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New insight into puroindoline function inferred from their subcellular localization in developing hard and soft near-isogenic endosperm and their relationship with polymer size of storage proteins

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ABSTRACT

Wheat endosperm texture is correlated with one major locus, *Ha*, located on the short arm of chromosome 5D, which comprises several genes among which are two puroindoline genes, *Pina* and *Pinb*. In this study, we used two near-isogenic lines, the hard-textured line lacking *Pina* and the soft-textured line containing both *Pina* and *Pinb* wild-type genes. Hard and soft endosperms were observed at four kernel developmental stages, from 180 °Cd to 750 °Cd. Puroindolines were located within protein bodies at the onset of prolamin accumulation by transmission electron microscopy and immunolabelling. *Ab initio* modeling showed a closer structural relationship between puroindolines and 2S storage proteins from dicots than between puroindolines and other cysteine-rich wheat proteins, i.e. LTP and amylase inhibitors. Compared to the soft line, storage protein polymers in the hard line exhibited higher molecular mass (increase of from 6 to 93%) and polydispersity indices (increase of from 26 to 63%) over the course of the 4-year experiment. This suggests that puroindolines might impact the aggregation of storage proteins. Finally, these data pave the way for investigation of the role of protein–protein interactions in the texture of wheat endosperm.

1. Introduction

Puroindolines are small (about 13 kDa), basic (p*I* 10–11), cysteine-rich (five disulphide bonds) seed-specific proteins that display an amphiphilic tryptophan-rich domain responsible for their capability of penetrating lipid membranes or oily phases *in vitro*. They share structural characteristics, i.e. a similar cysteine signature in 8 out of 10 cysteines and high helix secondary structure, with non-specific lipid transfer proteins (nsLTP), but, in contrast to the proteins in this family of ubiquitous plant proteins, they are only found in the seeds of plants from the *Triticeae* and *Avenae* tribes (Douliez et al., 2000; Morris, 2002). Their cellular location also differs from nsLTP, which are synthesized and stored in the aleurone layer (Dubreil et al., 1998), whereas puroindolines are mainly located in the starchy endosperm cells, although a possible location in the aleurone layer is under discussion (Digeon et al., 1999). Puroindolines display antimicrobial activity likely through their ability to form ion channels in biological membranes (Charnet et al., 2003) and in other cereals, their expression is related to an increase in their resistance to microbial pathogens (Krishnamurthy et al., 2001; Luo et al., 2008).

Soft kernel texture is closely associated with the presence of the two puroindoline 'wild-type' alleles, *Pina-D1a* and *Pinb-D1a*, on the short arm of chromosome 5D that co-localize with the locus carrying the most significant hardness QTL (Morris, 2002). Variable hardness of bread wheat kernel is observed in many cultivated varieties, due to the absence and/or mutations in one or two of these genes (Bhave and Morris, 2008a). The molecular basis of the relationship between these proteins and endosperm texture is still unknown. They were initially described as proteins associated with

Abbreviations: AFFFF, Asymmetrical Flow Field-Flow Fractionation; °Cd, degree Celsius days; daa, days after anthesis; GH, grain hardness; MWD, molecular weight distribution; NILs, near-isogenic lines; NIRS, near-infrared reflectance spectrometry; PINa, puroindoline-a; PINb, puroindoline-b; PC, protein content; QTL, Quantitative Trait Loci; TEM, Transmission Electron Microscopy.

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starch granules (Oda and Schofield, 1997) in mature kernel flour, but it has also been suggested that this location could be an artefact resulting from extraction processes (Gautier et al., 1994; Jolly et al., 1993). Stronger adhesion between starch granules and surrounding protein matrix in hard wheat was thought to be responsible for hardness and the higher number of damaged starch granules after milling (Barlow et al., 1973; Greenwell and Schofield, 1986). Puroindolines could limit the protein–starch interactions by an unknown mechanism perhaps involving their lipid binding properties. This role of puroindoline–lipid interaction in kernel texture is supported by previous results showing that hardness was also correlated with a decrease in free polar lipid contents (Morrison et al., 1989).

Although puroindolines and their impact on flour end uses have been extensively studied (Bhave and Morris, 2008a, 2008b), the biological role of these proteins is still an enigma. In the present work, we investigated the subcellular location of puroindolines in the developing hard and soft endosperms of near-isogenic wheat lines and the relationship between puroindolines and polymer size of storage proteins.

2. Materials and methods

2.1. Genetic material

Experiments were carried out on the Australian near-isogenic lines hard and soft *Triticum aestivum* L. cv Falcon, obtained by six generations of back crossing, selecting only for differences in grain hardness. Falcon hard and soft NILs have the same genetic background, and differ only in the presence or absence of the *Pina* gene. Falcon hard has a *Pina-D1b* (*Pina* null) and *Pinb-D1a* genotype (Ikeda et al., 2005). Falcon soft has a *Pina-D1a* and *Pinb-D1a* genotype (data not shown). Grain hardness values of Falcon lines grown at Clermont Ferrand in 2008 were 98 for Falcon hard and 42 for Falcon soft, assessed by NIRS (AACC method, 39-70A, 1999), from wholemeal flour ground in a Cyclotec 14,920 mill (Hilleröd, Denmark).

2.2. Culture conditions and sampling

Seeds were sown in pots in a glasshouse. After three weeks, plantlets were subjected to cold in a growth chamber for two weeks at 6 °C with an 8 h photoperiod. Plants were then grown in a plastic greenhouse in 2008 with natural photoperiod under irrigated conditions. At anthesis, ears were tagged and air temperatures were recorded every 30 min in four locations near the spikes in the greenhouse. Daily average temperatures were calculated and summed, allowing the expression of grain development in thermal time (°Cd). This process provides more precise information on the grain developmental stage than only recording days after anthesis. Developing caryopses were collected from the middle part of the ear at 180 °Cd, 300 °Cd, 500 °Cd and 750 °Cd corresponding in our conditions to respectively 11, 19, 32 and 44 daa (days after anthesis). These four stages correspond to the end of endosperm cellularization, fast starch accumulation, slow starch accumulation and maturity before desiccation, respectively. For each of the four developmental stages, three samples of 12 kernels (two kernels from six different plants) were collected, immediately frozen in liquid nitrogen and stored at −20 °C.

2.3. Determination of puroindoline content

Puroindoline content was determined by ELISA using polyclonal and specific monoclonal antibodies against PINa and PINb as previously described (Turnbull et al., 2000).

2.4. Immunolabelling and transmission electron microscopy

Samples were cut into 1 mm^3 pieces with a razor blade and fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 4 h at 4 °C and then washed several times in phosphate buffer and deionized water. They were then subjected to dehydration, resin infiltration and polymerization as reported by Dubreil et al. (1998).

For electron microscopy, 80 nm sections were prepared using an ultramicrotome (MICROM MT-7000, Oklahoma City, USA).

PINa and PINb polyclonal antibodies have already been characterized (Dubreil et al., 1998; Turnbull et al., 2000). Wheat grain sections were collected on carbon-coated 200-mesh nickel grids. Immunogold labelling and silver enhancement were performed by floating each grid on a 50 μl droplet of solution. The grids were first floated on 3% (w/v) BSA in phosphate-buffered saline (PBS 0.1 M, pH 7.2) for 30 min at room temperature to block non-specific binding sites. The sections were incubated for 1 h at room temperature with either anti-PINa or anti-PINb rabbit polyclonal antibodies diluted 1/200 and 1/100 respectively in PBS containing 1% BSA and 0.05% Tween 20. Control sections were incubated with an anti-pealegumin serum diluted 1/100 in the same solution. The sections were washed six times for 5 min with PBS, 1% BSA, 0.05% Tween 20, and were then incubated for 1 h with a 1/20 dilution of goat anti-rabbit Nanogold (1 nm) conjugated antibody (Aurion, Biovalley, France). Sections were washed three times for 5 min each in 1% BSA, 0.05% Tween 20 in PBS, and three times for 5 min each in PBS. The samples were post-fixed in 2.5% glutaraldehyde in PBS for 10 min at room temperature and rinsed three times in PBS and three times in deionized water. After incubation in Enhancement Conditioning (EC) solution, the size of the gold particles was amplified by silver enhancement for 40 min in darkness. The reaction was stopped by incubating the sections for 5 min in sodium thiosulfate (0.03 M in EC solution). After exchanges in EC solution and water, the samples were stained with uranyl acetate (2% in water) for 15 min in darkness. Finally, the grids were washed in deionized water, air-dried and stored.

Electron microscopy was carried out with a JEOL JEM 1230 transmission electron microscope running at 80 keV.

2.5. Structure prediction of puroindolines

Structure prediction was carried out using iterative threading assembly refinement (I-TASSER) server (http://zhanglab.ccmb.med.umich.edu/I-TASSER). Three-dimensional (3D) atomic models were generated from multiple threading alignments of amino acid sequences and iterative structural assembly simulations (Roy et al., 2010). Protein structures were visualized with the PyMOL molecular graphics software.

2.6. Asymmetrical flow field-flow fractionation (AFFFF)

Proteins were extracted from wholemeal flour following the protocol of Lemelin (Lemelin et al., 2005) with some modifications. Briefly, flour samples (30 mg) were dispersed in 1 ml of 0.1 M sodium phosphate buffer (pH 6.9) containing 2% (w/v) SDS and incubated at 60 °C for 15 min. The extracts, containing both soluble and insoluble storage protein polymers, were sonicated for 15 s at an amplitude setting of 30% using an Ultrasonic Processor (Bioblock Scientific, France). The fractionations were carried out using for the AFFFF an Eclipse3 F System (Wyatt Technology, Santa Barbara, CA, USA) serially connected to a UV detector (Aglient 1200, Agilent Technologies, Germany), MALS detector (Dawn multi-angulaire Heleos TM, Wyatt Technology Corporation, Europe) and an