Protein extraction and identification of puroindoline in 97-1 transgenic rice seeds

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INTRODUCTION

Puroindolines (PINs), expressed in endosperm tissue of wheat, oat and barley seeds, have demonstrated antimicrobial properties against several fungal pathogens.

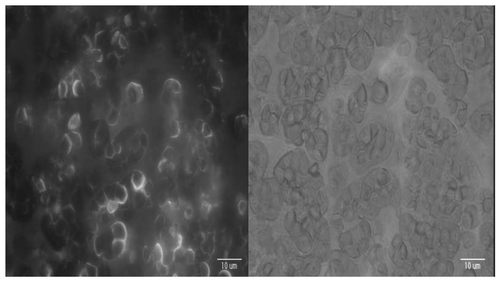


Figure 1. Immunolocalization of puroindoline-expressing transgenic rice flour. Antibody tagged PINs localized to the surface of starch granules appear as fluorescent "halos" and can be compared to a light micrograph image on the right (no Ab control).

- A transgenic 97-1 rice line was created containing PIN, in attempt to improve microbial resistance in rice (Krishnamurthy et al, 2001).
- Puroindoline contains a unique tryptophan-rich domain and has a molecular mass of 13kDa.
- Studies show that PIN localizes to the wheat starch granule surface (Wall et al, 2010).

OBJECTIVE

- The purpose of this research project was to purify puroindoline from 97-1 transgenic rice seeds, and verify its identity to see if it also targets the granule surface in rice.
- A specific primary antibody has successfully recognized puroindoline in some wheat varieties and can be used to verify the presence of PINs in transgenic rice seeds.

ATTTTCCGATCACCTGGCCATGGAAATGGTGGAAGGGTGGTTGCGAGGAG

F | P I T W P W K W W K G G C E | E

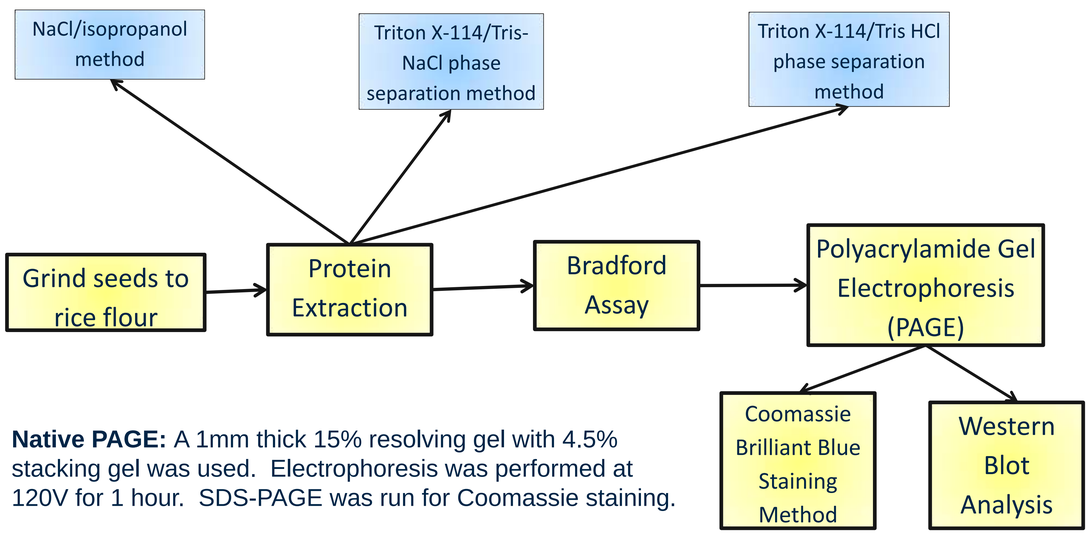
Fig 2: Tryptophan-rich sequence in 3B3-7 cDNA (boxed-in). Nucleotide sequence is located above the corresponding amino acid sequence (Tanchak et al, 1998).

METHODS

NaCl/isopropanol Method: PIN solubilisation solution (50% isopropanol (v/v), 50mM NaCl) was added to flour and supernatant was collected.

Triton X-114/Tris-NaCl Phase Separation Method: 1% Triton X-114 in Tris buffered saline (10mM Tris, 150mM NaCl, pH 7.5) was added to flour. Lower detergent phase was collected.

Triton X-114/Tris-HCl Phase Separation Method: Tris-HCl buffer (5mM EDTA, 100mM KCl, 4% Triton X-114, pH 7.8) added to flour. After a series of 8 hour incubation periods, the lower detergent phase was collected.



Native PAGE: A 1mm thick 15% resolving gel with 4.5% stacking gel was used. Electrophoresis was performed at 120V for 1 hour. SDS-PAGE was run for Coomassie staining.

Western Blot: Native polyacrylamide gels were transferred to nitrocellulose membranes using a BioRad wet electro-blotting system.

- Antibodies→ A 1:1,000 dilution of a polyclonal rabbit anti-PIN primary antibody and a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was used.

RESULTS

A Bradford assay was used to quantify proteins in a series of protein extracts.

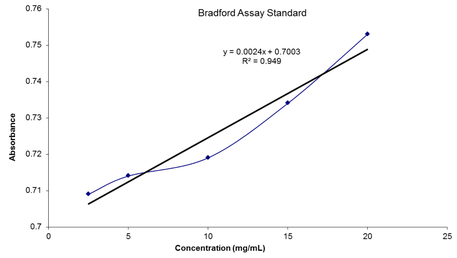


Figure 3: Protein concentration determined using Bradford assay standard curve. Concentrations range from 2.5mg/mL to 20mg/mL. Linearity is defined by a coefficient of correlation of 0.949.

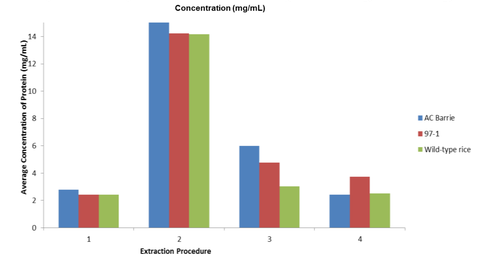


Figure 4: Average mass of total protein extracted from AC Barrie wheat seeds, 97-1 rice seeds and wild-type rice seeds using 1)NaCl/isopropanol, 2-3) Triton X-114/Tris-NaCl bottom phase and top phase and 4) Triton X-114/Tris-HCl methods.

Samples were analyzed using SDS-PAGE to test for the presence of puroindoline. Proteins with molecular masses of 13kDa are found in all extraction samples (lanes 1-4).

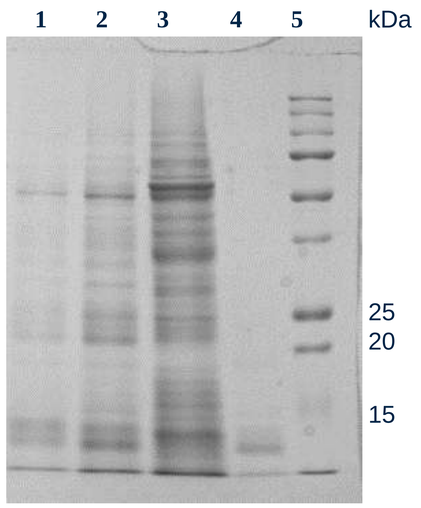


Figure 5: Protein extractions of various rice and wheat varieties. Wild-type rice was used as a negative control and AC Barrie wheat was used as a positive control. Triton X-114/Tris-NaCl extraction method was performed on wild-type rice (lane 1), 97-1 rice (lane 2) and AC Barrie wheat (lane 3). Isopropanol/NaCl extraction was performed on 97-1 rice (lane 4). Biorad precision ladder is used (lane 5).

Fractionation on native-PAGE verified the presence of unreduced puroindoline in protein extracts. A faint 13kDa signal was present in the Triton X-114/Tris-HCl extract of 97-1 seeds (lane 3). A darker signal was present in this extract of AC Barrie wheat (lane 2).

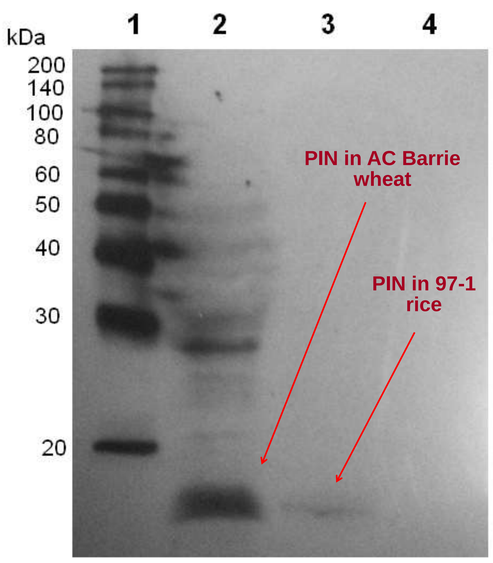


Figure 6: Immunodetection of PIN in transgenic rice flour. Triton X-114/Tris-HCl buffered phase partitioning PIN extracts from wheat (positive control), wild type rice cultivar Kaybonnet (negative control), and PIN+ rice were ran on a native PAGE. 1) Ladder, 2) AC Barry PIN extract, 3) PIN+ rice extract, 4) wild type rice flour PIN extract.

DISCUSSION AND CONCLUSION

•The NaCl/isopropanol and the Triton X-114/Tris-NaCl methods extracted the smallest and largest mass of total protein from all seed varieties.

•Approximately equal amounts of protein were extracted from each rice and wheat seed type for all three extraction methods (Figure 4).

•Poor resolution makes it difficult to analyze banding pattern differences between samples and controls (Figure 5).

•Other proteins may be present with the same molecular mass as puroindoline.

• Western blot analysis confirmed the presence of puroindoline in 97-1 transgenic rice seeds (Figure 6, lane 3). A fainter signal in the transgenic rice compared to the positive control indicates the need for optimization of extraction methods.

•A signal in AC Barrie present at 26kDa suggests the possibility of non-specific binding of antibodies (Figure 6, lane 2).

PIN was difficult to purify because of the following reasons:

1. Tight association to the surface of the starch granule
2. Native vs. denaturing conditions
3. Low abundance relative to other endogenous proteins

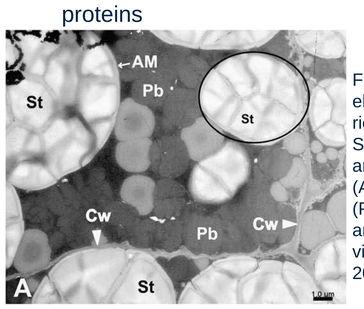


Figure 7. Transmission electron micrograph of rice starchy endosperm. Starch granules (St), amyloplast membranes (AM), protein bodies (Pb), and cell walls (Cw) are the only components visible. (Hong et al, 2009).

FUTURE WORK

•Optimize extraction methods in order to more efficiently purify puroindoline from the surface of transgenic 97-1 seeds.

•Run a higher concentration SDS-PAGE for better separation of smaller proteins in order to distinguish 13kDa proteins.

•Mass spectrometry can sequence proteins of various sizes and verify the presence/absence of puroindoline.

•Obtain protein concentrator columns with specified molecular weight cutoff (ie 17kDa) to eliminate larger proteins.

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