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# Puroindolines co-localize to the starch granule surface and increase seed bound polar lipid content☆

L. Feiz [a], H.W Wanjugi [a,1], C.W. Melnyk [b,2], I. Altosaar [b], J.M. Martin [a], M.J. Giroux [a,*]

[a] Department of Plant Sciences and Plant Pathology, Montana State University, P.O. Box 173150, Bozeman, MT 59717-3150, United States
[b] Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5

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ABSTRACT

Endosperm texture in wheat is controlled by the *Pina* and *Pinb* genes that comprise the *Hardness* (*Ha*) locus. Studies have shown that soft and hard varieties differ in the amount of starch bound polar lipids but have not addressed whether PINs are directly involved and whether the presence of one particular PIN affects seed polar lipid levels and cellular localization. Here, we determined the effect of over-expressing PINA or PINB on seed bound polar lipids and PIN localization. $F_3$ recombinants homozygous for either a *Pina* or *Pinb* null *Ha* locus with or lacking a transgenically added *Pina* or *Pinb* were analyzed for grain hardness, PIN abundance, and seed bound polar lipid levels. Overexpressed PINs resulted in reduced hardness, increased starch bound PINs, and increased seed bound polar lipids. Addition of PINA to the PINA nulls or PINB to the PINB nulls resulted in higher bound polar lipid levels than the addition of the alternative PIN. Both PINs localized to the starch surface in the presence or absence of the other protein. Our results indicate that PIN overexpression results in reduced endosperm texture and increased seed bound polar lipids and that PINs independently localize to the surface of starch granules.



## 1. Introduction

The '*Hardness*' (*Ha*) (Symes, 1965, 1969) locus on chromosome 5DS (Law et al., 1978; Mattern et al., 1973) determines whether wheat (*Triticum aestivum* L.) is soft (*Ha*) or hard (*ha*) textured (Morris and Rose, 1996). The first evidence for which proteins comprised the *Ha* locus came when a 15 kDa complex called friabilin was found to be associated with endosperm texture with higher amounts on soft wheat starch than hard wheat starch (Greenwell and Schofield, 1986). Friabilin was found to be a mixture of puroindoline a (PINA) and puroindoline b (PINB) since friabilin N terminal sequences (Jolly et al., 1993; Morris et al., 1994) matched PINs (Blochet et al., 1993). The *Pina* and *Pinb* genes (Gautier et al., 1994) were reported as tightly linked to *Ha* (Giroux and Morris, 1998; Sourdille et al., 1996) and are located within a 25 kb segment of DNA (Chantret et al., 2005). Soft wheats possess the wild type *Pina-D1a* and *Pinb-D1a* alleles; while hard wheats contain a mutation in either *Pin* (Giroux and Morris, 1997, 1998; Morris et al., 2001). PINA and PINB are 60% homologous and are unique in having a tryptophan-rich domain thought to be involved in binding polar lipids (Blochet et al., 1993; Marion et al., 1994). Data supportive of this idea came from Clifton et al. (2007a,b) who showed that the degree of PINB penetration into lipid bilayers whether as a single protein or in the presence of PINA, is affected by mutating a tryptophan in the tryptophan-rich domain of PINB. However, Evrard et al. (2008) demonstrated that amino acid residues other than tryptophan are involved in PINB binding to yeast membranes.

The genetic role of *Pins* in controlling endosperm texture has been demonstrated in rice (Krishnamurthy and Giroux, 2001) and wheat (Beecher et al., 2002; Hogg et al., 2004; Martin et al., 2006). In rice, which lacks puroindoline homologues (Caldwell et al.,

*Abbreviations:* DGDG, digalactosyl diglyceride; DOPC, (1,2-dioleoyl-sn-glycero-3-phosphocholine); DOPE, (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine); FNLs, free non-polar fraction lipids; GL, glycolipid; *Ha*, hardness; MGDG, monogalactosyl diglyceride; LSD, least significant difference; PINA, puroindoline a protein; *Pina*, *puroindoline a* gene; PINB, puroindoline b protein; *Pinb*, *puroindoline b* gene; PL, phospholipid; SKCS, single kernel characterization system; TLC, thin layer chromatography; TX-114, Triton X-114.

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* Corresponding author. Tel.: +1 406 994 7877; fax: +1 406 994 7600.
*E-mail address:* mgiroux@montana.edu (M.J. Giroux).
[1] Current address: USDA, ARS, PWA, WRRC-GGD, 800 Buchanan Street, Albany, CA 94710, United States.
[2] Current address: The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

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2004; Gautier et al., 2000), transgenic expression of the "soft type" *Pina-D1a* and *Pinb-D1a* alleles resulted in significantly softer endosperm texture (Krishnamurthy and Giroux, 2001). The mechanism of grain hardness or endosperm strength is most likely determined by the degree of adhesion between starch granules and the surrounding protein matrix (Barlow et al., 1973). Soft wheats have less starch granule–protein matrix interaction and hence release a high proportion of intact and less damaged starch granules after milling (Barlow et al., 1973; Simmonds, 1974). On the contrary, in hard wheats, the starch granule–protein matrix interaction is stronger and larger irregularly shaped particles mainly composed of whole endosperm cells are produced during milling (Pomeranz and Williams, 1990). The starch granule–protein matrix interaction is apparently regulated by friabilin (PINA and PINB) (Beecher et al., 2002). Promoter analysis of *Pina* and *Pinb* has shown that these genes are expressed solely in endosperm cells (Wiley et al., 2007) consistent with immunolocalization studies which have found that PINs are localized to the starch granule surface (Capparelli et al., 2005; Wiley et al., 2007).

Greenblatt et al. (1995) observed high levels of bound polar lipids (glycolipids and phospholipids) associated with water washed starch of soft but not hard wheat varieties. This led them to conclude that friabilin components associate with starch through residual bound polar lipid mediated interactions. Further results have shown that PINA and PINB interact on the surface of starch to form friabilin and create soft texture (Hogg et al., 2004). Among soft wheats, PINB limits friabilin formation (i.e., 'softness') more than PINA (Swan et al., 2006). More recently, PINA and PINB have been reported to either associate with starch independently to confer intermediate texture or together leading to full friabilin function and soft endosperm texture (Wanjugi et al., 2007). The association of PINs with the surface of starch granules has been speculated to be either direct, involving lipid bridges between the starch and protein, or indirect where lipids cause conformational change in the protein allowing starch matrix binding (Oda and Schofield, 1997).

In wheat kernels, the distribution of various lipid classes within tissue fractions varies significantly (Hargin and Morrison, 1980) with separation achieved by thin layer chromatography (TLC) (Greenblatt et al., 1995; Morrison et al., 1980) or HPLC (Ohm and Chung, 1999). In wheat, most lipids are free lipids (FLs), that can be extracted by non-polar solvents. Bound lipids have associations with other entities like starch granules or proteins and can be partly extracted using polar solvents (Ruibal-Mendieta et al., 2002). The total lipid composition of wheat flour consists of free and bound polar phospholipids (PLs) and glycolipids (GLs), and free non-polar fraction lipids (FNLs) (Morrison et al., 1980). PL in wheat flour consists primarily of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (McMurray and Morrison, 1970). The major GLs of wheat seeds are monogalactosyl diglycerides (MGDGs) and digalactosyl diglycerides (DGDGs) (Hargin and Morrison, 1980; McMurray and Morrison, 1970; Morrison et al., 1980; Prieto et al., 1992).

Despite Greenblatt et al. (1995) having reported that water washed starch prepared from soft wheat varieties had more starch bound polar lipids than hard wheat water washed starch, a definitive link between PIN expression and levels of total seed polar lipids has not been demonstrated. In this study, we determined the effect of overexpressing PINA or PINB on seed bound polar lipids as well as PIN immunolocalization.

## 2. Experimental

### 2.1. Genetic materials

The genotypes used in this study were a subset of the recombinant lines described by Wanjugi et al. (2007) and were developed by crossing a transgenic isoline overexpressing PINA (transgenic event HGA3) or PINB (transgenic event HGB12) created in the variety Hi-Line (HL) (Hogg et al., 2004; Lanning et al., 1992) to either a PINB null, 'Canadian Red' (CR), hard white spring (Clark et al., 1926) or a PINA null, 'McNeal' (McN), hard red spring (Lanning et al., 1994) variety. The transgenic events were selected from the events described by Hogg et al. (2004, 2005) as having good plant vigor and relatively unaltered plant yield, seed size, and seed protein content. Canadian Red has the soft type *Pina-D1a* and a mutant *Pinb-D1e* allele that contains a point point mutation changing Trp-68 to a stop codon (Morris et al., 2001). The PINA and PINB amino acid numbering system used in this paper is counted from the methionine start codon for the immature proteins. McNeal possesses the soft type *Pinb-D1a* and a mutant *Pina* allele (*Pina-D1b*) which is an apparent deletion of the *Pina* coding sequence (Giroux and Morris, 1998). Hi-Line has the soft type *Pina-D1a* and the mutant *Pinb-D1b* allele which contains a glycine to serine substitution at residue 75 of PINB (Giroux and Morris, 1998). The crosses generated populations in the HL/McN and in the HL/CR background, with each population segregating for the native *Ha* locus from HL, CR, or McN and the presence or absence of the transgene (*Pina* or *Pinb*). A subset of three random lines from two of the genotype classes used by Wanjugi et al. (2007) were used in the experiments presented here. The two classes were those inheriting the McN *Ha* locus in the crosses to McN or the CR *Ha* locus in crosses to CR in combination with the presence or absence of an added transgene. In this paper the McN *Ha* locus lines with the added PINA, PINB and without transgene are called McNA+, McNB+ and McN− respectively. The CR *Ha* locus lines with the added PINA, PINB and without transgene are called CRA+, CRB+ and CR− respectively. The classes inheriting the native *Ha* from HL were not included as the effect of PIN overexpression in the presence of the HL *Ha* locus has been previously reported (Hogg et al., 2004, 2005).

### 2.2. Field planting and seed traits

Three randomly selected lines per genotype class homozygous for the transgene and *Ha*, two negative lines per genotype class and one parental line were planted in 2007 at the Montana State University–Bozeman Arthur H. Post Field Research Farm under irrigated conditions. Each entry was planted in single-row plots that were 3 m long and were planted with 10 g of seed with 30 cm between rows in mid April. Plots were irrigated with 2.5 cm of water one week before and one week after anthesis. In mid August, after all plots had reached physiological maturity, plots were cut with a binder (Mitsubishi Agricultural Machinery, Tokyo, Japan), the grain was threshed with a Vogel bundle thresher (Bill's Welding, Pullman, WA), and the seeds were cleaned and weighed. Grain hardness and seed weight of each line were determined using the Single Kernel Characterization System 4100 (SKCS, Perten Instruments, Springfield, IL) on a subsample of 50 seeds from each line. A subsample of mature seeds harvested from each plot was ground on an UDY mill fitted with a 0.5 mm screen (UDY Co., Fort Collins, Co). Between 170 and 180 mg of the ground samples were used to determine the protein content using a FP 528 Leco N analyzer (Leco Corporation, St. Joseph, MI) with an N to protein conversion factor of 5.7. The ground meal obtained from mature seeds was also analyzed for PIN protein levels and bound polar lipid content.

### 2.3. Extraction of total and starch bound puroindolines

Extraction and fractionation of total TX-114 soluble puroindoline proteins were done as described by Giroux et al. (2003) with quantification as described by Wanjugi et al. (2007). Water washed