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Inactive Fluorescently Labeled Xylanase as a Novel Probe for Microscopic Analysis of Arabinoxylan Containing Cereal Cell Walls

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ABSTRACT: A new technique to visualize cereal cell walls by fluorescence microscopy was developed. The novel staining technique is based on an inactive fluorescently labeled xylanase binding to arabinoxylan (AX), an important polysaccharide in grain cell walls in terms of the technological and physiological functionalities of grain. The xylanase probe could stain AX in the seed coat, nucellar epidermis, aleurone layer, and starchy endosperm, but not the highly substituted AX of the pericarp layer. The advantage of this new staining technique over the existing immunolabeling techniques is that the staining procedure is clearly faster and less laborious, and uses a smaller probe that can easily be produced by marking a well characterized enzyme with a fluorescent label. In the future, the here proposed technology can be used to develop probes having specificity also for cell wall components other than AX and thus to study plant cell walls further through fluorescence microscopy.

KEYWORDS: Cereal, arabinoxylan, cell wall, xylanase, fluorescence, microscopy

INTRODUCTION

Arabinoxylan (AX) is a major nonstarch polysaccharide in cereal grains. It is a main cell wall constituent that is built up of a linear backbone of β-(1,4)-linked xylose residues that can be un-, mono-, or disubstituted with arabinose residues, some of which, in turn, carry a phenolic acid residue.[1,2] Other substituents, such as glucuronic acids and acetyl groups, can also be present.[3] AX is an important component in grain both from a technological as well as from a nutritional point of view. It is well-known that AX has a strong impact on flour functionality in cereal-based processes and on end-product properties, despite its relatively low abundance in wheat flour (1.5−2.0%).[4] Therefore, xylanases are often used to improve processing and product quality by modifying the AX population and hence its functionality.[4] Epidemiological studies have shown that consuming elevated levels of dietary fiber may reduce the risk of cancers, type-2 diabetes, and cardiovascular diseases, and assist in regulation of body weight.[5]

Nowadays, several imaging methods are available that permit the visualization of AX in cereal cell walls (Table 1). Both monoclonal and polyclonal antibodies against AX have been developed and used for immunolabeling to investigate cell wall structures by light and electron microscopy.[6−8] The suitability of the antibody to visualize AX depends not only on the amount of AX in the sample but also on the number of potential binding sites for the probe on the target compound. The different antibodies generated against AX clearly differ in specificity. The polyclonal anti-X3 antibody requires a sequence of three unsubstituted xylose residues for maximum affinity, while the monoclonal anti-AX1 antibody can better handle arabinose substituents.[6] Also, the LM10 and LM11 monoclonal antibodies differ in their capacity to bind substituted xylans. While LM10 only binds to un- or lowly substituted xylans, such as birch wood and oat spelt xylan, LM11 is capable of binding strongly to wheat AX and, to a lesser extent, to maize glucurono-AX.[7]

Immunolabeling of AX is a two-step procedure in which the AX epitope is first recognized by a primary antibody that specifically binds to AX, which in turn, is recognized by a second antibody that is conjugated to a fluorescent label or gold particle. Furthermore, xylanases[9] and xylan-specific carbohydrate-binding modules[10,11] have been used as primary probes for AX. The location of these primary probes is then visualized by immunolabeling using primary antibodies that recognize the xylanase or the polyhistidine tag of the CBM as secondary probes, which, in turn, are recognized and visualized by tertiary probes, i.e., secondary antibodies recognizing the primary antibodies carrying a fluorescent label or gold particle. Different CBM probes show different affinities for xylan substrates, and there seems to be a relationship between the affinity of the CBM for xylan and its microbial origin.[10]

The reported AX staining techniques, so far, are based on immunolabeling and thus are quite laborious and time-consuming. However, simpler indirect staining techniques, which do not stain AX as such, but other cereal cell wall components, have been used to evaluate changes in AX after xylanase treatment. Calcofluor staining of β-1,4 linkages in β-glucan and cellulose is an example of such an indirect AX staining. As both AX and β-glucan/cellulose are located close to each other in cereal cell walls, changes in AX have been suggested to be visualized with Calcofluor staining. Van Craeyveld and co-workers[12] proposed that the increased brightness of the Calcofluor staining was attributed to the disappearance of AX by xylanase, resulting in increased β-glucan/cellulose fluorescence by Calcofluor. Similar results were

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Table 1. Overview of the Currently Used Microscopic Staining Techniques for Arabinoxylan (AX) in Cereal Cell Walls

	technique	staining protocol	probe	reference
direct	immunolabeling of AX	two-step: recognition of AX with primary and secondary antibodies	LM10	7
			LM11	
			Anti-AX1	6
			Anti-X3	
	immunolabeling of carbohydrate binding module (CBM) probes	three-step: recognition of the His-tag of CBM bound to AX with primary and secondary antibodies	*Ct*CBM6	11
			*Cf*CBM2b-1-2	10
			*Rm*CBM4-2	
			*Cj*CBM15	
			*Ct*CBM22-2	
			*Cj*CBM35	
	immunolabeling of xylanase probes	three-step: recognition of xylanase bound to AX with primary and secondary antibodies	*Thermobacillus xylanilyticus* xylanase (WT and inactive mutant)	9
indirect	Calcofluor staining of β-1,4 linkages in β-glucan and cellulose	one-step	increased brightness in signal due to AX removal	12,13
	UV-induced blue autofluorescence of ferulic acid residues	no staining step	decreased blue signal due to AX removal	9

obtained by Autio and co-workers[13] upon incubation of barley sections with xylanase. Another simple indirect approach to visualize AX is the use of UV-induced blue autofluorescence of ferulic acid residues that are covalently bound to AX.[9,14]

Fast and simple direct staining techniques using probes that are covalently bound to a label exist for other cell wall components. This way, the multistep immunolabeling procedure can be replaced by one simple incubation step in which the section is incubated with the probe followed by a washing step. This has already proven to be successful for the staining of cellulose, starch, and xyloglucan with CBMs that were conjugated to fluorescent tags.[15,16]

In the current study, a new technique for the visualization of AX in wheat cell walls is presented using a fluorescently labeled xylanase as a simple, fast, and direct AX probe.

MATERIALS AND METHODS

Materials. All chemicals, solvents, and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. Wheat (*Triticum aestivum*) grain kernels of cultivar Selekt were obtained from Clovis-Matton NV (Avelgem-Kerk-hove, Belgium). Bran material was derived from mixed wheat varieties (Mühle Rüningen GmbH & Co. KG, Braunschweig, Germany) that were peeled to remove 2−3% of the grain outer layers before milling in order to reduce the level of contaminating microbes and enzymes on the kernel surface. The wheat kernels and bran material had total AX contents of 7.3% and 21.7% and β-glucan contents of 0.6% and 2.8%, respectively. Alexa Fluor488 C_5 maleimide dye was from Molecular Probes (Invitrogen, Carlsbad, CA).

Sample Preparation. The bran was ground in batches of about 4 kg by passing each batch three times through a mill (Hosokawa Alpine, 100 UPZ, Retsch GmbH, Germany; mill sieve size 0.3 mm). After grinding, the mean particle size was about 100 μm, and 90% of the particles were smaller than 390 μm as determined by a Coulter Particle size analyzer dry module (Coulter Corporation, Miami, FL). Bran was moistened in fixing solution containing 3.0% paraformaldehyde and 1.0% glutaraldehyde in 0.10 M sodium potassium phosphate buffer at pH 7.0, and small bran pellets were made. These bran pellets were then embedded into 2% agar for further fixation and dehydration steps. Wheat kernels, of which the embryo was removed, were moistened between wetted filter papers for 16 h at 4 °C, and subsequently, kernel ends were removed to facilitate infiltration.

Bran pellets embedded in agar and kernels were fixed overnight with 3.0% paraformaldehyde and 1.0% glutaraldehyde in 0.10 M sodium potassium phosphate buffer at pH 7.0. The samples were then washed, dehydrated in a series of ethanol solutions (50, 70 and 95% for 2 times 1.0 h and 100% overnight), and infiltrated with Historesin Embedding Kit (Leica Microsystems, Bensheim, Germany) for five days. Two bran pellets or four grain kernels were embedded per sample block and cut into semithin sections (4 μm) with a rotary microtome HM355 (Microm Laborgeräte GmbH, Walldorf, Germany) using a steel knife. The sections were then transferred onto 2-welled microscopy slides (Thermo Scientific Cel-line; SSG Braunschweig, Germany) and dried on a metal heating plate at 40 °C prior to staining.

Production of the Fluorescently Labeled Xylanase Probe. Two mutants of the *Bacillus subtilis* xylanase (XBS) (UniProtKB P18429) were created. In the first mutant, a cysteine residue was introduced with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to replace a serine residue in the α-helix of the enzyme (S155C). The second mutant also contained the S155C mutation but had an additional E172A mutation which makes the enzyme catalytically inactive.[17,18] Template DNA and pairs of complementary oligonucleotide primers used to create these mutants are shown in Table 2. The insertion of the mutations was verified by sequence analysis (Genetic Service Facility, VIB, Wilrijk, Belgium). The two enzymes were expressed in *Escherichia coli*·BL21 (DE3) pLysS cells, purified and characterized as described previously.[19]

The purified xylanases were then labeled by making use of the Alexa Fluor488 C_5 maleimide dye (Invitrogen, Carlsbad, CA). The 100 μL reaction mixture contained 100 μM enzyme, 1.0 mM tris(2-carboxyethyl)phosphine (TCEP) reducing agent, and 1.0 mM Alexa Fluor488 C_5 maleimide in sodium Hepes buffer (50 mM, pH 7.0). TCEP was used to break up dimers formed between different XBS enzymes through

Table 2. Template DNA and Oligonucleotide Primers Used in Site-Directed Mutagenesis of the *Bacillus subtilis* Xylanase

mutation	template DNA	forward primer (5′→3′)	reverse primer (5′→3′)
E172A	pEXP5-CT-*xynA*	CCAAGTCATGGCGACAGCTGGATATCAAAGTAGTG	CACTACTTTGATATCCAGCTGTCGCCATGACTTGG
S155C	pEXP5-CT-*xynA*	GAACGCATGGAAGTGCCATGGAATGAATC	GATTCATTCCATGGCACTTCCATGCGTTC

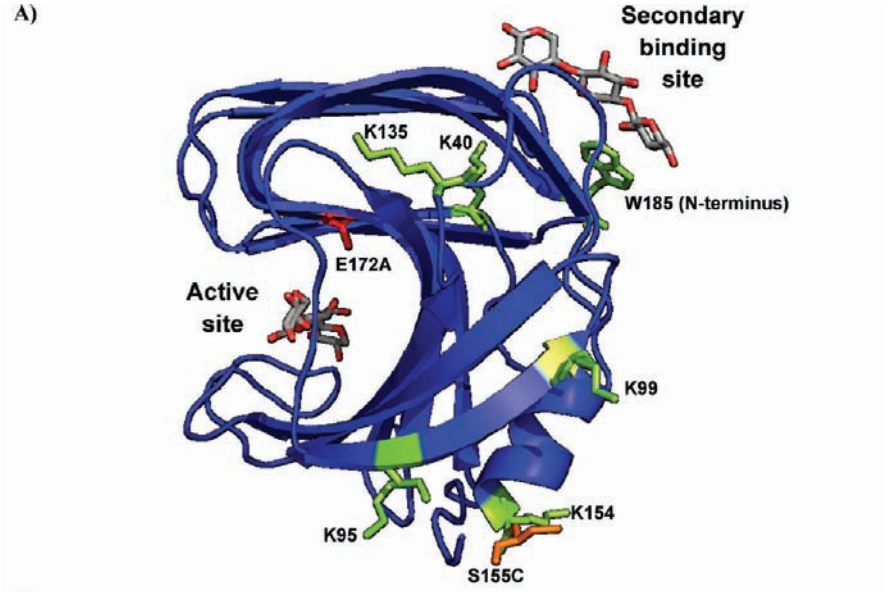

Figure 1. (A) Structure of the *Bacillus subtilis* xylanase A showing the active site and the surface binding site where the substrate can bind. The lysine residues (K40, K95, K99, K135, and K154) as well as the N-terminus (W185) are marked in green. The E172A and S155C mutations are marked in red and orange, respectively. The figure was drawn using PyMOL (DeLano Scientific; http://www.pymol.org/) based on PDB 2QZ3.[17] (B) Characteristics and structure of the Alexa Fluor488 C5 maleimide dye.

disulfide bonds.[20] Since TCEP does not contain a thiol group itself, it is compatible with the maleimide dye used for labeling. This reaction mixture was incubated in the dark for 120 min at room temperature. To remove residual free dye and TCEP from the labeled enzyme, samples were extensively rinsed with sodium Hepes buffer (50 mM, pH 7.0) using Amicon Ultracell-10 filters (Millipore, Billerica, MA). By measuring the extinction at 280 and 493 nm, with the Nanodrop-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), the molar ratio of dye over xylanase was calculated as described by the manufacturer. For both xylanase mutants, approximately 1 mol of dye was incorporated per mole of xylanase.

Staining of AX with the Xylanase Probe. Staining of microscopic sections was done by incubating the sections with 100 μL of Alexa Fluor488-labeled xylanase, prepared in sodium Hepes buffer (25 mM, pH 7.0). Different staining conditions were tested. Both the effect of incubation time (1.0, 4.0, and 24.0 h) and xylanase probe concentration (0.01 μM, 0.1 μM, 0.5 μM, and 1.0 μM) were studied. All incubations were performed in moisturized chambers in the dark and at room temperature. For each staining, two replicate sections containing either four individual wheat kernels or two individual bran pellets were stained. All stained sections were inspected carefully through a microscope to ensure that the staining was uniform and reproducible and that no artifacts were present.

The stained sections were examined with an Olympus BX-50 epifluorescence microscope (Olympus Corp., Tokyo, Japan), and micrographs were taken using a PCO SensiCam CCD camera (PCO AG, Kelheim, Germany) and cell^P imaging software (Olympus). The xylanase probe was imaged using mirror cube U-MSWB (Olympus; excitation 420−480 nm, emission >515 nm). Exposure times were 1600, 800, and 400 ms at 4×, 10×, and 20× magnification, respectively. Furthermore, bright field images have been taken of the same regions for comparison.

RESULTS AND DISCUSSION

Production and Characterization of the Probe. XBS was used as the AX binding probe as it is one of the best characterized xylanases. The main advantages of this xylanase are that it has a relatively high affinity for insoluble AX and that it is quite stable. The choice for the Alexa Fluor488 dye was based on the fact that

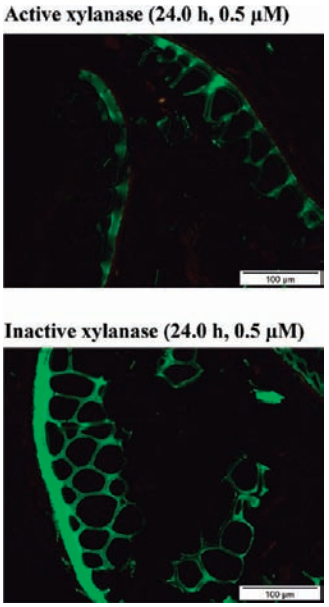

Figure 2. Wheat bran sections stained with an active and an inactive fluorescently labeled xylanase probe.

Alexa Fluor dyes are believed to exhibit more intense fluorescence than other dyes,[21] allowing the use of shorter exposure times and reducing the interference of autofluorescence. In addition, these dyes are also more photostable than several other fluorescent dyes,[21] giving less fading of the signal upon image capturing. Other advantages are their good water solubility and stability during storage.[21]

The two most common ways of attaching commercially available dyes to proteins make use of amine-reactive or thiol-reactive probes. Control of the position where the probe is attached and obtaining 1:1 labeling are difficult when an amine-reactive probe is used, since XBS contains 5 lysine residues as well as the N-terminus where labeling can take place (Figure 1A). However, as the wild-type XBS does not contain cysteine residues, introduction of a sole cysteine residue in the enzyme by site-directed mutagenesis will allow a controlled attachment of a thiol-reactive fluorescent label to the xylanase. Residue S155 on the α-helix structure of the enzyme was selected to be mutated into a cysteine as this residue is located far enough from both the active site and the secondary substrate binding site where interactions between the enzyme and the substrate occur[17,19] (Figure 1A). Alexa Fluor488 C_5 maleimide dye (Figure 1B) was chosen as the fluorescent probe for enzyme labeling as it can be specifically attached to the inserted cysteine residue, and it is a relatively small fluorescent label which should not hinder the enzyme in substrate binding. Alexa Fluor conjugates are available in several fluorescent colors.[21] The choice for the 488 nm excitation wavelength was only based on the fact that this is a quite common wavelength for excitation.

Expression of the XBS mutants in *E. coli* followed by enzyme purification from the cell lysates by cation exchange chromatography gave yields of purified enzyme of around 20−30 mg recombinant XBS per L cell culture. Cuyvers and co-workers clearly demonstrated that the xylanase activity of XBS did not decrease upon incorporation of the S155C mutation and upon labeling with the Alexa Fluor488 C5 maleimide dye.[20]

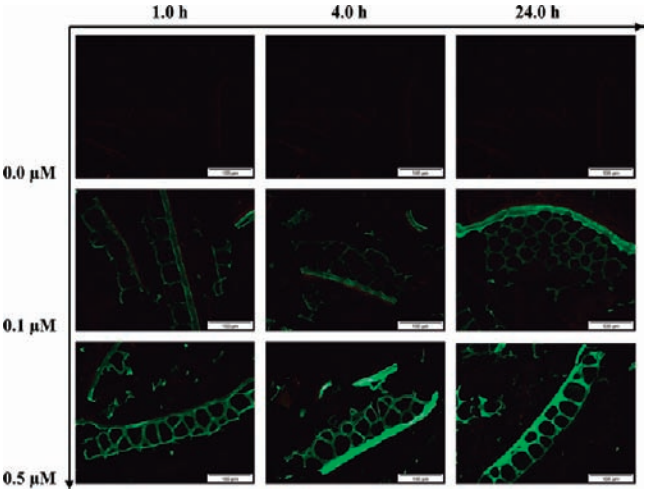

Figure 3. Wheat bran sections stained for different times and with different concentrations of the inactive fluorescently labeled xylanase probe.

Optimization of the Xylanase Probe Staining Technique. Staining of AX in wheat bran material was tested both with an active and an inactive (E172A mutant) variant of XBS. The Alexa Fluor488 label was attached to these enzymes at a cysteine residue at position 155 (S155C), which was introduced by site-directed mutagenesis. Both xylanases could stain bran AX, but the inactive variant clearly gave a much more intense signal at the same concentration after the same incubation time (Figure 2). This can most likely be explained by the fact that the active enzyme is able to hydrolyze AX, resulting in less substrate available and hence in less intensive staining. The inactive variant, in contrast, cannot hydrolyze the substrate. In addition, it has been demonstrated that the inactive xylanase hardly comes loose from the substrate once it is bound.[20] This can be linked to the suggestion by Pollet and co-workers[22] that after hydrolysis, thumb movement of the enzyme is necessary to catapult the substrate out of the active site. The absence of hydrolysis probably prevents this action from occurring.

The optimization of the staining technique for cereal cell wall material was continued with the inactive xylanase variant. To this end, microscopic sections of wheat bran were incubated for different times, and different concentrations of the inactive xylanase probe were tested. At very low enzyme concentration (0.01 μM), the signal was not noticeable due to the too strong autofluorescence of the cell wall material. At concentrations above 0.1 μM, the staining of the cell wall material was very intense, enabling the use of shorter exposure times at which the cell wall autofluorescence signal was negligible. Increasing enzyme concentration and increasing incubation time gave rise to more intensively stained structures at the same camera exposure time and hence a stronger fluorescent signal (Figure 3). Optimal staining of AX was achieved by incubating sections for 1.0 h with 100 μL of a 0.5 μM inactive Alexa Fluor488-labeled XBS. These conditions were used for the rest of the experiments in this study.

Using the Xylanase Probe for the Localization of AX in Cell Walls of Wheat Kernel. The localization of AX in cell wall polymers in the wheat grain was examined by microscopic analysis of kernel cross-sections. Structural differences in cell walls of wheat kernel cross-sections were analyzed in three different regions, i.e., the outer kernel layers, the central starchy endosperm, and the crease region of the grain kernel. Figure 4

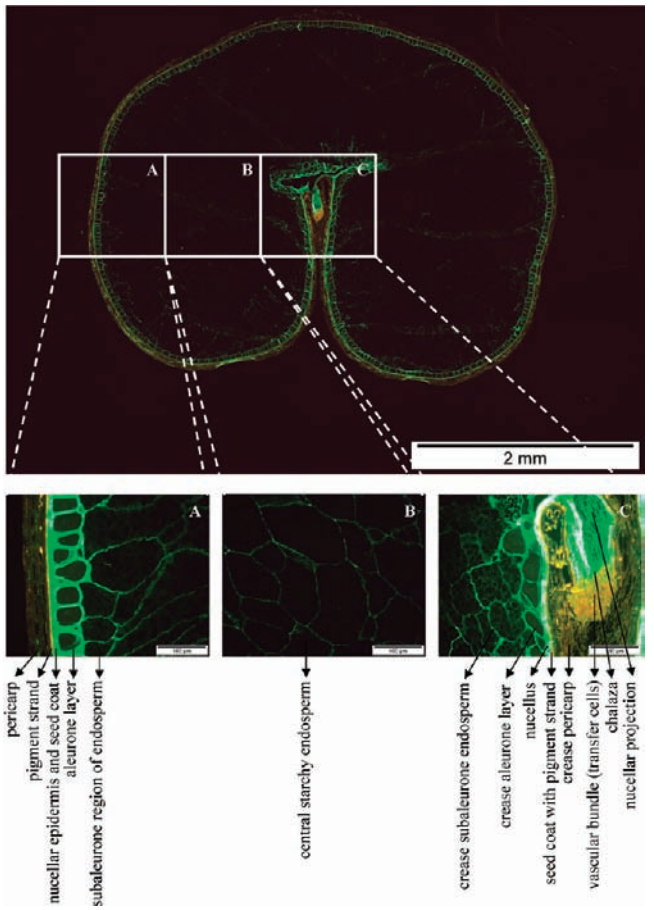

Figure 4. Cross-section of a wheat kernel stained with the inactive fluorescently labeled xylanase probe. The different zones in which images are taken are indicated: (A) outer grain kernel layers, (B) central starchy endosperm, and (C) crease region. In addition, the different tissues within each zone are appointed on the figure.

shows a wheat kernel cross-section in which the different zones that were analyzed are indicated. From the exterior to the interior, the outer layers of the grain kernel consist of outer pericarp, inner pericarp (cross and tube cells), seed coat (two cuticle layers and a pigment strand), nucellar epidermis, aleurone layer, and subaleurone starchy endosperm (Figure 4A). The starchy endosperm was analyzed in the central part of the kernel, as endosperm cell shape and cell wall composition are known to differ in the different parts of the grain endosperm (Figure 4B).[23] Figure 4C shows the crease region of the kernel. The main function of the tissues in this region is the transport of nutrients to the developing grain.[24] The crease regions consist of the vascular bundle (containing the transfer cells), the chalaza, and nucellar projection. These tissues are surrounded by modified aleurone, nucellus, seed coat, and pericarp material.

When the cross-sections of wheat kernels were stained with the xylanase probe, the cell walls in the samples stained with the xylanase probe showed an intense green signal, while in the control samples, no green color was detected (Figure 5). The result indicates that the fluorescent green signal originates from the xylanase probe. Bright field pictures from the same regions showing where the cell walls are were taken for comparison (Figure 5). There were differences in staining between the different wheat tissues. The pericarp layer was not stained, but

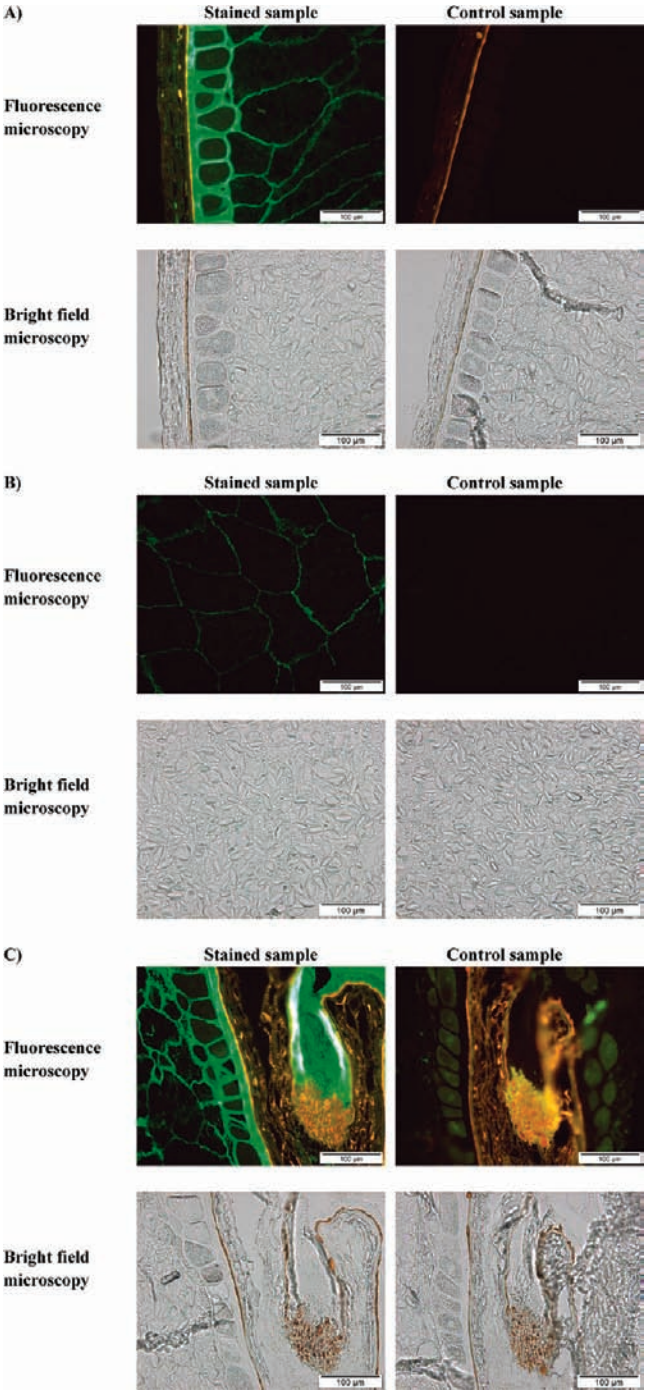

Figure 5. Fluorescence and bright field images of (A) outer kernel layers, (B) central starchy endosperm, and (C) crease region of wheat kernel cross-sections stained with the inactive fluorescently labeled xylanase probe or incubated with buffer for control.

it could be detected as a brownish-yellow structure due to autofluorescence (Figure 5A). The nucellar epidermis was colored very brightly with xylanase staining. The aleurone cell walls were also stained brightly, and the staining intensity appeared to be quite similar in anticlinal and periclinal cell walls. Cells in the central starchy endosperm had much thinner cell walls compared to those in aleurone layer cells (Figure 5B). In the crease region (Figure 5C), the nucellar projection was stained very intensively by xylanase probe staining. The aleurone and

subaleurone cells in the crease region were stained similarly to these in the outer kernel layers. The chalaza, vascular bundles, and pericarp material remained unstained, and the pericarp material was again seen as a brownish-yellow structure due to autofluorescence.

The efficiency of the xylanase probe to stain the AX in the different tissues can be explained by both the level of AX in the tissue and their degree of arabinose substitution. According to Barron and co-workers,[25] AX levels (calculated as the sum of arabinose and xylose) in the outer pericarp are around 45−47%, while in the intermediate layer, consisting of the nucellar epidermis, seed coat, and inner pericarp, the levels are around 37−40%. The nucellar epidermis was also tested separately and was found to be very rich in AX with levels of around 53−67%. The aleurone layer contains about 18−24% AX, while the endosperm only contains around 1.5−1.7%. Of course, the lower AX levels in the latter tissues are due to the presence of lower levels of cell walls and higher levels of cell content compared to those of the outer kernel layers. Higher AX levels can be expected to result in higher staining intensities of cell walls. However, the different tissues differ not only in the levels but also in the structure of AX. AXs in the nucellar epidermis have a very low arabinose to xylose (A:X) ratio (A:X = 0.10−0.13), while those in the aleurone (A:X = 0.3−0.5) and endosperm (A:X = 0.5−0.7) are moderately substituted and in the pericarp are highly substituted (A:X > 1.0).[25−27] The specificity and the selectivity of the AX probe can be expected to depend on the AX structure. In the case of the XBS enzyme, the active site of XBS can only accommodate substrates containing at least three consecutive unsubstituted xyloses.[17] The consequence of this is that the xylanase is seriously hindered when a lot of arabinose substituents are present on the xylan chain, as has been demonstrated previously. Indeed, the degradation of AX by XBS is increasingly hindered by the presence of arabinose substituents. With increasing A:X ratio of the substrate, the average degree of polymerization of the hydrolysis end product became larger, indicating that these substrates are less XBS degradable.[28] This is caused by the existence of fewer hydrolysis sites available for the enzyme on AX substrates with higher A:X ratios and thus also fewer enzyme binding sites.

Evaluation of the Xylanase Probe and Perspectives. The fluorescent xylanase probe allows a relatively fast and simple procedure for visualizing AXs in the cell wall material of different wheat tissues. Compared to existing AX staining techniques based on immunolabeling of AX or immunolabeling of probes that bind AX, this new staining method has several advantages. First, the xylanase probe can easily be made. It only requires recombinant expression and purification of the xylanase mutant, followed by a simple incubation step with the Alexa Fluor488 C5 maleimide dye and thorough rinsing to remove free dye. Although one could argue that recombinant expression, purification, labeling, and characterization of this probe is quite laborious, the same holds for the production of CBMs as probes. The production of antibodies is even more complex and time-consuming and requires more specific equipment than that needed for standard molecular biology techniques. Second, the staining protocol is relatively fast and easy. It only requires one incubation step with the xylanase probe, whereas immunolabeling is at least a two-step staining procedure, using both a primary and a secondary antibody. In addition, immunolabeling requires blocking of unspecific binding sites with BSA or other blocking agents, while this was not needed for AX staining with the xylanase probe, as we did not observe any unspecific binding in the sections. Another advantage of the xylanase staining is that the probe is only about 21 kDa, which is much smaller than the antibody probes. Therefore, the fluorescent signal of the xylanase probe was very intense compared to the signal obtained by immunolabeling (results not shown), allowing short exposure times and hence little or no interference of autofluorescence.

In the future, the here introduced technology could be used to develop other fluorescently labeled inactive xylanases with different AX binding properties as probes to visualize cell walls. Indeed, similar to that for antibodies, the efficiency of the xylanase probe is dependent on the degree of substitution of AX, and thus xylanase probes with different capacities to bind more or less substituted AX could/should be developed to increase the efficiency of the enzyme probes for the visualization of AX in different grains tissues. In addition, enzymes of other classes and specific for other cell wall components of grain, such as β-glucanases, cellulases, ferulic acid esterases, and arabinofuranosidases, could be coupled to fluorescent labels and tested as probes for more detailed cell wall visualization with fluorescence microscopy.

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