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Cell wall polysaccharides in differentiating anthers and pistils of *Lolium perenne*

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Summary. We are presenting the pattern of distribution of several carbohydrate epitopes, which constitute an important component of cell walls, within the anthers and pistils of a monocot grass species, perennial ryegrass (*Lolium perenne* L.). The results of immunocytochemical studies revealed that the flower organs are rich in (1→3, 1→4)-β-D-glucans and possess surprisingly high amounts of methylesterified pectic domains that bind JIM7 antibody and pectin side chains rich in (1→4)-β-D-galactose residues which react with LM5 antibody. The presence of arabinogalactan protein epitopes binding JIM13 is restricted to microspores and ovule integuments. The results are discussed in terms of possible functions of cell wall polysaccharides and arabinogalactan proteins in the differentiation of flower organs.

Keywords: Arabinogalactan protein; Glucan; Pectin; Ryegrass; Flower.

Introduction

Cell walls have been demonstrated to play an essential role in the differentiation of tissues and organs. Composition and macromolecular organisation of particular cell wall components change in the course of development and specialisation of tissues, the events that reflect alternations in both temporal synthesis and spatial distribution of molecules contributing cell wall polysaccharides, proteins, or glycoproteins (Carpita et al. 2001b, Willats et al. 2001).

The flower is an especially interesting organ to study the rearrangements of cell wall accompanying the differentiation process because it is composed of two totally different tissue types. They represent not only different ploidy levels but also variable functions. Sporophytic cells, which at a definite developmental phase give rise to the haploid gametophytic generation, later on play mainly nutritional and protective roles and may also be a source of some structural or signalling molecules. Gametophytic cells are predestined to differentiate into gametes after a relatively long developmental period that involves both expansion growth, mitotic cell divisions, and maturation of egg cells and pollen grains, the latter containing one or two sperm cells (Pennell et al. 1991, McCormick 1993).

Among numerous cell wall compounds, pectins and arabinogalactan proteins (AGPs) have been reported not only to be abundantly present within the flower tissues but also to appear and disappear at definite developmental stages (Pennell and Roberts 1990, Gołaszewska and Bednarska 1999, Kawaguchi and Shibuya 2000, Aoulai et al. 2001). Moreover, some of these molecules were shown to mark certain types of cells and tissues at very precise stages, indicating their role in either proliferation or expansion (Willats et al. 1999, 2001; Ermel et al. 2000; McCartney et al. 2000; Majewska-Sawka et al. 2004). The role of pectin components in plant morphogenesis has also been evidenced by studies of mutants and transgenic plants, with altered expression of enzymes involved in pectin metabolism. Some of such plants show a dwarf phenotype (His et al. 2001), do not form side shoots and flowers (Skjøt et al. 2002), or produce sterile microspores or pollen grains (Rhee and Somerville 1998).

The chemical structure of grass cell walls differs greatly from that of other monocotyledonous and dicotyledonous plants (Carpita and Gibeaut 1993, Gibeaut and Carpita 1993, Cosgrove 1999, Carpita et al. 2001a). They are predominantly composed of glucan complexes that tightly bind to cellulose microfibrils. The major cross-linking glycans are glucuronoarabinoxylan and unbranched mixed-linkage (1→3, 1→4)-β-D-glucan. A strong framework of

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glycan and cellulose in grasses is embedded in a complex matrix of pectic polysaccharides and structural proteins. Pectins, however, have been reported to be a rather scarce component of grass cell walls (Carpita 1996, Darley et al. 2001, Willats et al. 2001).

We are presenting the results of studies on the presence and distribution of (1→3, 1→4)-β-D-glucan and several oligosaccharidic epitopes typifying pectins and arabinogalactan proteins within the anthers and pistils of perennial ryegrass (*Lolium perenne* L.). The specific localisation of particular antigens is discussed in relation to the previous biochemical data on cell wall composition in grasses, as well as to the known characteristics of cell walls in dicotyledonous species.

Material and methods

Anthers and pistils of perennial ryegrass (*Lolium perenne* L.) were fixed and embedded in either paraplast or London Resin Gold as described elsewhere (Majewska-Sawka and Nakashima 2004).

Immunocytochemistry

Both paraplast- and LR Gold-embedded material was cut with a RM 2155 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Federal Republic of Germany) into 10 μm and 0.5 μm sections, respectively.

LR gold sections were blocked in 5% bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS) buffer, pH 7.3, for 36 h. Then, the material was incubated with primary antibodies (Table 1) diluted in 0.01 M PBS supplemented with 5% BSA: anti-(l→3, l→4)-β-D-glucan was diluted 1 : 50, JIM5, JIM7, LM5, LM6, and JIM13 were diluted 1 : 5. The reaction was performed during 12 h, followed by extensive overnight washing in buffer. In the next step the tissues were incubated for 7 h with the secondary antibodies diluted 1 : 40 in 0.01 M PBS supplemented with 0.2% BSA (anti-mouse fluorescein isothiocyanate [FITC] for detection of (l→3, l→4)-β-D-glucans and anti-rat FITC for detection of all other antigens). Control reactions were performed by omitting the primary antibody. After overnight washings in several changes of buffer, the slides were briefly rinsed in distilled water and air-dried. Then they were covered with anti-fade solution composed of 0.5% *p*-phenyldiamine in 0.01 M PBS, pH 12.0, mixed with glycerine 1 : 4, and observed under a fluorescence microscope equipped with a blue filter. Pictures were taken with Kodak 400 ASA colour film.

Paraplast was removed by passing the slides through several changes of xylene and ethanol, followed by tissue rehydration in a series of alcohol and distilled water solutions. The initial blocking was carried out for 2 h in TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween-20), pH 8.0, supplemented with 1% BSA and 1% nonfat milk. Then the slides were covered with primary antibodies: anti-(1→3, 1→4)-β-D-glucan, JIM7, and LM5, all diluted 1 : 10 in TBST containing 1% BSA and 1% nonfat milk. The reaction was performed overnight at 4 °C, followed by 5 h washing in TBST buffer. Incubation with secondary antibodies diluted 1 : 100 in TBST with 0.5% BSA lasted for 2 h (anti-mouse alkaline phosphatase for detection of (1→3, 1→4)-β-D-glucans and anti-rat alkaline phosphatase for detection of pectic epitopes). Then the material was washed in several changes of TBST for 2 h and subsequently in AP buffer for 30 min (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$), pH 8.5. Antibody binding was visualised with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium substrate, resulting in a purple reaction product. After the slides were air-dried, they were mounted with Vecta Mount and observed under an Olympus CK2 microscope. The pictures were taken with Kodak 100 ASA colour film.

Results

The results of immunocytochemical reactions with a set of monoclonal antibodies directed against (1→3, 1→4)-β-D-glucan and against carbohydrate epitopes typifying pectins and AGPs revealed characteristic patterns of distribution of definite cell wall components within the flower organs of *Lolium perenne*, in the stage of uninucleate microspores.

Detection of cell wall components within the anthers

β-Glucans are abundantly present within the anther, where they can be precisely localised within cell walls of three tissue layers that contribute the anther wall, i.e., epidermis, endothecium, and middle layer (Fig. 1A). At this stage, the tapetum is already compressed and does not display the signs of labelling. The anther locule shows peripherally arranged

Table 1. Antibodies used to detect glucans, pectins, and AGPs in *Lolium perenne* flower organs

Antibody	Epitope	Source or reference
Anti-(1→3, 1→4)-β-D-glucan	(1→3, 1→4)-β-D-glucan	commercial, Australia Biosupplies
JIM7	unknown, optimal binding to pectins (HGA) showing 15–80% esterification, containing epitopes composed of methyl-esterified residues with adjacent or flanking unesterified residues	Clausen et al. 2003
JIM5	unknown, optimal binding to pectins (HGA) showing 31–40% esterification, containing epitopes composed of four or more contiguous unesterified residues adjacent to or flanked by residues with methyl-ester groups	Clausen et al. 2003
LM5	four (1→4)-β-D-galactose residues	Jones et al. 1997
LM6	five (1→5)-α-L-arabinose residues	Willats et al. 1999
JIM13	unknown, optimal binding to GlcpA-β (1→3)-D-GalpA-α (1→2)-L-Rha	Yates et al. 1996

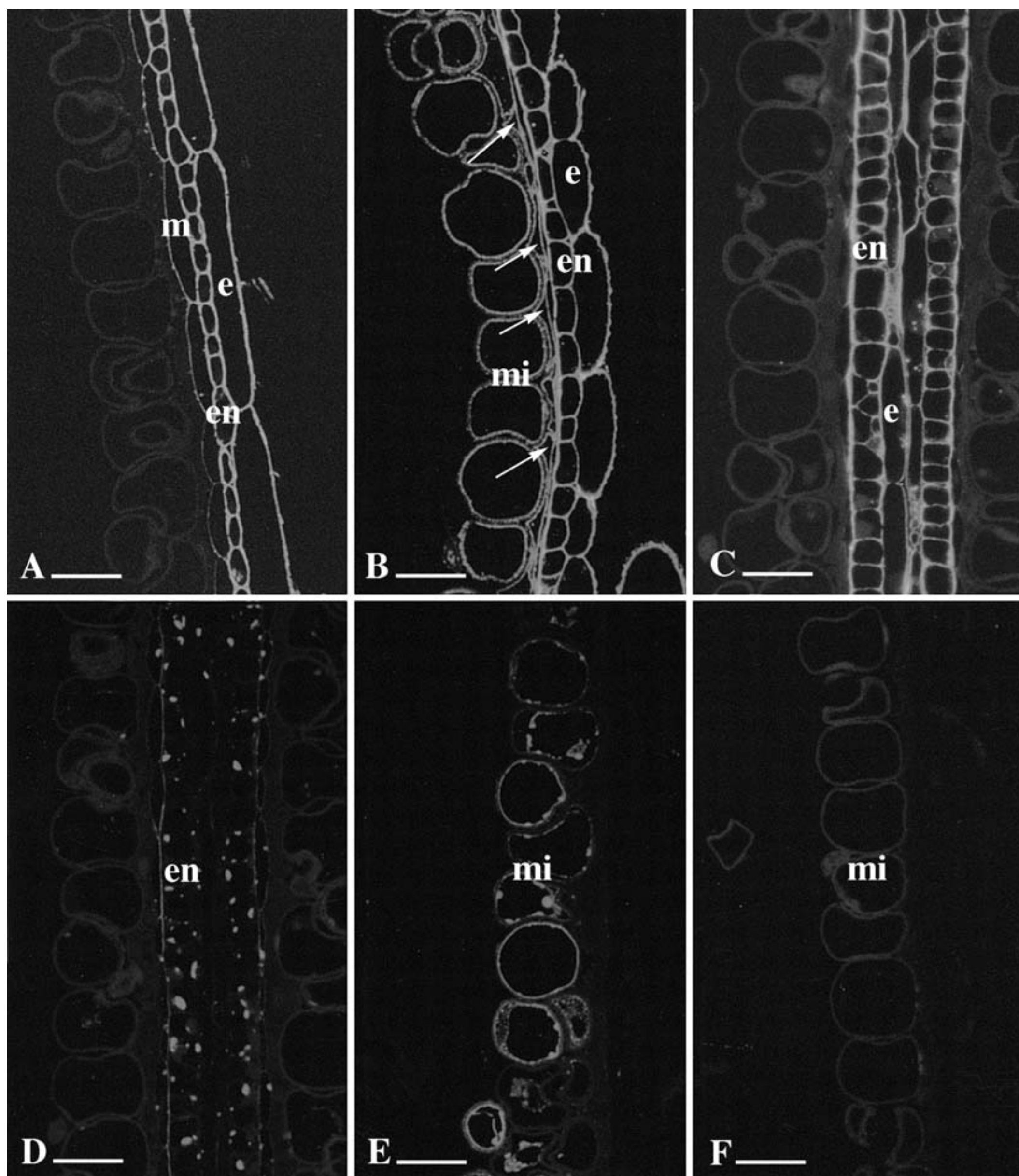

Fig. 1 A–F. Distribution of cell wall polysaccharides within *Lolium perenne* anthers. **A** (1→3, 1→4)-β-D-Glucans are visible in the epidermis (*e*), endothecium (*en*), and middle layer (*m*) of the anther wall. **B** Pectic epitopes that bind to JIM7 are present in all layers of the anther wall, in the tapetum remnants (arrows) and also in the microspore walls (*mi*). **C** Pectins that react with LM5 are located in epidermis (*e*) and endothecium (*en*). **D** Pectins that bind LM6 show scattered distribution within endothecium only (*en*). **E** Carbohydrate epitopes of AGP that react with JIM13 are seen within the micropores (*mi*), whereas anther wall layers are devoid of signal. **F** Control reaction results in lack of fluorescence. Bars: 25 µm

microspores – the typical feature of grasses – which obviously do not possess β-glucans in their walls (Fig. 1A).

Some pectic epitopes are richly present within the anther tissues. Pectins with a high level of esterification, which bind JIM7 antibody, are the most widely spread throughout the organ. They have been found in all layers of the anther wall, in tapetum remnants, as well as in the walls of microspores (Fig. 1B). Epitopes that react with LM5 antibody can be detected in the two outer tissues of the anther wall, epidermis and endothecium, but are absent in the middle layer and in the microspores (Fig. 1C). Pectins binding LM6 show a spotted pattern of distribution only within the cells of the endothecium (Fig. 1D), and epitopes with a low level of esterification, which react with JIM5 antibody, cannot be detected in any of the anther tissues (not shown).

Carbohydrate epitopes of AGPs that are detectable with JIM13 are not present in anther wall layers but are distributed exclusively within the micropores (Fig. 1E). Control

reactions performed by omitting the primary antibodies and applying only FITC-conjugated secondary antibody resulted in a complete lack of signal (Fig. 1F).

Detection of cell wall components within the pistils

β-Glucans are abundant in the pistils of *Lolium perenne* and show a characteristic spatial pattern of distribution reflected in the presence of variable quantities of this polysaccharide within the cells constituting different regions of the organ. This event is evidenced by a different intensity of labelling obtained for particular pistil parts with both FITC and alkaline phosphatase markers. The regions most intensively labelled correspond to the rachilla and the base of the ovary, and also to the specific zones in the upper ovary part, lying just above the ovule cavity (Fig. 2A). The ovule itself shows only trace labelling in the part of the nucellus, and not even traces of signal within integuments (Fig. 2A). Control sections show no signs of purple coloration (Fig. 2E).

Lolium perenne pistils show very prominent labelling with the two anti-pectin antibodies JIM7 and LM5. Both bind to all cell walls throughout the organ and to some lesser extent to the ovule tissues (Fig. 2B, C). For LM5, the labelling is most intense in the ovary part but decreases gradually towards the style and stigma (Fig. 2C). The reaction with LM6 resulted in trace labelling only, whereas JIM5 did not bind to any of the pistil tissues (not shown).

The epitopes of AGPs that bind JIM13 proved to be very scarce within the pistil. Nevertheless, their location was very precise, restricted to the ovule integuments (Fig. 2D).

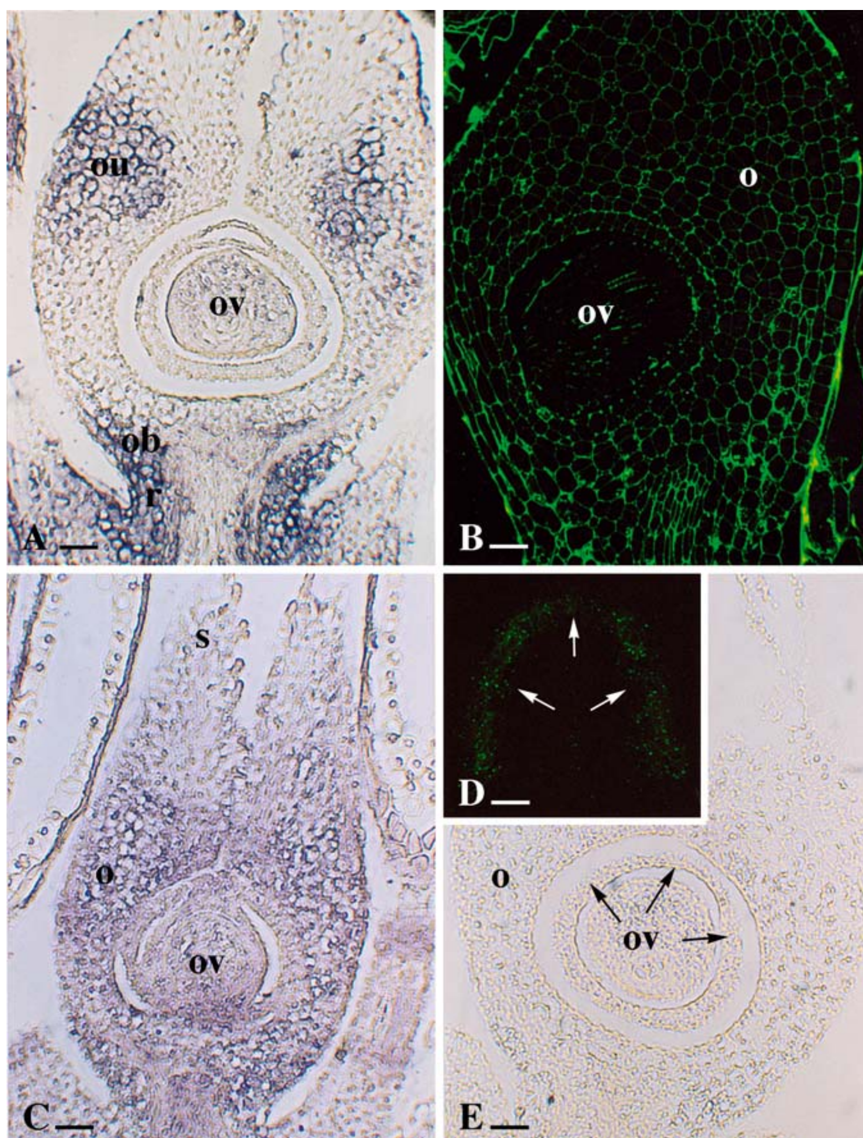

Fig. 2 A–E. Distribution of cell wall polysaccharides within *Lolium perenne* pistils is visualized by alkaline phosphatase- (A, C, and E) or FITC-conjugated antibodies (B and D). **A** (1→3, 1→4)-β-D-Glucans accumulate in the rachilla (*r*), in the ovary base (*ob*), and in the upper ovary part (*ou*), above the ovule cavity, whereas other parts of the organ as well as the ovule (*ov*) show only trace labelling or no labelling at all. **B** Pectin epitopes that bind JIM7 are widely distributed throughout the whole ovary (*o*) and ovule (*ov*). **C** Epitopes that bind LM5 are abundant in the ovary (*o*) and ovule (*ov*) tissues, but decrease towards the style and stigma (*s*). **D** Labelling with JIM13 results in fluorescence in the tissue corresponding to ovule integuments (arrows in **D** and **E**). **E** Control paraplast section obtained by incubation with anti-rat alkaline phosphatase secondary antibody, followed by reaction with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium substrate. Bars: 50 μm

Discussion

In this report we presented the spatial distribution of β-glucans and carbohydrate epitopes that characterise pectins and AGPs within the flower organs of *Lolium perenne*, and we showed their precise location within definite tissues of anther and pistil. The cells in which these particular compounds are present only in trace amount or not present at all are also pointed out.

The (1→3, 1→4)-β-D-glucans are found only in members of the order Poales, the single order that includes cereals and grasses. These polysaccharides have been found in several vegetative tissues such as coleoptiles, leaves, or endosperm, constituting more than 70% of the total cell wall material in the last tissue (Carpita 1996). β-Glucans have been previously reported to be absent from actively dividing cells, but they have been noted to accumulate when the cells enlarge or elongate. After the cell growth is stopped, these polymers are quickly hydrolysed (Kim et al. 2000).

The widespread distribution and relatively high content of some pectic epitopes within the cell walls of anther and pistil seem to be somewhat surprising, especially when data evidencing low amounts of pectins within cell walls of grasses are considered (Carpita 1996, Willats et al. 2001). We demonstrated that the *Lolium perenne* flower organs show a prominent presence of low-methylesterified pectins that react with JIM7, as well as pectin side chains bearing (1→4)-β-D-galactose residues binding LM5 antibody. The structural pectin epitopes that react with RGII, JIM5, and LM5 antibodies have also been reported to be synthesised by root cells of maize (Baluška et al. 2002, Yu et al. 2003). Moreover, it has been evidenced that – at least in maize – some pectin domains undergo rapid endocytosis and are internalised to intracellular compartments, an event that may reflect dynamic turnover of pectic compounds (Baluška et al. 2002). Galactose-rich pectin side chains have been found to appear specifically in *Arabidopsis thaliana* root cells that are at the phase preceding rapid elongation (McCartney et al. 2003) and in carrot root cells at the differentiation phase (Willats et al. 1999). The deposition of the epitope binding LM5 antibody has been related to increased cell wall firmness, a feature that typifies well differentiated cells (McCartney et al. 2000). This interpretation corresponds well to the (1→4)-β-D-galactose presence within ryegrass anther epidermis and endothecium, which are supposed to protect developing microspores against mechanical injury or harmful influence of environmental factors.

AGPs have important functions in gamete differentiation, as well as in the course of pollination and fertilisation in numerous plant species (Pennell and Roberts 1990, Du et al. 1996, Cheung and Wu 1999, Acosta-Garcia and Vielle-Calzada 2004). Defined AGP epitopes have been localised in the microspores and pollen grains of several dicots, where they are predominantly located within the intine, at the plasma membrane of vegetative cells, and at the boundary of generative cells and sperm cells (Pennell et al. 1991, van Aelst and van Went 1992, Li et al. 1995, Southworth and Kwiatkowski 1996, Ferguson et al. 1999, Mogami et al. 1999, Nothnagel et al. 2001). The presence of AGP epitopes within *Lolium perenne* microspores reported herein allows to broaden the list of species for which the involvement of these proteoglycans in sexual reproduction is postulated.

Female flower organs are particularly rich in different classes of AGPs that are developmentally regulated (Gane et al. 1995, Majewska-Sawka and Nothnagel 2000). They have been found in abundance in the extracellular matrix of transmitting tracts or in the extracellular mucilage of both dicot and monocot species (Coimbra and Duarte 2003). AGPs are also present in ovaries, especially in epidermal cells of placenta and septum (Pennell et al. 1991, Gane et al. 1995, Lennon et al. 1998). Depending on the species, the stage of development, and the AGP epitope examined, the labelling of the ovules shows a variable spatial pattern. In rape flowers, at the stage when the embryo sac was two- or four-nucleate, the JIM8 epitope could be detected in the nucellar epidermis close to the micropyle region, whereas as the ovule matured, the presence of this AGP extended towards the chalazal end and became additionaly noticable in the membrane of the egg cell and within the synergids. Similarly, in *Amaranthus hypochondriacus*, both MAC207- and JIM8-responsive molecules could be initially detected only in nucellar cells in the region of the future micropyle, whereas later on labelling was also observed within the embryo sac and integuments (Coimbra and Salema 1997, Coimbra and Duarte 2003). In *Pisum sativum*, the epitope that binds MAC207 appears only in the ovule integuments (Pennell and Roberts 1990) and shows a pattern similar to that described by us for *Lolium perenne* for JIM13.

The results of our studies show that floral organs of *Lolium perenne*, a monocot grass species, possess high amounts of β-glucans and also high amounts of pectins that are highly methylesterified and rich in (1→4)-β-D-galactose residues. The latter findings are contradictory to the previously described composition of the wall of members of the Poales, according to which pectins are rather poorly represented within the cell walls of various vegetative organs (Carpita 1996, Willats et al. 2001). Whether the high pectin content reported by us is a species-specific

feature or is rather a phenomenon characteristic for the process of differentiation of flower organs only, will be determined in future studies.

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