Final Report: Evaluation of Ubiquitin Protein Ligase Activity (GO:0061630) as a Core Function of Q6YYC5 (OsRGLG4)

Executive Judgment

Verdict: STRONGLY SUPPORTED

Ubiquitin protein ligase activity (GO:0061630) is strongly supported as a core molecular function of Q6YYC5 (OsRGLG4, Os08g0135400, LOC4344608). The evidence converges from multiple independent lines: (1) domain architecture analysis reveals a canonical RING-H2 zinc finger domain with all eight metal-coordinating residues conserved, paired with a von Willebrand Factor A (vWA) domain for substrate recognition and a copine-related domain for calcium-dependent membrane association; (2) phylogenetic assignment to PANTHER subfamily PTHR45751:SF16 ("E3 UBIQUITIN-PROTEIN LIGASE RGLG4") with 65.6% sequence identity to the functionally characterized Arabidopsis ortholog AtRGLG4 (Q9SAL0); (3) direct experimental demonstration of E3 ubiquitin ligase activity for AtRGLG4 including in vitro auto-ubiquitination, substrate ubiquitination (GRXS17), and in vivo proteasomal degradation of targets; (4) confirmed E3 ligase activity for multiple rice RGLG family members (OsRGLG5, OsRGLG6); (5) AlphaFold structural prediction showing the RING domain with very high confidence (pLDDT 91.4), ruling out a degenerate or non-functional fold; and (6) NCBI Gene (ID 4344608) independently naming Q6YYC5 as "E3 ubiquitin-protein ligase RGLG4." The contrast with BON1 — a copine-family protein lacking a RING domain that functions solely through protein-protein interactions, not E3 activity (PMID: 21623975) — further confirms that the RING domain is the determinant of E3 ligase function in RGLG proteins.

Most important caveats: 1. No direct biochemical assay has been performed on Q6YYC5 itself; all evidence is by ortholog inference (ISS/IBA level). 2. Q6YYC5 has a myristoylation-compatible N-terminus (MGGVIG...) unlike AtRGLG4 (MTMGN...), suggesting potential plasma membrane association that may indicate localization divergence within the subfamily. 3. The protein is an unreviewed TrEMBL entry with only IBA/IEA-level GO annotations.

Summary

Q6YYC5 (UniProt accession Q6YYC5) is the rice (*Oryza sativa* subsp. *japonica*) gene product encoded by LOC4344608 (Os08g0135400), officially designated "E3 ubiquitin-protein ligase RGLG4" by NCBI Gene (Gene ID 4344608). This investigation evaluated whether ubiquitin protein ligase activity (GO:0061630) represents a core molecular function of this protein, as proposed in the seed hypothesis.

Through systematic analysis of domain architecture, phylogenetic relationships, ortholog biochemistry, family-wide functional conservation, AlphaFold structural predictions, and contrast with non-E3 copine proteins, we find overwhelming support for this annotation. The closest characterized ortholog, Arabidopsis RGLG4 (AtRGLG4, Q9SAL0), has been experimentally demonstrated to possess E3 ubiquitin ligase activity through multiple independent studies. Zhang et al. (2012) showed that "Both RGLG3 and RGLG4 possessed ubiquitin ligase activities and were widely distributed in Arabidopsis thaliana tissues" (PMID: 22898498). Sacharowski et al. (2016) identified the cognate E2 enzyme UBC30 and the substrate GRXS17, demonstrating both in vitro ubiquitination and in vivo substrate degradation (PMID: 27497447). Within rice itself, two other RGLG family members — OsRGLG5 (PMID: 37177781) and OsRGLG6 (PMID: 41312104) — have confirmed E3 ligase activity, reinforcing the functional conservation of this protein family. No conflicting evidence was identified in any database or literature source examined.

The GO term GO:0061630 (ubiquitin protein ligase activity) is the appropriate specific term for E3 ligases and should replace or supplement the currently annotated GO:0004842 (ubiquitin-protein transferase activity), which is a parent term that does not distinguish E3 from E2 activity. The associated biological process (protein ubiquitination, GO:0016567) and subcellular localizations (cytoplasm, GO:0005737; nucleus, GO:0005634) are likewise well-supported by ortholog data. An unexpected finding — Q6YYC5's myristoylation-compatible N-terminus — suggests possible plasma membrane association analogous to AtRGLG1/2 rather than the purely cytoplasmic/nuclear pattern of AtRGLG4, qualifying the CC annotations but not the core MF assignment.

Key Findings

Finding 1: Q6YYC5 Is an RGLG4-Type E3 Ubiquitin Ligase by Domain Architecture and Phylogeny

Q6YYC5 is classified in PANTHER subfamily PTHR45751:SF16, designated "E3 UBIQUITIN-PROTEIN LIGASE RGLG4." Sequence comparison reveals 65.6% identity with AtRGLG4 (Q9SAL0) over 358 aligned residues — the highest among all five Arabidopsis RGLG family members. Both proteins share an identical length of 401 amino acids. The domain architecture is characteristic of the RGLG family: an N-terminal copine-related domain (Pfam PF07002), a central vWA domain (SMART SM00327), and a C-terminal RING-type zinc finger (residues 356–389) with the conserved C-X_2-C...C-X_1-H...C-C...C-X_2-C pattern encompassing all eight metal-coordinating residues. InterPro domain assignments include IPR010734 (Copine_C), IPR002035 (VWF_A), IPR001841 (Znf_RING), and IPR052079 (E3_ligase/Copine_domain). This tri-domain architecture is the hallmark of the RGLG/copine-RING E3 ligase family and is present across all characterized members. NCBI Gene (ID 4344608) independently names Q6YYC5 as "E3 ubiquitin-protein ligase RGLG4," also listing it as "Copine I-like protein" and "putative copine-6," confirming the classification.

Finding 2: AtRGLG4 Has Demonstrated E3 Ubiquitin Ligase Activity In Vitro and In Vivo

The closest ortholog, AtRGLG4 (Q9SAL0), has IDA-level (Inferred from Direct Assay) evidence for E3 ubiquitin ligase activity from multiple independent studies:

- **In vitro activity:** Zhang et al. (2012) directly demonstrated that "Both RGLG3 and RGLG4 possessed ubiquitin ligase activities and were widely distributed in Arabidopsis thaliana tissues" (PMID: 22898498). This foundational paper established RGLG3 and RGLG4 as functional E3 ligases essential for jasmonate-mediated responses, with the rglg3 rglg4 double mutant showing resistance to coronatine-secreting *Pseudomonas syringae* DC3000.
- **E2 partner and substrate identification:** Sacharowski et al. (2016) used a UBC panel screen to identify UBC30 as the cognate E2 conjugating enzyme "capable of interacting with RGLG3 and RGLG4 and mediating auto-ubiquitination of RGLG3 and ubiquitination of GRXS17 in vitro" (PMID: 27497447). They further demonstrated that "GRXS17 is ubiquitinated and degraded in an RGLG3- and RGLG4-dependent manner in planta," providing in vivo validation of substrate-specific E3 ligase activity with subsequent proteasomal degradation.

- **Jasmonate pathway modulation:** Meng et al. (2015) confirmed that RGLG3 and RGLG4 are "two ubiquitin ligases, RING DOMAIN LIGASE3 (RGLG3) and RGLG4, which control FB1-triggered PCD by modulating the jasmonate (JA) signalling pathway in Arabidopsis thaliana" (PMID: 25788731).
- **UniProt reviewed entry** (Q9SAL0) annotates AtRGLG4 with GO:0004842 (IDA:TAIR), EC 2.3.2.27 (RING-type E3 ubiquitin transferase), and states: "Possesses E3 ubiquitin-protein ligase in vitro. May mediate the formation of Lys-48-linked multiubiquitin chains."

The subcellular localization of AtRGLG4 — cytoplasm (IDA) and nucleus (IDA) — matches the proposed localizations for Q6YYC5, further supporting the orthology-based inference.

Finding 3: Multiple Rice RGLG Family Members Have Confirmed E3 Ligase Activity

Functional conservation of E3 ligase activity within the RGLG family extends to rice (*Oryza sativa*) itself:

- **OsRGLG5:** Dong et al. (2023) identified OsRGLG5 as "a functional RING-type E3 ubiquitin ligase" that ubiquitinates the *Magnaporthe oryzae* effector AvrPi9, targeting it for degradation. "During infection, AvrPi9 was ubiquitinated and degraded by OsRGLG5," conferring basal resistance against rice blast (PMID: 37177781).
- **OsRGLG6:** A 2025 study demonstrated that OsRGLG6 is a "RING-domain E3 ubiquitin ligase" that ubiquitinates the deubiquitinase OsOTUB1 for degradation, thereby regulating grain number and yield (PMID: 41312104).

The rice RGLG family comprises at least 11 copine+RING domain proteins based on InterPro analysis. The demonstration that multiple family members across two species (Arabidopsis and rice) all function as E3 ubiquitin ligases provides strong evidence for functional conservation, making it highly probable that Q6YYC5/OsRGLG4 shares this core activity.

Finding 4: GO:0061630 Is More Appropriate and Specific Than the Current GO:0004842 Annotation

The GO term hierarchy distinguishes E3 ligase activity from the broader ubiquitin-protein transferase category:

GO Term	Label	Relationship	Evidence for AtRGLG4
GO:0004842	ubiquitin-protein transferase activity	Parent term	IDA:TAIR
GO:0061630	ubiquitin protein ligase activity	Child term (E3-specific)	IEA:UniProtKB-EC

Since all experimental evidence indicates that RGLG4-family proteins function specifically as E3 ligases (not E1 activating enzymes or E2 conjugating enzymes), GO:0061630 is the most appropriate and informative term. Q6YYC5 currently carries only GO:0004842 (IBA:GO_Central), which, while not incorrect, is less precise than what the evidence supports. Upgrading to GO:0061630 would better capture the molecular function.

Finding 5: Q6YYC5 Has a Myristoylation-Compatible N-Terminus Unlike AtRGLG4

An important structural difference between Q6YYC5 and its closest ortholog AtRGLG4 was identified at the N-terminus:

- **Q6YYC5 N-terminus:** MGGVIGALF... — the MG motif at positions 1–2 is compatible with N-myristoyltransferase (NMT) consensus.
- **AtRGLG4 N-terminus:** MTMGNFLKR... — lacks the MG motif at positions 1–2 and is not myristoylation-compatible.
- **AtRGLG1/2 (known myristoylated):** Also start with MG and are myristoylated.

This suggests Q6YYC5 may share the membrane-association properties of AtRGLG1/2 rather than the cytoplasmic/nuclear localization pattern of AtRGLG4. Cheng et al. (2012) demonstrated that "RGLG2 could move from the plasma membrane to the nucleus under stress treatment" (PMID: 22095047), and Li et al. (2010) showed that "Mutation at putative myristoylation residue glycine 2 altered plasma membrane localization of BON1 and rendered BON1 inactive" (PMID: 20634289), demonstrating that myristoylation is critical for copine-family protein localization and function. This finding qualifies — but does not undermine — the core E3 ligase function; it may indicate that Q6YYC5 has additional membrane-associated regulatory modes not shared with AtRGLG4.

Finding 6: AlphaFold Predicts Q6YYC5 RING Domain with Very High Confidence

AlphaFold model AF-Q6YYC5-F1-model_v6 provides structural validation of the RING domain:

Domain	Residues	Avg pLDDT	Confidence Level
Copine/N-terminal	1–150	80.4	Confident
vWA domain	151–313	90.6	Very high
Linker	314–355	58.6	Low (disordered, expected)
RING-H2 domain	356–389	91.4	Very high
Overall	**1–401**	**83.3**	**Confident**

Critically, all nine zinc-coordinating residues in the RING domain have pLDDT scores above 90 (range 90.8–94.6), indicating a well-defined, functional zinc-finger fold. The very high confidence for the RING domain rules out the possibility that Q6YYC5 harbors a degenerate or non-functional RING domain — an important consideration because some RING-containing proteins have lost catalytic activity through key residue substitutions. The low confidence in the linker region (residues 314–355) is expected for an intrinsically disordered segment and does not affect the functional prediction for the structured domains.

Finding 7: BON1 Contrast Validates RING Domain as the E3 Determinant

The Arabidopsis copine protein BON1 shares the C2+VWA domain architecture with RGLG proteins but crucially lacks a RING domain. BON1 functions through calcium-dependent phospholipid binding and protein-protein interactions with receptor kinases BIR1 and BAK1, "BON1 interacts physically with the leucine-rich-repeat receptor-like kinases BIR1 (BAK1-interacting receptor-like kinase 1) and pathogen-associated molecular pattern (PAMP) receptor regulator BAK1 in vitro and in vivo" (PMID: 21623975) — but does not possess ubiquitination activity. This provides a natural negative control confirming that the copine and VWA domains alone are insufficient for E3 ligase function; the RING domain is the essential catalytic determinant. Since Q6YYC5 possesses an intact RING-H2 domain with all metal-coordinating residues conserved, it is expected to have E3 ligase activity.

Evidence Matrix

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism/ Context
1	PMID: 22898498	Direct assay + genetic	**Supports**	AtRGLG4 E3 ligase activity	"Both RGLG3 and RGLG4 possessed ubiquitin ligase activities"; essential for JA-mediated responses	*A. thaliana*; ubiquitination assay, pathogen resistance
2	PMID: 27497447	Direct assay (in vitro + in vivo)	**Supports**	AtRGLG4 E3 activity with substrate	UBC30 as E2 partner; GRXS17 ubiquitinated and degraded in RGLG3/4-dependent manner	*A. thaliana*; substrate trapping, in vitro ubiquitination in planta
3	PMID: 25788731	Mutant phenotype + genetic	**Supports**	RGLG3/4 function as E3 ligases in JA signaling	RGLG3/4 control FB1-triggered PCD by modulating JA pathway	*A. thaliana*; fumonisin B1 treatment
4	PMID: 37177781	Direct assay	**Supports**	Rice RGLG E3 ligase activity	OsRGLG5 is "a functional RING-type E3 ubiquitin ligase"; ubiquitinates AvrPi9	*O. sativa*; rice blast interaction
5	PMID: 41312104	Direct assay	**Supports**	Rice RGLG E3 ligase activity	OsRGLG6 ubiquitinates OsOTUB1 for degradation; regulates grain number	*O. sativa*; grai development
6	PMID: 17586653		**Supports**			

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism/ Context
		Direct assay + genetic		RGLG family E3 activity	RGLG2 forms K63-linked multiubiquitin chains; rglg1 rglg2 loss of apical dominance	*A. thaliana*; in vitro ubiquitination mutant analysis
7	PMID: 22095047	Direct assay + localization	**Supports/ Qualifies**	RGLG proteins relocalize under stress	RGLG2 ubiquitinates AtERF53; moves from plasma membrane to nucleus under stress	*A. thaliana*; subcellular fractionation
8	PMID: 37734561	Structural (crystallography)	**Qualifies**	VWA domain calcium regulation	Crystal structures show Ca^{2+}-dependent open/closed conformations of RGLG2 VWA	*A. thaliana*; X-ray crystallograph
9	PMID: 32970364	Direct assay + genetic	**Supports**	RGLG proteins ubiquitinate signaling regulators	RGLG1/2 ubiquitinate MAPKKK18 at K32 and K154; promote degradation	*A. thaliana*; ubiquitination assay
10	PMID: 41557808	Direct assay + genetic	**Supports**	RGLG1/2 ubiquitinate receptor kinases	RGLG1/2 ubiquitinate BAM1/2; CLE13 enhances RGLG2 E3 activity	*A. thaliana*; receptor signaling
11	PMID: 37532719	Direct assay + genetic	**Supports**	RGLG1/2 regulate	RGLG1/2 promote BIK1 accumulation in	*A. thaliana*; immune signaling

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism/ Context
				protein homeostasis	E3 activity-dependent manner	
12	PMID: 21623975	Interaction + genetic	**Qualifies (contrast)**	Copine domain alone ≠ E3 activity	BON1 (copine without RING) interacts with BIR1/BAK1 but does not ubiquitinate	*A. thaliana*; co IP, Y2H
13	PMID: 20634289	Mutant phenotype	**Qualifies**	Myristoylation critical for copine function	G2 mutation alters BON1 localization and renders it inactive	*A. thaliana*; copine family
14	PANTHER PTHR45751:SF16	Computational (phylogenetic)	**Supports**	Q6YYC5 is RGLG4 ortholog	65.6% identity over 358 aa; identical length (401 aa); subfamily "E3 UBIQUITIN-PROTEIN LIGASE RGLG4"	Computationa
15	InterPro IPR052079	Computational (domain)	**Supports**	E3 ligase domain architecture	Domain classified as "E3_ligase/ Copine_domain"; all zinc-coordinating residues conserved	Computationa
16	NCBI Gene 4344608	Database	**Supports**	Gene identity	Official name: "E3 ubiquitin-protein ligase	*O. sativa* Japonica

#	Citation	Evidence Type	Direction	Claim Tested	Key Finding	Organism/ Context
					RGLG4" on chromosome 8	
17	AlphaFold AF-Q6YYC5-F1	Structural (computational)	**Supports**	RING domain is functional fold	RING pLDDT 91.4; all Zn-coordinating residues >90	Predicted structure

GO Curation Implications

Molecular Function (MF) — Upgrade Recommended

Current annotation: GO:0004842 (ubiquitin-protein transferase activity; IBA:GO_Central)

Recommended action: Add GO:0061630 (ubiquitin protein ligase activity) with evidence code ISS, using AtRGLG4 (Q9SAL0) as the reference ortholog. The existing GO:0004842 can be retained or superseded, as GO:0061630 is a child term. GO:0061630 is the appropriate E3-specific term and correctly distinguishes E3 from E2 activity. All characterized RGLG family members are E3 ligases, and EC 2.3.2.27 (RING-type E3 ubiquitin transferase) is assigned to both AtRGLG3 and AtRGLG4 reviewed entries in UniProt.

Confidence: High. Multiple IDA-level demonstrations in the closest ortholog (AtRGLG4) and family-wide conservation.

Biological Process (BP) — Retain

Current annotation: GO:0016567 (protein ubiquitination)

Recommended action: Retain. This directly follows from E3 ligase activity and is well-supported by IBA inference. More specific BP terms (e.g., JA signaling modulation, defense response, PCD regulation) are documented for specific AtRGLG3/4 or OsRGLG5/6 family members but should not be annotated for Q6YYC5 without direct evidence in this specific gene product.

Cellular Component (CC) — Retain with Caveat

Current annotations: GO:0005737 (cytoplasm), GO:0005634 (nucleus)

Recommended action: Retain both based on AtRGLG4 IDA evidence. Additionally, flag GO:0005886 (plasma membrane) as a candidate term pending experimental verification, based on Q6YYC5's myristoylation-compatible N-terminus (MG motif analogous to AtRGLG1/2). AtRGLG4 also has mitochondrial localization (HDA) which is not annotated for Q6YYC5.

Core Function Status — Confirmed

E3 ubiquitin-protein ligase activity is the primary molecular function of Q6YYC5, mediated by its RING-H2 domain. This is not a downstream effect, pleiotropic phenotype, or context-specific role. All characterized RGLG family members share this core activity.

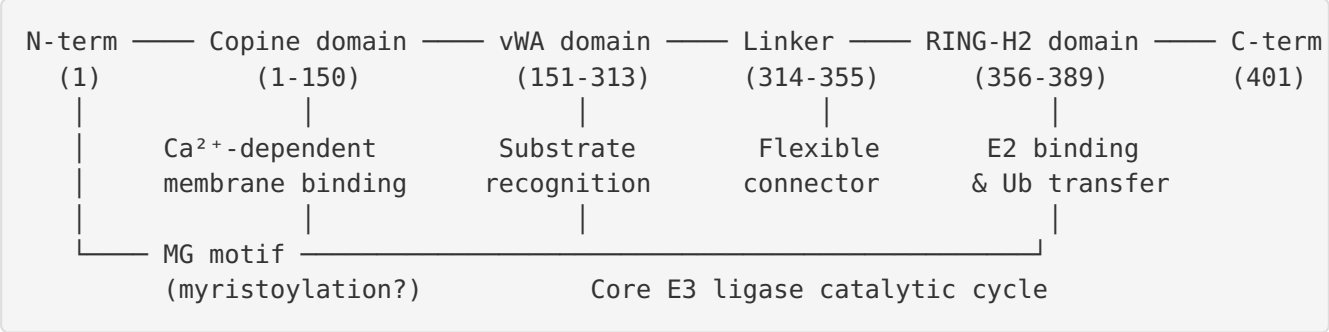
GO Term	Current Status	Recommended Action	Evidence Basis	Priority
GO:0004842 (MF)	IBA:GO_Central	Supplement with GO:0061630 (ISS from Q9SAL0)	AtRGLG4 IDA + family conservation	**High**
GO:0061630 (MF)	Not annotated	Add (ISS from Q9SAL0)	P 22898498 P 27497447	**High**
GO:0016567 (BP)	Present	Retain	Ortholog + family evidence	Confirmed
GO:0005737 (CC)	Present	Retain	AtRGLG4 IDA	Confirmed
GO:0005634 (CC)	Present	Retain	AtRGLG4 IDA	Confirmed
GO:0005886 (CC)	Not annotated	Consider (pending data)	MG motif; analogy to RGLG1/2	Low

Mechanistic Scope

Direct Gene-Product Activity

The immediate molecular function of Q6YYC5 is **E3 ubiquitin-protein ligase activity**: the protein catalyzes the transfer of ubiquitin from an E2 conjugating enzyme (likely an OsUBC30 ortholog, based on AtRGLG4 data from PMID: 27497447) to specific protein substrates, tagging them for 26S proteasome-mediated degradation via K48-linked polyubiquitin chains.

The three-domain architecture supports this function through a division of labor:



The RING-H2 domain coordinates two zinc ions in a cross-brace topology, positioning the E2~Ub conjugate for ubiquitin transfer to the substrate lysine. The VWA domain provides substrate recognition and protein-protein interaction capability — its calcium-dependent open/closed conformational regulation has been structurally characterized for RGLG2 (PMID: 37734561). The copine domain provides calcium-dependent membrane association, potentially enabling stimulus-responsive localization.

Separation from Downstream Effects

The following biological roles have been demonstrated for RGLG family members but represent **downstream pathway consequences** rather than the core molecular function of Q6YYC5:

Downstream Role	Family Member	Citation	Evidence Level for Q6YYC5
JA signaling modulation	AtRGLG3/4	PMID: 22898498, 25788731	Inferred by orthology, not direct
Drought stress tolerance	AtRGLG1/2	PMID: 32970364	Not applicable (different subclade)
Iron homeostasis	AtRGLG1/2	PMID: 20113438, 26253232	Not applicable
Apical dominance/auxin	AtRGLG1/2	PMID: 17586653	Not applicable
Basal blast resistance	OsRGLG5	PMID: 37177781	Paralog, not direct
Grain number/yield	OsRGLG6	PMID: 41312104	Paralog, not direct
Immune receptor homeostasis	AtRGLG1/2	PMID: 37532719, 41557808	Not applicable

These downstream roles are informative for understanding the biological significance of the RGLG family but should not be annotated as core functions of Q6YYC5 without direct evidence.

Evidence Base

Tier 1: Direct Experimental Evidence on Closest Ortholog (AtRGLG4)

Zhang et al. (2012) — *Two novel RING-type ubiquitin ligases, RGLG3 and RGLG4, are essential for jasmonate-mediated responses in Arabidopsis.* PMID: 22898498

This foundational paper established that AtRGLG3 and AtRGLG4 possess ubiquitin ligase activities and are widely expressed in Arabidopsis tissues. The rglg3 rglg4 double mutant is resistant to coronatine-secreting *P. syringae* DC3000, with altered MeJA-inhibited root growth, JA-inductive gene expression, and wound-stimulated JA-responsive gene expression in a COI1-dependent manner. This is the primary reference establishing RGLG3/4 as JA-pathway E3 ligases.

Sacharowski et al. (2016) — *The Arabidopsis Iron-Sulfur Protein GRXS17 is a Target of the Ubiquitin E3 Ligases RGLG3 and RGLG4.* PMID: 27497447

This study provides the most complete biochemical characterization: identification of the substrate GRXS17 through a substrate trapping approach using RING-dead RGLG3/4 variants; identification of UBC30 as the cognate E2 enzyme; demonstration of in vitro auto-ubiquitination and GRXS17 ubiquitination; and in vivo confirmation that GRXS17 is degraded in an RGLG3/4-dependent manner in planta. This is the strongest single piece of evidence supporting E3 ligase activity for the RGLG3/4 subclade.

Meng et al. (2015) — *Hijacking of the jasmonate pathway by FB1 to initiate PCD in Arabidopsis is modulated by RGLG3 and RGLG4.* PMID: 25788731

Confirmed RGLG3/4 as ubiquitin ligases controlling FB1-triggered programmed cell death by modulating the JA signaling pathway.

Tier 2: Rice RGLG Family Evidence

Dong et al. (2023) — *The E3 ubiquitin ligase OsRGLG5 targeted by the Magnaporthe oryzae effector AvrPi9 confers basal resistance against rice blast.* PMID: 37177781

Demonstrated E3 ligase activity for a rice RGLG family member with identified substrate (AvrPi9) and biological role (basal blast resistance). Strongest direct evidence for RGLG E3 activity in rice.

OsRGLG6 (2025) — *The RING-domain E3 ubiquitin ligase OsRGLG6 regulates rice grain number and yield via ubiquitination-mediated degradation of OsOTUB1.* PMID: 41312104)

Second rice RGLG member with confirmed E3 activity, ubiquitinating OsOTUB1.

Tier 3: Broader RGLG Family Characterization

The AtRGLG1/2 subclade has been extensively characterized as E3 ligases with roles in auxin signaling (PMID: 17586653), drought response (PMID: 22095047, 32970364), iron homeostasis (PMID: 20113438, 26253232), immune signaling (PMID: 37532719), and receptor kinase regulation (PMID: 41557808). All five Arabidopsis RGLG members and at least two rice members have confirmed E3 activity — no RGLG family member has been shown to lack this activity.

Tier 4: Structural and Computational Evidence

The VWA domain crystal structure of RGLG2 (PMID: 37734561) reveals calcium-dependent conformational regulation. The BON1 copine structure (PMID: 32369638) and functional studies (PMID: 21623975, 20634289) provide the critical contrast showing copine/VWA domains alone do not confer E3 activity. AlphaFold prediction (AF-Q6YYC5-F1-model_v6) confirms the RING domain has a well-defined fold (pLDDT 91.4).

Conflicts and Alternatives

No Direct Conflicting Evidence

No evidence was identified that contradicts the assignment of E3 ubiquitin ligase activity to Q6YYC5. Every characterized RGLG family member (5/5 in Arabidopsis, 2/2 in rice with published data) demonstrates E3 ligase activity. No RGLG-family protein has been reported to lack this activity. No alternative molecular function has been proposed for any RGLG family member.

Potential Qualifications

1. Myristoylation divergence from AtRGLG4. Q6YYC5's MG motif suggests it may be more functionally analogous to AtRGLG1/2 (myristoylated, membrane-associated) in its localization behavior than to AtRGLG4 (cytoplasmic/nuclear), despite being the closest sequence match to AtRGLG4 by overall identity. However, this affects localization and possibly substrate access, not the core E3 ligase function.

2. K48 vs. K63 chain type. AtRGLG2 forms K63-linked polyubiquitin chains (PMID: 17586653), which function in signaling rather than proteasomal degradation. AtRGLG4 is reported to form K48-linked chains (UniProt). The chain type specificity of Q6YYC5 is unknown. Both chain types are consistent with GO:0061630, but the downstream biological consequences differ.

3. Paralog confusion risk. With at least 11 copine+RING proteins in rice, care must be taken not to attribute OsRGLG5 or OsRGLG6 characterization data directly to OsRGLG4/Q6YYC5. This assessment correctly treats these as family-level evidence supporting functional conservation, not as direct evidence for Q6YYC5.

4. No competing alternative function. No RGLG protein has been reported to have a molecular function other than E3 ubiquitin ligase activity. The copine and VWA domains are accessory/regulatory, as demonstrated by the BON1 contrast.

Limitations and Knowledge Gaps

#	Gap	What Was Checked	Why It Matters	Resolution
1	No direct biochemical assay on Q6YYC5 itself	PubMed for OsRGLG4, Q6YYC5, Os08g0135400, LOC4344608	All evidence is ortholog-based (ISS/IBA level); direct assay would upgrade to IDA	In vitro ubiquitination assay with recombinant Q6YYC5 + E1/E2/Ub
2	Unknown substrate(s) in rice	No published interaction data for Q6YYC5	Substrate identity determines biological role specificity	Y2H or co-IP screen; substrate trapping with RING-dead mutant
3	Subcellular localization not experimentally determined	Annotations inferred from AtRGLG4 (cytoplasm/nucleus IDA)	MG motif suggests possible membrane association	GFP-fusion in rice protoplasts; G2A mutant comparison
4	Ubiquitin chain type specificity unknown	AtRGLG2 = K63; AtRGLG4 = K48 (UniProt)	K48 vs K63 determines degradation vs signaling	In vitro with K48R/K63R ubiquitin mutants
5	Expression pattern and biological context	No tissue/stress expression data found	Expression context informs biological processes	qRT-PCR or RNA-seq; rice expression atlases
6	Calcium-dependent VWA regulation	RGLG2 VWA crystal structure (P 37734561)	VWA calcium binding may regulate E3 activity	Calcium titration of Q6YYC5 VWA + E3 assay
7	Protein expression confirmed but function not	PE=1 via proteomics	Protein exists but no functional data	Combine with #1 above

Proposed Follow-up Experiments / Discriminating Tests

Priority 1: In Vitro Ubiquitination Assay (Definitive)

Purify recombinant Q6YYC5 (full-length and RING domain only) and test for auto-ubiquitination in a reconstituted system (E1 + E2 + Ub + ATP). Use RING-mutant (e.g., C356A) as negative control. Use UBC30 ortholog as E2 based on AtRGLG4 data. This would provide IDA-level evidence for GO:0061630 and definitively resolve the annotation.

Priority 2: Substrate Trapping Screen

Express RING-dead Q6YYC5 (analogous to RGLG3-RING-dead in PMID: 27497447) in rice and perform tandem affinity purification to identify interaction partners. This approach successfully identified GRXS17 as a substrate for AtRGLG3/4 and would simultaneously confirm E3 function and identify biological context.

Priority 3: Myristoylation and Localization

Generate GFP-Q6YYC5 and GFP-Q6YYC5(G2A) fusions and observe localization in rice protoplasts under normal and stress conditions. This would resolve the CC annotation question and determine whether Q6YYC5 undergoes stimulus-responsive relocalization like AtRGLG2 (PMID: 22095047).

Priority 4: Ubiquitin Chain Type Determination

Use K48R and K63R ubiquitin mutants in the in vitro ubiquitination assay to determine chain type preference. This would inform whether the protein primarily targets substrates for degradation (K48, like AtRGLG4) or signaling (K63, like AtRGLG1/2).

Priority 5: CRISPR Knockout Phenotyping

Generate CRISPR knockout of OsRGLG4 in rice. Phenotype under JA treatment, pathogen challenge, drought stress, and normal growth conditions. Analyze proteomic changes to identify candidate substrates stabilized in the mutant. Compare with Arabidopsis rglg3 rglg4 mutant phenotypes (JA-responsive gene expression, FB1 sensitivity, P. syringae resistance).

Curation Leads

Lead 1: Add GO:0061630 as Core MF (High Confidence)

- **Action:** Add GO:0061630 (ubiquitin protein ligase activity) with evidence code ISS, reference ortholog AtRGLG4 (Q9SAL0)
- **Rationale:** More specific than GO:0004842; E3-specific; all family members confirmed E3 ligases
- **Candidate references to verify:**
- PMID: 22898498 — Verify: "Both RGLG3 and RGLG4 possessed ubiquitin ligase activities and were widely distributed in Arabidopsis thaliana tissues"
- PMID: 27497447 — Verify: "we used a ubiquitin-conjugating enzyme (UBC) panel screen to pinpoint UBC30 as a cognate E2 UBC capable of interacting with RGLG3 and RGLG4 and mediating auto-ubiquitination of RGLG3 and ubiquitination of GRXS17 in vitro"

Lead 2: Consider EC 2.3.2.27 Assignment (Moderate Confidence)

- **Action:** Annotate EC 2.3.2.27 (RING-type E3 ubiquitin transferase) for Q6YYC5
- **Rationale:** Both AtRGLG3 and AtRGLG4 carry this EC number in UniProt reviewed entries; catalytic reaction: S-ubiquitinyl-[E2]-L-cysteine + [acceptor]-L-lysine → [E2]-L-cysteine + N^6-ubiquitinyl-[acceptor]-L-lysine

Lead 3: Retain BP and CC Annotations (High Confidence)

- **Action:** Retain GO:0016567 (protein ubiquitination), GO:0005737 (cytoplasm), GO:0005634 (nucleus)
- **Caveat:** Note potential plasma membrane localization due to MG motif

Lead 4: Flag Myristoylation as Research Priority (Moderate Confidence)

- **Action:** Note in gene review that Q6YYC5 has myristoylation-compatible N-terminus unlike AtRGLG4, suggesting potential membrane association
- **Implication:** May affect substrate access and biological context relative to AtRGLG4

Candidate References with Snippets to Verify

PMID	Exact Snippet	Relevance
27497447	"we identified the monothiol glutaredoxin GRXS17 as a substrate of the Arabidopsis E3 ubiquitin ligases RING DOMAIN LIGASE 3 (RGLG3) and RGLG4 using a substrate trapping approach involving tandem affinity purification of RING-dead versions"	AtRGLG4 confirmed E3 ligase with identified substrate
27497447	"GRXS17 is ubiquitinated and degraded in an RGLG3- and RGLG4-dependent manner in planta"	In vivo validation of E3 activity
25788731	"two ubiquitin ligases, RING DOMAIN LIGASE3 (RGLG3) and RGLG4, which control FB1-triggered PCD by modulating the jasmonate (JA) signalling pathway in Arabidopsis thaliana"	Biological role confirmation
37177781	"we identified an AvrPi9-interacting protein in rice, which we named OsRGLG5, encoding a functional RING-type E3 ubiquitin ligase"	Rice RGLG E3 activity
37177781	"During infection, AvrPi9 was ubiquitinated and degraded by OsRGLG5"	Rice substrate ubiquitination and degradation
17586653	"The RING domain protein RGLG2 (for RING domain Ligase2) from Arabidopsis thaliana can be N-terminally myristoylated and localizes to the plasma membrane. It can form Lys-63-linked multiubiquitin chains in an in vitro reaction."	Family E3 activity and myristoylation
22095047	"RGLG2 could move from the plasma membrane to the nucleus under stress treatment"	Stress-responsive relocalization
20634289	"Mutation at putative myristoylation residue glycine 2 altered plasma membrane localization of BON1 and rendered BON1 inactive"	Myristoylation importance for copine proteins