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Changes in the starch-protein interface depending on common wheat grain hardness revealed using atomic force microscopy

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ABSTRACT

The atomic force microscope tip was used to progressively abrade the surface of non-cut starch granules embedded in the endosperm protein matrix in grain sections from wheat near-isogenic lines differing in the puroindoline b gene and thus, hardness. In the hard near-isogenic wheat lines, starch granules exhibited two distinct profiles corresponding either to abrasion in the surrounding protein layer or the starch granule. An additional profile, only identified in soft lines, revealed a marked stop in the abrasion at the protein-starch transition similar to a lipid interface playing a lubricant role. It was related to the presence of both wild-type puroindolines, already suggested to act at the starch-protein interface through their association with polar lipids.

This study revealed, for the first time, *in situ* differences in the nano-mechanical properties at the starch-protein interface in the endosperm of wheat grains depending on the puroindoline allelic status.

1. Introduction

The major part of common wheat (*Triticum aestivum* L.) grains corresponds to the starchy endosperm (80–85% of the dry grain mass) and is composed of cells filled with at least two distinct size populations of starch granules, between 8 and 30 μm, embedded in a protein matrix, mainly composed of storage proteins able to form the so-called gluten network [1]. Changes in the interface between the endosperm composing polymers are now recognized as the major factor to explain distinct hardness wheat classes [2,3]. It was found to be related to differences in the amount and allelic form, at the starch granule surface [4], of basic low-molecular weight cysteine rich proteins carrying a unique tryptophan domain [5], called Puroindoline a (PINA) and Puroindoline b (PINB), whose corresponding genes were found to be located within the main genetic locus (*Ha*) controlling hardness in the short arm of chromosome 5D [6–8]. Expression of the wild-type alleles *Pina-D1a* and *Pinb-D1a,* encoding PINA and PINB, respectively, was found to lead to a soft mechanical behavior whereas mutation or deletion in one or both of the PIN encoding genes results in a harder grain texture [9]. In Europe and North America, the most common mutation in PINB is encoded by the gene allele *Pinb-D1b* and corresponds to a single amino acid change (Gly46Ser), which leads to a hard grain phenotype [10–12].

These differences in grain mechanical resistance has a main impact on the wheat grain end-use properties as it plays a role in the energy required for starchy endosperm isolation during processing, in the obtained particle sizes and final flour yield, as well as in starch granule dissociation from the protein matrix or starch granule damage [13–17]. More recently, construction of a numerical model mimicking the endosperm granular structure showed that lowering the adhesion strength between starch granules and the protein network leads to a decrease of the starch damage as observed in soft grains [18,19].

If differences in a soft and a hard grain phenotype have been clearly linked to the presence of both of the wild-type PIN genes thanks to the use of near-isogenic lines (NIL) [20,21] and complementation of null [22] or mutated alleles [23], introduction of PIN genes in other cereals, such as durum wheat [24], rice [25] or

Abbreviations: AFM, atomic force microscopy; A_T, projected frontal abrasion area; ESEM, environmental scanning electron microscopy; F_N, normal force; F_T, tangential force; *H*, polymer hardness value; PIN, puroindoline; *N*, number of abrasive scans; n_l, number of AFM tip scan lines; NILs, near isogenic lines; *R*, tip radius; *V*, abraded volume; V_T, tip velocity; *z*, abrasion depth; z_{norm}, normalized depth.

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corn [26] lacking the corresponding genes, was moreover found to reduce grain hardness. In addition, RNAi-mediated silencing of one of the PIN genes was shown to decrease the expression of the others and led to a significant increase of grain hardness [27]. However, the mechanisms by which PINs impact on the grain texture and have a role in the starch protein interface remain unclear.

The main hypothesis suggests that interaction of PINs with the starch granule surface is mediated by polar lipids from the remnant amyloplasts [28]. This hypothesis is supported by the differences in polar (phospho- and glyco-) lipid amount found associated with starch depending on PIN composition [29–31] and by *in vitro* demonstration of the tight bonding of PINs with polar lipids [32], as well as possible insertion into the lipid bilayer [33,34]. PINs are thus, believed to impair starch-protein adhesion *via* their lipid interaction and thus, affect grain hardness, but evidence of mechanical changes at the starch granule interface was still missing. Probing the starch granule interface in the wheat endosperm from near-isogenic lines differing in hardness thus, appears interesting to study to better understand the differences in the interactions between the main endosperm polymers.

As a method to investigate both the local structure and mechanical properties [35,36], atomic force microscopy (AFM) appears appropriate to investigate the endosperm structure and hardness properties. Indeed it was used to reveal imaging differences between hard and soft endosperm structure [37] but no mechanical studies were pursued. Recently, using an AFM tip as an abrasive tool to abrade the surface of tablets made of starch or the storage protein matrix isolated from wheat grains, distinct mechanical properties of the two main endosperm components were determined and revealed relatively high respective hardness values of around 2.4 and 0.6 GPa [38]. Therefore, this original AFM method which proceeds by scratching of the surface layer-by-layer appeared the most appropriate to both explore the wheat endosperm polymer properties *in situ* and investigate the starch granule interface with the surrounding protein matrix.

2. Materials and methods

2.1. Plant material and growth conditions

NIL of *T. aestivum* L. displaying either a or b allelic forms of *Pinb-D1* encoding the wild-type or mutated (Gly46Ser) PINB protein, respectively, and thus, differing in grain hardness were described previously [39]. Genetic similarity (>97%) between the NILs was confirmed with the use of Diversity Array Technology markers [40] generated by Triticarte Pty. Ltd. (www.triticarte.com.au). Grains were harvested in summer 2008, cleaned to remove broken kernels and impurities, and then stored at 4 °C before analysis. Grain hardness was evaluated using a Percon NIRS apparatus (method 39-70A [41]) on randomly chosen grain sub-samples of each near-isogenic line and results were respectively equal to 17 for soft NIL (*Pina-D1a/PinbD1a* genotype) and 63 for hard NIL (*Pina-D1a/PinbD1b* genotype).

2.2. Sample preparation for microscopy

Wheat grain sections were obtained with a cryotome (Microm HM 520Cryostat, Thermo Scientific, USA). Grains were picked up randomly in the grain population of each near-isogenic line and cut at both ends to eliminate the germ and the brush then glued on a sample holder using a Frozen Section Medium (Neg-50, Richard-Allan Scientific, Thermo Scientific, USA). The grains were fixed from the widest part corresponding to the germ and placed into the cryotome at −28 °C before sectioning, until the glue was frozen (2 min). Sections of 50 μm were removed progressively to reach the middle of the grain. Then continuous cutting of the sample surface was performed by removing thinner sections of 5 μm. The remaining 500 μm grain sections were finally fixed on glass slides, protected with parafilm and kept under controlled temperature and humidity conditions (20 °C, 30% relative humidity) before microscopy analysis.

2.3. Environmental scanning electron microscopy (ESEM) and AFM microscopy

ESEM (Fei Quanta 200 FEG, FEI Co, Hillsboro, OR, USA) without sputter coating was used for grain section imaging.

AFM assays were performed as previously described [38] with a Nanoscope V atomic force microscope (Bruker instruments, Santa-Barbara, CAUSA), operating in the contact mode under controlled conditions of temperature and humidity (20 °C, 30% relative humidity). Commercial silicon tips (Ref. FMV, 2.8 N/m, Bruker, Camarillo, CA, USA) mounted on a rectangular cantilever with stiffness ranging between 1 and 5 N/m were chosen to preserve reasonable measurement sensitivity and to exert sufficiently large forces to abrade the samples. Before each measurement, the normal (F_N) and tangential forces (F_T) were calibrated and the contacting areas of the tips were characterized before and after calibration and at the end of AFM measurements, as described previously [38]. The contacting areas of the tips were characterized through reverse imaging obtained with AFM on a calibrating grid of equally-spaced sharp points of apex radius around 10 nm (TGT01, Mikromasch, Inc., Estonia). These measurements clearly showed that the AFM tip apex can be well fitted after calibration by a sphere from the extremity to 20 nm high, with an average radius for the set of tips $R = 82 \pm 32$ nm. This value is significantly above the initial tip radius of 10 nm due to wear off occurring during the calibration process and was checked to remain stable during abrasion assays.

AFM assays were performed on wheat grain sections after selection of a working area *i.e.*, at the surface of a small starch granule. A first topographic image (5×5 μm) was acquired at a low applied normal force ($F_N = 100$ nN) and a scan tip velocity $V_T = 10$ μm/s (512×512 pixels) and was used as the reference of the undamaged surface. The abrasion process was then initiated in the central area ($L \times L = 1 \times 1$ μm) with an increase of the applied normal force ($F_N > 200$ nN) and a decrease of the scan velocity $V_T = 2$ μm/s (256×256 pixels). Both the trace and retrace F_T force maps were acquired (respectively, scanning from left side to right side of the image and from right side to left side) to determine the average force sustained by the sample in the direction of displacement. Then, the normal force was lowered back to its initial value (100 nN) and a second topographic image (5×5 μm) was recorded at $V_T = 10$ μm/s before again increasing the normal force to further abrade the material. A progressive and controlled abrasion of the sample was ensured by repeating the abrasion step at least twenty times with a mean number of abrasion scans $N = 50$, depending on the abraded area, interrupted by regular acquisitions of larger topographic images. Large topographic images (5×5 μm) were acquired after each scan until five scans were reached and then after each group of five scans. Repetitions of the abrasion process were made on different starch granules (at least twelve) chosen in the middle of the grain cheek section from randomly selected soft or hard NIL samples. The acquired AFM images were visualized and analyzed using the Gwyddion 2.26 software (Department of Nanometrology, Czech Metrology Institute, Brno, CZ) in order to evaluate the abrasion depth and the friction force F_T. The depth increment between two successive abrasion steps was obtained by subtracting the two corresponding topographic images after careful lateral positioning through correlation techniques and sensible leveling of the height to account for instrumental drift. The evolution of abrasion depth was then determined by summing all depth