Current potential and limitations of immunolabeling in cereal grain research

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Abstract

Immunolabeling techniques have made a valuable contribution to cereal grain research during the past decade in terms of precise localization of specific compounds. While these techniques have several limitations, such as the availability and specificity of the antibodies, immunolabeling has proven especially useful in cereal studies seeking a better understanding of grain development and characterization. According to the literature reviewed in this paper, immunolabeling techniques will continue to be a useful tool in the characterization and localization of cereal grain components.

Keywords
Microscopy; grain development; storage protein; antibody

1. Introduction

Immunolabeling has emerged as a powerful investigative tool to localize specific cell components *in situ* within the complexity of cereal tissues and to integrate tissue-based analysis with proteomic information. Immunolabeling is a method for qualitative or quantitative determination of the presence of a target in a sample, where antibodies are utilized for their specific binding capacity. The antibodies form a complex with the target (antigen), with a detectable label being present on the antibody or on a secondary antibody. The label is a microscopically dense marker that provides a measurable signal by which the binding reaction is monitored, providing a qualitative and/or quantitative measure of the degree of binding. The relative quantity and location of signal generated by the labeled antibodies can serve to indicate the location and/or concentration of the target. The principal differences in immunolabeling methods and materials reside in the type of antibodies generated against the epitopes (monoclonal, polyclonal), the way that the label is attached to the antibody-antigen complex (direct, indirect), the type of label used (e.g., particles such as colloidal gold, fluorescent or phosphorescent compounds, and enzymes such as peroxidase or alkaline phosphatase), and the means by which the antibody-antigen complex is detected (e.g., electron microscopy, light microscopy or fluorescence microscopy).

Both direct and indirect antibody labeling are used for immunolabeling. Direct labeling utilizes only a primary antibody, which is specific for the target and is already bound to the label (Fig. 1a). This simplifies the staining procedure and provides minimal nonspecific staining and less background. Additionally, the direct labeling technique allows the use of two or more primary antibodies of the same species, avoiding the problems with secondary antibody staining. However, each different primary antibody must be tagged, which requires an abundant supply of purified antibody, and the resulting signal is weak since only one labeled primary antibody binds to each antigen. These are the main reasons why, despite the advantages of direct immunolabeling, the indirect approach is more commonly used instead. Indirect immunolabeling involves a multi-step process in which a secondary antibody bound to the label and raised against the γ globulin of the primary species is used, e.g. a goat anti-mouse antibody (Fig. 1b). Several labeled secondary antibodies can bind to each primary antibody and therefore the signal is amplified.

To date, no examples of direct labeling can be found in cereal studies. However, some alternative techniques, such as labeled enzymatic probes, have been developed for *in situ* cell wall analysis and are based on the same philosophy as direct immunolabeling (Dornez, Holopainen, et al., 2011; Wilson, et al., 2006).

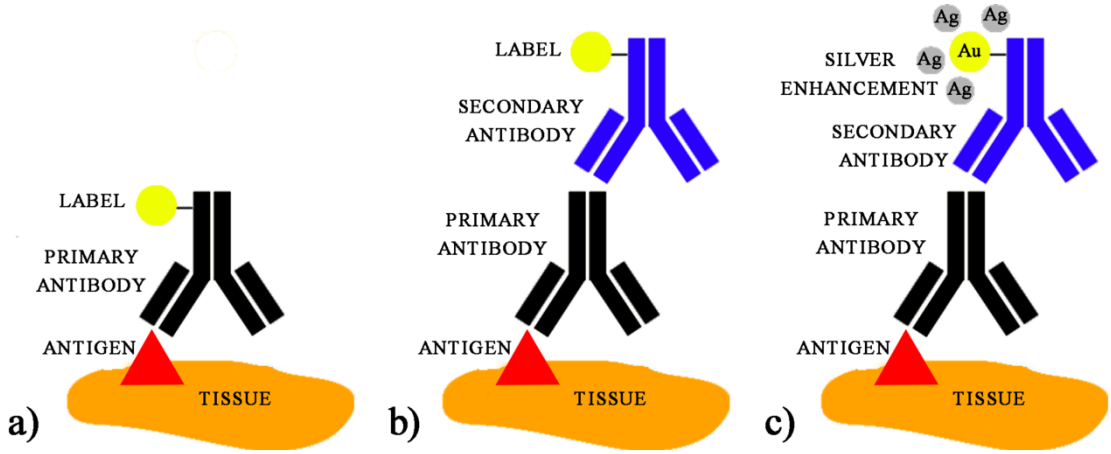

Fig. 1. Schematic representation of immunolabeling mechanisms. a) Direct labeling. b) Indirect labeling; c) Silver-enhanced indirect immunogold labeling.

In most cereal studies, immunogold and immunofluorescence techniques are mainly applied. Figure 2 shows examples of the application of different immunolabeling techniques to localize gluten proteins in wheat endosperm. In the case of immunogold labeling, the cell components are detected using secondary antibodies tagged with electron-dense colloidal gold particles that can be observed by transmission electron microscopy (TEM). The size of the colloidal gold marker bound to the secondary antibody can also be increased using silver enhancement technology (Fig. 1c), so that it can be detected at magnifications appropriate for accurate identification of the labeled compounds in the scanning electron microscope (Mills, et al., 2005) (Fig. 2a), in the light microscope (Van Herpen, et al., 2008) (Fig. 2b) or by confocal laser scanning microscopy (CLSM) (Galuszka, et al., 2005). Silver enhancement is also necessary when the gold particles bound to the secondary antibody are too small to be detected even by TEM (Lesage, et al., 2011). Observation of highly contrasted labeling by light microscopy is also possible using an epipolarization system in which gold particles are visualized as bright spots (Beaugrand, et al., 2005).

In the immunofluorescent labeling technique, fluorochrome-labeled secondary antibodies are used to tag the primary antibodies (Fig. 2c). Immunofluorescent labeled cells are analyzed using a conventional fluorescence microscope or by CLSM, which provides images with high resolution and a 3D reconstruction with a minimum of background noise.

Double immunolabeling allows the detection of two different compounds in the same sample (e.g., two different storage proteins of the grain endosperm) by using two different fluorochrome-conjugated antibodies (Furukawa, et al., 2003; Saulnier, Guillon, Sado, & Rouau, 2007; Washida, et al., 2009) or two antibodies conjugated to gold particles of different sizes (Holding, et al., 2007; Loussert, Popineau, & Mangavel, 2008) (Fig. 2c and d).

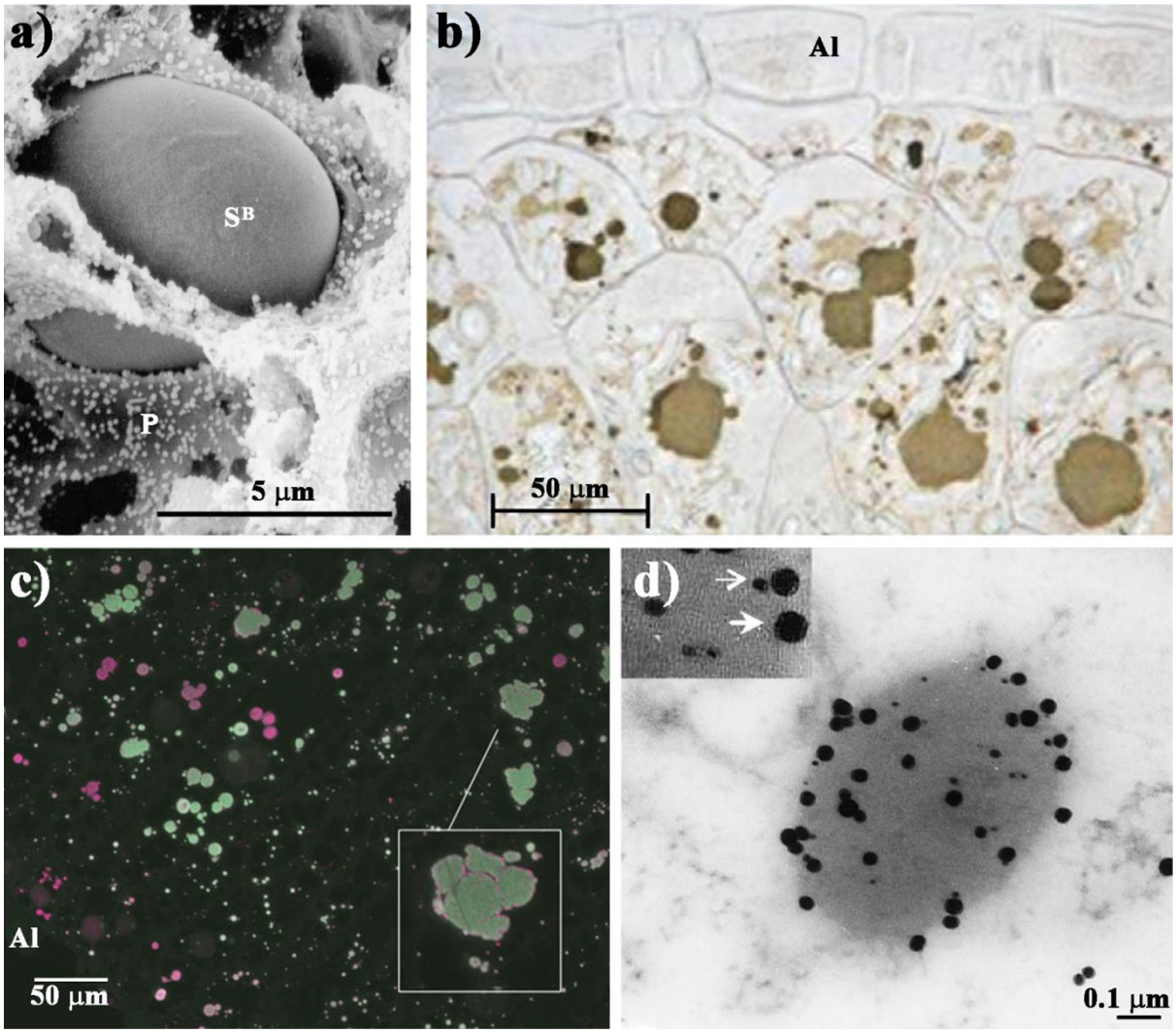

Fig. 2. Immunolocalization of gluten proteins in wheat grain endosperm using different immunolabeling techniques. a) Silver-enhanced immunogold labeling using an anti-gliadin monoclonal antibody observed by scanning electron microscopy in mature starchy endosperm cells. S^B: small starch granule; P: protein matrix (Mills, et al., 2005)[1]; b) Silver-enhanced immunogold labeling using anti-α-gliadin-specific antibodies observed by light microscopy, 18 days after anthesis (daa) (Van Herpen, et al., 2008)[2]; c) Immunofluorescence double labelling of α-gliadin (magenta) and low-molecular weight (LMW) glutenin, 20 daa. (Tosi, et al., 2009)[3]; d) Double immunogold labeling observed by transmission electron microscopy. Small gold particles (empty arrows) were labeled with a monoclonal anti-α/β-gliadin

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[2] Reprinted from Van Herpen, T.W.J.M. et al., Detailed analysis of the expression of an alpha-gliadin promoter and the deposition of alpha-gliadin protein during wheat grain development, Annals of Botany, 2008, 102, pp. 331-342, by permission of Oxford University Press.
[3] Reprinted from Tosi, P. et al., Trafficking of storage proteins in developing grain of wheat, Journal of Experimental Botany, 2009, 60, 3, pp. 979-991, by permission of Oxford University Press.

antibody. Large gold (full arrows) particles were labeled with a polyclonal anti-LMW glutenin subunit antibody, 15 dAA (Loussert, et al., 2008)[4].

In addition to immunogold and immunofluorescence labeling, several studies have been carried out using peroxidase- or alkaline phosphatase-linked detection systems (Chen, Chyan, Jiang, Chen, & Tzen, 2012; Wiley, et al., 2007).

Immunological tissue printing is a simple new technique that consists of transferring cellular material from the freshly cut surface of tissues to appropriate substrate material such as nitrocellulose membranes. The location of proteins, nucleic acids, carbohydrates, and small molecules in a tissue-specific mode can be identified with this technique. Plant tissues can be used to produce prints revealing a remarkable amount of anatomical detail, even without staining, which can be useful in recording developmental changes over time. This technique has been used, for instance, to localize puroindolines in starchy endosperm cells of wheat grain (Wiley, et al., 2007).

Immunolabeling techniques also allow quantitative analyses of compounds by means of quantification of the labeling, usually expressed as the density of gold particles (number of gold particles/μm^2). When immunofluorescence is applied, the fluorescence intensity can be quantified by image analysis. Ohdaira, et al. (2011) clarified the location and quantity of 13 kDa prolamin and 23 kDa glutelin in different rice cultivars by evaluating relative distance from the outer surface and relative fluorescence intensity, respectively.

The main applications of immunolabeling in grain characterization studies during the past decade are shown in Figure 3. These applications, together with an analysis of the strengths and limitations of this technique in the field of cereal research, are discussed below.

[4] Reprinted from Journal of Food Science, 47, Loussert, C. et al., Protein bodies ontogeny and localization of prolamin components in the developing endosperm of wheat caryopses, pp. 445-456. Copyright 2008, with permission from Elsevier.

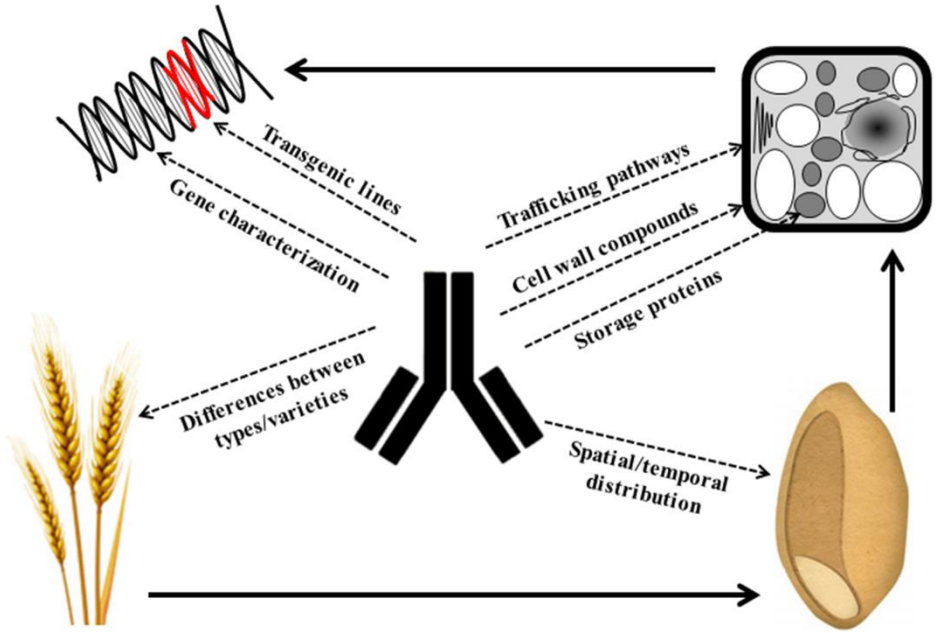

Fig. 3. Schematic representation of the main applications of immunolabeling on cereal grain studies during the last decade.

2. Localization and characterization of grain components

Combining the specificity of antibodies with topographical information obtained using microscopy techniques provides information on the distribution of components within cereal grains.

Investigation of plant cells using nondestructive approaches such as immunolabeling has already provided specific information about the distribution of compounds at ultrastructural levels. According to results produced in previous and recent immunolabeling studies on cereals, cell wall polysaccharides and storage proteins have been the main focus (Table 1). Moreover, specific antibodies against other proteins, such as puroindolines (Capparelli, et al., 2005), and phenolic compounds, such as p-coumaric acids (Tranquet, Saulnier, Utille, Ralph, & Guillon, 2009) have been developed.

2.1. Cell walls

For a better understanding of the distribution and functional roles of cell wall polymers, antibodies are required to analyze individual components *in situ* in relation to intact cell wall architecture. Monoclonal antibodies are specific and sensitive reagents that have been used to show that cell wall composition varies in the walls surrounding a single cell, amongst cell types, and during grain development. Frozen, fractured grains are particularly useful for immunolabeling studies, because the aleurone layer and outer (sub-aleurone) and inner endosperm can be clearly identified on the fracture face (Mills, et al., 2005).

Cereal endosperm cell walls are essentially composed of arabinoxylans (AX) and (1→3),(1→4)-β-D-glucans or mixed-linked glucans with minor amounts of cellulose and mannans. Immunolabeling techniques have been applied to improve understanding of the structure and

properties of cereal cell wall polysaccharides due to their significant commercial value in the end-use of cereal grains and their impact on the nutritional quality of cereal foods as a major constituent of dietary fiber (Saulnier, Guillon, et al., 2007).

The number of antibodies directed against cell wall epitopes has grown steadily over the years. However, until the past decade few antibodies against the cell wall polysaccharide components of the grain had been developed. Polyclonal and monoclonal antibodies had been generated against (1→3),(1→4)-β-D-glucans and arabinogalactan proteins (AGP), whereas only polyclonal antibodies had been produced against AX, p-coumaric acid, or feruloyl-arabinose. Some monoclonal antibodies had also been produced against galactomannan, xyloglucan, homogalacturonan and hydroxyproline-rich glycoprotein (Guillon, et al., 2004; Willats & Knox, 2003).

Despite this development in antibody production, until recently only a fraction of the molecular structures that make up cell walls were known and understanding of cell wall components was limited. In the past decade, great efforts have been invested in developing antibodies against specific cell wall components. Polyclonal and monoclonal antibodies against different motifs encountered in xylans and AX have now been designed (Guillon, et al., 2004; McCartney, Marcus, & Knox, 2005; Ordaz-Ortiz, et al., 2004). Monoclonal antibodies against (1→3),(1→4)-β-D-glucans (Rampitsch, Ames, Storsley, & Marien, 2003), xyloglucans (Marcus, et al., 2008), pectic homogalacturonan (Clausen, Willats, & Knox, 2003; Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, & Knox, 2009), xylogalacturonan (Willats, et al., 2004) and p-coumaric acid (Tranquet, et al., 2009), and polyclonal antibodies against ferulic acid (Philippe, Tranquet, Utille, Saulnier, & Guillon, 2007) have been generated and characterized. Pattathil, et al. (2010) described a comprehensive toolkit of approximately 180 plant cell wall-directed monoclonal antibodies, of which approximately 130 were newly generated. This represents an invaluable increase in the global collection of cell wall glycan-directed monoclonal antibodies and has increased the number of detailed studies on the structure, dynamics, function, and biosynthesis of cell walls in cereals.

Immunolabeling has proven to be very useful in studying the structure and composition of the starchy endosperm and the aleurone of cereal grains (Table 1). However, the outer layers (bran without aleurone), transfer cells, and the embryo (germ) remain less well characterized (Beaugrand, et al., 2005; Chen, et al., 2012; Jerkovic, et al., 2010; Robert, et al., 2011).

Table 1. Detection of cereal grain components by using immunolabeling techniques.

Part of grain	Compound	Crop & References
Outer layers / Bran without aleurone	Arabinoxylan	Wheat (Beaugrand et al., 2005; Beaugrand, Reis, et al., 2004; Dornez, Holopainen, et al., 2011; Philippe et al., 2006; Philippe et al., 2007); oat, rye, barley (Dornez, Holopainen, et al., 2011)
	(1→3),(1→4)-β-D-glucan	Wheat (Philippe et al., 2006)
	Callose	Wheat (Philippe et al., 2006)
	Ferulic acid	Wheat (Philippe et al., 2007)
	Xylanase	Wheat (Beaugrand, Cronier, et al., 2004; Beaugrand et al., 2005; Beaugrand, Reis, et al., 2004)
	Oxalate oxidase	Wheat (Jerkovic et al., 2010)
	Xylanase inhibitor I protein	Wheat (Jerkovic et al., 2010)
	Wheatwin1	Wheat (Jerkovic et al., 2010)
	Cytokinin dehydrogenase	Maize (Galuszka et al., 2005)
Aleurone	Arabinoxylan	Wheat (Beaugrand et al., 2005; Beaugrand, Reis, et al., 2004; Dornez, Holopainen, et al., 2011; Guillon et al., 2004; Lovegrove et al., 2013; Philippe et al., 2006; Philippe et al., 2007; Robert et al., 2011; Saulnier, Guillon, et al., 2007; Suliman et al., 2013); oat, rye (Dornez, Holopainen, et al., 2011); barley (Dornez, Holopainen, et al., 2011; Wilson et al., 2012)
	Xylan	Wheat (Lovegrove et al., 2013)
	Xyloglucan	Barley (Wilson et al., 2012)
	(1→3),(1→4)-β-D-glucan	Wheat (Guillon et al., 2004; Philippe et al., 2006; Robert et al., 2011; Saulnier, Guillon, et al., 2007); barley (Wilson et al., 2012)
	Callose	Wheat (Philippe et al., 2006); barley (Wilson et al., 2012)
	Mannan	Barley (Wilson et al., 2012)
	Ferulic acid	Wheat (Philippe et al., 2007; Robert et al., 2011)
	p-Coumaric acid	Wheat (Robert et al., 2011; Tranquet et al., 2009)
	Xylanase	Wheat (Beaugrand, Cronier, et al., 2004; Beaugrand et al., 2005; Beaugrand, Reis, et al., 2004)
	Glycosyltransferase	Wheat (Suliman et al., 2013)
	Oxalate oxidase	Wheat (Jerkovic et al., 2010)
	Xylanase inhibitor I protein	Wheat (Jerkovic et al., 2010)
	Wheatwin1	Wheat (Jerkovic et al., 2010)
	Cytokinin dehydrogenase	Maize (Galuszka et al., 2005)
	Prolamin	Wheat (Gil-Humanes et al., 2011); maize (Reyes et al., 2011); rice (Saito et al., 2009)
	Globulin	Wheat (Van Herpen et al., 2008; Wiley et al., 2007)
	Oleosin / Caleosin	Rice (Chen et al., 2012); oat (Heneen et al., 2008)
	Puroindolines	Wheat (Capparelli et al., 2005)
Transfer cells	Arabinoxylan	Wheat (Dornez, Cuyvers, et al., 2011; Dornez, Holopainen, et al., 2011; Lovegrove et al., 2013; Robert et al., 2011); oat, rye, barley (Dornez, Holopainen, et al., 2011)

Part of grain	Compound	Crop & References
	Xylan	Wheat (Lovegrove et al., 2013)
	(1→3),(1→4)-β-D-glucan	Wheat (Robert et al., 2011)
	Ferulic acid	Wheat (Robert et al., 2011)
	Cell wall invertase	Maize (Kang et al., 2009)
Starchy endosperm	Arabinoxylan	Wheat (Dornez, Cuyvers, et al., 2011; Dornez, Holopainen, et al., 2011; Guillon et al., 2004; Lovegrove et al., 2013; Philippe et al., 2006; Philippe et al., 2007); oat, rye (Dornez, Holopainen, et al., 2011); barley (Dornez, Holopainen, et al., 2011; Wilson et al., 2006; Wilson et al., 2012)
	Xylan	Wheat (Lovegrove et al., 2013)
	Xyloglucan	Wheat (Pellny et al., 2012); barley (Dwivany et al., 2009; Wilson et al., 2012)
	(1→3),(1→4)-β-D-glucan	Wheat (Guillon et al., 2004; Philippe et al., 2006); barley (Wilson et al., 2006; Wilson et al., 2012); maize (Carpita and McCann, 2010)
	Callose	Wheat (Pellny et al., 2012; Philippe et al., 2006); barley (Wilson et al., 2006; Wilson et al., 2012)
	Mannan	Wheat (Pellny et al., 2012); barley (Wilson et al., 2006; Wilson et al., 2012)
	Cellulose	Barley (Wilson et al., 2006)
	Ferulic acid	Wheat (Philippe et al., 2007)
	Arabinogalactan proteins	Barley (Wilson et al., 2006)
	Prolamin	Wheat (Gil-Humanes et al., 2011; Loussert et al., 2008; Mills et al., 2005; Tosi et al., 2011; Tosi et al., 2009; Van Herpen et al., 2008); rice (Furukawa et al., 2003; Nagamine et al., 2011; Ohdaira et al., 2011; Saito et al., 2009; Saito et al., 2008; Takahashi et al., 2005; Tian et al., 2013; Washida et al., 2009; Yasuda et al., 2009); maize (Arcalis et al., 2010; Chikwamba et al., 2003; Holding et al., 2007)
	Globulin	Wheat (Arcalis et al., 2004); rice (Takahashi et al., 2005; Tian et al., 2013); maize (Arcalis et al., 2010)
	Glutelin	Wheat (Arcalis et al., 2004; Wang et al., 2013); rice (Hennegan et al., 2005; Kawagoe et al., 2005; Takahashi et al., 2005; Tian et al., 2013; Washida et al., 2009)
	Albumins	Wheat (Arcalis et al., 2004); rice (Washida et al., 2009)
	Oleosin / Caleosin	Oat (Heneen et al., 2008)
	Puroindolines	Wheat (Capparelli et al., 2005; Lesage et al., 2011; Wiley et al., 2007)
	Tryptophanin	Oat (Mohammadi et al., 2007)
	Binding protein (BiP)	Rice (Takahashi et al., 2005)
Embryo and scutellum	Dehydrin	Quinoa (Carjuzaa et al., 2008)
	Oleosin / Caleosin	Rice (Chen et al., 2012); oat (Heneen et al., 2008); barley (Liu et al., 2005)
	Prolamin	Rice (Ohdaira et al., 2011)
	Cytokinin dehydrogenase	Maize (Galuszka et al., 2005)

Specific antibodies have been applied to determine the spatial and temporal distribution of cell wall polysaccharides in different development stages of wheat endosperm (Philippe, et al., 2006) and barley endosperm (Wilson, et al., 2006). Observation of immunogold-labeled cell walls by TEM has been particularly useful in these studies. Some examples of micrographs obtained with this technique are shown in Figure 4.

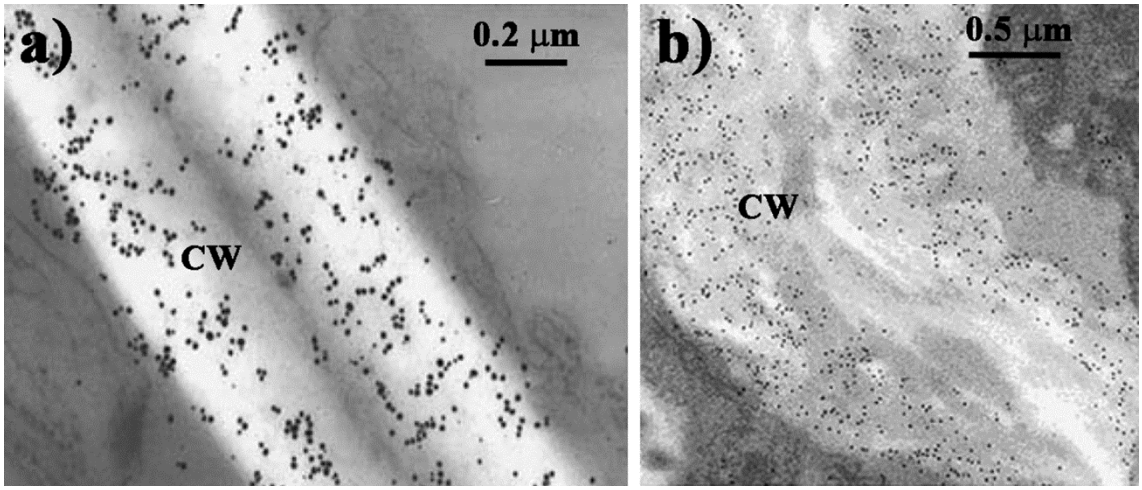

Fig. 4. Transmission electron micrographs of cereal endosperm cell wall immunogold-labeled with anti-carbohydrate antibodies. a) (1→3),(1→4)-β-D-glucans in cell walls in central region of wheat starchy endosperm at 446 degree-days (temperature accumulated daily from anthesis) (Philippe, et al., 2006)[5]; b) Arabinoxylan antibody (LM11) labeling in barley endosperm 8 days after pollination: cell walls near the crease were strongly labeled (S. M. Wilson, et al., 2006)[6]. CW: cell wall.

The application of immunolabeling techniques has been useful in identifying significant variations in the amount and structure of non-starch polysaccharides depending on cereal type (Dornez, Holopainen, et al., 2011), tissues (Dornez, Holopainen, et al., 2011; Guillon, et al., 2004; Philippe, et al., 2007; Zhang, et al., 2011), and stages of grain development (Pellny, et al., 2012; Philippe, et al., 2006; Wilson, et al., 2006). Understanding the meaning of such variability and identifying genes controlling the level and structure of cell wall polysaccharides are two key challenges for the development of new cultivars with enhanced nutritional and technological properties (Saulnier, Sado, Branlard, Charmet, & Guillon, 2007). Guillon, et al. (2004) generated and characterized monoclonal and polyclonal antibodies against AX and were able to detect variations in structural features of AX, such as the degree of xylose substitution by arabinose within the wheat grain according to cell type. The same antibodies have helped to provide a better

[5] Reprinted from Planta, vol. 224, 2006, pp. 456, Arabinoxylan and (1→3),(1→4)-β-glucan deposition in cell walls during wheat endosperm development, Philippe et al., Fig. 6f, copyright 2006, with kind permission from Springer Science and Business Media.

[6] Reprinted from Planta, vol. 224, 2006, pp. 664, Temporal and spatial appearance of wall polysaccharides during cellularization of barley (*Hordeum vulgare*) endosperm, Wilson et al., Fig. 11c, copyright 2006, with kind permission from Springer Science and Business Media.

understanding of the AX biosynthesis process (Philippe, et al., 2006; Suliman, et al., 2013) and the role of AX substitution in the regulation of water movement within the endosperm (Robert, et al., 2011). Two additional monoclonal antibodies against different epitopes of the xylan backbone, named LM10 and LM11, have been developed recently. LM10 is specific to unsubstituted or low-substituted xylans, whereas LM11 binds to wheat AX in addition to unsubstituted xylans (McCartney, et al., 2005). Consequently, LM11 has proven useful in localizing AX in different cereal grains (Dornez, Holopainen, et al., 2011; Wilson, et al., 2012; Wilson, et al., 2006). Localization of poorly substituted AX has also been achieved by means of anti-(1→4)-β-unsubstituted xylan antiserum (Beaugrand, et al., 2005; Beaugrand, Reis, et al., 2004). Using immunolabeling, AX has been detected in the outer layers, aleurone, starchy endosperm, and transfer cells of different cereals (Table 1).

The distribution of (1→3),(1→4)-β-D-glucans in the cell walls of mature wheat grains has been investigated using immunofluorescence (Dornez, Holopainen, et al., 2011; Guillon, et al., 2004; Philippe, et al., 2006; Robert, et al., 2011) and immunogold electron microscopy (Guillon, et al., 2004; Philippe, et al., 2006), using a specific monoclonal antibody. The location of (1→3),(1→4)-β-D-glucans has also been identified by immunolabeling in other cereals such as barley (Dornez, Holopainen, et al., 2011; Wilson, et al., 2006), maize (Carpita & McCann, 2010), oat (Dornez, Holopainen, et al., 2011) and rye (Dornez, Holopainen, et al., 2011). In addition to their localization, a better understanding of (1→3),(1→4)-β-D-glucan biosynthesis (Carpita & McCann, 2010; Robert, et al., 2011; Wilson, et al., 2006) and function (Guillon, et al., 2004; Robert, et al., 2011) has been achieved by immunolabeling.

The immunolabeling of AX, (1→3),(1→4)-β-D-glucans, and other minor polysaccharides in cereal cell walls, such as xyloglucans, mannans, callose, and cellulose, has allowed monitoring of cell wall formation in the endosperm of developing wheat (Pellny, et al., 2012; Philippe, et al., 2006) and barley (Wilson, et al., 2006), in order to define the temporal and spatial pattern of deposition of cell wall components. In combination with quantitative (q)-PCR or quantitative reverse transcription (qRT)-PCR analyses, it has also helped to identify candidate genes responsible for cell wall composition (Pellny, et al., 2012; Wilson, et al., 2012). Wilson, et al. (2006) shed some light on the sequence of deposition of individual cell wall polysaccharides in developing barley endosperm by immunolabeling of polysaccharides, which showed initial deposition of callose and cellulose, followed by (1→3),(1→4)-β-D-glucans, hetero-(1→4)-β-mannans, AGP, and AX. Similar sequencing in wheat endosperm for callose, (1→3),(1→4)-β-D-glucan, and AX has been reported using immunolabeling (Philippe, et al., 2006).

In contrast to the aleurone layer and the starchy endosperm, other parts of the grain such as the transfer cells, which are involved in nutrient transfer from the maternal tissues to the developing endosperm, have not been extensively studied in terms of structure and composition. Robert, et al. (2011) focused on characterization of the polysaccharides (1→3),(1→4)-β-D-glucans and AX and on the aromatic composition of transfer cell walls of wheat by immunolabeling, and Fourier transform infrared (FT-IR) and Raman micro-spectroscopy. However, AX and (1→3),(1→4)-β-

D-glucans were not stained in the transfer cells when four different grain cell wall staining techniques, including immunolabeling were applied in a different study on wheat, barley, oat, and rye (Dornez, Holopainen, et al., 2011).

A better understanding of the outer layers of cereal grain has been obtained in the past few years (Beaugrand, Cronier, et al., 2004; Beaugrand, et al., 2005; Beaugrand, Reis, et al., 2004; Dornez, Holopainen, et al., 2011; Jerkovic, et al., 2010; Philippe, et al., 2007). For example, Beaugrand, Cronier, et al. (2004) evaluated the impact of outer-layer changes across grain ripening on *in situ* degradation of AX in the peripheral grain tissues by immunolocalization of a (1→4)-β-endo-xylanase. Beaugrand, et al. (2005) revealed a degree of cellular heterogeneity in wheat bran based on differences in AX labeling density.

Some other cell wall compounds have been localized by immunolabeling. AGP-specific monoclonal antibodies have helped to reveal the developmental dynamics of AGP glycan structure and represent a diagnostic tool for AGPs. Frequently used anti-AGP monoclonal antibodies are JIM8, JIM13, JIM14, LM2, and CCRC-M7. In primary and secondary walls of cereals, ferulic acid and p-coumaric acid are predominant among the hydroxycinnamic acids. They contribute to cell wall assembly, promote tissue cohesion, and restrict cell expansion. Immunocytochemistry is very sensitive and potentially more specific for the *in situ* analysis of cell wall phenolic constituents than other methods such as Ramman spectroscopy (Tranquet, et al., 2009). The characterization of a polyclonal antibody against 5-O-(trans-feruloyl)-L-arabinose and two monoclonal antibodies useful for the detection of p-coumaric acid has recently been reported (Philippe, et al., 2007; Tranquet, et al., 2009). These antibodies have been used to localize ferulic acid and p-coumaric acid, to compare the degree of feruloylation of AX in transfer and aleurone cells, and to monitor changes in p-coumaric acid esters in the cell wall of wheat grains during development (Philippe, et al., 2007; Robert, et al., 2011; Tranquet, et al., 2009).

2.2. Grain proteins

Depending on their function, cereal proteins can be classified into storage, structural, metabolic and protective proteins. The storage proteins are the most abundant proteins in cereal grains and have important impacts on the nutritional quality for humans and livestock and on the functional properties in food processing. Immunolabeling has contributed to the study of storage proteins in cereals (Table 1).

Gluten proteins are the major storage protein fraction in the mature wheat grain and interact during grain development to create large polymers, which form a continuous proteinaceous network when flour is mixed with water to make dough. Immunofluorescence and immunogold labeling of gluten proteins has allowed determination of quantitative and qualitative gradients in gluten protein composition (Tosi, et al., 2011), their trafficking pathways (Tosi, et al., 2009), and their processes of expression and deposition (Van Herpen, et al., 2008; Wang, et al., 2013) during grain development. These findings are of particular interest because of the major role played by

the gluten proteins in determining grain processing quality. Immunolabeling has revealed that prolamin proteins are heterogeneously deposited, not only throughout the wheat endosperm, with the outer endosperm containing a much greater proportion of prolamins than the inner endosperm (Mills, et al., 2005). Prolamin proteins are also heterogeneously deposited within the endosperm cells themselves (Tosi, et al., 2009). These protein gradients have been subsequently investigated in detail using antibodies recognizing specific gluten protein types (Tosi, et al., 2011). However, according to Loussert, et al. (2008) immunochemical labeling of prolamins has failed to reveal in TEM analyses any particular internal organization in protein bodies, since all prolamins studied occurred in the same protein bodies, without any segregation according to type.

In other cereals, immunochemical experiments have been carried out to identify the distribution of prolamin proteins and characterize the accumulation process of these proteins in developing and mature rice grains (Nagamine, et al., 2011; Saito, et al., 2009; Saito, et al., 2008; Takahashi, et al., 2005). Storage protein distribution in different rice cultivars has also been compared (Furukawa, et al., 2003; Ohdaira, et al., 2011). Moreover, these techniques have been used to characterize the structure of processed rice protein and relate it to its digestibility (Kumagai, et al., 2006). Meanwhile, Reyes, et al. (2011) studied the synthesis and transport of storage proteins in maize aleurone cells and concluded that their trafficking pathway may be a widespread mechanism for vacuolar delivery of prolamins in cereals. Double immunolabeling in combination with image analysis has been used to determine differences in the distribution of zeins between wild and mutant maize cultivars (Holding, et al., 2007).

In order to investigate the mechanisms of synthesis and deposition of storage proteins and to gain an insight into the relationship between RNA and protein localization, immunolabeling has been applied to transgenic lines of wheat (Arcalis, et al., 2004; Gil-Humanes, et al., 2011; Tosi, et al., 2009; Van Herpen, et al., 2008), rice (Kawagoe, et al., 2005; Kawakatsu, Hirose, Yasuda, & Takaiwa, 2010; Saito, et al., 2009; Shigemitsu, Masumura, Morita, & Satoh, 2013; Tian, et al., 2013; Wakasa, Yang, Hirose, & Takaiwa, 2009; Wakasa, et al., 2011; Washida, et al., 2009; Yasuda, et al., 2009), and maize (Arcalis, et al., 2010). These lines have been essential in studies of the formation and fusion of protein bodies in developing grains.

Other group of proteins related to grain softness, and therefore affecting both milling and baking properties, are puroindolines. The location of puroindolines by immunolabeling has been used in studies of their antibacterial properties (Capparelli, et al., 2005) and their impact on the aggregation of storage proteins (Lesage, et al., 2011). Wiley, et al. (2007) provided clear evidence on the synthesis and accumulation of puroindolines by applying immunolocalization with alkaline phosphatase labeling of tissue prints. Antibodies against puroindolines and friabilin have been used to localize their counterparts in oat seed, tryptophanins, and study their changes during seed development and germination (Mohammadi, et al., 2007).

The lipid matrix of the oil bodies from cereal grain is surrounded by a monolayer of phospholipids embedded with unique proteins. This has permitted oleosin and caleosin to be

immunolocalized in rice grains for their characterization (Chen, et al., 2012) and in oat (Heneen, et al., 2008) and barley (Liu, et al., 2005) in order to elucidate their role in oil body formation.

The metabolism of cell wall polysaccharides requires the involvement of numerous enzymes. Suliman, et al. (2013) recently identified glycosyltransferases involved in wheat endosperm cell wall formation. Immunogold labeling and silver enhancement were used in their study in order to localize AX and a polypeptide associated to the Golgi apparatus using TEM analyses. The flavoprotein cytokinin dehydrogenase controls the degradation of cytokinins, which are plant hormones that contribute to the regulation of numerous developmental processes. Histochemical localization of cytokinin dehydrogenase by activity staining and immunochemistry using optical and confocal microscopy have shown that cytokinin dehydrogenase is most abundant in the aleurone layer of maize kernels and in phloem cells of seedling shoots (Galuszka, et al., 2005).

3. Challenges and limitations of immunolabeling

3.1. Availability of specific antibodies

Polyclonal antibodies are sera containing a heterogeneous complex mixture of antibodies with different specificities and epitope affinities against one antigen and are therefore non-specific. Monoclonal antibodies consist of only one antibody subtype which detects only one specific epitope of the antigen (Fig. 5). Generation of antisera is easier and less time consuming compared to monoclonal antibodies. However, generation of hybridoma monoclonal antibodies derived from cell-based isolation procedures is currently the most effective way to generate antibodies with higher specificity.

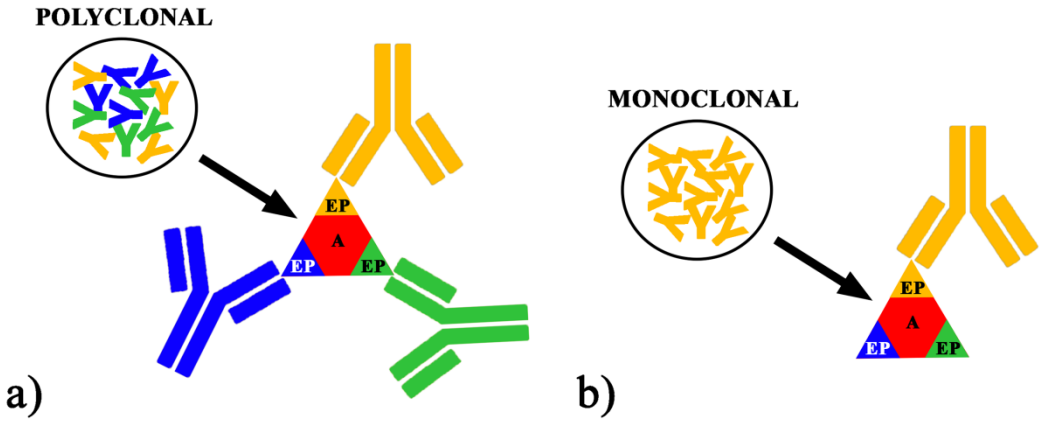

Fig. 5. Schematic representation of a) polyclonal and b) monoclonal antibodies. A: antigen; EP: epitope.

Different information may be obtained depending on whether monoclonal or polyclonal antibodies are used, as occurred in recent studies regarding the location of puroindolines in the aleurone of wheat grain (Capparelli, et al., 2005; Wiley, et al., 2007). Both polyclonal and monoclonal antibodies have their advantages which make them useful depending on the application. Polyclonal antibodies can help amplify signal from target proteins with low

expression level, since they can bind each target molecule on multiple epitopes. However, polyclonal antibodies are not useful in studies probing specific domains of the antigen or quantifying labeling. The high specificity of monoclonal antibodies, on the other hand, decreases background noise and cross-reactivity and helps provide reproducible results. However, this also makes monoclonal antibodies more sensitive to antigen changes and loss of reactivity.

The generation of antibodies to the polysaccharide and phenolic components of cell walls is generally less straightforward than the generation of antibodies to proteins and the peptide components of glycoproteins (Willats & Knox, 2003). The lack of appropriate antibodies for specific purposes has been a limitation in recent studies on cell wall characterization (Philippe, et al., 2006; Robert, et al., 2011) and metabolic processes (Galuszka, et al., 2005). The amount of commercial antibodies available for cereal research purposes is limited (Table 2) and the development of specific antibodies in the laboratory is a long and complicated process. Despite this, the specificity and variety of available antibodies have progressively increased during recent years, offering new opportunities in cereal research. The available antibodies are becoming more sophisticated and permit description of spatial and temporal variations in the structure that could not be determined by other chemical analysis techniques (Wilson, et al., 2012).

Table 2. Commercial antibodies used in cereal research during the last decade.

Supplier	Compound	Antibodies	References
Biosupplies[a]	(1→3,1→4)-β-D-glucan	(mAb, Ms)	(Wilson, et al., 2006)
	(1→3)-β-D-glucan	(mAb, Ms)	(Wilson, et al., 2006)
	(1→4)-β-D-mannan	(mAb, Ms)	(Wilson, et al., 2006)
Plant Probes[b]	Xylan	LM10 (mAb, Rt)	(McCartney, et al., 2005)
	Arabinoxylan, xylan	LM11 (mAb, Rt)	(McCartney, et al., 2005)
	Xyloglucan	LM15, LM24, LM25 (mAb, Rt)	(Marcus, et al., 2008)
	Mannan	LM21, LM22 (mAb, Rt)	(Marcus, et al., 2010)
	Homogalacturonan	LM7, LM18, LM19, LM20 (mAb, Rt)	(Clausen, et al., 2003; Verhertbruggen, et al., 2009)
	Xylogalacturonan	LM8 mAb (rat IgM)	(Willats, et al., 2004)
	Feruloylated (1-4)-β-D-galactan	LM9 (mAb, Rt)	(Clausen, et al., 2004)
	Arabinogalactan protein	LM2, LM14 (mAb, Rt)	(Wilson, et al., 2006)
	Feruloylated polymers	LM12 (mAb, Rt)	(Zhang, et al., 2011)

mAb: monoclonal antibody; Rb: rabbit; Ms:mouse; Rt: rat.

[a] Biosupplies Australia Pty Ltd., PO Box 835, Parkville 3052 Australia. Fax: +613 9347 1071, enquiries@biosupplies.com.au

[b] PlantProbes, ULCL, 3 Cavendish Road, Leeds, LS2 9JT, UK. Fax: +44–113–34–33144, plantprobes@leeds.ac.u

However, the intermolecular interactions of (1→3),(1→4)-β-D-glucans and AX and their role in cell wall properties have not been extensively explored (Philippe, et al., 2006; Robert, et al., 2011). The development of new antibodies against cell wall compounds, such as highly substituted AX, would contribute to a better understanding of the role of these polysaccharides in

cereal cell wall. Although the monoclonal antibody LM11 binds to a certain extent to maize glucuronoarabinoxylans (McCartney, et al., 2005), an antibody specific against these compounds has yet to be developed. In the same way, antibodies directed against ferulic acid dehydrodimers are needed to better understand their role in cell wall structure and tissue adhesion. To date, the polyclonal antibody anti-5-O-Fer-Ara has been used, based on the assumption that the amount of dehydrodimers is proportional to that of esterified ferulic acid (Philippe, et al., 2007).

3.2. Antibody specificity

Despite the superior specificity of the antibodies compared with conventional histological staining agents, the suitability of an antibody depends on the number of potential binding sites for the probe on the target compound. This makes it more difficult to detect diffuse proteins than aggregated proteins by immunocytochemistry (Saito, et al., 2009). In the case of AX, the antibodies developed clearly differ in specificity and react with different AX epitopes. The monoclonal antibody anti-AX1 and the polyclonal serum anti-X3 both bind unsubstituted β-(1→4) xylosyl sequence, but differ in their capacity to recognize substituted AX (Guillon, et al., 2004; Ordaz-Ortiz, et al., 2004). The polyclonal anti-X3 antibody requires a sequence of three unsubstituted xylose residues for maximum affinity, while the monoclonal anti-AX1 antibody has higher affinity for xylosaccharides containing arabinose, and the monoclonal LM10 only binds to un-substituted or low substituted xylans (Guillon, et al., 2004; McCartney, et al., 2005). The recently developed LM11 monoclonal antibody had originally been shown to bind to wheat arabinoxylan in addition to unsubstituted xylans (McCartney, et al., 2005). However, its effectiveness in detecting arabinoxylans is highly influenced by their degree of substitution, and this has led to different conclusions about the temporal and spatial deposition of these polysaccharides during grain development (Wilson, et al., 2006). Nevertheless, this limitation has been solved by pretreating sections with α-L-arabinofuranosidase, therefore exposing the epitopes needed for antibody recognition (Wilson, et al., 2012). An antibody binding to highly substituted AX remains to be developed. In certain organs, large sets of xyloglucan and mannan epitopes are masked by the presence of pectic homogalacturonan, which limits the understanding of xyloglucan function in primary cell walls (Marcus, et al., 2010; Marcus, et al., 2008). The use of enzymatic degradation in conjunction with cell wall probes is likely to be an important analytical tool in studies on the developmental regulation of links between pectic polymers and xyloglucan (Marcus, et al., 2008).

Some authors have suggested that low intensity labeling of AX and (1→3),(1→4)-β-D-glucan could be due to their epitopes being masked by other deposited polymers or substituents (Philippe, et al., 2006; Wilson, et al., 2006).

Discrepancies between immunogold labeling and other immunoanalyses such as ELISA and Western blot analysis, have been reported and two different explanations have been proposed. The antibody-reactive epitopes could be affected by the tissue preparation procedures required for

microscopy observation, or the sensitivity level of the immunogold detection technique could be higher than that of the other immunotechniques (Chikwamba, et al., 2003).

In order to avoid false positives when applying indirect immunolabeling, all experiments should be performed with a series of controls conducted simultaneously. In general, not all the different types of control are evaluated in each study, but at least one or two types of control are tested. The most common control tests carried out are the omission of primary or secondary antibodies to certify that the labeling intended is the result of the antibody-antigen interaction, and incubation with preimmune serum instead of the primary antibody to ensure that no component in the immune serum other than the specific IgG is responsible for the binding. Other types of control consist of reaction of the primary antibody with an excess of antigen to show that only the antigen is responsible for the localization seen, or the use of an unlabeled antibody to determine that the specific properties of the labeled antibody are responsible for the localization.

In the case of polyclonal antiserum, antibodies that can react with other antigens may induce nonspecific labeling during immunostaining of the compound of interest. Therefore, treatment of the antiserum in order to remove the nonspecific binding groups is necessary (Beaugrand, Reis, et al., 2004).

3.3. Tissue preparation and methodology limitations

Preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. Frozen, fractured grains are particularly useful for immunolabeling studies, because the aleurone, sub-aleurone and inner endosperm layers can be clearly identified on the fracture face (Mills, et al., 2005). Ultrastructural preservation in immunoelectron microscopy is usually compromised because the fixatives used may prevent the antibody-epitope reaction. For example, formalin fixation renders epitopes inaccessible to antibody recognition, while glutaraldehyde and/or osmium tetroxide does not interfere with carbohydrate epitope labeling, but high concentrations of these fixatives should be avoided for protein labeling. Moreover, the inherent features of plant cells, such as their hydrophobic surfaces, rigid cell walls, and large vacuoles, complicate the exchange of reagents. Hence, achieving ultrastructural preservation while protecting the antigenicity of molecular epitopes has proven difficult in plant tissues. Wilson and Bacic (2012) examined a traditional, chemical-based protocol and a method based on cryofixation techniques, including high-pressure freezing, and freeze-substitution, and found that both methods provide good ultrastructural detail in plant cells, while preserving the binding capacity of carbohydrate and protein epitopes. However, immunolabeling in combination with cryofixation and freeze-substitution techniques provides more detailed information on the immunoelectron-microscopic localization of molecules in the plant cell and superior immunolocalization of protein antigens than can be obtained from chemically fixed tissues. Takeuchi, Takabe, and Mineyuki (2010) also introduced methods for immunoelectron microscopy of post-embedded, cryofixed plant tissues by applying an antibody to a thin plastic resin-embedded section prepared by cryofixation followed by freeze-substitution.

One of the limitations of immunolabeling plastic sections is difficulty in penetration of the labels after embedding. For formalin-fixed paraffin-embedded tissues, antigen retrieval is often necessary, and involves exposing the tissue sections to heat or proteolytic enzyme. The preparation of thin sections of most mature cereal grains for cytochemical analysis is difficult. Rice is especially difficult in this regard, because its endosperm tissue is hard. Saito, et al. (2008) recently developed the frozen film method, which consists of using an adhesive film to avoid damaging frozen sections during cutting. This allows histological sections of mature rice grains to be obtained for morphometric investigations and for immunohistochemical experiments.

Although immunolabeling can provide a semiquantitative assessment of gene expression levels, the limitations of this method prevent fully stoichiometric assessment of protein expression per cell. Wiley, et al. (2007) provided clear evidence that puroindolines are only synthesized and accumulated in the starchy endosperm cells of the wheat grain by immunolocalization with alkaline phosphatase labeling of tissue prints. However, the immunochemical analysis measured the total accumulated proteins and was not able to confirm the difference in distribution between the central and outer layers of the starchy endosperm shown by β-glucuronidase (GUS) expression assays.

Despite its limitations, immunolabeling is a complementary technique to other analytical and staining methods in revealing the location and distribution of the major cereal components (Dornez, Holopainen, et al., 2011). Immunolabeling in combination with Fourier-transform infrared microspectroscopy (Mills, et al., 2005; Toole, et al., 2012) has helped to provide a more complete picture of the changes in AX structure during grain development and its distribution within the grain. A combination of immunolabeling and N-glycan analysis of recombinant proteins has proven useful in elucidation of protein trafficking pathways in cereals (Arcalis, et al., 2004; Arcalis, et al., 2010). Similarly, Jerkovic, et al. (2010) applied immunofluorescence overlaid on differential interference contrast (DIC) images to confirm the location of three major defense proteins identified in different wheat bran fractions and to place them in a functional framework.

4. Future perspectives and conclusions

Immunolabeling has been applied consistently in a wide variety of cereal grain studies during the past decade. According to the literature included in this review, these techniques can be expected to remain in use, representing a useful tool in the characterization and localization of cereal components. Although fine spatial and temporal tuning of the biosynthesis of cell wall polymers has been achieved in recent years, further studies are needed to fully understand the heterogeneity of cell wall structure in cereal endosperm, and immunolabeling will be essential in this regard. Combined with the great advances in the past decade, the future development of more antibodies against specific fiber and protein fractions will contribute even more to a deeper understanding of cereal grain structure. Another step forward will be to study the relationship between this spatial,

compositional information and grain properties, such as biomechanical properties that can affect the milling process.

Progress towards establishing plants as a vehicle for the production of plant-made pharmaceuticals is likely to accelerate in the coming years (Hensel, 2011). The achievement of appropriate levels of expression remains somewhat empirical, and to a large extent varies from one recombinant protein to another (Hensel, 2011). Therefore, immunolabeling of recombinant proteins will be necessary in order to understand pathways and biochemical processes yet to be unraveled (Finnie, Sultan, & Grasser, 2011).

It can also be concluded from this review of the literature that there are areas in cereal research where the potential of immunolabeling has not been properly explored as yet. New allergen-specific antibodies may be developed and used to localize allergens in cereal grains by immunolabeling, as has been already done in the characterization of a wheat serine proteinase inhibitor (Constantin, et al., 2008) and different gluten fractions (Mitea, et al., 2008; Tranquet, Larré, & Denery-Papini, 2012). Immunolabeling may also be useful to evaluate the degradation of different components of cereals and cereal products during digestion. Moreover, most of the applications of immunolabeling have focused on wheat, rice, and maize, while studies using this technique for other cereals such as oat or rye have been relatively scarce. The potential of immunolabeling techniques could also be applied to cereal-derived products such as bread, porridge or extruded cereal.

The use of *Arabidopsis thaliana* and rice as model plants has greatly accelerated research in cereal biology (Pattathil, et al., 2010). However, these plant models present drawbacks to addressing biological questions related to temperate grasses. Several studies using immunolabeling techniques have sought to gain insights into the usefulness of *Brachypodium distachyon* as a model for studies on cell walls (Guillon, et al., 2011) and storage proteins (Larré, et al., 2010) in cereal grains. Therefore, immunolabeling techniques are expected to be useful in future studies on *B. distachyon*.

To sum up, immunolabeling techniques have made a valuable contribution to cereal grain characterization during the past decade when precise localization of specific compounds has been required. These techniques are especially useful in cereal studies seeking a better understanding of grain development and characterization.

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