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Distribution of Arabinogalactan Protein (AGP) Epitopes on the Anther-derived Embryoid Cultures of *Brassica napus*

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ABSTRACT

The anther-derived embryoid cultures of *Brassica napus* is stably embryogenic and has an extracellular matrix (ECM) layer covering the surface of the developing embryoids. In this study, the distribution of arabinogalactan protein (AGP) epitopes in the ECM layer and the embryogenic tissue of winter oilseed rape were investigated by immuno-labelling with anti-AGP monoclonal antibodies (mAb JIM4, JIM8, and JIM13). There was no labelling by the JIM4 and JIM8 mAbs in the ECM layer, unlike what was reported in other plant species. JIM13 epitope is developmentally regulated because it was only present in the ECM layer of the mature embryogenic tissue. These observations indicate a possible variability in the AGP epitopes present in the ECM layer among the different plant species. JIM8 and JIM13 epitopes were found in some epidermal cells of embryogenic tissue, but not in the non-embryogenic tissue, implying that AGPs might have a specific role in embryogenic competency or determining the cell fate of the *B. napus* embryogenic cells.

Keywords: Arabinogalactan protein, AGP, somatic embryogenesis, immunolocalisation, *Brassica napus*, embryogenic tissue, extracellular matrix layer

INTRODUCTION

Arabinogalactan proteins (AGPs) are proteoglycans containing over 90% (w/w) carbohydrate and can be found membrane bound, cell wall associated, in the intercellular spaces of the tissue or secreted into the medium in cell cultures (Fincher *et al.*, 1983; Knox, 1996; Nothnagel, 1997). The carbohydrate moiety of AGPs consists mainly of arabinose and galactose with minor amounts of uronic acids, while polysaccharides are O-linked to the protein core (Seifert and Roberts, 2007). AGPs have been shown to contribute to various aspects of plant development (Gao and Showalter, 2000; Rumyantseva, 2005) including cell division (Serpe and Northnagel, 1994), cell expansion (Willats and Knox, 1996; Ding and Zhu, 1997), programmed cell death (Chaves *et al*., 2002; Guan and Nothnagel, 2004), sexual reproduction (Coimbra *et al.*, 2007; Pereira *et al.*, 2006) and somatic embryogenesis (Pennell *et al.*, 1992; Kreuger and van Holst, 1993; van Hengel *et al.*, 2001; Samaj *et al.*, 2008).

The use of monoclonal antibodies that bind specific/individual epitopes of arabinogalactan has made it possible to analyse the occurrence

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of AGPs localised in membranes, cell walls and of those secreted to the culture medium (Malinowski and Filipecki, 2002). Previous immunolocalisation studies have shown specific epitopes of AGPs as a marker for transitional states during carrot somatic embryogenesis (Stacey *et al*., 1990; Pennell *et al.*, 1992; McCabe *et al*., 1997; Toonen *et al*., 1997; Schultz *et al*., 1998). In *Picea abies*, it was reported that the lack of staining for the arabinogalactan protein epitope recognised by the monoclonal antibody JIM13 was an efficient marker for distinguishing proembryogenic masses (PEMs) from somatic embryos (Filonova *et al*., 2000). The expression of the JIM4 epitope was studied during the formation of embryos in continuous cultures of callus cells from hypocotyl explants of carrot (Stacey *et al*., 1990). Meanwhile, differential expression of the JIM4 epitope by cells at all stages, from PEMs to the mature embryo, indicated that plasma membrane AGPs are correlated with the position of cells in emerging plant forms.

Following that, AGPs have been shown to be released in the growth medium by cells in an embryogenic suspension culture that can stimulate somatic embryo development in a non-embryogenic culture (Kreuger and van Holst, 1993; Egertsdotter and von Arnold, 1995). This suggests that certain AGPs can be signalling molecules. Some researchers have suggested that a special class of AGPs, containing N-acetylglucosamine (the chitin monomer), can serve as a substrate for the activity of chitinase. Therefore, a pre-treatment of AGPs with EP3 endochitinase was found to have resulted in an optimal somatic embryo-forming activity (van Hengel *et al.*, 2001).

All these studies reinforce the message that AGPs, associated with the plasma membrane and cell wall of plant cells, contain information and can act as signalling molecules to neighbouring cells (cell-cell communication) in the form of surface epitopes produced by distinct tissues or groups of cells during the early stages of somatic embryogenesis. In their previous study, Namasivayam *et al.* (2006) reported the presence of an ECM layer in *Brassica napus* embryogenic cultures. This layer is composed of a fibrillar network that covers the surface of embryoids of a winter oilseed rape embryogenic culture from the pre-embryogenic stage until the early globular stage. Nonetheless, this ECM layer was not present in the non-embryogenic tissue grown under the same experimental conditions. The composition of this layer needs to be elucidated as the first step towards investigating its role during the early events of secondary embryogenesis. Although there have been several attempts to characterise the molecular components of the ECM layer in other plants, including maize (Samaj *et al.*, 1995), coconut (Verdeil *et al.*, 2001), Cichorium (Chapman *et al.*, 2000a: 2000b), wheat (Konieczny *et al.*, 2007) and hybrid fir (Samaj *et al.*, 2008), its composition is still poorly understood even now. It is also not clear if the same components that make up the ECM layer of all the other plant species are also present in the ECM layer of *Brassica napus*.

Therefore, the aim of this study was to find out if AGPs are amongst the ECM components, as well as if and how their epitopes are developmentally regulated during winter oilseed rape secondary embryogenesis. Therefore, an attempt to detect the presence of AGP epitopes in the ECM layer, embryogenic cell clusters in the hypocotyls of winter oilseed rape embryoids, and to compare with non-embryogenic tissue, was carried out by immuno-labelling with anti-AGP monoclonal antibodies (JIM4, JIM8, and JIM13).

MATERIALS AND METHODS

Antibodies

The monoclonal antibodies anti-AGPs (JIM4, JIM8, and JIM13) were kindly provided by Dr. Maureen McCann from the Department of Cell Biology, John Innes Centre, Norwich, UK.

Plant Materials

Sources and preparation of the plant materials for the pre-embryogenic (PEC), mature embryogenic (MEC), and non-embryogenic

(NEC) of *Brassica napus* ssp. *oleifera* cv. Primor tissue were generated using a method identical to the procedure described in Namasivayam *et al.* (2006).

Fluorescence Immunochemistry

All the tissue samples were fixed in 4% (w/v) formaldehyde in 0.1M PIPES buffer, pH 7.2 at room temperature for 4 hr. The tissues were rinsed in the same buffer, dehydrated in a graded series of ethanol solutions and embedded in LR White resin (Agar Scientific Ltd., Essex, UK). Sections were cut at 1 micrometre using a Leica Ultracut UCT (Viennna, Austria) and mounted in groups of 5 on the SuperFrost plus slides (VWR International, Strasbourg, France). Labelling with each antibody was carried out on duplicate slides.

The sections were incubated with 100 µl of blocking buffer (1% (w/v) Bovine Serum Albumin (BSA), 5% (v/v) normal goat serum, 0.01% (v/v) TritonX-100, 0.01% (v/v) Tween-20) in Tris buffered saline (TBS) pH 7.4 for 30 min at room temperature. The blocking buffer was replaced with primary antibody diluted in TBS containing 0.01% (v/v) Tween-20 and 0.01% (v/v) TritonX-100 (TBSTT), with a dilution factor of 1:20. A control without any primary antibody was incubated with blocking buffer. The sections were given 5 X 15 min washes with TBSTT on the next day and then incubated in the secondary antibody; goat anti-rat IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma, UK; 1:100 dilution) with TBSTT, and left for an hr in the dark. The sections were given 6 X 15 min washes with TBSTT, followed by 2 final washes in distilled water. The dried slides were mounted in CITIFLUOR glycerol solution and sealed with clear nail varnish. After that, the slides were viewed under a Leica DM RXA confocal microscope. FITC was excited by the 488 nm wavelength of an argon laser, and emission was collected at 515-530 nm. Photographs were taken using a digital camera (Nikon Cool Pix 950, Japan).

RESULTS

Immunolocalisation with Anti-AGPs

Immunolocalisation of AGP epitopes was carried out with three different monoclonal antibodies (mAbs), JIM4, JIM8, and JIM13 on three different tissues, namely pre-embryogenic tissue (PEC), mature embryogenic tissue (MEC), and non-embryogenic tissue (NEC). Concurrently, negative control labelling experiments with the goat anti-rat FITC conjugate only (no primary antibodies), and without the secondary antibodies, were included. These negative controls did not show any specific bright green FITC immunofluorescence signal but a greenish yellow (*Fig. 1A*) or yellow fluorescent background signal was observed (*Fig. 1B*). This was attributed to the phenolic compounds present in the cell walls, and was mainly found

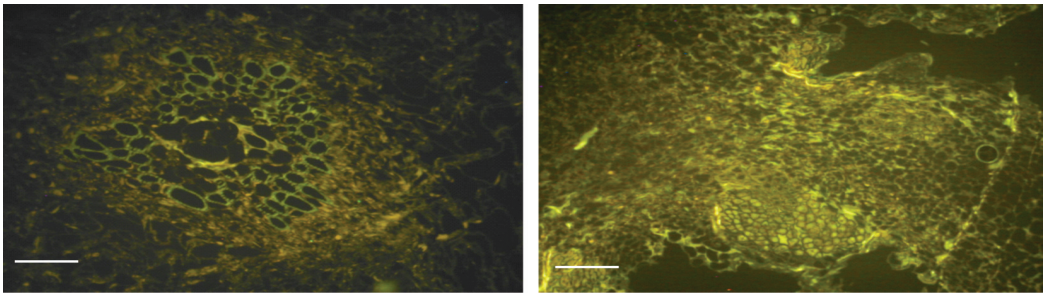

Fig. 1: Negative controls of the immunocytochmical reactions without primary antibodies. Negative controls for both non-embryogenic (A) and m ature embryogenic tissues (B) showed only greenish yellow autofluorescence in the xylem tissues and yellow fluorescence overall. Bars = 100 µm and 150µm respectively. MEC, mature embryogenic tissue; NEC, non-embryogenic tissue; C, cortex; P,pith; e, epidermis

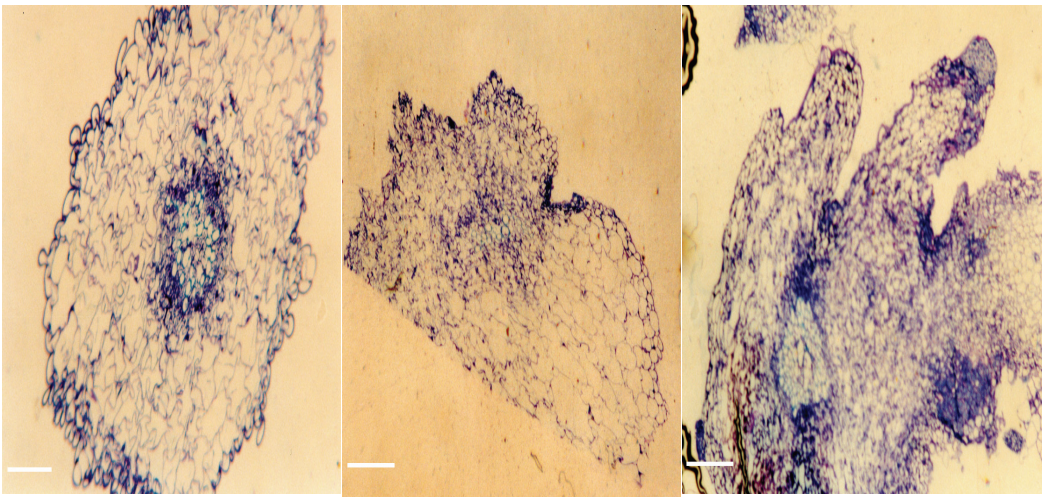

Fig. 2: Light micrographs of sections of NEC, PEC and MEC. Longitudinal and cross sections of 3 different samples for immunolocalisation studies, stained with AzureA/ methylene blue for anatomical analysis and to assist in identification of tissues and cells. NEC, non-embryogenic tissue, Bar= 80μm; PEC, pre-embryogenic tissue, Bar=250μm; MEC, mature embryogenic tissue, Bar=400μm. e, epidermis; C, cortex; P, pith

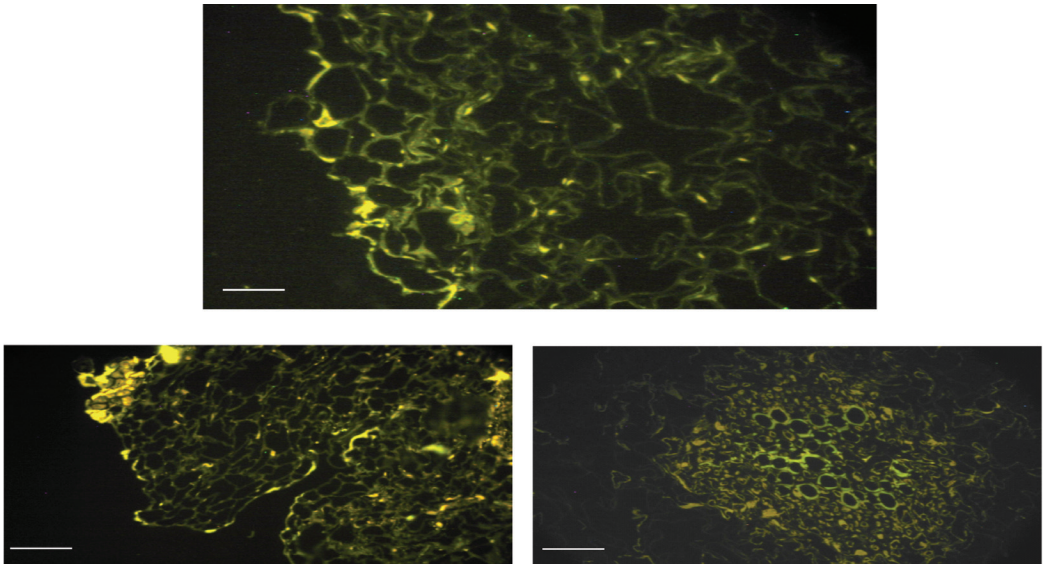

Fig. 3: Immunolocalisation of JIM4 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilseed rape. A, Immunolabelling of longitudinal sections of PEC tissue showing no signal except for yellow background fluorescence. Bar=40μm. B, Immunolabelling of longitudinal sections of MEC tissue showing no signal except for yellow fluorescence. Bar=60μm. C, Immunolabelling of cross sections of NEC hypocotyl showing greenish yellow autofluorescence in xylem tissues and yellow fluorescence overall. Bar=100μm. C, cortex; ep, epidermis; P, pith; pe, proembryoid

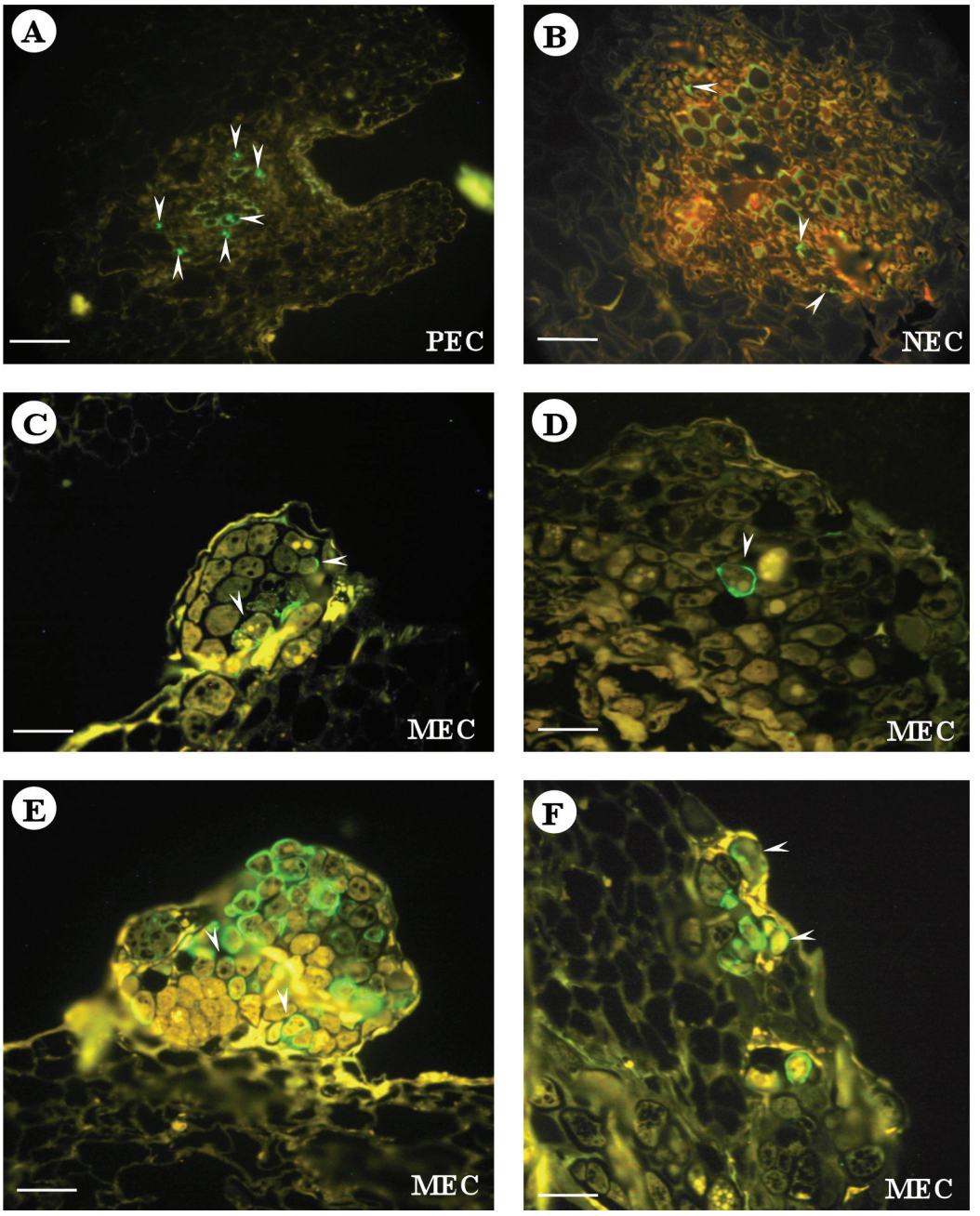

*Fig. 4: Immunolocalisation of JIM8 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilsedd rape. A, B, Hypocotyls of PEC and NEC showing bright green fluorescence only in the vascular tissues. Bars=200*µm *and 100*µm *respectively. C, E, F, Immunofluorescence over preembryoid on the epidermal cell layer of MEC tissue and arrows denote uneven labelling on the cell wall. Bars= 50*µm, *30*µm *and 50*µm *respectively. D, uneven immunofluorescence signal in the cell wall of one of the subepidermal cells. Bar=50*µm. *C, cortex; ep, epidermis; P, pith; pe, proembryoid*

in the vascular tissues or in the walls of large clusters of cells in the epidermis. Transverse and longitudinal sections of the sample tissues were stained with Azure A/methylene blue for anatomical studies (*Fig. 2*).

Immunolocalisation with JIM4

Immunolocalisation of the JIM4 epitope showed no immunolabelling signal in all tested samples (*Fig. 3A-C*). The mAb JIM4 was originally generated by immunisation with carrot protoplasts. The antibody has been shown to recognise AGP epitopes from the medium of suspension cultured carrot cells, gum arabic, and other AGP-like molecules (Knox *et al.*, 1989). Although fluorescence was observed in the vascular tissues in NEC tissue (*Fig. 3C*) this was attributable to autofluorescence since untreated sections also gave similar results (*Fig. 1A*). An attempt at immunolocalisation of the JIM4 epitope was also negative for the extracellular matrix layer of the PEC (*Fig. 3A*) and MEC (*Fig. 3B*) tissues.

Immunolocalisation with JIM8

JIM8 epitopes were detected in all the tested samples and the pattern of recognition varied between different types of tissues and stages of development. No immunolabelling was ever detected in the ECM layer of all the tissues tested. mAb JIM8 was originally developed from an immunization with sugar beet protoplasts (Pennell *et al*., 1991). JIM8 recognizes a carbohydrate epitope present in the plasma membrane arabinogalactan proteins in sugar beet leaves (Pennell *et al*., 1991) and labels several cell types in carrot embryogenic suspension cultures at the cell wall (Pennell *et al*., 1992; Toonen *et al*., 1996; McCabe *et al*., 1997). In this study, two types of labelling patterns were observed with mAb JIM8. This antibody labelled the walls of certain cells in a few proembryoids of the mature embryogenic tissue (*Fig. 4C, E* and *F*), and also labelled the vascular tissues (*Fig. 4A, B*) (*see* arrowheads) of all the tested samples. In the MEC tissue (*Fig. 4C, E, F*), proembryoids are actually clusters of embryogenic cells comprising small, spherical or oval-shaped cells with dense cytoplasm. Nonetheless, only some of those cells had JIM8-reactive cell walls although all the cells were parts of the same cell cluster. Moreover, no obvious morphological/histological difference was found between the JIM8 reactive and non-reactive cells. In addition, some of the JIM8 reactive cells only contained patches (*Fig. 4 C-F* see arrowheads) of FITC fluorescence in the cell wall. No immunolabelling was observed in any of the randomly distributed and densely cytoplasmic cells in the pre-embryogenic tissue or in epidermal or cortical parenchyma cells of NEC tissues (data not shown). However, bright green fluorescence labelling was detected in vascular tissues of PEC and NEC tissues, as shown in *Fig. 4A* and *B* (*see* arrowheads) and the same labelling pattern of JIM8 was observed in the vascular tissues of MEC (data not shown).

Immunolocalisation with JIM13

Immunofluorescence examination indicated that JIM13 epitope could be detected in all the tested tissues but it showed a pattern of expression restricted to specific tissues and developmental stage. JIM13 mAb was raised against the AGP fraction isolated from conditioned medium of an embryogenic cell suspension of carrot (Knox *et al*., 1991). In particular, JIM13 epitope was frequently found in the MEC tissue, almost throughout the whole region of the pre-embryo stage, labelling majority of the cell walls, cytoplasm and occasionally tiny patches of bright green fluorescence were found in the vacuole (*Fig. 5C*). There was also weak immunofluorescence detected in the ECM layer coating the proembryoid (indicated by the arrowheads in *Fig. 5C*), but not on the ECM layer over the epidermal cells (*see* tiny arrows in *Fig. 5C*). Based on the labelling intensity and binding pattern of JIM13, it appeared that the more vacuolated cells were reactive to JIM13 as compared to densely cytoplasmic cells in proembryoids (compare *Fig. 5C* and *Fig. 5D*). Another notable feature was that

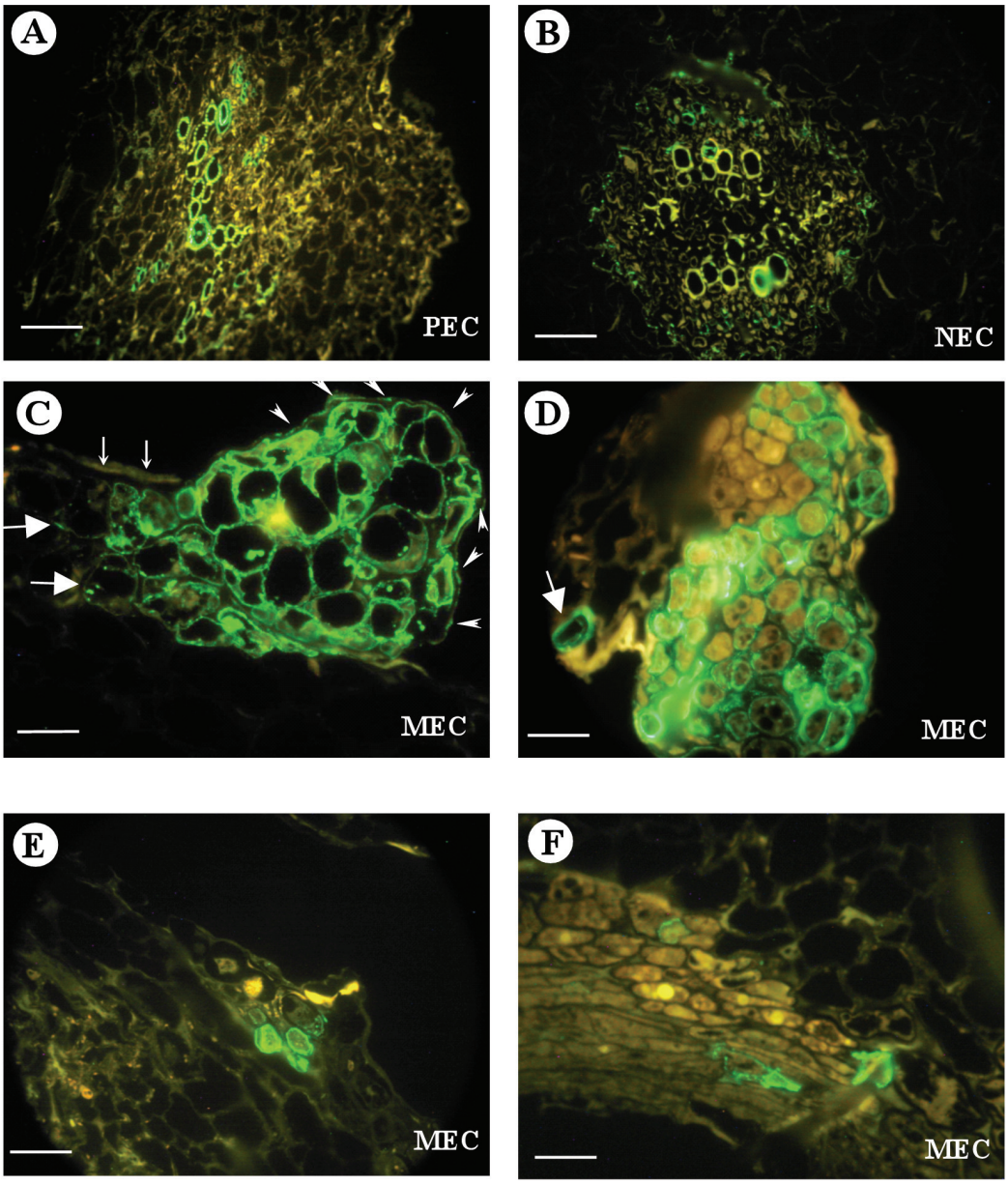

*Fig. 5: Immunolocalisation of JIM13 epitope in sections of pre-embryogenic (PEC), mature embryogenic (MEC) and non-embryogenic (NEC) tissue of oilseed rape. A, B, Immunolabelling of PEC and NEC hypocotyls showing bright green fluorescence particularly in xylem and putative phloem cells in the vascular tissues. Bars = 120um and 50um respectively. C, A proembryoid with bright green fluorescence in all the cells and weak immunofluorescence (shown by arrowheads) in the ECM layer coveing the proembryoid. Bar=25*μ*m. D, A, proembryoid with only some cells that showed positive signal to JIM13 antibodies. Bar=25*μ*m. E, F, Immunolabelling in a few epidermal and subepidermlal cells at the periphery of the MEC tissue. Bars=120*μ*m. C, cortex; e, epidermis; se, sub-epidermis; P, pith; pe, proembryoid*

immunolabelling was not detected throughout the mature or later stages of somatic embryoids, but restricted to some regions of the embryoid (*Fig. 5D*). Furthermore, immunofluorescence was also found in cells adjacent to the developing embryo (*Fig. 5C, D* see big arrows). Bright green fluorescence signal was also observed in a few epidermal and sub-epidermal cells at the periphery of MEC tissue (*Fig. 5E*). In the pre-embryogenic tissue, JIM13 epitope was not detected in the ECM layer or any of the epidermal cells or cells with dense cytoplasm (*Fig. 5A*), indicating that the epitope is not expressed during the early stages of embryogenic tissue development. In addition, for all the tested samples, the labelling experiment also showed the presence of JIM13 epitope in vascular tissues, particularly xylem cells with thick cell walls (*Fig. 5A, B*).

DISCUSSION

In the winter oilseed rape embryogenic culture, immunofluorescent light microscopy investigations using mAbs JIM4, JIM8, and JIM13 revealed that only JIM13 epitope was present within the ECM layer covering the proembryoid. Interestingly, JIM13 epitope was not detectable in the ECM layer at an earlier stage in the pre-embryogenic tissue, suggesting that JIM13 epitope is developmentally regulated. Similar observations were reported for Cichorium where an immunolabelling investigation using mAb JIM13 revealed that AGPs are present within the outer cell layer of young globular somatic embryos and as the embryos developed further, AGP expression was observed in the inner region of the walls of embryogenic cells (Chapman *et al*., 2000). In hybrid fir, specific AGPs containing β-(1→6)galactotetraosyl group was reported to be a component of ECM covering embryogenic cells of gymnosperm (Samaj *et al.*, 2008).

It is surprising that there was no labelling by JIM4 mAb in the ECM layer of the winter oilseed rape embryogenic culture because JIM4 epitope has been shown to be present in the ECM layer of maize embryogenic callus cells (Samaj *et al.*, 1999b). The authors also suggested that JIM4 antibody could be used as an early marker for embryogenic competence in maize callus cells. The absence of JIM4 epitope in the ECM layer of the winter oilseed rape embryogenic culture indicates a possible variability in the composition of the ECM layer among the different plant species. Similarly, JIM4 epitope was not detected in the ECM layer of androgenic callus of wheat (Konieczny *et al*., 2007) and AGPs of sugar beet cultures (Wisniewska and Majewska-Sakwa, 2007). Moreover, there was no positive control for immunolabelling with JIM4. Without it, it is possible that the activity of JIM4 mAb may be too low to detect the presence of JIM4 epitopes in the tissue samples tested.

The monoclonal antibody JIM8 has been shown to react with AGP epitopes in sexual organs, the eight celled embryo and a very limited number of other cell types in oilseed rape (Pennell *et al*., 1991). Recently, Coimbra and Salema (1997) have shown that young zygotic embryos and suspensor cells of *Amaranthus hypochondriachus* are reactive to JIM8. In carrot suspension cultures, JIM8 epitope has been localised on three different cell membrane AGPs (Pennell *et al.*, 1991) and on AGPs secreted into the suspension culture (Knox *et al*., 1991). It has been postulated that JIM8 epitope is a marker for embryogenic capacity of a cell culture as a whole, rather than for a competent cell state (Toonen *et al*., 1996). All these studies support the idea that AGPs play an important role in morphogenesis during zygotic and somatic embryogenesis.

The detection of the green fluorescent labelling with JIM8 and JIM13 in the vascular tissues of both embryogenic and non-embryogenic hypocotyls is consistent with the findings of some previous studies. Among other, JIM13 epitope was found to correlate with the early development of xylem in the roots of Arabidopsis (Dolan *et al*., 1995) and carrot (Knox *et al*., 1991) as well as xylem maturation in maize coleoptiles (Schindler *et al*., 1995), radish roots and carrot roots (Casero *et al*., 1998), and it has also been proposed that AGPs can identify cells committed to programmed cell

death. More recently, JIM8 and JIM13 epitopes were detected in protophloem sieve elements in maize roots (Samaj *et al*., 1998).

JIM8 and JIM13 epitopes were found scattered randomly in some of the densely cytoplasmic cells in the proembryoid and globular stage embryoids in mature embryogenic tissues. Interestingly, those cells expressed these epitopes on the cell wall or plasma membrane only at the later stages of development and not at the earlier pre-embryogenic stage. This implies that the expression of AGP epitopes is being developmentally regulated during the secondary embryogenesis in the winter oilseed rape embryogenic culture, as observed in other plant systems including *Picea abies* (Filonova *et al*., 2000; Egertsdotter and von Arnold, 1995), carrot cultures (Toonen *et al*., 1996; McCabe *et al*., 1997; Stacey *et al*., 1990; Pennel *et al*., 1992) and Cichorium (Chapman *et al*., 2000). However, the significance of AGP epitopes in some of the proembryoid or cytoplasm-rich cells or globular stage embryoids is not clear. There is a possibility that the cells with specific AGP epitopes may be involved in cell-cell recognition and maintenance of close cell contacts during the formation of proembryoids. The fact that anti-AGP mAbs (JIM8 and JIM13) labelled some of the epidermal or sub-epidermal cells in the embryogenic tissue and not the epidermal cells of the non-embryogenic tissue is an interesting observation, implying that AGPs may have a specific role in embryogenic competency or determining the cell fate of embryogenic cells. For future work, it will be interesting to see the three-dimensional immunolocalisation of the AGP antigens on the ECM layer using silver enhanced immunogold scanning electron microscopy (Samaj *et al.*, 1999a).

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