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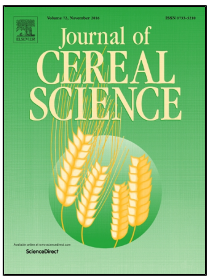

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Assessment of biochemical markers identified in wheat for monitoring barley grain tissue

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Highlights

- Para-coumaric acid is an appropriate marker of the presence of hulls in barley grains
- Alkylresorcinols are specifically located in barley grains in a composite layer including testa
- Phytic acid measurement could be used to assess the aleurone proportion in barley grains

List of abbreviations

- p-CA, para-coumaric acid
- d.m., dry matter
- FAt, dehydrotriferulic acid (8-O-4',5'-5'' form)
- AL+NE, composite isolated layer made of the aleurone layer and the residue of nucellar epidermis
- T+P, composite isolated layer made of the testa and pericarp

Abstract

The possible use of specific biochemical compounds identified in wheat grains was evaluated for monitoring barley grain tissues during fractionation. First barley grain anatomy was studied through microscopic observation and quantification of the relative proportion of each anatomical part in four distinct barley samples from both hulled and hulless genotypes. As expected from cereal phylogeny and irrespective of the possible presence of hull, common features were observed between barley and wheat grains, but the aleurone layer predominated in the outer layers. The specific location of the coumpounds identified in wheat was established. Phytic acid was specifically localized in the aleurone layer and alkylresorcinols in the composite layer containing the testa, even if their concentration differed from that observed in wheat grain tissues. Thus, these two markers identified in wheat can be used to monitor the corresponding barley tissues, independent of the presence of hulls. Conversely, phenolic compounds, either ferulic acid trimer or *p*-coumaric acid, cannot be used to monitor respectively the outer pericarp or the aleurone cell walls in barley grains. *p*-coumaric acid was identified as an efficient marker of the hull and could be used to distinguish hulled or hulless barley grains and to help monitor the dehulling process.

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1. Introduction.

Barley (*Hordeum vulgare* L.) belongs to the grass family of Poaceae, more specifically to the Triticeae tribe, like the main small cereal grains, wheat (*Triticum* spp.) and rye (*Secale cereale*). Barley is closely related to these two cereal species although it diverged approximately 11.6 million years ago and has further diversified into several subspecies (Chalupska et al., 2008). The major anatomical parts of the barley plant are typical of other members of Triticeae, in particular the kernel, which is an indehiscent fruit type, called caryopsis. In this grain, the future plant is accompanied by the endosperm comprising a storage tissue, called the starchy endosperm, and the aleurone layer. Both the embryo and the endosperm are surrounded by protective layers of maternal origin (the residue of nucellar epidermis, the testa and the pericarp). As also observed in wheat and rye, the outer layers, i.e. from the aleurone layer to the pericarp, are partly enclosed in the grain ventral side and create a crease dividing the grain in half longitudinally, but the crease is shallower in barley grains (Evers et al., 1999). The outermost layers of the barley grain were composed of structures resulting from spikelet differentiation, the palea and lemma. They are usually tightly stuck to the pericarp, which is considered desirable for selection of varieties for malting (Hoad et al., 2016). In the specific case of barley genotypes classified as hulless, this hull could be easily removed by combining threshing and cleaning of the grain.

Barley is primarily used for animal feed (60-70% of total production), then for malting and only small amounts (<1%) are used for human food (Sahlstrom and Knutsen, 2010). But today there is a growing interest in completely or partially replacing wheat with barley flour given the potential nutritional health benefits of barley grains (Baik and Ullrich, 2008). Barley grains are relatively rich in β-glucan, a soluble fibre whose ability to reduce cholesterol and blood glucose after a meal, allowed a health claim to be recently approved by the European Food Safety Authority. Moreover, like other whole cereal grains, they are rich in mineral, fibres or micronutrients. The introduction of barley as an ingredient is not straightforward and the use of barley in palatable and acceptable food products implies the use of different barley types (hulless, proanthocyanidin-free grains) or a different method

of processing the grain (Baik, 2014). Since the constituents are not evenly distributed in the grain, dry milling could be used to produce fractions rich in starch and protein, fibres or other bioactive compounds (as reviewed in Baik (2014)). Dry processing of barley grains is mainly based on abrasion/pearling in order to sequentially remove the hulls and the outer layers before possible grinding steps. If wheat milling facilities are to be used, barley grain specificities, e.g. grain hardness, bran brittleness and endosperm composition, will need to be taken into account (Baik, 2014). The rational development of efficient processes to obtain value added ingredients requires the ability to monitor the abrasion/pearling process and the fate of grain tissues, in the same way as for wheat grain (Hemery et al., 2007). This strategy relies on the identification of tissue-specific compounds whose concentration measured in mill streams could be directly linked to the proportion of a tissue, knowing their concentration in pure isolated tissues (Hemery et al., 2009). In wheat grains, phenolic compounds (a ferulic acid trimer and *p*-coumaric acid), alkylresorcinols and phytic acid have been shown to be specifically located in the outer pericarp, aleurone cell walls, testa and aleurone cell content, and were successfully used to monitor tissue distribution during traditional milling (Hemery et al., 2009; Raggiri et al., 2016), pearling process (Hemery et al., 2009) or innovative dry processing (Chen et al., 2013; Hemery et al., 2011). The close genetic relationship between barley and wheat grains and hence the similarity in grain structure and composition led to the hypothesis that the same biochemical tools previously identified for wheat grain tissue could also be used to monitor barley tissues.

The objective of this study was to evaluate the feasibility and the limits of this strategy. First, the barley (hulled or hulless) grain anatomy was studied through microscopic observation and the relative proportion of each anatomical part was measured. Second, distribution and quantification of the biochemical markers identified in wheat were studied in barley grain tissues to assess the efficiency of these markers for monitoring the barley grain tissue.

2 Experimental

2.1 Materials

Four different norwegian produced 2-rowed barley varieties were used in this study, including two hulled barley genotypes (Olve and Marigold) and two hulless barley genotypes (Pihl and Pirona). Their thousand kernel weight (TKW) was determined by weighing a 30 g-sample and counting the grains using an electronic seed counter. Results are reported as mean mass (g) of thousand kernels (ISO 108 International Standard 520:2010). At least three determinations were performed for each sample.

Barley grain tissues were isolated from these grains for chemical analyses and microscopic observations. Grains were cut to remove the germ and the brush. They were soaked in distilled water overnight to facilitate separation of the endosperm. For hulled grains, the loosely attached hulls were first removed and palea and lemma easily differentiated and separated into different samples. The crease was then removed and starchy endosperm scraped away. The aleurone layer + the residue of nucellar epidermis (AL+NE) were isolated from the testa+pericarp (T+P) layer with a scalpel. Dissected tissues were dried at 25°C over phosphorus pentoxide (P_2O_5). The tissues were then ground in liquid nitrogen with a Spex CertiPrep 6750 laboratory impact grinder and further dried before chemical analysis.

2.2 Determination of the relative amount of main tissues within barley grains

The relative proportion of the main barley grain tissues was determined through hand-isolation and gravimetric measurements according to the procedure of Raggiri et al. (2016) slightly modified. The following tissues were hand-isolated: the embryonic axis, the scutellum, the starchy endosperm, the outer layers (made of the aleurone layer, the residue of the nucellar epidermis, the testa and the pericarp), and the hull (if present). Twenty barley grains were hand dissected in quadruplicate and the recovered barley tissues were weighed after drying on P_2O_5 until a constant mass was obtained

or freeze-drying (in the case of the starchy endosperm). The relative proportion of each tissue was calculated by dividing the weight of the tissue by the weight of the initial grains (each in dry matter). The composition of the outer layers was deduced, as detailed by Barron et al. (2007), from the combined weight of AL+NE and T+P layer, dissected from outer layers especially prepared without the crease. To compare the means, Fisher's least significant difference (LSD) test was conducted at the 5% significance level.

2.3 Chemical analyses

2.3.1 Phytic acid

The phytic acid content was measured at 500 nm from acidic extracts according to a colorimetric method (Hemery et al., 2009). A standard curve was obtained with corn phytate (P-8810, Sigma) solutions of known concentrations. Samples were analysed in duplicate with a relative mean deviation < 5%.

2.3.2 Alkylresorcinols

Total alkylresorcinols were extracted with two successive extractions with n-propanol, for 2 h each at 20°C and an aliquot fraction measured through a colorimetric method (Hemery et al., 2009), using the coupling of alkylresorcinol to fast blue reagent and evaluation of the absorbance changes at 520 nm. The total amount of alkylresorcinols was estimated by comparison with a calibration curve (0-100 $\mu g.mL^{-1}$) prepared with olivetol (5-pentyl-1,3-benzediol) as the reference molecule. Samples were analysed in duplicate with a relative mean deviation < 5%.

2.3.3 Phenolic acids

Ester-linked phenolic acids were saponified under argon (oxygen-free) at 35°C in 2N sodium hydroxide. An internal standard (2,3,5-trimethoxy-(E)-cinnamic acid (TMCA), T-4002, Sigma Chemical Co., St Louis, USA) was added before adjusting the pH to 2. Phenolic acids were then extracted with

diethyl ether and quantified by RP-HPLC as described by Hemery et al. (2009). The response factors of the para-coumaric acid (*p*-CA) and the 4-O-8', 5'-5'' dehydrotriferulic acid (ferulic acid trimer, FAt) relative to the internal standard were determined at 320 nm with pure compounds. All analyses were performed at least in duplicate with a relative mean deviation < 5% (mean relative deviation of 4.3% for *p*-CA and 3.8% for FAt).

2.4 Microscope observations

Sections of barley grain tissue samples were prepared, stained and imaged as described by Hemery et al. (2011) with following exceptions: polymerized samples were sectioned (2 µm thick) in a rotary microtome HM 355S (Microm Laborgeräte GmbH, Walldorf, Germany) using a tungsten carbon knife. Protein and cell walls (β-glucan and cellulose) were stained with acid fuchsin, and calcofluor white. In exciting light (excitation, 400-410 nm; emission, > 455 nm) intact cell walls stained with calcofluor appeared blue and proteins stained with acid fuchsin appeared red, while starch was not stained and appeared black.

3 Results and discussion

3.1. Barley grain anatomy

3.1.1. Microscope observations and tissue identification of hand isolated layers

The thousand kernel weights of Olve, Marigold, Pirona and Pihl were respectively 41.7± 0.1 g, 43.5± 0.3 g, 42.9 ± 0.1 g and 44.6 ± 0.4 g. These values are relatively high for barley grains (Evers et al., 1999) but in the same range as those obtained for barley samples used for malt production (Griffey et al., 2010). No differences in kernel weights between the two hulled and hulless samples were observed as expected from previous studies (Griffey et al., 2010).

Barley grain microstructure was revealed through epifluorescence microscopy (Figure 1) using specific staining for cell wall β-glucans and proteins (Andersson et al., 1999). Observation of cross-section of the four barley grain samples revealed the usual oval shape, and the crease was more clearly visible in hulless samples (Figure 1a and 1b). The major part of the barley grains were the starchy endosperm, the germ and the outer layers. From the inside to the outside, different tissues were distinguished in the whole outer layers: first the aleurone layer with 2-3 rows of cells with thick cell walls, then the residue of nucellar epidermis, the seed coat or testa, surrounded with the pericarp and finally the hull comprising palea or lemma in the hulled samples (Figure 1c and 1d). On the basis of visual examination, the presence or absence of a hull did not have major influence on the overall thickness of each grain tissue layer.

Light microscopy examination of cross sections cut from embedded barley tissue layer fragments (Figure 2) were compared with the histological structure of the outer layers of the barley grain (Figure 1) in order to identify the tissue composition of each dissected layer.

In the hulled barley grains, lemma, the outer glume, was observed on the dorsal side of the barley grains and palea, the inner glume, on the ventral side (Figure 1a). The glumes consisted of the epidermis, layers of bast fibres, spongy parenchyma and inner epidermis (Figure 1c). These glumes were first isolated and were easily separated due to the low adhesion at the distal end of the grain, which made it possible to insert a scalpel. The lemma was recognizable thanks to the presence of five vessels, whereas only two were present in the palea (Briggs, 1978). Spongy parenchyma and inner epidermis were not intact in all the tissue fragments (Figure 2b) showing they had been damaged during the separation of the layers. Occasionally, the lemma and palea also contained a supplementary layer which was identified as the outermost layer of the pericarp (Figure 2a). The tight adherence between the lemma or palea and the pericarp had already been described in detail by Olkku et al. (2005). Accordingly, the composite layer of pericarp and testa (T+P), which was further dissected, contained in some places fragments of spongy parenchyma and inner epidermis of lemma

197 or palea (Figure 2c) or the outer layers of pericarp were partially missing. Below the testa, some
198 fragments of nucellar epidermis were sometimes present, but the nucellar epidermis was mainly
199 recovered at the outer surface of the last hand dissected layer containing the aleurone layer (Figure
200 2d). The aleurone layer was also associated with the sub-aleurone cell walls rich in β-glucan.
201 In the hulless barley grains, the outer layers were separated into two composite layers. The first
202 layer, including the most external tissues (Figure 2e) was made of the whole pericarp and the layer
203 underneath, the testa. The residue of the nucellar epidermis and the aleurone were isolated as the
204 other composite layer. Three to four rows of aleurone cells, especially rich in proteins, were observed
205 in the aleurone layer and some sub-aleurone cell walls with or without cell content from the starchy
206 endosperm still adhering to the dissected layer (Figure 2f). Strong coloration with calcofluor was
207 observed in the cell walls of starchy endosperm, indicating large amounts of β-glucan in this tissue.
208
209 To summarise, hand dissection enabled isolation of pure lemma and palea tissues (Fig. 2a and 2b)
210 from hulled barley grains. The following outer layers were divided into two composite layers. One
211 layer was made of the testa and the pericarp (called T+P in the following) and the other layer
212 comprised the aleurone layer and the residue of nucellar epidermis (called AL+NE). Some slight
213 differences were observed within barley grain samples, depending on the presence or absence of the
214 hull. In particular, the presence of the outer pericarp was not proved in the T+P layer when dissected
215 from the hulled barley samples. The separation of the outer layers during hand isolation was not
216 affected by the presence of hulls. The barley tissues did not separate in the same way as in wheat,
217 although the dissection procedure was similar (Barron et al., 2007). Still, considering the thinness of
218 the residue of nucellar epidermis in comparison with the aleurone layer, the composition of the
219 AL+NE in barley should be approximately the same as the composition of pure aleurone layer and
220 thus be comparable with pure wheat aleurone layers.
221
222 *3.1.2 Proportions of tissue within the barley grain.*

Meticulous hand dissection of barley grain under a stereomicroscope allowed the separation of pure or composite tissues whose identity was established by optical microscopy based on known barley grain anatomy. Their relative proportions were determined through hand isolation and gravimetric measurements (Table 1). The outer layers were quantified as a whole because they were too sparse to be weighed separately and because of the difficulty of isolating them in the crease. The calculated sum of the different isolated tissues (germ, endosperm, outer layers and hull in hulled samples only) ranged from 97.5% to 99.9%, i.e. negligible loss during hand dissection. Moreover, the mean relative standard deviation obtained from four independent measurements was 2.3%, indicating good repeatability and validating the data obtained. The relative proportion of the two main dissected layers, the T+P on one side, and the AL+NE on the other, was assessed in dedicated experiments, using outer layers without the crease, as also done with wheat grains (Barron et al., 2007).

The barley grains we studied mainly comprised starchy endosperm (74.1% to 84.7%), surrounded by the outer layers (11.0% to 12.8%), the hull (for hulled grains 7.7% to 8.0%), and the future plant accounted for 3.1% to 3.5% (split into the embryonic axis 2.0% and the scutellum 1.1% to 1.5%). The overall tissue composition was in agreement with the typical proportion of grain parts as reviewed by Evers et al. (1999). Total fibrous tissues (hulls plus outer layers) were in the same order of magnitude (about 20% compared to 21%). The hulled barley cultivars analysed in this study contained a small amount of hulls (8%), which is in the lowest part of the observed range (7% to 25%, Evers et al., 1999). Considering the ease of separation, the purity of the dissected hulls or outer layers in this study cannot be questioned. Moreover, even if the exact location of the outer pericarp, which was crushed during grain development (Briggs, 1978), was not easy to detect in the layers dissected in the hulled barley cultivars, its presence or absence could not explain this low amount. Instead it could be related to the barley type (2 rowed), the growing location (northern latitudes) and the kernel size (high thousand kernel weight), since all these characteristics tend to lower the hull proportions within the barley grain (Evers et al., 1999).

Interestingly, the proportion of outer layers was similar among the four barley samples, regardless of the presence of hulls. Therefore, in hulled barley grains, the relative amount of starchy endosperm is lower (74.1% to 76.3%) than in hulless grains (82.8% to 84.7%). This was not offset by the grain weight, as equivalent thousand kernel weights were measured in hulled (41.7 to 43.5g) or hulless samples (42.9 to 44.6g). The smaller quantity of starchy endosperm could explain the smaller quantity of starch in the hulled barley grains, as observed by Holtekjølen et al. (2006a) and Griffey et al. (2010) as starch is specifically located in the starchy endosperm.

Within the hull, the relative proportions of palea and lemma were similar between the two hulled barley samples. The proportion of lemma was higher than that of palea, respectively 58% compared to 42%. The proportion of the T+P and AL+NE layers in the outer layers was also observed to remain constant regardless of the barley sample, with respective values of 20% and 80%. In agreement with Evers et al. (1999), the relative proportion of the aleurone layer within the whole outer layer was therefore higher in barley grains than in wheat grains, where the value was about 45% (Barron et al., 2007; Evers et al., 1999). This could be linked to the relative thinness of the other barley grain maternal tissues, as the amount of aleurone layer in the barley grain is comparable to that in the wheat grain (Barron et al., 2007).

3.2 Location in barley grains of biochemical markers identified in wheat

The potential use of specific biochemical markers identified in wheat (*Triticum aestivum*, Hemery et al. (2009) or *Triticum durum*, Raggiri et al. (2016)) to monitor the distribution of barley grain tissue was studied. In wheat grain, phytic acid, *p*-coumaric acid, alkylresorcinols, and 4-O-8', 5'-5'' dehydrotriferulic acid (FAt) were used to monitor the aleurone cell content and cell walls, the testa and the outer pericarp, respectively. The corresponding amounts in the different layers dissected in barley are reported in Table 2. Based on the proportion of each tissue within the barley grain, the specificity of their location in barley grains is discussed.

FAt concentrations in the barley grains ranged from 0.024 to 0.038 mg.g^{-1} d.m. with the highest amounts observed in the two hulled barley varieties (Table 2). Independent of barley genotype, all the barley grain FAt originated from the whole outer layers. FAt was observed in each dissected layer, with lower values in the AL+NE layer (0.06-0.12 mg.g^{-1} d.m.) and higher in the T+P (0.45-0.93 mg.g^{-1} d.m.). Intermediate values were observed in the hulls, with similar values in lemma and palea (about 0.21 mg.g^{-1} d.m.). In the hulled barley varieties, FAt was evenly distributed in the most external part of the barley grains, from the testa to the hull, containing 70% to 88% of the barley grain's total FAt amount. Considering the mass proportion of the aleurone layer in barley grain, the amount (around 20%) of FAt originating from this tissue was not negligible. Therefore, in barley, this marker was not specific to the most external tissue as it is in wheat grain (Hemery et al., 2009). In addition, the small difference in FAt concentration between hulls and the T+P layer would also make it difficult to use it to monitor either the hulls or the outer pericarp, particular during processes such as pearling, which successively removes the most external tissues. In the hulless barley samples, about 65% to 80% of grain FAt originated from the T+P layer, including the pericarp layer, in accordance with and similar to its known location in wheat grain (Hemery et al., 2009). However, the ratio of the concentration in the AL+NE to that in the T+P layer in the hulless barley varieties (7-8) is lower than those observed between the aleurone layer and the outer pericarp (11-22) in wheat grain (Hemery et al., 2009). Thus, this marker's specificity is low in comparison with those in wheat, although this needs confirming through further analysis of other barley samples representing other genotypes, for example.

The concentration of para-coumaric acid (p-CA) measured in the hulled barley grains was between 0.21 and 0.39 mg.g^{-1} d.m., which is in agreement with results of previous studies (Andersson et al., 2008b; Hernanz et al., 2001, Holtekjølen et al., 2006b) (Table 2). However, it was 10 to 20 times lower in the hulless barley varieties (0.02 mg.g^{-1} d.m.) than in hulled ones, as also observed in Andersson et al. (2008b) and Holtekjølen et al. (2006b). This could be linked to the high amount of *p*-CA measured both in lemma and palea (between 1.8 and 4 mg.g^{-1} d.m.), as suggested by Nordkvist et

al. (1984) when they analysed pearled fractions. Hull tissues are known to be highly lignified and *p*-CA has been used as a marker of lignification in *Poaceae* plants (Grabber et al., 2004). More than 95% of the *p*-CA marker encountered in the whole outer layers of hulled barley cultivars originated from the hulls. Similar proportions of hulls in the whole outer layers were calculated using the quantification of pCA as a marker of hulls (35%) than using relative mass proportion from hand-dissected tissues (40%). In the whole hulled barley grains, about 80% of the total *p*-CA content was found in the hulls and only 2% in the AL+NE layer. Therefore, this marker should not be used to monitor aleurone cell walls in hulled barley varieties (as is done in wheat), but rather to check the presence of hulls in fractions. Further, the amount of *p*-CA measured in the hulls of the two different hulled barley varieties differed. This is in accordance with the known hydroxycinnamic acid content variability observed in barley grains (Hernanz et al., 2001; Zupfer et al., 1998). Consequently, to determine the proportion of hull using the marker methodology with the amount of *p*-CA, absolute amounts of *p*-CA in dissected hulls are necessary. In the hulless barley samples, the highest *p*-CA amount was observed either in the AL+NE layer (Pirona) or in T+P layer (Pihl). Thus, no conclusion can be drawn on the specific location of this constituent in the outer layers of hulless barley grains.

Total amounts of alkylresorcinols measured in barley grain samples ranged between 0.058 and 0.082 $mg.g^{-1}$ d.m. (Table 2), in agreement with the results previously published by Andersson et al. (2008a) and Ross et al. (2003). Analysing more specifically the dissected tissues including the testa layer, which is known to contain the alkylresorcinols in wheat or rye grain (Landberg et al., 2008), the concentration of alkylresorcinols measured in the T+P layer dissected from barley grains ranged between 1.89 and 3.60 $mg.g^{-1}$ d.m.. These values are low compared to those found in wheat (Barron et al., 2011; Landberg et al., 2008) in similar tissue, but should be seen in the context of the generally low total amount of alkylresorcinols in barley grains. Considering the measured T+P proportion within the grains (Table 1), the alkylresorcinols detected in this dissected layer account for 80% to 110% of the total alkylresorcinols measured in the whole barley grain. Therefore a specific location of

the alkylresorcinols was confirmed for barley grains even if microscopic observation was not sensitive enough to detect it (Landberg et al., 2008).

The amount of phytic acid was measured in whole barley grains and in the AL+NE dissected layer (Table 2) and ranged from between 11.9 and 18.9 $mg.g^{-1}$ d.m. and 126 to 169 $mg.g^{-1}$ d.m., respectively. These values are in the same order of magnitude as others observed for barley (Dai et al., 2010) or for wheat (Barron et al., 2011). As expected, palea, lemma and starchy endosperm do not contain any phytic acid (amount < 2 $mg.g^{-1}$ d.m.) whereas germ tissues contain part of total phytic acid (around 20-30 $mg.g^{-1}$ d.m. in the embryonic axis and 47 $mg.g^{-1}$ d.m. in the scutellum). Considering the proportion of each tissue in the whole barley grain, 82% to 88% of the total phytic acid found in the whole barley grain originated from the barley AL+NE layer (Table 3) the same as in wheat (O'Dell et al., 1972, Hemery et al., 2009). The remaining part (6.6%) originated from the barley germ. Surprisingly, a non-negligible proportion of phytic acid was also detected in the starchy endosperm of the different barley samples. However, this result was probably due to contamination during grain hand-dissection as all the soaking waters used to soften the starchy endosperm were pooled with the extracted starchy endosperm to recover the overall tissue. Nevertheless, phytic acid appeared to be a good marker to track the aleurone layer, more specifically aleurone cell content, considering its well-known location in the aleurone globoids (Jacobsen et al., 1971).

4. Conclusions

Barley grain presented a similar structure to other small cereals grains, but differed from wheat in the relative proportion of tissue within the outer layers. Indeed, the aleurone layer was found to be the main tissue in the outer layer (about 80%), and the pericarp tended to be present in lower proportion than in wheat, even in hulless barley cultivars.

The amounts of phytic acid and alkylresorcinols measured in barley grains or individual barley tissues differed from those measured in wheat. However, these two compounds were shown to be

specifically located in the aleurone layer and in the layer containing the testa, respectively, as also observed in wheat grains. Thus, these components could be used as markers to monitor the corresponding tissues during barley grain processing independently of the presence or absence of hulls. In contrast, phenolic compounds, either FAt or *p*-coumaric acid in barley, are not suitable to monitor the outer pericarp and the aleurone cell walls respectively. Interestingly, *p*-coumaric acid was identified as an efficient marker for barley grain hulls and could be used to detect its presence in barley grains and/or to monitor hull removal during processing.

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Table 1. Relative proportion of the main barley grain tissues estimated from relative dry mass.

Barley type	Cultivars	Embryonic axis	Scutellum	Outer layers (%)			Hull (%)			Starchy endosperm	Sum
		(%)	(%)	(%)	*% AL+NE**	*%T+P**	(%)	*% lemma*	*% palea*	(%)	(%)
Hulled	Marigold	2.0 (±0.03) [a]	1.1 (±0.01) [a]	11.0 (±0.6) [a]	*81.9*	*18.1*	7.7 (±0.2)	*58.3*	*41.7*	76.3 (±0.9) [b]	98
	Olve	2.0 (±0.05) [a]	1.1 (±0.03) [a]	12.3 (±0.5) [b,c]	*80.8*	*19.2*	8.0 (±0.1)	*57.8*	*42.2*	74.1 (±0.9) [a]	97.5
Hulless	Pihl	2.0 (±0.01) [a]	1.5 (±0.1) [c]	11.7 (±0.3) [a,b]	*78.5*	*21.5*				84.7 (±0.3) [d]	99.9
	Pirona	2.0 (±0.04) [a]	1.3 (±0.03) [b]	12.8 (±0.5) [c]	*80.0*	*20.0*				82.8 (±0.9) [c]	99

* determined on dissected outer layers without the crease. AL+NE: aleurone layer and the residue of nucellar epidermis; T+P : testa and pericarp, Means in the same column with the same letter are not significantly different ($p > 0.05$) on the basis of Fisher's least significant difference (LSD) test.

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Table 2. 4-O-8', 5'-5'' dehydrotriferulic (FAt), *p*-coumaric acid (p-CA), alkylresorcinols and phytic acid concentrations in grains and tissues of four barley samples (values in $mg.g^{-1}$ d.m.).

Barley type	Cultivar	Grain or tissue	FAt	p-CA	Alkylresorcinols	Phytic acid
Hulled	Marigold	grains	0.037	0.394	0.066	11.9
		AL+NE	0.118	0.071	-	133.0
		T+P	0.454	0.084	3.448	-
		lemma	0.206	4.143	-	0.6
		palea	0.238	3.944	-	0.2
		Embryonic axis	-	-	-	20.0
		Scutellum	-	-	-	47.2
		Starchy endosperm	-	-	-	1.2
	Olve	grains	0.038	0.214	0.075	14.6
		AL+NE	0.066	0.048	-	126.8
		T+P	0.627	0.075	3.601	-
		lemma	0.213	2.208	-	1.2
		palea	0.236	1.806	-	1.0
		Embryonic axis	-	-	-	20.9
		Scutellum	-	-	-	47.7
		Starchy endosperm	-	-	-	2.3
Hulless	Pirona	Grains	0.024	0.021	0.058	18.9
		AL+NE	0.086	0.217	-	145.4
		T+P	0.616	0.060	1.886	-
		Embryonic axis	-	-	-	30.8
		Scutellum	-	-	-	42.0
		Starchy endosperm	-	-	-	1.99
	Pihl	grains	0.029	0.016	0.082	16.7
		AL+NE	0.114	0.077	-	169.1
		T+P	0.934	0.137	2.636	-
		Embryonic axis	-	-	-	20.8
		Scutellum	-	-	-	46.6
		Starchy endosperm	-	-	-	1.1

(-) not determined

Table 3. Proportion (%) of grain phytic acid located in the different tissues of four barley samples.

Barley type	Barley cultivars	Hand-isolated tissues	% of grain phytic acid present in the indicated tissue
Hulled	Marigold	AL+NE	86.4
		Embryonic axis	2.9
		Scutellum	3.7
		Starchy endosperm	6.8
		Palea	0.0
		Lemma	0.2
	Olve	AL+NE	82.0
		Embryonic axis	2.7
		Scutellum	3.4
		Starchy endosperm	11.2
		Palea	0.2
		Lemma	0.4
Hulless	Pirona	AL+NE	84.1
		Embryonic axis	3.5
		Scutellum	3.1
		Starchy endosperm	9.3
	Pihl	AL+NE	88.4
		Embryonic axis	2.4
		Scutellum	4.0
		Starchy endosperm	5.3

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Figure captions

Figure 1. Microstructural comparison of hulled and hulless barley grains. Cross sections of a whole (a) hulled barley grain (cv. Olve) showing outer and inner glume or lemma (L) and palea (P), and (b) hulless barley grain (cv. Pirona). Peripheral grain layers of (c) hulled barley grain (cv. Marigold) and hulless barley grain (cv. Pirona) including hull (E epidermis, Bf bast fibres, Sp spongy parenchyma, Ie inner epidermis), pericarp (H hypodermis, Cc cross cells), testa and endosperm (A aleurone, Sa sub-aleurone, Se starchy endosperm). Sections were stained with acid fuchsin and calcofluor showing protein red and β-glucan bright blue, respectively. The pericarp and hull appear greenish yellow due to autofluorescence.

Figure 2. Cross section of each hand-isolated layer of tissue of a hulled (cv. Marigold) and hulless (cv. Pihl) barley grain stained with acid fuchsin and calcofluor. Hulled grain enabled us to obtain (a) lemma, (b) palea, (c) a composite layer of pericarp and testa (T+P), and (d) aleurone with the residue of nucellar epidermis and some cell walls of subaleurone (AL+NE). The tissues separated from hulless grain were respectively (e) a composite layer of pericarp and testa (T+P), and (f) aleurone with the residues of nucellar epidermis and some cell walls of sub-aleurone (AL+NE). Please see Fig. 1 for explanations concerning the colours.

Figure 1

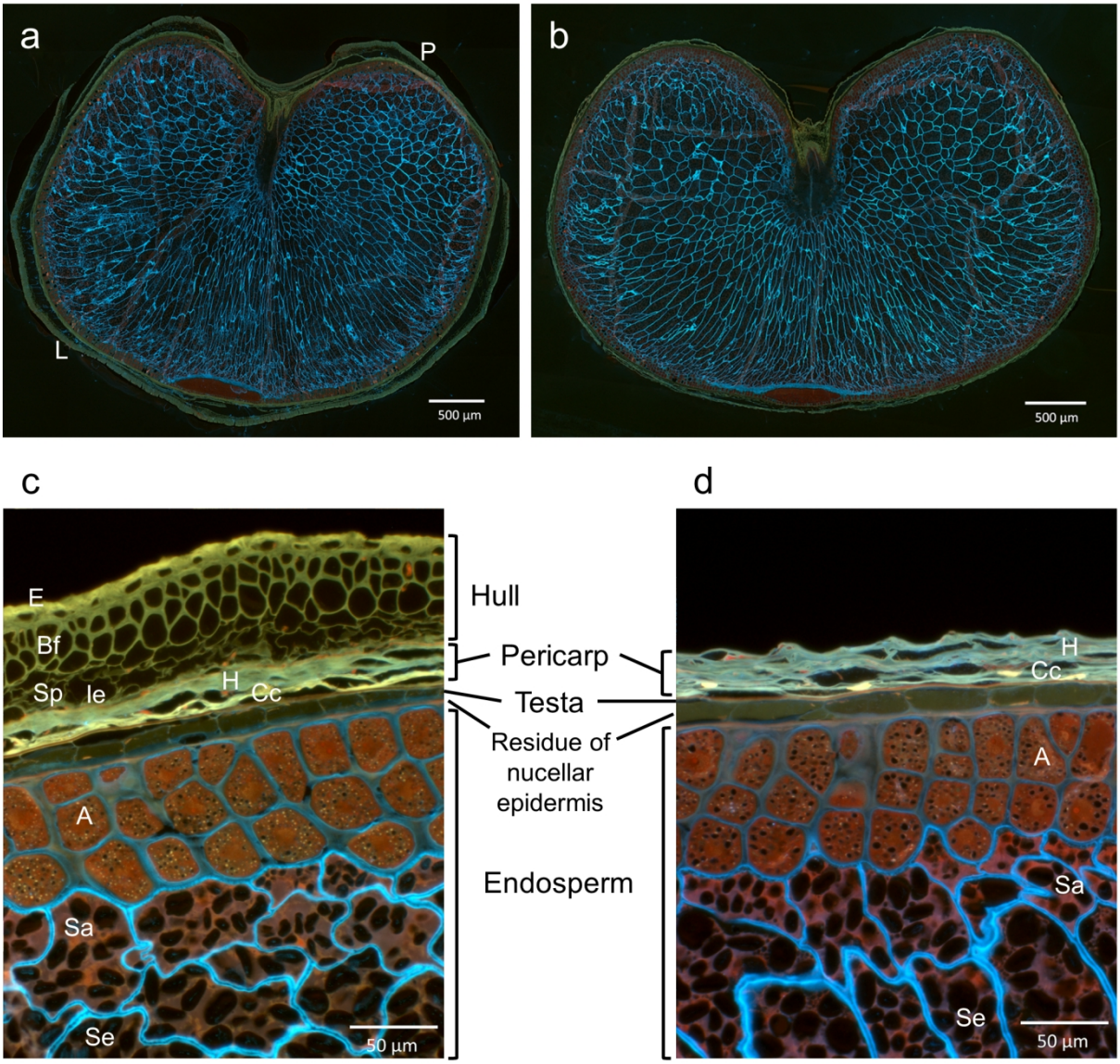

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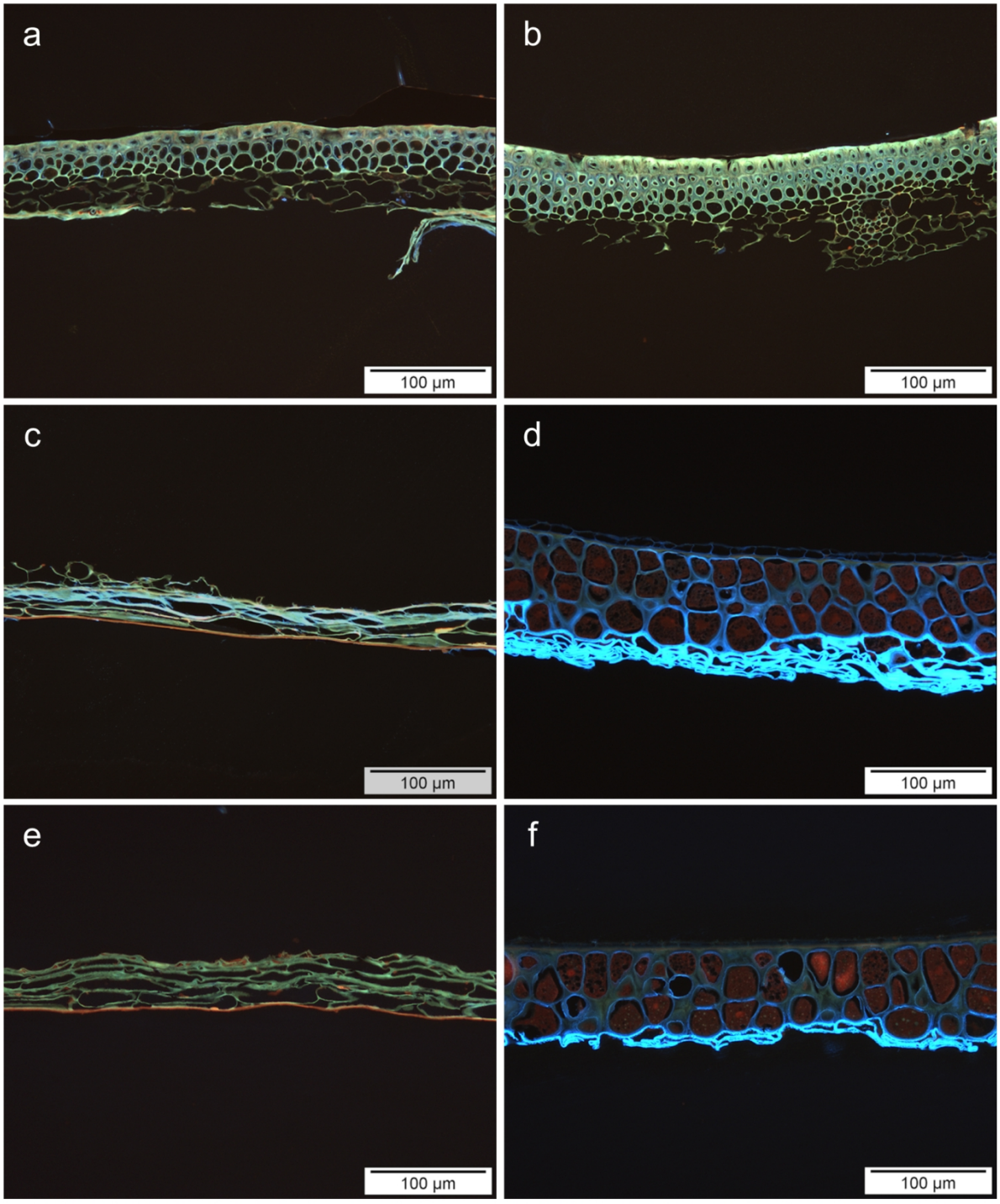
Figure 2



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