Generation of Recombinant Antibodies against Orchardgrass Acidic nsLTP-Like Proteins

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Embryogenic and non-embryogenic suspension cultures of orchardgrass (*Dactylis glomerata* L.) secreted into the culture medium a set of proteins, among which low molecular mass (11/12 kDa) proteins were found. However, only the 11/12 kDa proteins from the embryogenic suspension cultures reacted specifically with an antiserum raised against the carrot EP2 non-specific lipid transfer protein (nsLTP). Two-dimensional (2-D) electrophoretic analysis revealed that the extracellular nsLTP-like proteins from the embryogenic lines were acidic proteins, with pI values ranging between 4.3 and 6.4, and the 11/12 kDa proteins of the non-embryogenic lines were basic ones (pI 8–9.3). This is only the second case to report on the accumulation of extracellular acidic nsLTP-like proteins in the culture medium during somatic embryogenesis. A naïve phage display Griffin1. library was used to select single-chain phage antibodies, which specifically bind to acidic nsLTP-like proteins. Nine phage clones were selected after four rounds of biopanning of the target proteins blotted on a nitrocellulose membrane. Three soluble monoclonal single-chain phage antibodies, expressed in the non-suppressor *E. coli* strain HB2151, were purified by metal affinity chromatography and found to be highly specific for the acidic nsLTP-like proteins from the embryogenic suspension cultures. The application of the selected monoclonal antibodies for localization and elucidation of the role of the acidic nsLTP-like proteins *in vivo* is discussed.

Key words: Lipid Transfer Proteins, Somatic Embryogenesis, Recombinant Antibodies

Introduction

The ability of plants to initiate embryo development from somatic cells by somatic embryogenesis is based on cellular totipotency that is unique in higher plants. How somatic cells undergo a change of fate to become embryogenic is still largely unknown (for reviews see Fehér *et al.*, 2003; Quiroz-Figueroa *et al.*, 2006). Somatic embryogenesis in cell suspension cultures offers an alternative way to study this problem. Suspension cultures secrete into the medium glycoproteins derived mainly from the plant cell wall, which play an important role in somatic embryogenesis by their ability to either promote or inhibit embryo development (for review see Matthys-Rochon, 2005). One of the secreted proteins shown to play a key role in carrot somatic embryogenesis was identified as a 10 kDa lipid transfer protein designated EP2 (Sterk *et al.*, 1991).

Non-specific lipid transfer proteins (nsLTPs) are ubiquitous lipid-binding proteins from plants (for reviews see Kader, 1996; Carvalho and Gomes, 2007). They are characterized by their ability to transfer phospholipids between membranes and to bind fatty acids *in vitro*. The nsLTPs are small, basic proteins (pI 8.8–10.0) ranging in size from 7 to 12 kDa. Several functions have been proposed for the role that nsLTPs play in plant physiology including cutin synthesis and somatic embryogenesis (Sterk *et al.*, 1991; Sabala *et al.*, 2000), plant signalling (Blein *et al.*, 2002; Maldonado *et al.*, 2002), plant defense against phytopathogens (Molina *et al.*, 1993; Regente *et al.*, 2005) and allergenicity (Marion *et al.*, 2004). Expression of LTPs is a well-known marker of somatic embryo induction (Sterk *et al.*, 1991; Sabala *et al.*, 2000). It is also a marker for embryo differentiation, as it has been detected in the protoderm of developing somatic and zygotic embryos (Vroemen *et al.*, 1996; Toonen *et al.*, 1997). Taken together, a correct expression of *ltp* genes is required for normal embryo development. Still, the *in vivo* function of nsLTPs remains to be elucidated.

Studies on the role of secreted proteins and their use as markers for somatic embryogenesis in

monocots are relatively scarce (Nielsen and Hansen, 1992; Stirn *et al.*, 1995; Tchorbadjieva *et al.*, 2005; Tchorbadjieva, 2006). In search for embryogenic markers, we identified 11/12 kDa proteins constitutively secreted in the medium of embryogenic suspension cultures as acidic nsLTP-like proteins with as yet unknown function. Secretion of 9 kDa acidic and basic nsLTP-like proteins in the culture medium of *Cichorium* has been reported (Blanckaert *et al.*, 2002). The authors suggested that the acidic LTP-like protein played a role in the early induction of somatic embryogenesis. To our best knowledge, this is the second case to report on the secretion of acidic nsLTP-like proteins in the medium of embryogenic suspension cultures and it remains to elucidate their role in somatic embryogenesis.

Phage display has proven a useful technique for rapid selection of high affinity antibody fragments from large repertoires to any protein of interest (Willats, 2002; Hoogenboom, 2005). The technique involves the display of a library of single-chain fragment variable (scFv) fragments on the surface of filamentous phages followed by selection of the desired recombinant phages by means of specific binding to an antigen of interest. The scFv fragments are small heterodimers comprising the antibody heavy-chain (V_H) and light-chain (V_L) variable domains that are connected by a peptide linker to stabilize the molecule. The expression of scFv antibodies inside the cell (intrabodies) provides an effective strategy to interfere with the function of the target molecule *in vivo* ("immunomodulation") (De Jaeger *et al.*, 2000).

In this report, we describe the use of a synthetic phagemid library for the generation of monoclonal scFv antibodies directed against acidic nsLTP-like proteins. The specificity of binding was confirmed by Western blot. Furthermore, we discuss the possible application of the selected scFvs as intrabodies to study the function of acidic nsLTP-like proteins *in vivo*.

Materials and Methods

Plant material and suspension cultures

Callus-derived suspension cultures from four embryogenic (E_1, E_2, E_3, E_4) and three non-embryogenic (NE_1, NE_2, NE_3) cell lines of orchardgrass (*Dactylis glomerata* L.) were initiated according to Conger *et al.* (1989) and maintained in a liquid SH30 medium essentially as previously described (Tchorbadjieva and Odjakova, 2001).

Protein preparation

Suspension-cultured cells at day 14 after transfer onto fresh medium were centrifuged at $500 \times g$ for 5 min and the culture medium was recovered by passing the supernatant through a Millipore 0.22 μm filter. Extracellular proteins in the medium were prepared according to De Vries *et al.* (1988). Briefly, proteins were precipitated by the addition of 2.5 volumes of ethanol, and after standing overnight at 4 °C the precipitate was collected by centrifugation (12000 $\times$ g at 4 °C for 30 min), vacuum-dried and stored at −70 °C or dissolved in water for immediate use. The cell wall proteins were isolated from sedimented living cells and the proteins were precipitated as described for extracellular proteins (Tchorbadjieva and Odjakova, 2001). The protein content was determined according to Bradford (1976). As molecular mass standards the LMW-SDS marker kit (GE Healthcare/Amersham Biosciences, Uppsala, Sweden) and Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Okadjima *et al.* (1993). Analytical flat bed isoelectric focusing (IEF) of extracellular proteins was carried out on 5% (w/v) polyacrylamide gels containing ampholines in the pH range of 3.5 to 9.5 (GE Healthcare/Amersham Biosciences). After IEF the excised lanes were equilibrated for 20 min in equilibration buffer [50 mM dithiothreitol (DTT), 2% (w/v) SDS, 0.01% (w/v) Bromphenol Blue and 62.5 mM tris(hydroxymethyl)aminomethane (Tris) adjusted to pH 6.8 with HCl] and separated by SDS-PAGE in the second dimension. Proteins were stained with silver nitrate according to Blum *et al.* (1987).

Immunoblotting of extracellular proteins

After SDS-PAGE, the proteins from the medium were electrotransferred at 1 mA cm^{-2} for 1 h onto 0.2-μm PVDF membranes using a semi-dry apparatus (Hoefer Semi-Phor). The membrane was blocked for 1 h in TBST-0.5 (10 mM Tris, pH 7.4, 150 mM NaCl and 0.5% Tween 20) and incubated for 1 h with a 1:1000 dilution of rabbit anti-

serum raised against carrot EP2 nsLTP (a kind gift of Prof. Sacco de Vries, University of Wageningen, The Netherlands) in the same buffer, but with 0.05% Tween 20 (TBST-0.05). After three washes in TBST-0.05 (5 min each) the membrane was incubated for 45 min in a 1:10000 dilution of alkaline phosphatase-labelled goat anti-rabbit IgG. Detection was achieved with SIGMA *FAST*™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets according to the manufacturer's instructions.

Selection of anti-acidic nsLTP-like protein phagemids

Extracellular proteins from the embryogenic suspension culture E_1 were separated by SDS-PAGE and blotted onto a nitrocellulose (NC) membrane using the conditions described in *Gel electrophoresis*. One strip of the membrane was excised and stained with 0.1% (w/v) Amido Black in 25% *i*-propanol/10% CH_3COOH to visualize the transferred proteins, and the band corresponding to the 11/12 kDa proteins was subjected to phage panning (Nakamura *et al.*, 2001). Phage antibodies were selected through four rounds of panning from the human synthetic scFv Griffin.1 library which is in phagemid format (Griffiths *et al.*, 1994). The library was obtained as a generous gift from Prof. Greg Winter, MRC, Cambridge, UK. Basic manipulations of phages were performed according to the library protocol. For each round of panning, one piece of the membrane containing the blotted 11/12 kDa proteins, after blocking with 5% skimmed milk in PBS (MPBS), was incubated for 120 min at room temperature with phages $(1 \cdot 10^{12}$ cfu) in 1 ml of 5% MPBS containing 0.1% Tween 20 (MPBST). After washing 10 times with PBS containing 0.1% Tween 20 (v/v) and 10 times with PBS, bound phages were eluted with 100 mᴍ triethylamine, neutralized with 1 ᴍ Tris-HCl (pH 7.4), and used to infect *E. coli* (TG1 strain). Infected bacteria were plated on tryptone yeast extract (TYE) containing 1% glucose and 100 μg/ml ampicillin and grown overnight at 30 °C. The phages were rescued by helper phage VCS M13, and the excised phagemids were harvested by polyethylene glycol/sodium chloride precipitation and used for the next rounds of selection.

Selection of monoclonal phage antibodies

After four rounds of panning, 42 single colonies from the final polyclonal population were ran-domly selected, individually grown and screened for acidic nsLTP-like protein-binding activity by Western blot. Extracellular proteins from *D. glomerata* L. embryogenic suspension culture E_1 were electrotransferred onto a NC membrane after SDS-PAGE. After blocking with 0.5% TPBS, the membrane was incubated with the scFv-displaying phages $(1 \cdot 10^{12}$ cfu) in 1 ml TPBS overnight at 4 °C. Then the NC membrane was thoroughly washed with 0.1% TPBS and incubated with anti-M13-HRP conjugate (GE Healthcare/Amersham Biosciences) in 0.05% TPBS for 2 h at room temperature. After subsequent washes of the membrane with 0.05% TPBS and PBS, the bound recombinant phages were visualized with 0.5 mg/ml DAB in PBS.

Soluble expression and purification of the phage-displayed antibodies, scFv #3, scFv #8 and scFv #12

The recombinant antibodies in the Griffin.1 library have been inserted in the pHEN2 vector that allows the glucose-sensitive expression of myc and His_6-tagged scFvs. In the amber-suppressor *E. coli* strain TG1 scFvs are produced as fusion proteins with the pIII phage protein whereas in the non-suppressor *E. coli* strain HB2151 only soluble scFvs are produced. The scFvs with the PelB leader sequence at the *N*-terminus were expressed periplasmically from the pHEN2-scFv vector in *E. coli* HB2151 using 0.5 mᴍ IPTG for 3 h at 30 °C. After induction soluble scFvs were prepared essentially as described by Kipriyanov *et al.* (2003). The His-tagged scFvs were purified by nickel affinity chromatography using a His-Trap column (GE Healthcare) as recommended by the manufacturer. Western blot of recombinant soluble phage antibodies was done using anti-c-myc-HRP antibodies (Invitrogen, San Diego, CA).

Results and Discussion

Detection of LTP-like proteins in D. glomerata L. embryogenic suspension cultures

In search for embryogenic markers among the extracellular proteins of suspension cultures, polyclonal antibodies against a carrot nsLTP designated EP2 (Sterk *et al.*, 1991) were used to probe Western blots after SDS-PAGE separation of extracellular proteins from four embryogenic (E_1, E_2, E_3, E_4) and their corresponding non-embryogenic (NE_1, NE_2, NE_3) cell lines (Fig. 1). Silver-
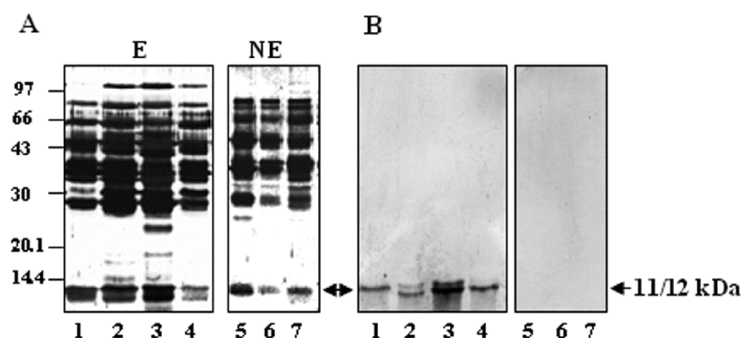
Fig. 1. SDS-PAGE protein pattern of extracellular proteins secreted into the medium of embryogenic E_1 (1), E_2 (2), E_3 (3), E_4 (4) and non-embryogenic NE_1 (5), NE_2 (6), NE_3 (7) suspension cultures of *D. glomerata* L. (A) Silver stain; (B) immunoblot with anti-EP2 antibody after transfer on a PVDF membrane. Protein mass markers are given in kDa on the left.

stained 1-D gels (Fig. 1A) showed the presence of 11/12 kDa proteins in both embryogenic and non-embryogenic cultures. However, Fig. 1B (left panel) shows that the anti-EP2 antibody strongly and specifically cross-reacts with an 11/12 kDa protein band from the culture medium of embryogenic lines only. Competent single cells from the embryogenic cell lines go through three phases of somatic embryo development, from microclusters to proembryogenic masses (PEMs) to somatic embryos, while the non-embryogenic single cells divide to form microclusters whose further development is blocked. LTP-like proteins were found in the medium during all phases of somatic embryogenesis of the embryogenic cell lines showing a constitutive expression of these proteins during development (data not shown). As the induced suspension cultures of the embryogenic and non-embryogenic cell lines share the same explant origin and were cultured under the same conditions, the LTP-like proteins detected in the embryogenic cultures could possibly be used as markers for embryogenic potential.

Secretion of an LTP (EP2) from carrot somatic embryos and their precursor cells PEMs has been reported by Sterk *et al.* (1991) who have proposed a role for nsLTPs in cutin biosynthesis by effecting the transport of cutin monomers through the extracellular matrix. Cutin is only present in embryogenic regions and on embryos as a homogenous and continuous layer. A possible role for cutin in the cell wall of embryogenic cells could be the physiological isolation of embryogenic competent cells from their neighbours as a prerequisite for organized development. Expression of lipid trans-

fer proteins is an early marker of somatic embryo induction in different systems (Sterk *et al.*, 1991; Toonen *et al.*, 1997; Sabala *et al.*, 2000). Our data is in accordance with the study of Coutos-Thévenot *et al.* (1993), who found four basic LTP isoforms in the medium of grapevine embryogenic cell cultures only, and that of Poulsen *et al.* (1996), who detected an EP2-like protein in alfalfa embryogenic suspension cultures. We suppose that the LTP-like proteins in the culture medium of *D. glomerata* L. embryogenic cell lines might contribute to normal somatic embryo development by playing a role similar to that suggested for the carrot lipid transfer protein EP2.

Two-dimensional (2-D) electrophoretic analysis of D. glomerata L. extracellular proteins

For more detailed characterization of the LTP-like proteins the extracellular proteins from all lines were separated by 2-D gel electrophoresis (Fig. 2). It was found that the 11/12 kDa proteins from the embryogenic suspension cultures consisted of five acidic and slightly neutral proteins (pI 4.3, 4.5, 4.8, 5.3 and 6.4) (Fig. 2A). The non-embryogenic cell lines secreted basic 11/12 kDa proteins (pI 8−9.3) (Fig. 2B). The 2-D patterns of the other lines were identical and are not presented here. During somatic embryogenesis, basic nsLTPs have been found to be secreted in the culture medium of embryogenic cell cultures of carrot (Sterk *et al.*, 1991) and grapevine (Coutos-Thévenot *et al.*, 1993). Secretion of a 9 kDa acidic nsLTP-like protein in the culture medium of *Cichorium* has been reported with a proposed role
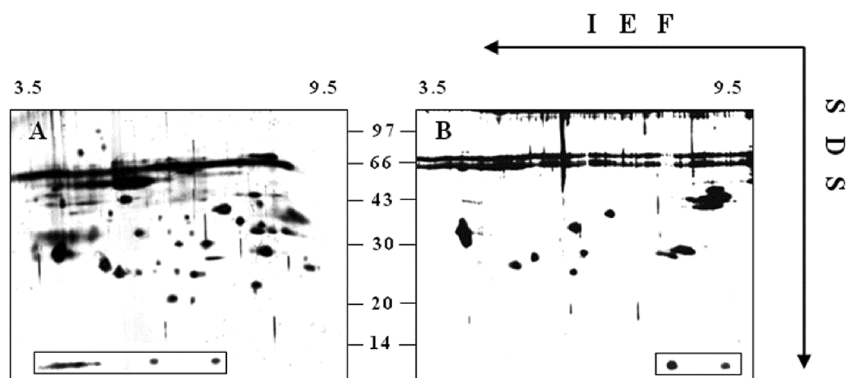
Fig. 2. Two-dimensional gel electrophoresis of extracellular proteins from the culture medium of (A) embryogenic E_1 and (B) non-embryogenic NE_1 suspension cultures of *D. glomerata* L. (silver stain). The 11/12 kDa proteins are presented in boxes.

for the induction of somatic embryogenesis (Blanckaert *et al.*, 2002). To our best knowledge, this is the second case to report on the secretion of acidic nsLTP-like proteins in the medium of embryogenic suspension culture.

In most species, several LTP genes exist which are often differentially regulated (Coutos-Thévenot *et al.*, 1993; Vignols *et al.*, 1997; Arondel *et al.*, 2000). The various *ltp* genes show complex expression patterns and are temporarily and spatially controlled. Besides the transfer of lipids, other functions have been assigned to LTPs, such as protection from biotic and abiotic stress. Interestingly, Arondel *et al.* (2000) were the first to report on three acidic plant ltps from *Arabidopsis* with pI values very close to those of the acidic nsLTP-like proteins from suspension cultures of *D. glomerata* L., and it remains to be elucidated what role the acidic nsLTP-like proteins might play in the process of somatic embryogenesis.

Selection of phage clones

The aim of the present study was the generation of scFvs directed against acidic nsLTP-like proteins from *D. glomerata* L. embryogenic suspension cultures which could be later used as intrabodies in loss-of-function experiments. For this purpose, we used a method to rapidly assign monoclonal antibodies from the human synthetic scFv Griffin.1 library for Western-blotted antigens (Nakamura *et al.*, 2001). To identify individual specific scFv phage binders, the eluted scFv phages after the fourth round of NC membrane panning were used to infect TG1, which were then plated

to form single colonies. Next, 42 colonies selected at random were picked and tested for binding to acidic nsLTP-like proteins by means of Western blot. Of these, 9 phage antibodies recognized different protein bands in Western blot (Fig. 3). ScFv #3, scFv #8 and scFv #12 specifically recognized the 11/12 kDa proteins as expected. Interestingly, scFv #13, scFv #17 and scFv #18 recognized a 24 kDa band only, and the other three phage antibodies – scFv #14, scFv #15 and scFv #16 – recognized both of them the 11/12 kDa and the 24 kDa bands. Experiments in our laboratory (unpublished data) have shown 11/12 kDa proteins form dimers in solution and this is probably the reason to select phage antibodies to the monomeric, dimeric or both forms. It is yet unclear what type of bonds holds the dimers together; they must
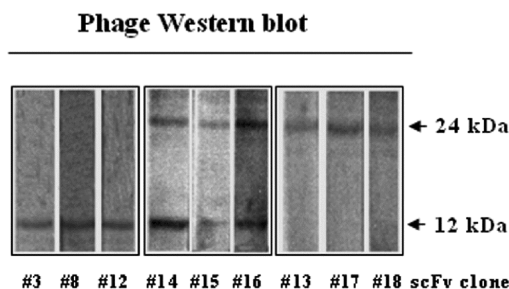
Phage Western blot

Fig. 3. Monoclonal phage Western blots against acidic nsLTP-like proteins. Extracellular proteins from *D. glomerata* L. embryogenic suspension culture E_1 were blotted on the nitrocellulose membrane after SDS-PAGE and incubated with antibody-displaying phages produced by randomly chosen clones from the fourth round of selection.
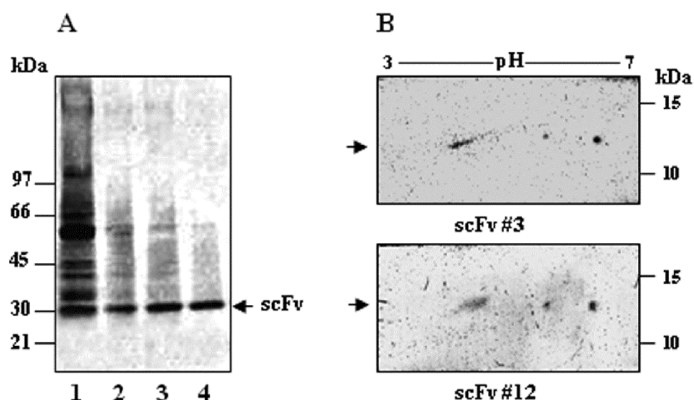
Fig. 4. Purification and binding activity of soluble scFv #3 and scFv #12. (A) Coomassie Blue-stained 10% SDS-PAGE showing the purification of soluble scFv # 3 and scFv #12 by nickel affinity chromatography. 1, Total cell lysate from IPTG-induced *E. coli* HB2151; 2, purified scFv #3; 3, purified scFv #8; 4, purified scFv #12. (B) Detection of acidic nsLTP-like proteins from *D. glomerata* L. embryogenic suspension culture E_1 with soluble scFv fragments after 2-D gel electrophoresis. Both soluble scFvs recognize all five acidic nsLTP-like proteins (arrows).

be rather strong ones if they still resist the denaturing and reducing conditions of SDS-PAGE. The antibodies to the dimeric form could be used to study the possible involvement of these dimers in the control of the protein's functional activity. Traditionally, immunotubes have been used for the immobilization of target proteins. To immobilize the acidic nsLTP-like proteins, we used an NC membrane, and our results showed that this is a highly effective method, which requires only a small amount of transferred protein (about 100/200 ng/cm² per round of selection) and no preliminary purification of the target protein.

Expression, purification and binding activity of soluble monoclonal scFvs

In many cases monoclonal phage antibodies can be used directly as antigen binders in ELISA or Western blot analysis, without being expressed in soluble form and further purified. However, their relatively large size makes them poor probes for immunocytochemistry and, as Manfield *et al.* (2005) have shown, the soluble, phage-free scFv format allows immunolocalization of plant cell wall antigens with high resolution. For this reason the scFv #3, scFv #8 and scFv #12, recognizing the acidic nsLTP-like proteins (11/12 kDa), were chosen for further analysis. The corresponding scFv phages were used to infect the non-suppressor strain *E. coli* HB2151 for the soluble expression of the selected antibodies. After induction of expression with IPTG, the soluble scFvs (containing a

polyhistidine tag) were purified from crude *E. coli* periplasmic fractions by metal affinity chromatography and their purity was evaluated in 10% SDS-PAGE stained with Coomassie Blue (Fig. 4A). Each of the purified antibodies was visualized as a 30 kDa band. The production yielded 2 mg of each pure scFv per liter of culture medium. The reactivity of the purified soluble scFv #3 and scFv #12 antibodies with extracellular proteins was tested by Western blot analysis (Fig. 4B). Both antibodies were expressed in functional and stable form and recognized all five acidic nsLTP-like proteins.

Mostly all known nsLTPs in dicots and monocots are basic proteins with pI values of 8.8–10 (Sterk *et al.*, 1991; Kader, 1996; Vroemen *et al.*, 1996; Wang *et al.*, 2004) with much diversified functions. In the present study, we are the second to report on the secretion in the culture medium of acidic nsLTP-like proteins during somatic embryogenesis. The presence of *D. glomerata* L. acidic nsLTP-like proteins in the cell wall (unpublished results) and culture medium of embryogenic suspension cultures at early stages of somatic embryo development, their low molecular mass (11/12 kDa) and overall cross-reactivity to antibodies against carrot LTP EP2 makes it probable that they play a role similar to that proposed for the carrot nsLTP EP2.

Immunomodulation is a molecular technique which makes use of the possibility for ectopic expression of genes encoding phage antibodies. As a

result, interference with cellular metabolism, signal transduction or pathogen infectivity can be accomplished in plant cells (De Jaeger *et al.*, 2000). The monoclonal scFvs selected to acidic nsLTP-like proteins, when fused to fluorescent proteins, would be a valuable tool to study and modulate their function, as well as to track them through the cell *in vivo*.

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