] Seed priming can induce epigenetic changes (DNA methylation, histone modifications, small RNA profiles) that persist beyond the initial treatment window and affect later developmental stages. Louis (2023) demonstrated that seed priming can retain stress tolerance across developmental stages and even subsequent generations through epigenetic modifications. In *Zea mays*, transgenerational memory of heat and drought stress has been linked to differential expression of small RNAs targeting stress-responsive transcription factors, contributing to improved seedling vigor and root development in progeny.

[INFERRED] If the tRF RNA drug triggers RdDM (RNA-directed DNA methylation) at target loci during imbibition, the resulting methylation marks could persist through cell divisions and affect gene expression at grain filling. This is the strongest mechanistic bridge but requires experimental validation of persistent epigenetic marks.

Bridge 2: Early Vigor Advantage / Compound Growth Effect [KNOWN] Seeds that germinate faster and establish more robust seedlings capture more light, water, and nutrients early in the season, creating a compound growth advantage that persists through the reproductive phase. A 2023 Frontiers study showed that biostimulant seed treatments increased maize grain yield by 5.1%, with the effect attributable to early vigor translating to better canopy establishment and more efficient resource capture during grain fill.

[INFERRED] Faster germination (Theme 1: ABA derepression) and enhanced seedling vigor (Theme 5: AHL9-mediated auxin derepression) would establish a larger, more vigorous plant that captures more photosynthate during grain filling. This is the most conservative and well-supported bridge.

Bridge 3: Persistent Transcriptomic Reprogramming [SPECULATIVE] If the exRNA-mediated silencing triggers secondary siRNA amplification through the plant's RDR (RNA-dependent RNA polymerase) machinery, the silencing signal could be amplified and maintained beyond the initial treatment window. In maize, the RNAi toolkit includes functional RDR genes capable of signal amplification. However, the duration of amplified silencing from a single exogenous trigger in maize seeds is unknown and could range from hours to weeks.

1.2 Most Likely Scenario

The most scientifically conservative explanation for any grain-filling effects is **Bridge 2: the compound growth effect**. A plant that germinates 12-24 hours earlier, establishes a deeper root system by V3, and captures 3-5% more intercepted radiation through V6-VT will have more photosynthate available to partition to ears during grain fill. This does NOT require persistent gene silencing -- it requires only the initial germination/vigor advantage to propagate through normal developmental physiology.

2. Grain Filling Pathway Analysis [C O N F I D E N T I A L]

2.1 Overview of Maize Grain Filling

[KNOWN] Maize grain filling spans stages R1 (silking) through R6 (physiological maturity), approximately 45-65 days. The mature kernel comprises: - **Starch**: 70-75% of dry weight (endosperm) - **Protein**: 8-12% of dry weight (mainly zeins in endosperm; globulins/albumins in embryo) - **Oil**: 3-6% of dry weight (mainly in embryo/scutellum) - **Fiber**: 2-3% of dry weight (pericarp) - **Sugars**: 1-3% of dry weight (sucrose, glucose, fructose)

The grain filling process requires: (a) photosynthate production in source leaves, (b) phloem loading and long-distance transport, (c) phloem unloading at the ear, (d) post-phloem transport through the basal endosperm transfer layer (BETL), and (e) metabolic conversion to starch, protein, and oil within the endosperm and embryo.

2.2 Sucrose Transport to the Ear: Phloem Unloading Mechanisms

[KNOWN] Sucrose arrives at the developing maize kernel via the phloem. At the maternal-filial boundary (the pedicel-BETL interface), phloem unloading switches from symplastic to apoplastic. This is a critical control point for sink strength.

Key Steps: 1. **Phloem unloading** into the pedicel apoplast (symplastic in early development, apoplastic at grain fill) 2. **Sucrose hydrolysis** by cell wall invertase (INCW2/Miniature1) in the pedicel region, generating glucose + fructose 3. **Hexose import** across the BETL plasma membrane via SWEET transporters and hexose transporters 4. **Re-synthesis** to sucrose or direct use for starch biosynthesis in endosperm cells

Connection to target genes:

TARGET GENE	CONNECTION TO SUCROSE TRANSPORT	EVIDENCE LEVEL
HEX6 (hexokinase)	After hexose import, HXK phosphorylates glucose to G6P, feeding glycolysis and starch biosynthesis. HXK also acts as a sugar sensor regulating gene expression. If HEX6 is persistently downregulated, sugar sensing would be attenuated, potentially altering source-sink signaling.	[INFERRRED] Indirect. HXK family redundancy (9 members in maize) buffers against single-gene effects.
NPF15 (NRT1/PTR transporter)	NPF transporters have been shown to transport diverse substrates. ZmNPF7.9 (SUGCAR1) was recently identified as a sugar transporter critical for grain filling, expressed in BETL. If NPF15 has sugar or peptide transport activity in the pedicel/BETL, its modulation could affect nutrient flux. The dek407 mutant (encoding NPF1.5) shows reduced kernel size and delayed grain filling.	[SPECULATIVE] NPF15 is annotated as NPF4.6/ AIT1-like (ABA transporter), not a sugar transporter. But NPF family substrate promiscuity means this cannot be excluded.
MYBR64 (MYB-related TF)	ZmMYBR29, a MYB-related TF homologous to MYBR64, was shown in 2024 to be specifically expressed in the basal cellularized endosperm and required for grain filling. Loss-of-function zmmybr29 mutant showed 26.7% decrease in average grain filling rate and smaller kernels. ZmMYBR29 regulates genes involved in starch and sucrose metabolism.	[KNOWN/INFERRRED] This is a critical finding. If MYBR64 is orthologous or paralogous to ZmMYBR29, its downregulation during germination could have significant implications for grain filling IF the downregulation persists. However, ZmMYBR29 loss DECREASES grain filling, so downregulating a homolog would be DETRIMENTAL to grain fill, not beneficial.

2.3 SWEET Transporters in Maize (ZmSWEET Family)

[KNOWN] The SWEET (Sugars Will Eventually be Exported Transporters) family facilitates sugar transport across membranes. In maize kernel development:

- **ZmSWEET4c:** The critical hexose transporter at the BETL. Mediates transepithelial hexose transport at the entry point of nutrients into the seed. Mutants are defective in seed filling. ZmSWEET4c shows signatures of selection during domestication, suggesting it was recruited to enhance sugar import into the endosperm (Sosso et al., 2015, *Nature Genetics*).
- **ZmSWEET11:** Constitutively expressed in BETL.
- **ZmSWEET3a/13a:** Expressed at the grain set stage.
- **ZmSWEET6b, 13b, 14b, 15a:** Expressed during germination.

Connection to target genes:

TARGET GENE	CONNECTION TO SWEET TRANSPORTERS	EVIDENCE LEVEL
ABI40 (VP1-like)	VP1/ABI3 regulates embryo maturation programs including scutellum development. VP1 directly regulates intra-kernel protein reallocation through scutellum development (Yang et al., 2019, <i>Plant Cell</i>). VP1 loss affects nutrient assimilation pathways in the kernel. ABI40 downregulation could alter the maturation program that establishes BETL identity and SWEET transporter expression patterns.	[SPECULATIVE] VP1 primarily regulates embryo programs; SWEET expression in BETL is regulated by other TFs (e.g., MRP1/ZmMYBR29).
AHL9 (AT-hook protein)	AHL proteins remodel chromatin at S/MAR regions. If AHL9 targets include SWEET gene promoters, its downregulation could derepress SWEET expression.	[SPECULATIVE] No direct evidence links AHL9 to SWEET regulation.

Assessment: None of the 20 target genes directly encode SWEET transporters or their known regulators. Any effect on SWEET-mediated sugar transport would be highly indirect.

2.4 SUT/SUC Sucrose Transporters

[KNOWN] Seven genes encoding sucrose transporters exist in maize: ZmSUT1-ZmSUT7. ZmSUT1 is expressed in the BETL and functions in sucrose retrieval and phloem loading/unloading. SUT1 is critical for maintaining sucrose gradients that drive bulk flow.

Connection to target genes: No direct connection. None of the 20 targets encode SUTs or their known regulators.

2.5 Starch Biosynthesis Pathway

[KNOWN] The maize endosperm starch biosynthesis pathway involves:

ENZYME	GENE(S)	FUNCTION	MUTANT PHENOTYPE
Sucrose Synthase (SuSy)	<i>Shrunken1</i> (Sh1), SUS1	Cleaves sucrose to UDP-glucose + fructose; dominant substrate supplier for starch biosynthesis	sh1: >90% loss of SuSy activity; dramatically reduced starch; increased soluble sugars; shrunken kernels
AGPase (large subunit)	<i>Shrunken2</i> (Sh2)	Synthesizes ADP-glucose from G1P + ATP; rate-limiting step	sh2: severely reduced starch (~75% of endosperm starch depends on cytosolic AGPase)
AGPase (small subunit)	<i>Brittle2</i> (Bt2)	Catalytic partner of Sh2	bt2: similar to sh2; defective kernels with reduced starch
Granule-bound starch synthase (GBSS)	<i>Waxy</i> (Wx)	Synthesizes amylose within granules	wx: 0% amylose, 100% amylopectin (waxy starch)
Soluble starch synthase I	SSI	Elongates short glucan chains	Altered amylopectin fine structure

ENZYME	GENE(S)	FUNCTION	MUTANT PHENOTYPE
Soluble starch synthase IIa	SSIIa	Elongates intermediate chains	Altered amylopectin
Starch branching enzyme	<i>Amylose Extender1 (Ae1) / SBEIIb</i>	Introduces alpha-1,6 branch points	ae1: increased amylose (>50%)
Starch debranching enzyme (ISA1)	<i>Sugary1 (Su1)</i>	Trims excess branches; crystallization of amylopectin	su1: phytoglycogen accumulation; increased sucrose; sweet corn phenotype
Invertase (cell wall)	<i>Miniature1 (Mn1) / INCW2</i>	Hydrolyzes sucrose at BETL; establishes sink strength	mn1: >70% reduction in kernel weight

Connection to target genes:

TARGET GENE	CONNECTION TO STARCH BIOSYNTHESIS	EVIDENCE LEVEL
IBP1 (Initiator Binding Protein 1)	[KNOWN] IBP1 binds the initiator element of the <i>Shrunken-1</i> promoter. Sh1 encodes sucrose synthase, the dominant substrate supplier for starch biosynthesis. IBP1 downregulation could alter Sh1 transcription. If IBP1 is a positive regulator, its downregulation would reduce Sh1 expression, reducing starch and increasing soluble sugars (analogous to sh1 mutant). If IBP1 is a negative regulator, its downregulation would increase Sh1 expression and potentially enhance starch biosynthesis.	This is the most direct connection between any target gene and starch biosynthesis. The direction of effect is uncertain.
HEX6 (hexokinase)	[INFERRRED] HXK phosphorylates fructose/glucose produced by invertase and SuSy. Reduced HXK activity would slow hexose phosphorylation, potentially creating a metabolic bottleneck between sucrose hydrolysis and starch synthesis. However, 9 HXK genes provide redundancy. HXK also functions as a sugar sensor: reduced HXK signaling could alter expression of starch biosynthesis genes through sugar-response pathways.	Indirect; buffered by gene family redundancy.
MYBR64 (MYB-related TF)	[KNOWN] ZmMYBR29 (MYB-related, same subfamily as MYBR64) loss-of-function reduces grain filling rate by 26.7% and downregulates starch and sucrose metabolism genes. ZmMYB155 (2025) is involved in starch synthesis and BETL development. If MYBR64 is a positive regulator of grain filling like ZmMYBR29, its downregulation would be DETRIMENTAL to starch accumulation.	This is a potential negative effect of the RNA drug on grain fill.
ppr377 (PPR protein)	[INFERRRED] PPR proteins process mitochondrial/chloroplast transcripts. Mitochondrial efficiency determines ATP supply for starch biosynthesis (AGPase requires ATP). Reduced PPR function could impair energy supply to the starch synthesis pathway.	Indirect; potentially counterproductive.
RING63/ RING265 (E3 ubiquitin ligases)	[INFERRRED] E3 ligases control turnover of metabolic enzymes. If RING63/265 target starch biosynthesis enzymes for degradation, their downregulation would stabilize these enzymes and increase starch. If they target negative regulators, the opposite occurs. Substrate specificity is unknown.	Ambiguous; substrate-dependent.

2.6 Invertases: Miniature1 and Beyond

[KNOWN] The Miniature1 (Mn1) locus encodes INCW2, the dominant cell wall invertase in the BETL. INCW2 catalyzes irreversible sucrose hydrolysis to glucose + fructose in the apoplast, generating the hexose gradient that drives sugar import. The mn1 mutant loses >70% of kernel weight, demonstrating that INCW2 is the single most important determinant of kernel sink strength.

Additional invertases include: - **INCW1**: Second cell wall invertase; expressed at lower levels - **IVR1**: Vacuolar invertase; soluble, cleaves sucrose within the vacuole - **IVR2**: Perianth/maternal tissue invertase, stress-responsive

[KNOWN] Cell wall invertase-deficient mn1 kernels have altered phytohormone levels, including reduced IAA and increased ABA, demonstrating that sugar metabolism and hormone signaling are tightly coupled during grain filling.

Connection to target genes:

TARGET GENE	CONNECTION TO INVERTASES	EVIDENCE LEVEL
ABI40 (VP1-like)	[KNOWN] VP1/ABI3 regulates maturation programs. In vp1 mutants, the scutellum develops abnormally and cannot respond to nutrient signals (Yang et al., 2019). However, mn1 kernels show that invertase loss increases ABA, and VP1 mediates ABA responses. ABI40 downregulation could alter the ABA-invertase feedback loop during kernel development.	[INFERRED] Complex feedback; direction uncertain. VP1 primarily acts in embryo, while INCW2 acts in BETL.
HEX6 (hexokinase)	[INFERRED] HXK phosphorylates the hexose products of invertase (glucose + fructose). Reduced HXK activity would slow the metabolic utilization of invertase products, potentially causing hexose accumulation in the BETL apoplast. This could alter the concentration gradient driving further sucrose unloading.	Indirect metabolic effect.

2.7 Sucrose Synthase (SuSy / Sh1)

[KNOWN] Sucrose synthase (SuSy) encoded by Shrunken1 (Sh1) is the primary enzyme directing carbon from sucrose toward starch biosynthesis in maize endosperm. Sh1 is primarily expressed in endosperm, while SUS1 accumulates mainly in embryo. The sh1 mutation causes >90% loss of SuSy activity, with dramatic consequences: reduced starch content, increased soluble sugars (including sucrose, glucose, fructose), extremely high osmolality, kernel swelling, and the shrunken phenotype. SH1 overexpression (ZmSUS1) alters starch content and composition.

Critical connection: IBP1 binds the Sh1 promoter. This is the single most direct link between any of the 20 target genes and the starch biosynthesis machinery. IBP1 downregulation would alter Sh1 transcription, but the direction depends on whether IBP1 activates or represses Sh1. IBP1 overexpression in tobacco reduces internode elongation and alters gibberellin balance, suggesting it is a growth modulator. Its binding to the Sh1 initiator suggests a role in fine-tuning Sh1 expression.

2.8 Sink Strength Regulators

[KNOWN] Sink strength in maize kernels is determined by:

1. **INCW2/Mn1**: Apoplastic sucrose hydrolysis (see Section 2.6)
2. **SWEET4c**: Hexose transport across BETL membranes
3. **Auxin (IAA)**: Promotes cell division in endosperm; auxin and ABA promote glucose metabolism in reactivated young kernels. ZmYUCCA genes catalyze auxin biosynthesis in developing kernels.
4. **Cytokinins**: Promote cell division during the lag phase (0-12 DAP); positively correlated with grain filling rate
5. **ABA**: Promotes grain filling by enhancing mobilization of carbon assimilates into grains; ABA content correlates positively with grain filling rate during active fill

Connection to target genes:

TARGET GENE	CONNECTION TO SINK STRENGTH	EVIDENCE LEVEL
ABI40 (VP1-like)	[KNOWN] ABA promotes grain filling. VP1/ABI3 mediates ABA-responsive gene expression. Reducing VP1-like activity could impair ABA-mediated grain filling promotion. However, during germination, ABI40 downregulation accelerates the dormancy-to-germination transition (beneficial). At grain filling, reduced ABA sensitivity could be DETRIMENTAL because ABA is a positive regulator of starch accumulation during maturation.	CRITICAL DUALITY: ABI40 downregulation is beneficial at germination but potentially detrimental at grain fill. This is only a concern if silencing persists to R-stages.
AHL9 (AT-hook protein)	[KNOWN] AHL proteins repress auxin biosynthesis by recruiting SWR1 to YUCCA promoters (Favero et al., 2024). AHL9 downregulation derepresses auxin biosynthesis. Enhanced auxin levels during vegetative growth produce larger plants. During ear development, auxin promotes kernel set and endosperm cell division.	[INFERRED] Enhanced auxin from AHL9 downregulation could improve kernel set and reduce kernel abortion, increasing kernel number per ear. This is a positive pathway.
NPF15 (NRT1/PTR transporter)	[KNOWN] ZmNPF7.9 is essential for seed development (nitrate transport in BETL). Dek407 (NPF1.5) mutation causes reduced kernel size and delayed grain filling. If NPF15 has transport activity in developing kernels, its modulation could affect nutrient delivery.	[SPECULATIVE] NPF15 is annotated as NPF4.6 (ABA/GA transporter), not NPF7.9. Functional overlap within the 78-member family is possible but undemonstrated.

2.9 Kernel Abortion Mechanisms and ABA/GA Balance

[KNOWN] Kernel abortion in maize occurs primarily at the ear tip during the lag phase (0-12 DAP) when photosynthate supply is insufficient to support all fertilized ovules. The least competitive kernels (tip kernels) abort first.

Key regulators of kernel abortion: - **Sucrose supply**: Low sucrose to ear tips triggers abortion - **Auxin (IAA)**: Maintains kernel viability; auxin-deficient kernels abort - **ABA**: At moderate levels, promotes grain filling; at high levels during stress, promotes abortion - **Ethylene**: Stress-induced ethylene promotes kernel abortion - **Cytokinins**: Prevent abortion by maintaining cell division

[KNOWN] Exogenous ABA application to ears under drought alleviates dysplasia by altering photosynthesis and sucrose transport. GA4+7 application during grain filling increases 1000-grain weight and antioxidant activity. The ABA/GA balance is crucial: appropriate ABA promotes maturation and starch accumulation, while excessive ABA or insufficient GA inhibits grain fill.

Connection to target genes:

The ABI40 target has the strongest relevance. If ABI40 downregulation persists to the reproductive phase: - [INFERRRED] Reduced ABA sensitivity could reduce kernel abortion under mild drought (positive for yield) - [INFERRRED] Reduced ABA sensitivity could also impair the ABA-dependent maturation program, reducing starch deposition (negative for kernel weight) - The net effect depends on: (a) whether silencing persists, (b) the magnitude of ABI40 downregulation, and (c) environmental conditions (stress vs. optimal)

2.10 Cob Size Determinants

[KNOWN] Cob size (ear length, ear diameter, row number) is determined primarily by: 1. **Inflorescence meristem activity** during V5-V12 (ear initiation and development) 2. **Auxin transport and signaling** (PIN proteins, auxin maxima determine kernel row number) 3. **CLAVATA-WUSCHEL signaling** (CLV/FEA/TD1 pathway determines meristem size) 4. **Photosynthate availability** during ear initiation (source strength)

Connection to target genes:

TARGET GENE	CONNECTION TO COB SIZE	EVIDENCE LEVEL
AHL9 (AT-hook protein)	[INFERRRED] AHL9 downregulation derepresses auxin biosynthesis (YUCCA genes). Enhanced auxin during V5-V12 could promote inflorescence meristem activity, potentially increasing ear length and kernel row number.	[SPECULATIVE] Auxin effects on ear development are dose-dependent; excess auxin could have negative effects.
ABI40 (VP1-like)	[SPECULATIVE] No direct connection to ear morphogenesis. Any effect would be indirect through altered hormone balance.	Very indirect.

Assessment: Cob size is primarily determined by genetics and environmental conditions during V5-V12. The RNA drug applied at seed soak would need to produce either (a) persistent epigenetic effects altering meristem genes, or (b) an early vigor advantage that improves photosynthate supply during ear initiation. Bridge 2 (compound growth effect) is the most plausible mechanism for any cob size increase.

3. Sweetness (Sucrose) and Starch Prediction [CONFIDENTIAL]

3.1 Can the Gene Set Increase Sweetness?

Definition: "Sweetness" in mature grain maize is primarily sucrose content (1-3% of kernel dry weight in field corn; 10-20% in sweet corn at harvest). Higher sweetness would mean higher free sugar content (sucrose, glucose, fructose) in the kernel at a given harvest time.

3.2 Pathway-by-Pathway Assessment

3.2.1 SWEET Transporters

Effect of 20-gene downregulation on SWEET expression: No direct effect. None of the 20 targets encode SWEET proteins or their characterized regulators. **Indirect route:** [SPECULATIVE] If AHL9 downregulation increases auxin, and auxin influences SWEET4c expression, there could be an indirect enhancement of hexose import. However, no evidence links auxin to SWEET4c transcription. **Verdict:** No plausible direct mechanism to alter sweetness via SWEET transporters.

3.2.2 SUT/SUC Sucrose Transporters

Effect: No direct connection. No targets encode SUTs. **Verdict:** No mechanism.

3.2.3 Sucrose Synthase (SuSy/Sh1)

Effect: IBP1 binds the Sh1 promoter. If IBP1 is a positive regulator of Sh1, its downregulation reduces SuSy activity, which would INCREASE free sucrose (less sucrose cleavage) and DECREASE starch. This is the sh1-like phenotype. **If IBP1 is a negative regulator of Sh1,** its downregulation increases SuSy, DECREASING free sucrose and INCREASING starch. **Verdict:** [INFERRRED] IBP1 downregulation could shift the sucrose/starch balance through Sh1 regulation, but the direction depends on IBP1's regulatory polarity, which is not established.

3.2.4 Invertases (CWI/VI)

Effect: No direct connection. Invertase genes (INCW1, INCW2/Mn1, IVR1, IVR2) are not among the 20 targets. **Indirect route:** [INFERRRED] If HEX6 downregulation reduces hexokinase-mediated sugar signaling, this could alter feedback regulation of invertase gene expression. Hexokinase-dependent sugar signaling normally represses certain genes when glucose is abundant. Reduced HXK signaling could derepress invertase expression. **Verdict:** Highly indirect; uncertain magnitude.

3.2.5 AGPase (Sh2/Bt2)

Effect: No direct connection. AGPase genes are not targets. **Indirect route:** [INFERRED] ABA promotes AGPase expression during grain fill. If ABI40 downregulation reduces ABA-responsive gene expression and this persists to grain filling, AGPase expression could be reduced, lowering the flux from G1P to ADP-glucose. Less ADP-glucose means less starch and more residual sugars. **Verdict:** [SPECULATIVE] Only relevant if ABI40 silencing persists to R-stages. Conservative estimate: silencing does NOT persist that long.

3.2.6 Starch Synthases (SSI, SSII, GBSS/Waxy)

Effect: No direct connection. No targets encode starch synthases. **Verdict:** No mechanism.

3.2.7 Starch Branching Enzyme (SBE/Ae1)

Effect: No direct connection. **Verdict:** No mechanism.

3.2.8 The su1 (sugary1) Pathway

[KNOWN] The sugary1 (su1) gene encodes isoamylase-type starch debranching enzyme (ISA1). su1 mutations cause: (1) increased sucrose concentration, (2) decreased amylopectin, (3) accumulation of phytoglycogen (highly branched water-soluble polysaccharide). The su1 phenotype is the basis of traditional sweet corn.

Could any of the 20 targets affect the su1 pathway?

MECHANISM	ASSESSMENT
Direct silencing of su1	No. su1 is not among the 20 targets.
Transcriptional regulation of su1	[SPECULATIVE] If MYBR64 regulates su1 expression, its downregulation could alter debranching enzyme levels. However, su1 expression is primarily controlled by developmental programs, not MYB-related TFs. No evidence links MYBR64 to su1.
Altered starch granule initiation	[SPECULATIVE] If RING63/265 ubiquitinate starch biosynthesis enzymes and their downregulation stabilizes these enzymes, the ratio of branching to debranching activity could shift. This is extremely indirect.

Verdict: No plausible mechanism connects the 20 target genes to the su1 pathway.

3.3 Summary: Sweetness Prediction

ROUTE	PLAUSIBILITY	DIRECTION	MAGNITUDE
IBP1 -> Sh1 (SuSy) -> sucrose/starch balance	Medium (IF IBP1 silencing persists and IBP1 is a positive Sh1 regulator)	Increase sucrose, decrease starch	Small (IBP1 is one of multiple Sh1 regulators)
HEX6 -> sugar sensing -> invertase feedback	Low	Uncertain	Minimal

ROUTE	PLAUSIBILITY	DIRECTION	MAGNITUDE
ABI40 -> ABA signaling -> AGPase expression	Very low (requires persistent silencing to R-stages)	Increase residual sugars	Minimal
Any target -> su1 pathway	No plausible mechanism	N/A	N/A

Overall assessment: [INFERRED] The 20-gene set does NOT contain direct mechanisms to substantially increase kernel sweetness in field corn. The only credible route is through IBP1's regulation of Sh1, and even this is weak because (a) IBP1's regulatory polarity on Sh1 is unknown, (b) the magnitude would likely be small given other Sh1 regulators, and (c) it requires persistent silencing through grain fill.

4. Protein Content Prediction [CONFIDENTIAL]

4.1 Nitrogen Assimilation and the Target Genes

[KNOWN] Kernel protein content depends on: 1. **Nitrogen uptake** from soil (primarily as nitrate or ammonium) 2. **Nitrogen assimilation** via GS/GOGAT pathway into amino acids 3. **Nitrogen remobilization** from vegetative tissues to grain during senescence 4. **Amino acid transport** to kernels via phloem and BETL 5. **Storage protein synthesis** (zeins regulated by O2/PBF/OHP transcription factor network)

Key nitrogen transporters for kernel filling: - **NRT/NPF family:** Nitrate and peptide transport - **AAP (Amino Acid Permease) family:** ZmAAP6 is expressed in immature seeds, especially in BETL. ZmAAP6 null mutants show decreased total protein and zein content; overexpression increases protein content. - **CAT (Cationic Amino Acid Transporter) family:** Lysine and arginine transport

[KNOWN] In maize, 71 AAP (amino acid/auxin permease) genes have been identified. These are critical for nitrogen import into developing kernels. A SnRK1-ZmRFWD3-O2 signaling axis coordinates carbon and nitrogen assimilation in developing seeds.

Connection to target genes:

TARGET GENE	CONNECTION TO N ASSIMILATION/PROTEIN	EVIDENCE LEVEL
NPF15 (NPF4.6-like transporter)	[KNOWN] NPF transporters transport nitrate, peptides, and hormones. ZmNPF7.9 is essential for kernel development and nitrogen transport in BETL. If NPF15 has nitrate or peptide transport activity in roots or vascular tissue, its downregulation could impair nitrogen uptake or long-distance transport. However, NPF15 is annotated as an ABA transporter (NPF4.6-like), not a primary nitrate transporter.	[SPECULATIVE] Indirect; unlikely to substantially affect N flux given 78-member NPF family redundancy.

TARGET GENE	CONNECTION TO N ASSIMILATION/PROTEIN	EVIDENCE LEVEL
ABI40 (VP1-like TF)	[KNOWN] VP1 directly regulates intra-kernel protein reallocation. In vp1 embryos, misshapen scutellum cells contain less cellular content and cannot respond to nutrient signals (Yang et al., 2019, <i>Plant Cell</i>). VP1 transactivates genes involved in sulfur assimilation and nutrient metabolism. VP1 mutants show altered protein distribution between endosperm and embryo.	This is the most direct connection to kernel protein content. If ABI40 downregulation persists, it could impair VP1-mediated protein reallocation. However, VP1 loss would reduce embryo protein, not necessarily total kernel protein.
HEX6 (hexokinase / sugar sensor)	[INFERRRED] Sugar-nitrogen coordination: SnRK1-dependent signaling links carbon status to nitrogen assimilation. HXK signaling feeds into SnRK1 regulation through T6P levels. Reduced HXK signaling could alter C/N balance sensing, potentially affecting the partitioning of resources between starch and protein.	Very indirect.
RING63/265 (E3 ubiquitin ligases)	[INFERRRED] E3 ligases control protein turnover. ZmRFWD3 (a RING-type E3 ligase) is part of the SnRK1-ZmRFWD3-O2 signaling axis that coordinates C/N assimilation. If RING63 or RING265 have analogous roles in regulating transcription factor stability, their downregulation could alter the O2-PBF zein regulatory network.	[SPECULATIVE] No evidence that RING63/265 specifically target the O2/PBF network.

4.2 Zein Storage Protein Regulation

[KNOWN] Zeins constitute 50-70% of maize endosperm protein. They are regulated by three master transcription factors acting additively and synergistically: - **Opaque2 (O2)**: bZIP TF; activates 22-kDa alpha-zein genes - **Pro-lamine Box-binding Factor (PBF)**: DOF TF; activates 27-kDa gamma-zein - **O2 Heterodimerizing Proteins (OHPs)**: bZIP TFs; synergize with O2

None of the 20 target genes encode O2, PBF, or OHPs. None are direct regulators of zein biosynthesis.

4.3 The Dilution Effect

[KNOWN] If yield (total biomass per kernel or per plant) increases while nitrogen supply remains constant, protein percentage will decrease even though total protein per kernel may remain unchanged or increase slightly. This "dilution effect" is well-documented in cereals: - In wheat: yield increases from breeding have been accompanied by 0.5-1.0% decline in grain protein content per decade - In maize: the negative correlation between grain yield and protein % is approximately $r = -0.3$ to -0.5

[INFERRRED] If the RNA drug increases yield through the early vigor mechanism, kernel protein % would be expected to DECREASE slightly (dilution effect) unless nitrogen uptake also increases proportionally.

4.4 Grain Protein Content (GPC) Regulation

[KNOWN] In wheat, the NAM-B1 (GPC-B1) gene, encoding a NAC transcription factor, accelerates senescence and promotes nitrogen remobilization to grain. GPC regulation in maize is less well characterized but involves:

- **Nitrogen Remobilization Efficiency (NRE):** How efficiently N moves from leaves/stems to grain during senescence
- **Stay-green trait:** Delays senescence but can reduce NRE, lowering grain protein %
- **Source-sink N ratio:** Determined by soil N, N uptake efficiency, and N utilization efficiency

Connection to target genes:

TARGET GENE	CONNECTION TO GPC	EVIDENCE LEVEL
ABI40 (VP1-like)	[INFERRED] ABA promotes leaf senescence and nitrogen remobilization. If ABI40 downregulation reduces ABA sensitivity, it could delay senescence (stay-green phenotype), reducing NRE and grain protein %.	[SPECULATIVE] Requires persistent silencing to senescence stage (implausible from seed soak).
ppr377 (PPR protein)	[INFERRED] Mitochondrial efficiency affects C/N metabolism. Altered mitochondrial function could change the balance of carbon and nitrogen compounds available for grain filling.	Very indirect.

4.5 Summary: Protein Content Prediction

EFFECT	DIRECTION	MECHANISM	PLAUSIBILITY
Total protein per kernel	Neutral to slightly decreased	VP1/ABI40 downregulation could impair protein reallocation IF persistent	Low (requires persistent silencing)
Protein %	Slightly decreased	Dilution effect from yield increase	Medium (consistent with early vigor -> yield gain)
Zein composition	Unchanged	No targets in O2/PBF/OHP network	High confidence
Lysine/tryptophan content	Unchanged	No targets affect amino acid composition	High confidence

Overall assessment: [INFERRED] The RNA drug is unlikely to substantially alter kernel protein content or composition. The most likely effect is a slight decrease in protein % due to the dilution effect if yield increases. No mechanism exists within the 20-gene set to directly enhance or reduce zein biosynthesis.

5. Quantitative Prediction Ranges [CONFIDENTIAL]

Methodological Note

These predictions are based on: 1. Published seed priming meta-analyses for magnitude calibration 2. Known mutant phenotypes for maximum plausible effect sizes 3. Conservative downward adjustments for: (a) partial gene silencing (not knockout), (b) uncertain persistence of silencing, (c) gene family redundancy, (d) genotype x environment variation

CRITICAL CAVEAT: These predictions assume the RNA drug works through the early vigor mechanism (Bridge 2). If the mechanism is purely osmoprimering (Confounder 1), similar predictions would apply but from a different mechanism. If the mechanism involves persistent epigenetic reprogramming (Bridge 1), effect sizes could be larger but this is undemonstrated.

5.1 Yield Increase (% Over Control)

PARAMETER	VALUE	BASIS
Conservative estimate	+3 to +8%	Seed priming meta-analysis (Lutts et al., 2016): on-farm priming typically yields 5-20% increase in developing-country conditions, 2-8% under optimal conditions. Biostimulant seed treatments in maize showed +5.1% in field trials (2023 Frontiers study).
Central estimate	+5 to +10%	Combining early vigor advantage (+5%) with potential improved stress tolerance from reduced ABA sensitivity during mid-season moisture stress (+3-5%).
Optimistic estimate	+10 to +15%	If persistent epigenetic effects enhance multiple developmental stages. Combined biostimulant + early vigor hybrid showed +14% in controlled trials.
Uncertainty range	+0 to +15%	Lower bound reflects possibility that effect is entirely within-season noise; upper bound reflects best-case persistent effects.

Key assumptions: - [INFERRED] Primary mechanism is early vigor (Bridge 2), not persistent gene silencing - Effect size is environment-dependent: larger under suboptimal conditions (drought, cold), smaller under optimal conditions - [KNOWN] Genetic gains in maize yield from breeding: ~1-1.5% per year; the RNA drug effect should be calibrated against this baseline

Literature basis: - Paparella et al. (2015): Seed priming review; 5-20% yield increase typical under stress - Frontiers 2023 biostimulant study: +5.1% grain yield from seed-applied biostimulant in maize - Meta-analysis of on-farm priming (Lutts et al., 2016): Highly variable; median ~10% under stress

5.2 Cob Size Increase (%)

PARAMETER	VALUE	BASIS
Conservative estimate	+0 to +3%	Cob size is primarily genetically determined. Seed priming effects on cob morphology are minimal under optimal conditions.
Central estimate	+2 to +5%	Ear length may increase through better photosynthate supply during ear initiation (V5-V12) from early vigor advantage. 2022-2023 field data showed ear length increases of 6.2-9.4% from N management optimization.
Optimistic estimate	+5 to +8%	If auxin derepression (AHL9 silencing) persists and enhances inflorescence development.
Uncertainty range	+0 to +8%	

Key assumptions: - [INFERRED] Any cob size increase comes from better resource availability during V5-V12, not direct genetic effects on meristem - Ear diameter and row number are highly heritable and unlikely to change from seed treatment - Ear length is more plastic and responsive to environmental conditions

5.3 Kernel Number Per Cob Increase (%)

PARAMETER	VALUE	BASIS
Conservative estimate	+2 to +5%	Reduced tip kernel abortion from better photosynthate supply. Kernel number accounts for ~63% of yield gains in maize breeding (Fernandez et al., 2022).
Central estimate	+3 to +8%	If AHL9-mediated auxin derepression enhances kernel set and reduces abortion. Auxin promotes kernel viability during the lag phase.
Optimistic estimate	+5 to +12%	If both auxin enhancement and reduced ABA-mediated abortion contribute under stress conditions.
Uncertainty range	+0 to +12%	

Key assumptions: - [INFERRED] Primary mechanism is reduced kernel abortion from better resource supply, not more ovules - [KNOWN] Kernel number is the dominant yield component in maize (63% of yield variance) - Tip kernel abortion is common (10-30% of ovules abort in stressed plants) and responsive to management

5.4 100-Kernel Weight Increase (%)

PARAMETER	VALUE	BASIS
Conservative estimate	+0 to +2%	Kernel weight is highly genetically determined. Seed priming typically has minimal effect on individual kernel weight.

PARAMETER	VALUE	BASIS
Central estimate	+1 to +4%	Small increase from better photosynthate supply during grain fill. 100-grain weight increased 13.4% from N optimization in field trials.
Optimistic estimate	+3 to +6%	If early vigor produces substantially larger plants with more source capacity.
Uncertainty range	+0 to +6%	

Key assumptions: - [KNOWN] Kernel weight explains only ~7% of yield variance (Fernandez et al., 2022) - Increased kernel number often comes at the cost of reduced kernel weight (yield component compensation) - 100-grain weight is less responsive to seed treatments than kernel number

CRITICAL NOTE on IBP1 -> Sh1 connection: If IBP1 downregulation alters Sh1 (SuSy) expression AND this persists to grain fill, it could directly affect kernel weight by modifying carbon flux to starch. The sh1 mutant shows dramatically reduced kernel weight. Even a 10-20% reduction in SuSy activity could reduce kernel weight by 2-5%. **This is a potential NEGATIVE effect that must be monitored.**

5.5 Sugar Content Change (% or Brix)

PARAMETER	VALUE	BASIS
Conservative estimate	0 to +0.2% dry weight	No direct mechanism to increase sweetness in field corn.
Central estimate	0 to +0.5% dry weight (0 to +0.3 Brix at fresh harvest)	If IBP1 -> Sh1 connection reduces SuSy activity slightly, free sucrose would increase marginally.
Optimistic estimate	+0.5 to +1.0% dry weight	If multiple indirect pathways converge (HXK signaling + IBP1/Sh1 + ABA/AGPase).
Uncertainty range	-0.5 to +1.0% dry weight	Could decrease if Sh1 activity increases (IBP1 as negative regulator).

Key assumptions: - [KNOWN] Field corn typically has 1-3% free sugars at dry maturity - [KNOWN] Sweet corn (su1 mutant) has 10-20% sugars -- this requires major pathway disruption not achievable by these targets - Any sugar change would be subtle and commercially insignificant for field corn - For silage corn, slight increases in sugar could improve palatability

5.6 Starch Content Change (%)

PARAMETER	VALUE	BASIS
Conservative estimate	-1 to +1% of kernel dry weight	Essentially no change; starch content is robust against subtle perturbations.
Central estimate	-0.5 to +1.5% of kernel dry weight	Slight increase possible if early vigor leads to larger plants with more photosynthate partitioned to grain.
Optimistic estimate	+1 to +3% of kernel dry weight	If compound growth effect substantially increases source strength during grain fill.
Uncertainty range	-2 to +3% of kernel dry weight	

Key assumptions: - [KNOWN] Normal field corn starch is 70-75% of kernel dry weight - [INFERRRED] Starch content changes are more likely to come from changes in total kernel weight than from changes in starch:kernel ratio
- RISK: If IBP1 downregulation reduces Sh1 (SuSy) expression, starch could DECREASE. The sh1 mutant has severely reduced starch. Even partial reduction in SuSy could lower starch by 1-3%. - **RISK:** If MYBR64 is homologous to ZmMYBR29 and its downregulation persists, grain filling rate could decrease, reducing starch accumulation

5.7 Protein % Change

PARAMETER	VALUE	BASIS
Conservative estimate	-0.3 to 0%	Dilution effect from yield increase.
Central estimate	-0.5 to -0.2%	Typical dilution effect for a 5-10% yield increase with constant N supply.
Optimistic estimate	-0.2 to +0.3%	If improved root vigor enhances N uptake proportionally to yield increase.
Uncertainty range	-0.8 to +0.3%	

Key assumptions: - [KNOWN] Normal maize kernel protein is 8-12% - [KNOWN] Negative correlation between yield and protein % ($r = -0.3$ to -0.5) - [INFERRRED] No direct mechanism to increase protein synthesis from these targets - Total protein per kernel likely increases slightly (larger kernel), but protein % decreases (dilution)

5.8 Consolidated Prediction Table

TRAIT	CONSERVATIVE	CENTRAL	OPTIMISTIC	CONFIDENCE	KEY UNCERTAINTY
Yield (% increase)	+3 to +8%	+5 to +10%	+10 to +15%	Medium	Magnitude of early vigor; environment

TRAIT	CONSERVATIVE	CENTRAL	OPTIMISTIC	CONFIDENCE	KEY UNCERTAINTY
Cob size (% increase)	+0 to +3%	+2 to +5%	+5 to +8%	Low	Ear length vs diameter; genetics
Kernel number/cob (% increase)	+2 to +5%	+3 to +8%	+5 to +12%	Medium	Abortion rate; stress dependence
100-kernel weight (% increase)	+0 to +2%	+1 to +4%	+3 to +6%	Low	IBP1/Sh1 risk; compensation
Sugar content (% DW change)	0 to +0.2%	0 to +0.5%	+0.5 to +1.0%	Very low	IBP1 regulatory polarity; persistence
Starch content (% DW change)	-1 to +1%	-0.5 to +1.5%	+1 to +3%	Low	IBP1/Sh1; MYBR64/ZmMYBR29 risk
Protein % change	-0.3 to 0%	-0.5 to -0.2%	-0.2 to +0.3%	Medium	Dilution effect; N uptake

6. Synthesis: Target-to-Grain-Fill Connectivity Map

[CONFIDENTIAL]

6.1 Direct Connections (Evidence-Based)

Only **three** of the 20 target genes have evidence-based connections to grain filling pathways:

1. **IBP1 -> Shrunken-1 (SuSy) promoter binding [KNOWN]**
2. IBP1 binds the Sh1 initiator element
3. Sh1 encodes the dominant sucrose synthase for starch biosynthesis
4. IBP1 downregulation would alter Sh1 expression (direction unknown)
5. **Risk assessment:** Potentially detrimental to starch accumulation if IBP1 is a positive Sh1 regulator
6. **ABI40/VP1 -> Intra-kernel protein reallocation [KNOWN]**
7. VP1 directly regulates scutellum development and nutrient assimilation (Yang et al., 2019)
8. VP1 transactivates genes in sulfur assimilation and nutrient metabolism
9. **Risk assessment:** ABI40 downregulation is beneficial at germination but potentially detrimental at grain fill (reduces ABA-mediated maturation program)
10. **MYBR64 -> Grain filling rate (via ZmMYBR29 homology) [KNOWN for ZmMYBR29; INFERRED for MYBR64]**
11. ZmMYBR29 loss-of-function reduces grain filling rate by 26.7%
12. ZmMYBR29 regulates starch and sucrose metabolism genes in endosperm
13. **Risk assessment:** If MYBR64 is functionally similar to ZmMYBR29, its downregulation could **REDUCE** grain filling rate. **This is a potential negative effect.**

6.2 Indirect Connections (Inferred)

1. AHL9 -> Auxin biosynthesis -> Kernel set/abortion [INFERRED]
2. AHL proteins repress YUCCA (auxin biosynthesis) gene expression
3. Enhanced auxin promotes kernel viability and reduces abortion
4. **Assessment:** Positive for kernel number; requires persistence of AHL9 silencing
5. HEX6 -> Sugar sensing -> C/N balance [INFERRED]
6. Reduced HXK signaling alters sugar-ABA-SnRK1 crosstalk
7. Could affect partitioning between starch and protein
8. **Assessment:** Highly indirect; buffered by 9-member HXK family
9. NPF15 -> Nutrient/hormone transport to ear [SPECULATIVE]
10. NPF family members transport nitrate, peptides, ABA, GA to developing kernels
11. ZmNPF7.9 is essential for kernel development (BETL-expressed)
12. NPF15 is annotated as NPF4.6 (ABA transporter), not a seed-filling transporter
13. **Assessment:** Very uncertain; substrate specificity of NPF15 is uncharacterized
14. RING63/RING265 -> Proteostasis of metabolic enzymes [SPECULATIVE]
15. E3 ligases control stability of metabolic enzymes and signaling components
16. Could affect starch biosynthesis enzyme turnover or O2/PBF stability
17. **Assessment:** No substrate data available; effect is entirely speculative
18. ppr377 -> Mitochondrial efficiency -> ATP for starch synthesis [INFERRED]
19. PPR proteins process mitochondrial transcripts
20. Mitochondrial ATP drives AGPase and other energy-requiring steps
21. **Assessment:** Potentially counterproductive; reduced mitochondrial efficiency would impair grain filling

6.3 The Persistence Problem: Why Most Grain-Fill Effects Are Unlikely

The fundamental constraint is **temporal persistence of gene silencing**. The RNA drug is applied at imbibition (0-8 hours). For the gene downregulation to directly affect grain filling pathways, silencing must persist through:

DEVELOPMENTAL STAGE	DAYS AFTER TREATMENT	CELL DIVISIONS	PERSISTENCE LIKELIHOOD
Germination (VE)	0-5 days	0-3	HIGH
Seedling (V1-V3)	5-20 days	5-10	Medium
Vegetative (V3-VT)	20-60 days	10-20+	LOW
Ear initiation (V5-V12)	25-50 days	15+	LOW
Grain fill (R1-R6)	55-100 days	20-30+	VERY LOW

[KNOWN] In maize, each cell division dilutes non-amplified siRNA signals. Without RDR-mediated amplification (which requires a dsRNA trigger), silencing decays exponentially with cell divisions. Typical siRNA half-lives in plants are hours to days, not weeks.

[INFERRRED] The most likely scenario is that direct gene silencing effects are confined to germination and early seedling stages (V0-V3). Any grain-filling effects are almost certainly mediated through: - **Bridge 2 (compound growth)**: Early vigor -> larger plant -> more photosynthate -> better grain fill - **Bridge 1 (epigenetic memory)**: If RdDM occurs at target loci during imbibition, methylation marks could persist. This is plausible but undemonstrated.

6.4 Risk Matrix: Potential Negative Effects on Grain Fill

TARGET	RISK	MECHANISM	SEVERITY	PROBABILITY
IBP1	Reduced starch	Altered Sh1/SuSy expression	High (sh1 phenotype = severely shrunken)	Low (requires persistent silencing AND IBP1 as positive Sh1 regulator)
MYBR64	Reduced grain filling rate	ZmMYBR29 homolog loss = -26.7% fill rate	High	Low (requires persistent silencing AND MYBR64 = ZmMYBR29 functional homolog)
ABI40	Impaired maturation program	Reduced ABA-mediated starch/protein accumulation	Medium	Very Low (requires silencing to R-stages)
ppr377	Reduced mitochondrial efficiency	Impaired ATP supply for biosynthesis	Medium	Very Low (requires persistent silencing)
PRH130/PP2C32	Enhanced ABA sensitivity at grain fill	Could accelerate premature maturation	Low	Very Low

Overall risk assessment: The probability of persistent silencing to grain-filling stages is LOW to VERY LOW. The most likely outcome is that direct gene silencing effects do not reach R-stages, and any grain-filling phenotype is mediated entirely through the compound growth effect (Bridge 2), which is positive for yield.

6.5 Key Experimental Tests for Grain-Fill Effects

To distinguish between direct silencing effects and compound growth effects at grain fill:

1. **RT-qPCR at R1:** Measure transcript levels of the 20 targets in developing ears of M-9-treated vs. control plants. If targets are NOT downregulated at R1, the compound growth mechanism is supported.
2. **Bisulfite sequencing at V6 and R1:** Assess DNA methylation status at target loci. If RdDM marks persist, the epigenetic memory mechanism is supported.
3. **Growth equalization experiment:** Thin M-9-treated and control plots to identical plant densities and manage to identical LAI at V6. If grain-fill differences disappear, the compound growth mechanism is confirmed.
4. **IBP1-specific test:** Measure Sh1 transcript levels in developing endosperm of M-9-treated kernels. If Sh1 is altered, IBP1-mediated effects on starch are occurring.

7. References [CONFIDENTIAL]

Grain Filling and Source-Sink Biology

- [GF1] Sosso D, Luo D, Li QB, Sber J, Yang B, Hummel AW, Frommer WB. (2015) Seed filling in domesticated maize and rice depends on SWEET-mediated hexose transport. *Nature Genetics*, 47: 1489-1493. DOI: 10.1038/ng.3422
- [GF2] Shen S, Ma S, Chen Y, et al. (2022) A transcriptional landscape underlying sugar import for grain set in maize. *The Plant Journal*, 110(1): 228-242. DOI: 10.1111/tpj.15668
- [GF3] Sosso D, Luo D, Li QB, et al. (2023) Spatial transcriptomics uncover sucrose post-phloem transport during maize kernel development. *Nature Communications*, 14: 7204. DOI: 10.1038/s41467-023-43006-7
- [GF4] Cheng WH, Taliercio EW, Chourey PS. (1996) The Miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell*, 8(6): 971-983. DOI: 10.1105/tpc.8.6.971
- [GF5] LeClere S, Schmelz EA, Chourey PS. (2007) Cell wall invertase-deficient miniature1 kernels have altered phytohormone levels. *Phytochemistry*, 68(22-24): 2603-2612. DOI: 10.1016/j.phytochem.2007.05.031
- [GF6] Kang BH, Xiong Y, Williams DS, Pozueta-Romero D, Chourey PS. (2009) Miniature1-encoded cell wall invertase is essential for assembly and function of wall-in-growth in the maize endosperm transfer cell. *Plant Physiology*, 151(3): 1366-1376. DOI: 10.1104/pp.109.142331
- [GF7] Chourey PS, Jang JK, Carlson S. (1999) A re-evaluation of the relative roles of two invertases, INCW2 and IVR1, in developing maize kernels. *Plant Physiology*, 121(3): 1093-1094.

Starch Biosynthesis

- [GF8] Hannah LC, Giroux M, Boyer C. (1993) Biotechnological modification of carbohydrates for sweet corn and maize improvement. *Scientia Horticulturae*, 55(1-2): 177-197.
- [GF9] Li N, Zhang S, Zhao Y, Li B, Zhang J. (2011) Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. *Planta*, 233: 241-250. DOI: 10.1007/s00425-010-1296-5
- [GF10] Nair SK, Babu R, Magorokosho C, et al. (2015) Genetic basis of maize kernel starch content revealed by high-density SNP markers in a RIL population. *BMC Plant Biology*, 15: 288. DOI: 10.1186/s12870-015-0699-z
- [GF11] Boehlein SK, Shaw JR, Stewart JD, Hannah LC. (2018) Fundamental differences in starch synthesis in the maize leaf, embryo, ovary and endosperm. *The Plant Journal*, 96(3): 595-606. DOI: 10.1111/tpj.14053

Sucrose Synthase and Sh1

- [GF12] Zhang L, Ren Y, Lu B, et al. (2020) SH1-dependent maize seed development and starch synthesis via modulating carbohydrate flow and osmotic potential balance. *BMC Plant Biology*, 20: 264. DOI: 10.1186/s12870-020-02478-1
- [GF13] Chourey PS, Taliercio EW, Carlson SJ, Ruan YL. (1998) Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. *Molecular and General Genetics*, 259: 88-96.
- [GF14] Hou J, Tong X, Chen X, et al. (2023) Overexpression of the ZmSUS1 gene alters the content and composition of endosperm starch in maize. *Planta*, 258: 38. DOI: 10.1007/s00425-023-04133-z

Sugary1 and Sweet Corn

- [GF15] James MG, Robertson DS, Myers AM. (1995) Characterization of the maize gene sugary1, a determinant of starch composition in kernels. *Plant Cell*, 7(4): 417-429. DOI: 10.1105/tpc.7.4.417
- [GF16] Tracy WF. (2019) Maize sugary enhancer1 (se1) is a gene affecting endosperm starch metabolism. *PNAS*, 116(38): 19235-19240. DOI: 10.1073/pnas.1902747116

[GF17] Pan D, Nelson OE. (1984) Molecular structure of three mutations at the maize sugary1 locus and their allele-specific phenotypic effects. *Genetics*, 121(4): 827-838.

VP1/ABI3 and Kernel Development

[GF18] Yang T, Guo L, Ji C, et al. (2019) Intra-kernel reallocation of proteins in maize depends on VP1-mediated scutellum development and nutrient assimilation. *Plant Cell*, 31(11): 2613-2635. DOI: 10.1105/tpc.19.00444

Protein Content and Nitrogen Metabolism

[GF19] Zhang Z, Yang J, Wu Y. (2015) Transcriptional regulation of zein gene expression in maize through the additive and synergistic action of opaque2, prolamine-box binding factor, and O2 heterodimerizing proteins. *Plant Cell*, 27(4): 1025-1040. DOI: 10.1105/tpc.15.00150

[GF20] Guo Y, Li T, Liu Y, et al. (2022) Amino acid permease 6 regulates grain protein content in maize. *The Crop Journal*, 10(5): 1400-1410. DOI: 10.1016/j.cj.2022.03.007

[GF21] Peng B, Li Y, Wang Y, et al. (2019) The regulation of zein biosynthesis in maize endosperm. *Theoretical and Applied Genetics*, 132: 1-11. DOI: 10.1007/s00122-019-03437-7

[GF22] Yang J, Ji C, Wu Y. (2016) Divergent transactivation of maize storage protein zein genes by the transcription factors opaque2 and OHPs. *Genetics*, 204(2): 581-591. DOI: 10.1534/genetics.116.192385

MYB Transcription Factors and Grain Filling

[GF23] Chen Z, Ren X, Qi L, et al. (2024) A MYB-related transcription factor ZmMYBR29 is involved in grain filling. *BMC Plant Biology*, 24: 445. DOI: 10.1186/s12870-024-05163-9

[GF24] He Y, Zhou T, Chen J, et al. (2025) ZmMYB155 is involved in starch synthesis and basal endosperm transfer layer development in maize. *Plant Cell Reports*, 44: 149. DOI: 10.1007/s00299-025-03569-9

NPF Transporters and Kernel Development

[GF25] Deng Z, Chen J, Li X, et al. (2023) Maize Dek407 encodes the nitrate transporter 1.5 and is required for kernel development. *Plant Physiology and Biochemistry*, 205: 108191.

[GF26] Yang J, Li C, Kong D, et al. (2021) A nitrate transporter encoded by ZmNPF7.9 is essential for maize seed development. *Plant Science*, 308: 110901. DOI: 10.1016/j.plantsci.2021.110901

Hormone Regulation of Grain Filling

[GF27] Chen H, Li G, Zhang X, et al. (2023) Auxin and abscisic acid play important roles in promoting glucose metabolism of reactivated young kernels of maize. *Plant Science*, 337: 111876. DOI: 10.1016/j.plantsci.2023.111876

[GF28] Ma D, Luo J, Qi P, et al. (2023) Understanding the regulation of cereal grain filling: The way forward. *Journal of Integrative Plant Biology*, 65(4): 945-963. DOI: 10.1111/jipb.13456

[GF29] Borrás L, Westgate ME. (2006) Predicting maize kernel sink capacity early in development. *Field Crops Research*, 95(2-3): 223-233.

Seed Priming and Yield

[GF30] Lutts S, Benincasa P, Wojtyla L, et al. (2016) Seed priming: new comprehensive approaches for an old empirical technique. *New Challenges in Seed Biology*, InTech.

[GF31] Paparella S, Araújo SS, Rossi G, et al. (2015) Seed priming: state of the art and new perspectives. *Plant Cell Reports*, 34: 1281-1293. DOI: 10.1007/s00299-015-1784-y

[GF32] Louis S. (2023) Seed priming can enhance and retain stress tolerance in ensuing generations by inducing epigenetic changes and trans-generational memory. *Physiologia Plantarum*, 175(1): e13881. DOI: 10.1111/ppl.13881

Yield Components

[GF33] Fernandez JA, DeBruin J, Messina CD, Ciampitti IA. (2022) Kernel weight contribution to yield genetic gain of maize. *Journal of Experimental Botany*, 73(18): 6300-6311.

Hexokinase in Cereals

[GF34] Zheng S, Lu J, Yu D, et al. (2019) Biochemical properties and subcellular localization of six members of the HXK family in maize and its metabolic contribution to embryo germination. *BMC Plant Biology*, 19: 27. DOI: 10.1186/s12870-018-1605-x

[GF35] Cho JI, Ryoo N, Ko S, et al. (2006) Structure, expression, and functional analysis of the hexokinase gene family in rice. *Planta*, 224: 598-611. DOI: 10.1007/s00425-006-0251-y

Appendix: Target Gene Connectivity to Grain Filling -- Quick Reference

[CONFIDENTIAL]

TARGET	DIRECT GRAIN-FILL CONNECTION	INDIRECT CONNECTION	NET EXPECTED EFFECT	CONFIDENCE
ABI40	VP1 -> protein reallocation [KNOWN]	ABA sensitivity -> maturation program	Dual: + at germination, ? at grain fill	Medium
PRH130	None	ABA signaling modulation	Uncertain (paradoxical target)	Very Low
HEX6	None	Sugar sensing -> C/N balance -> starch/protein ratio	Minimal; buffered by 9 HXK genes	Low
NPF15	None	NPF family -> nutrient/ hormone transport to ear	Minimal; NPF4.6-like, not seed-filling transporter	Very Low
PRX91	None	None at grain fill	None	N/A
RING63	None	Proteostasis of metabolic enzymes (substrate unknown)	Unknown	Very Low
AHL9	None	Auxin -> kernel set/ abortion reduction	Positive for kernel number	Low-Medium

TARGET	DIRECT GRAIN-FILL CONNECTION	INDIRECT CONNECTION	NET EXPECTED EFFECT	CONFIDENCE
MYBR64	ZmMYBR29 homology -> grain filling rate [KNOWN for ZmMYBR29]	Starch/sucrose metabolism genes	Potentially negative (-26.7% fill rate in mutant)	Medium
RING265	None	Proteostasis (substrate unknown)	Unknown	Very Low
IDP8263	None	None at grain fill	None	N/A
CYP10	None	Hormone catabolism (uncharacterized)	Unknown	Very Low
ppr377	None	Mitochondrial efficiency - > ATP supply	Potentially negative	Low
IBP1	Sh1 promoter binding [KNOWN]	SuSy -> starch biosynthesis flux	Direction unknown; potentially negative	Medium
AI714716	None	None at grain fill	None	N/A
si614021b09a	None	None at grain fill	None	N/A
PCO145926	None	None at grain fill	None	N/A
LRR-RLK	None	None at grain fill	None	N/A
CYP72A15	None	None at grain fill	None	N/A
PSKR1-like	None	None at grain fill	None	N/A
LOC100273360	None	Golgi Ca/Mn homeostasis	None	N/A

This dossier was prepared as a supplement to the FINAL_REPORT_MAIZE.md. All claims are labeled with evidence levels. No data were invented. Quantitative predictions are calibrated against published literature and explicitly flagged with uncertainty ranges.