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Introduction

Rice endosperm utilizes two types of storage proteins, prolamine and glutelin. Storage proteins are biological reserves for ions and metabolites, such as amino acids and iron, which are essential to plant growth and likewise the nutritional value of commonly consumed grains. Prolamine and glutelin are also categorized as RNA Binding Proteins (RBP's). The mRNA of prolamine is localized on the protein-body endoplasmic reticulum (PB-ER), whereas glutelin mRNA is targeted to the cisternal endoplasmic reticulum (C-ER). The mRNA that corresponds to these two proteins is responsible for the sorting of prolamine to the PB-ER. Glutelin is initially transported to the ER lumen, but is redirected to protein storage vacuoles and stored as irregularly shaped protein bodies. Exploitation of RNA sorting may afford means to determine where proteins are specifically accumulated within the cytoplasmic region (Hamada et al., 2003). It is important to elucidate the nature of RBP binding selectivity to specific zipcode sequences vs. a non zipcode control sequence. Biotinylated RNA constructs were created, bound to magnetic streptavidin beads, and used to probe the binding selectivity of glutelin proteins in seed extract. Beads were eluted with $MgCl_2$ and; elutions were analyzed on SDS-PAGE. No binding selectivity could be observed from data.

Methods

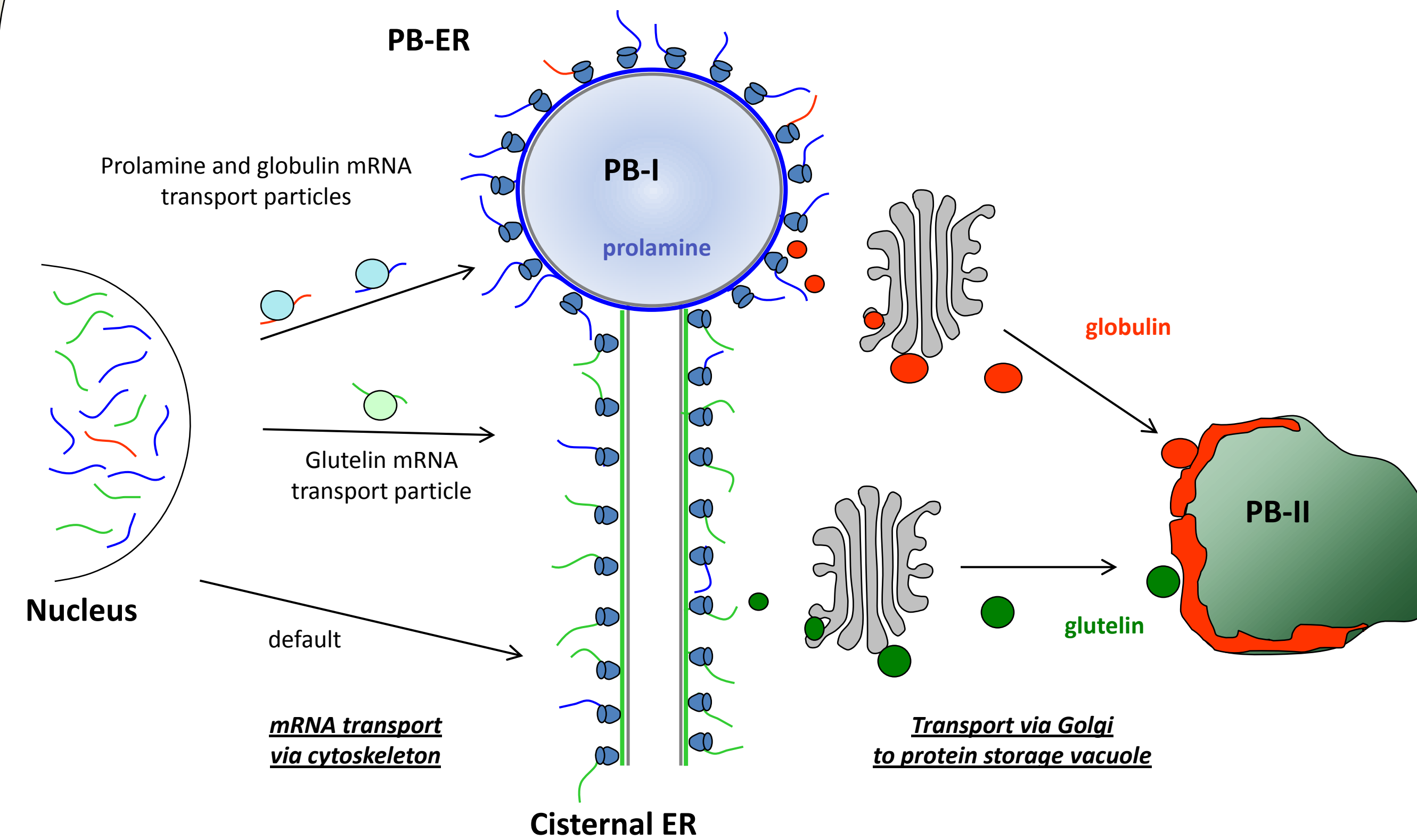
Work with glutelin was modeled after that conducted with prolamine, which consists of amplification of the glutelin (*gt2*) exon 1 sequence through PCR and *in-vitro* transcription utilizing a primer that contained a promoter sequence for a biotin-adenosine derivative (Huang F, He J, Zhang Y, Guo Y. 2008.). These fragments were then gel extraction purified. This biotinylated RNA was bound to magnetic streptavidin beads and used to probe zip-code binding selectivity of glutelin protein from the heparin binding fraction of seed extract as well as cytoskeleton-enriched seed extract per the protocol used in work with prolamine (Crofts A, et al. 2005). Seed extract was mixed and incubated with beads in solution; the mobile phase was removed by magnetically aggregating the beads. The proteins were eluted with washes of 0.3 M $MgCl_2$ followed by 1.0 M $MgCl_2$. The efficiency of RNA binding to the streptavidin beads was analyzed by 8 M Urea TBE gel, visualized with Ethidium Bromide under UV light. The eluted fractions were analyzed via silver stained 13% Acrylamide SDS-PAGE for presence of glutelin specific proteins.

DNA Construct

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GAGCTCAACCATTCAAGTTCATTAGTCCTACA
ACAACATGGCATCCATAAATCGCCCCATAGTT
TTCTTCACAGTTTGCTTGTTCTCTTGTGCAA
TGGCTCTCTAGCCCAGCAGCTATTAGGCCAG
AGCACTAGTCAATGGCAGAGTTCTCGTCGTG
GAAGTCCAAGAGAATGCAGGTTTCGATAGGTT
GCAAGCATTGAGCCAATTCGGAGTGTGAGG
TCTCAAGCTGGCACAACCTGAGTTCTTCGATGT
CCCTAATGAGCAATTTCAATGTACCGGAGTATC
TGTTGTCCGTCGAGTTATTGAACCTAGAGGCC
TTCTACTACCCATTACACTAATGGTGCATCTC
TAGTATATATCATCCAAG
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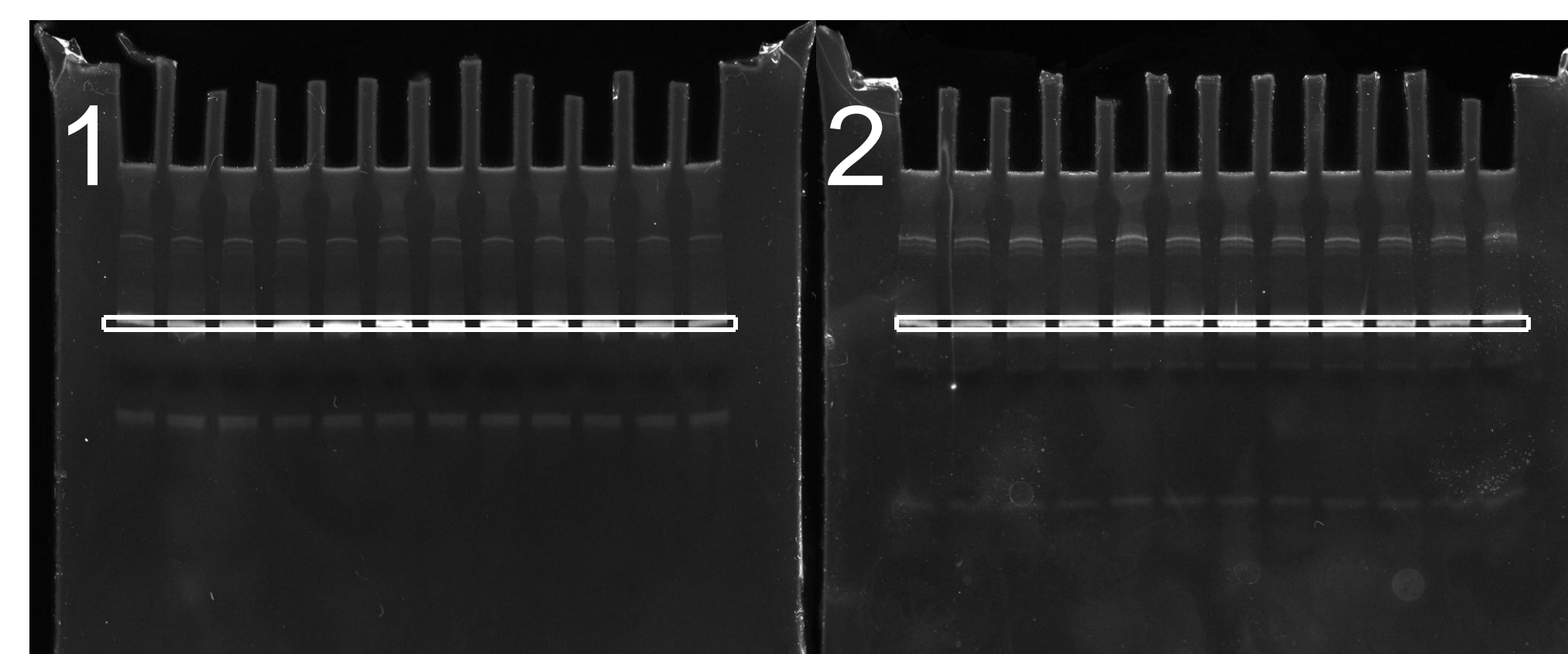
Bold underlined sequence was PCR amplified and used for *in vitro* transcription to make 5' biotinylated RNA. Green highlighted regions denote two putative zipcode regions within exon 1. The forward primer included a T7 ϕ 2.5 promoter sequence to incorporate the biotin-adenosine derivative (Huang et al., 2008).

RNA Localization



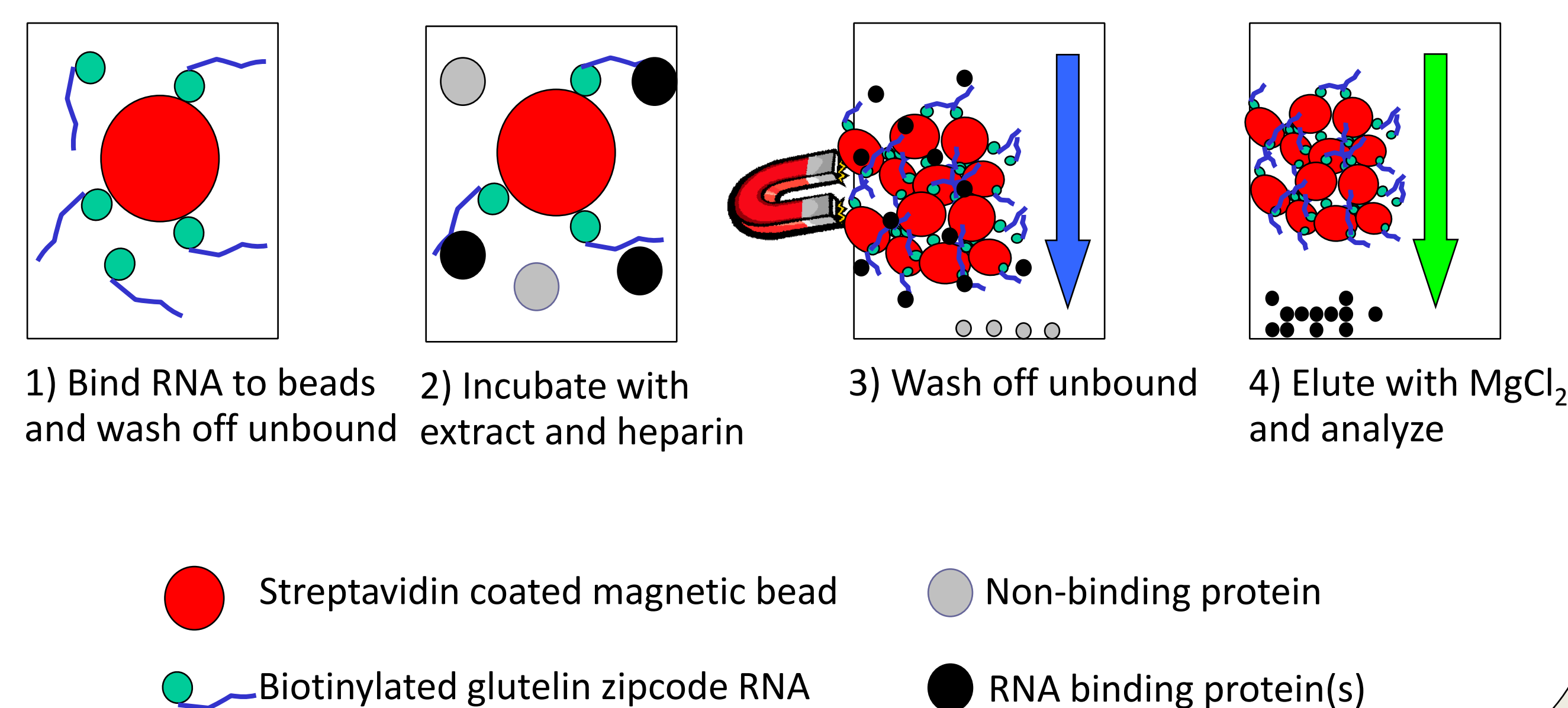
mRNA of prolamine storage proteins is localized on the PB-ER; prolamine storage proteins are likewise accumulated within the PB-I domain as inclusion granules 1-2 μ m in diameter. Glutelin mRNA is localized on the C-ER; glutelin proteins are transported via the Golgi body to irregularly shaped protein storage vacuoles (PB-II) 2-3 μ m in diameter.

PCR Amplification of *gt2* Sequence



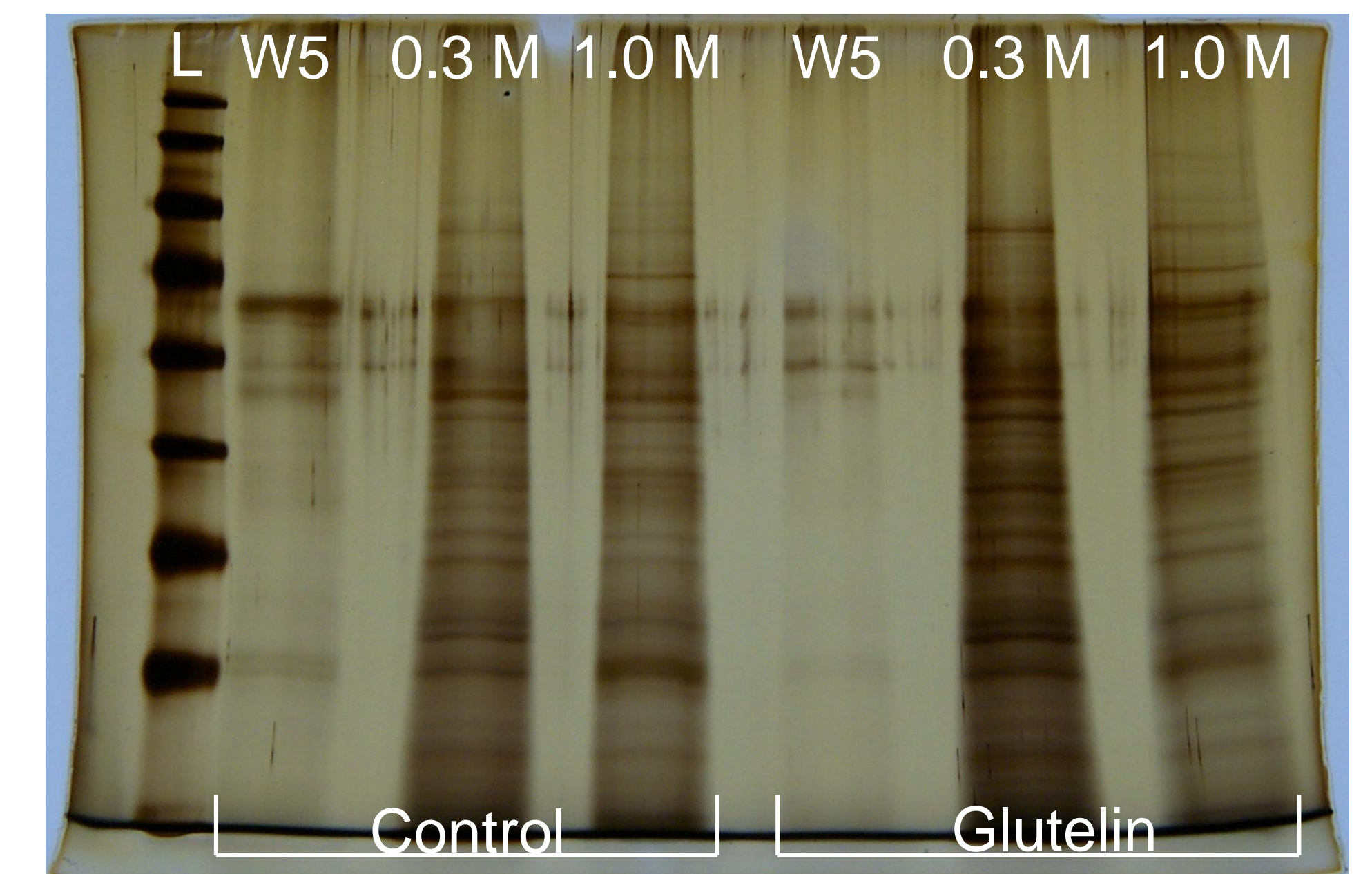
1,2: 1x TBE gel images, Control and Glutelin zip code DNA respectively. All 12 lanes seen containing 10 μ L of PCR reaction. Middle, highest intensity bands were scored from gel, and DNA was extracted. Template DNA was used in RNA transcription reaction.

RBP Capture Using Biotinylated RNA Bound to Magnetic Streptavidin Beads



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RBP Capture Silver Stained SDS Gel



Elution gel of RBP capture experiment:

Wash 5, 0.3 M $MgCl_2$, 1.0 M $MgCl_2$ for control then glutelin respectively. Corresponding bands are visible in both samples, without variation. Volume of RNA bound to beads were 5 and 3 μ L respectively, in order to account for concentration difference. RBP capture experiment performed in two phases: overnight capture and elution followed by 6 hour capture and elution. Elutions were acetone or TCA precipitated, boiled, and run on 13% Acrylamide SDS-PAGE gel.

Conclusions

DNA contaminants were removed from PCR amplified template sample; contaminants observed in transcription RNA product displayed no significant heparin binding and concluded to be inconsequential. Glutelin zipcode RNA products were observed to be higher yield, allowing more binding to streptavidin beads and thus effecting protein concentrations in elutions. RBP capture experiments with heparin-binding fraction samples displayed no significant specificity in glutelin sample vs. control. Use of salt gradient elution vs. heparin gradient elution in capture experiment provided similar data. Preliminary cytoskeleton-enriched fraction data revealed much lower protein concentration levels; even after RBP capture protocol was scaled up by a factor of five (only glutelin RNA tested), protein levels were insufficient. Seed extraction protocol will be modified, optimized for use in RBP capture experiments. Future experimentation will include processing control RBP capture experiment of cytoskeleton-enriched fraction and modification of seed extraction protocol, optimizing for use in RBP capture experiments. Glutelin zip-code RNA vs. control RBP capture protocol may be modified in several ways, including elution profile and RNA binding efficiency.

References

- Huang F, He J, Zhang Y, Guo Y. Synthesis of biotin-AMP conjugate for 5' biotin labeling of RNA through one-step in vitro transcription. Nature Protocols. 2008, 3(12):1848-1861
- Washida H, Kaneko S, Crofts N, Sugino A, Wang C, Okita TW. 2009 Identification of cis-localized elements that target glutelin RNAs to a specific subdomain of the cortical endoplasmic reticulum in rice endosperm cells. 2009 July 15. in press

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