Planta (1998) 205: 56–63

Planta

Extracellular β-1,3-glucanases are induced during early somatic embryogenesis in *Cichorium*

Stéphane Helleboid[1], Guy Bauw[2], Lionel Belingheri[1], Jacques Vasseur[1], Jean-Louis Hilbert[1]

[1]Laboratoire de Physiologie Cellulaire et Morphogenèse Végétales, USTL/INRA. Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq Cedex, France
[2]Laboratorium voor Genetica. Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium

Received: 5 May 1997 / Accepted: 25 September 1997

Abstract. In leaf tissues of the *Cichorium* hybrid clone '474' (*C. intybus* L. var. *sativum* × *C. endivia* L. var. *latifolia*), the acquisition and expression of embryogenic competence was characterised by the appearance of 15 polypeptides (Boyer et al., 1993, Plant Sci 93: 41–53). The 38-kDa proteins were found to be abundantly present in conditioned embryogenic medium after the first division of the induced cells. These proteins seemed to be glycosylated as indicated by general carbohydrate detection methods. Internal amino-acid sequences obtained after microsequencing tryptic peptides appeared to be 36–57% homologous with plant β-1,3-endoglucanases. In addition, these 38-kDa proteins were recognised by antibodies raised against the pathogenesis-related tobacco glucanase PR2a and their β-1,3-glucanase activity was demonstrated by direct detection in polyacrylamide gels after electrophoresis. These results strongly suggested that the 38-kDa somatic-embryogenesis-related (SER) polypeptides are β-1,3-glucanases. Moreover, the level of glucanase activity was nearly three times higher in the medium of the embryogenic '474' line than in the medium of a non-embryogenic line. The possible involvement of the extracellular 38-kDa proteins in callose degradation during somatic embryogenesis is discussed.

Key words: *Cichorium* – Extracellular polypeptide – β-1,3-Glucanase – Microsequencing – Somatic embryogenesis

Introduction

During asexual or somatic embryogenesis, somatic cells develop into whole plants via characteristic morphological stages that resemble sexual or zygotic embryos, such as globular, heart and torpedo stage embryos, in dicotyledons. This process does occur in vivo (i.e. "apomixis"), but mostly it is induced in vitro from explants through manipulation of the culture medium, as was initially described for carrot (Reinert 1958; Stewart et al. 1958). Since somatic embryogenesis in principle is a versatile method of rapidly and massively reproducing whole plants, ways have been developed to initiate somatic embryogenesis in many different plant species. For reasons unknown so far, the yields of somatic embryos in some plant species is low. This is also true for the economically interesting cultivars of chicory. In contrast to these recalcitrant cultivars, explants from the *Cichorium intybus* L., var. *sativum* × *C. endivia* L., var. *latifolia* hybrid clone '474' appeared to become highly embryogenic upon applying somatic embryogenesis-inducing culture conditions (Dubois et al. 1988, 1990, 1991; Guedira et al. 1989). This, together with the speed of embryo formation (less than 12 d) and the abundance of embryos produced (12 embryos per mm^2 of leaf tissue) made clone '474' attractive as a material to further investigate somatic embryogenesis in chicory, even more so, since by including glycerol in the embryo-culture medium (induction medium) the first cell division of the embryogenic cells can be delayed. Transferring the explants to medium without glycerol (expression medium) results in synchronised initial cell divisions of the induced cells (Robatche-Claive et al. 1992).

The initial step in our study of molecular events leading to somatic embryogenesis in *Cichorium* clone '474', was the analysis of changes in leaf-tissue protein patterns during the induction step. This resulted in the detection of 15 so-called somatic-embryogenesis-related proteins (SER-proteins) which appeared before the first cell division (Hilbert et al. 1992; Boyer et al. 1993). More recently, two extracellular 38-kDa proteins that may be

Abbreviations: AP-Hz = alkaline phosphatase-hydrazide; 1D, 2D = one-, two-dimensional; PAS = periodic acid-Schiff; PR = pathogenesis related; RBB = Remazol Brilliant Blue; SER = somatic embryogenesis related

Correspondence to: Jean-Louis Hilbert;
E-mail: hilbert@univ-lille1.fr; Fax: 33 03 20 33 72 44

related to somatic embryogenesis in chicory were detected in the medium of leaf explants during embryogenic induction and expression steps (conditioned-embryogenic medium; Helleboid et al. 1995). These medium-recovered proteins may be similar to two 38-kDa SER-proteins detected previously, because (i) they shared their positions on the gel after two-dimensional (2D) gel electrophoresis, (ii) they accumulated similarly at a low rate from day 3 of the induction step onward and after transfer to the expression medium, and (iii) their accumulation was similarly inhibited by the presence in the medium of α-difluoromethylarginine, an inhibitor of somatic embryogenesis in *Cichorium* (Helleboid et al. 1995). The involvement of extracellular glycosylated proteins in somatic embryogenesis has been reported in various plant species such as carrot (De Vries et al. 1988), grapevine (Coutos-Thevenot et al. 1992), citrus (Gavish et al. 1992) and pine (Domon et al. 1994). Because of their presence in explants tissues, we have previously suggested (Hilbert et al. 1992) that two proteins of 38 kDa could be correlated with the presence of an abundant fibrillar glycoprotein network specifically bound to the embryogenic cell walls surrounding the proembryos (Dubois et al. 1991).

In this paper, we report on the further characterisation of the 38-kDa extracellular SER-proteins in an attempt to elucidate their possible function during somatic embryogenesis in *Cichorium*.

Materials and methods

Plant material and culture conditions. Plantlets of chicory hybrid clone '474' (*Cichorium intybus* L., var. sativum × *C. endivia* L., var. *latifolia*) were grown in vitro as described previously (Helleboid et al. 1995). Leaf fragments of six-week-old plantlets were cultured for 4 d at 35 °C in darkness in 50 ml of an agitated liquid basal medium supplemented with sucrose (60 mM) and glycerol (330 mM) to induce somatic embryogenesis. The presence of glycerol during this induction step allowed activation of the mesophyll cells for embryogenic competence, without cell divisions (Robatche-Claive et al. 1992). This glycerol pretreatment synchronised the first divisions of the embryogenic cells, because these divisions only occurred after transfer of the 4-d-old induced leaf tissues to glycerol-free medium (expression medium).

Extraction of proteins from conditioned medium. Conditioned medium from embryo cultures was passed through a Faltenfilter (MN 7131/4; Osi, Elancourt, France) and then through a Millipore (Bedford, Mass., USA) 0.22-μm filter, dialysed for 72 h against distilled water, and lyophilised. Proteins were extracted from 50 ml of lyophilised medium by solubilisation in 1 volume of water and precipitation by adding 5 volumes of 10% trichloroacetic acid (TCA) in cold acetone overnight at −20 °C. After centrifugation (30 000 *g*, 20 min, 4 °C; 3K20 centrifuge; Sigma, Osterode, Germany), the supernatant was discarded and the pellet was washed with 2 ml cold acetone (−20 °C) for 45 min. The same step was repeated overnight. After centrifugation (30 000 *g*, 20 min, 4 °C), the acetone was discarded and the pellet was dried and resuspended in SDS sample buffer containing 20% glycerol, 100 mM DTT, 2% SDS and 62.5 mM Tris-HCl (pH = 6.8) or in lysis buffer (Damerval et al. 1986), and then centrifuged (30 000 *g*, 20 min, 22 °C). Supernatants were stored at −70 °C until analysis.

Denaturing and native electrophoresis of proteins. The extracellular-protein composition of conditioned medium was analysed using SDS-PAGE or 2D-PAGE. For SDS-PAGE, polyacrylamide slab gels (1 mm thick) were prepared as described by Laemmli (1970) with a 12.5% acrylamide separating gel and a 4% acrylamide stacking gel and run in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, Calif., USA) at 200 V constant voltage. The 2D-PAGE was performed as previously described by Boyer et al. (1993). Ampholytes were added to a final concentration of 4% and consisted of 90% ampholytes pH 3–10 and 10% ampholytes pH 5–7 (Bio-Rad). Proteins were silver-stained as described by Blum et al. (1987) or with Coomassie Brilliant Blue R-250. Native electrophoresis was performed as described for SDS-PAGE, except that running buffer was 50 mM Tris and 384 mM glycine (pH 8.4). The prestained marker proteins phosphorylase B (142.9 kDa), serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), trypsin inhibitor (29.7 kDa) and lysozyme (21.9 kDa) were from Bio-Rad.

Amino-acid sequencing of electroblotted protein (microsequencing). After SDS-PAGE, the gel was first pre-equilibrated in 100 ml of equilibration buffer (50 mM boric acid, 0.1% SDS, set to pH 8 with NaOH) for 1 h. Protein transfer was performed in a Mini-Protean II cell, using the Mini Transblot module, at 35 V constant voltage overnight on a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Transfer buffer consisted of 50 mM boric acid with 50 mM Tris. After transfer, the PVDF membrane was washed once in distilled water, stained for 5 min in staining solution [0.1% Naphthol Blue Black (w/v), 45% methanol (v/v), 7% (v/v) acetic acid], destained with distilled water, and dried in air. In-situ digestion of membrane-bound proteins was performed by a modified Bauw et al. (1989) method. Briefly, after electroblotting and visualisation, the band of interest was excised from the PVDF membrane using a razor blade in such a way that the surface where no protein is present is minimal. Membrane was then immersed in 500 μl of a 0.2% polyvinylpyrrolidone (PVP; 40 kDa) solution in methanol and stored at 4 °C. The quenching solution was diluted by an equal volume of distilled water and, after discarding the supernatant, the membrane pieces were washed twice with 500 μl water and once with 500 μl of 0.1 M Tris-HCl (pH 8.5). Electroblotted protein was digested by adding 1 μl of 1 M $CaCl_2$ and 1 μl of 1 mg · ml^{-1} porcine trypsin solution. After digestion at 37 °C for at least 4 h, buffer containing the eluted peptides was transferred into an Eppendorf tube. Then, membranes pieces were washed once with 100 μl of 80% (v/v) formic acid and twice with 100 μl of distilled water. All solutions were combined for reverse-phase HPLC separation using an HP 1050 Ti high-performance liquid chromatograph (Hewlett Packard, Palo Alto, Calif., USA). The enzymatically cleaved fragments were applied to a reverse-phase C4 column (0.46 i.d., 25 cm in length; Vydac Separations Group, Hesperia, Calif., USA) equilibrated with 0.1% trifluoroacetic acid in water. Peptides were eluted on an Waters-Millipore HPLC apparatus with a linearly increasing gradient of acetonitrile (from 0 to 70%), detected by UV absorbance at 214 nm and collected in Eppendorf tubes. The major peptides were dried in a Speed Vac (Savant, Farmingdale, N.Y., USA) concentrator and stored at −20 °C prior to sequence analysis. Automated peptide sequencing was performed with a 473A Applied Biosystems sequencer (Perkin Elmer, Foster City, Calif., USA) equipped with an on-line phenylthiohydantoin amino-acid-derivative analyser. The peptides generated were selected for amino-acid sequence analysis on the basis of peak height and peak resolution. The peptides were applied onto glass-fiber discs and placed in the chamber of the gas-phase protein sequencer. Amino-acid sequence comparisons were carried out using the SWISS-PROT data bank.

Glycoprotein staining on gel and after electroblotting. After SDS-PAGE, glycoproteins were identified directly on the gel by periodic acid-Schiff (PAS) staining as described by Dubray and Bezard (1982) except for final rinsings which were made in 0.5% sodium metabisulfite, or after transfer to nitrocellulose membranes using alkaline phosphatase-hydrazide (AP-Hz) according to Gershoni et al. (1985).

Antiserum production. Extracellular proteins were subjected to SDS-PAGE and the protein band corresponding to 38 kDa was cut out after Coomassie Brilliant Blue staining. The 38-kDa proteins were then eluted from the gel by a Bio-Rad Electroeluter and dialysed against water. After collection of preimmune serum, two white rabbits were immunised subcutaneously with 100 μg of the 38-kDa protein fraction emulsified in complete Freund's adjuvant and subsequently boosted fortnightly with 50 μg of protein. Two weeks after the last injection, the rabbits were bled by cutting the marginal ear vein and serum (P38-SH) was prepared. After clot removal, the serum was clarified by centrifugation at 6000 *g* for 10 min, divided into aliquots and stored at −20 °C until required.

Immunoblot assays. After electrophoresis, proteins in the gel were transferred onto a nitrocellulose membrane by electroblotting (100 V, 1 h). The electro-transfer buffer was 25 mM Tris, 192 mM glycine, 20% methanol. For immunoblot assay, the membranes were blocked by incubating overnight at room temperature in TBS (25 mM Tris-HCl, pH 7.4; 0.5 M NaCl) containing 2% PVP. The membrane was exposed to primary serum (1:2000 dilution) raised against the pathogenesis-related (PR) β-1,3-glucanase PR2a of tobacco or raised against 38-kDa polypeptides in TBST (25 mM Tris-HCl, pH 7.4; 0.5 M NaCl; 0.1% Triton X100) containing 1% PVP for 2 h. It was then washed with TBST three times (10 min each) and incubated for 2 h with alkaline-phosphatase-conjugated goat anti-rabbit antibodies and finally washed as mentioned before. The alkaline-phosphatase signal was developed using 0.03% nitroblue tetrazolium and 0.015% 5-bromo-chloro-3-indoyl phosphate in a solution 10 mM $NaCHCO_3$ and 1 mM $MgCl_2$ at pH 9.8.

Purification of β-1,3-glucanases. Conditioned medium of 11-d-old embryo cultures was collected, dialysed, concentrated and loaded onto an Econo-Pac cartridge (Bio-Rad). After elution of unbound protein, a 0.1–1 M NaCl gradient was applied to the column and all the collected fractions were assayed for β-1,3-glucanase activity.

Colorimetric β-1,3-glucanase activity assay. β-1,3-Glucanase activity was measured by the colorimetric assay of Kauffmann et al. (1987) using 0.25% laminarin obtained from *Laminaria digitala* (Sigma, St. Louis, Mo., USA) as a substrate in 100 mM Na-acetate buffer (pH 5.0). After addition of laminarin to the dialysed medium or to a purified fraction, the reaction was allowed to proceed for 30 min at 37 °C. The reaction was stopped by adding dinitrosalicylate and boiling for 10 min. The reducing sugar produced was determined colorimetrically at 500 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 mmol of glucose as reducing sugar.

Detection of β-1,3-glucanase activity by PAGE. Protein was solubilized in 25% glycerol and separated using native PAGE. Polyacrylamide gels were prepared as described by Laemmli (1970) with a 12.5% acrylamide separating gel and 4% acrylamide stacking gel, except that bidistilled water was replaced by a solution of 1.33 g CM-curdlan-Remazol Brilliant Blue (CM-curdlan-RBB; Loewe Biochemica, Sauerlod, Germany) per liter bidistilled water (Kalix and Buchenauer 1995). Gels were run in a Mini-Protean II electrophoresis cell (Bio-Rad) at 150 V constant voltage. After electrophoresis, the gel was incubated in acetate buffer (100 mM, pH 5.0) at 30 °C overnight. Glucanase activity was revealed as clear areas on a blue background.

Results

Accumulation of the 38-kDa protein band in conditioned media of the embryogenic '474' line. We compared patterns conditioned-medium proteins corresponding to 1- to 4-d-old induced leaf tissues of *Cichorium* clone '474', and after 4 or 7 d of transfer to expression medium

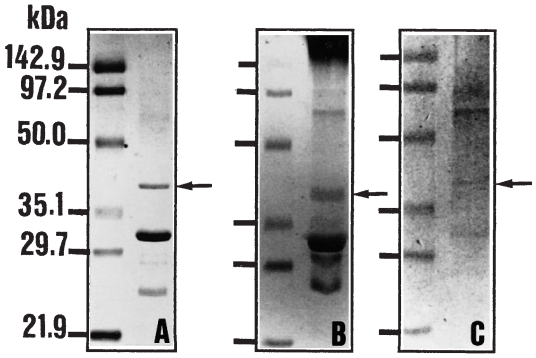

Fig. 1A–C. Detection of the 38-kDa extracellular protein band in the conditioned medium of a *Cichorium* embryo culture 7 d after transfer of 4-d-old induced leaf tissues to expression medium. SDS-PAGE gel after staining with Coomassie Brilliant Blue (**A**), PAS (**B**), and AP–Hz (**C**). *Left-hand lanes*, prestained low-range molecular-weight marker proteins: phosphorylase B (142.9 kDa), serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), trypsin inhibitor (29.7 kDa) and lysozyme (21.9 kDa). *Arrows*, 38-kDa extracellular protein

(see *Materials and methods* for details). Conditioned medium was collected and proteins corresponding to equal amounts of medium were subjected to SDS-PAGE. As shown on Fig. 1A, after Coomassie Brilliant Blue staining, three major proteins bands of 25, 30 and 38 kDa were detected in conditioned culture medium from 4-d-old induced leaf tissues transferred to expression medium for 7 d. The 38-kDa proteins were detected in 3-d-old conditioned medium by SDS-PAGE (Fig. 2A). They remained at a same low level during the induction step (days 3 and 4), but were detected at an up twofold higher level to day 11 of embryogenic culture, after transfer of induced tissues to expression medium. The 38-kDa band gave a strong positive signal after PAS gel staining (Fig. 1B) and was also visible after electroblotting and staining with AP-Hz (Fig. 1C), indicating that the 38-kDa proteins were glycoproteins.

Identification of the 38-kDa protein band. To identify the 38-kDa SER conditioned-medium glycoproteins, they were submitted to microsequencing. After electroblotting, the protein band was digested with trypsin and the tryptic peptides were separated by reverse-phase HPLC. From six tryptic peptides the amino acids could be unambiguously identified and compared with SWISS-PROT data base. The identified sequences, in total covering 63 amino acids, shared 36–57% identity with plant β-1,3-endoglucanases from *Arabidopsis thaliana* (Uknes et al. 1992), *Nicotiana plumbaginifolia* (Castresana et al. 1990), *Nicotiana tabacum* (Ori et al. 1990), *Lycopersicon esculentum* (Van Kan et al. 1992) and *Hordeum vulgare* (Wang et al. 1992; Fig. 3). This suggested that the 38-kDa proteins may be β-1,3-glucanases. The possibility that 38-kDa proteins are β-1,3-glucanases was further examined with a serum raised against PR2a (tobacco β-1,3-glucanase). As shown in Fig. 2B, the antiserum raised against PR2a recognised 38-kDa conditioned-medium proteins on a one-dimensional (1D) gel. To determine whether the

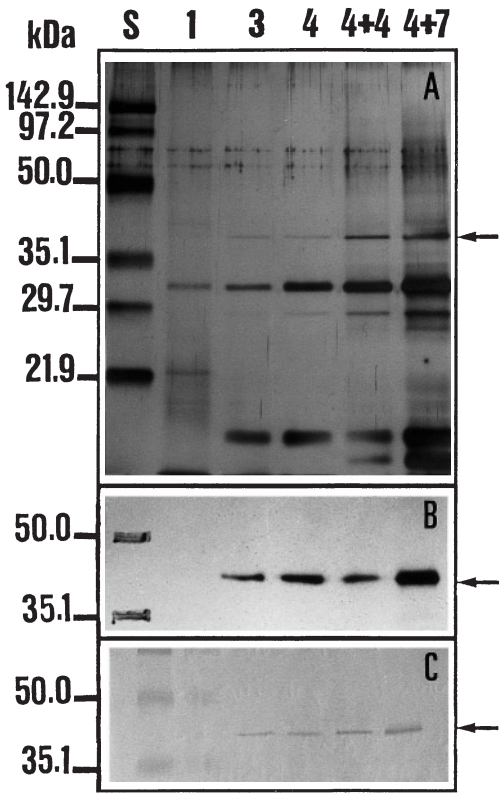

Fig. 2A–C. Detection of extracellular proteins in conditioned medium during embryogenesis in *Cichorium*. Proteins were from 1-d- (*lane 2*), 3-d- (*lane 3*), 4-d- (*lane 4*) old induced tissues, and 4 d (*lane 5*) and 7 d (*lane* 6) after transfer of 4-d-old induced leaf tissues to expression medium. **A** Silver-stained SDS-PAGE gel of proteins. **B** Cross-reactivity of tobacco PR2a antiserum with the extracellular 38-kDa proteins. **C** Detection of the 38-kDa extracellular proteins using P38-SH antiserum. *Lane 1*, prestained low-range molecular-weight marker proteins: phosphorylase B (142.9 kDa), serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), trypsin inhibitor (29.7 kDa) and lysozyme (21.9 kDa)

38-kDa proteins are indeed β-1,3-glucanase, the proteins present in embryo culture medium were fractionated and the fractions obtained tested for the presence of the 38-kDa proteins and β-1,3-glucanases activity. The secreted proteins were loaded onto an Econo-PAC Q cartridge column and eluted by a step NaCl gradient. β-1,3-Glucanases activity was found to be specifically high in the 0.1 M NaCl fraction (not shown). Analysis by SDS-PAGE revealed the presence of a 38-kDa protein

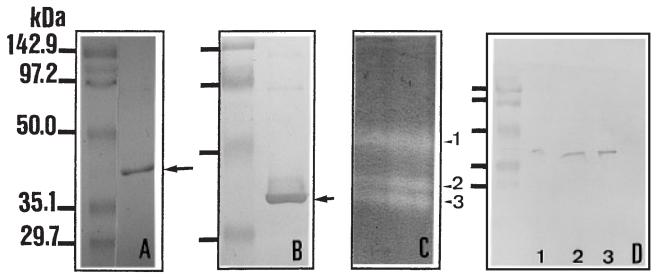

Fig. 4A–D. Glucanase activity of the 38-kDa proteins. **A** SDS-PAGE gel after staining with Coomassie Brilliant Blue. **B** Immunoblot of an SDS-PAGE gel using tobacco PR2a antiserum. **C** The 38-kDa purified protein fraction after separation in a CM-curdlan-RBB native gel. **D** Immunoblot after SDS-PAGE of the three bands showing glucanase activity on the CM-curdlan-RBB native gel, using PR2a antiserum

band in this fraction (Fig. 4A) which after immunoblotting was recognized by the PR2a antiserum (Fig. 4B). Three bands showed β-1,3-glucanase activity after separation in a native gel containing CM-curdlan-RBB (Fig. 4C). However, when Western blots were probed with PR2a antiserum, these three bands were not recognized (data not shown). Apparently, the PR2a antiserum only reacts with the 38-kDa proteins when they are denaturated. When each of the three bands was cut from a native CM-curdlan-RBB gel and separated under denaturing conditions (SDS-PAGE), each band corresponded to a 38-kDa protein recognized by the PR2a antiserum (Fig. 4D). These results show that the 38-kDa proteins are β-1,3-glucanases and that embryo-culture medium contains at least three 38-kDa proteins which exhibit β-1,3-glucanase activity.

The 38-kDa conditioned-medium proteins are 38-kDa leaf-induced SER proteins. Antiserum specific for 38-kDa extracellular proteins (P38-SH) was tested by immunoblotting. No proteins were detected with pre-immune serum (data not shown). As shown on immunoblots from conditioned-medium proteins, this antiserum recognised the 38-kDa protein band on a 1D-gel (Fig. 2C) and at least two 38-kDa extracellular conditioned-medium proteins on a 2D-PAGE gel (data not shown). Protein extracted from leaf explants developing somatic embryos were separated by SDS-PAGE and subjected to immunoblot analysis using P38-SH serum, to determine the relationship between 38-kDa extracellular proteins and

	Peak 1	Peak 2	Peak 3
Cichorium "474"	GTPAKPGR	SIETYQ	VAVGNEVDPD
Arabidopsis thaliana	--*RR**K	A****I	IN*****K*S
Nicotiana plumbaginifolia	--*R**-*	P****I	I*****IS*V
Nicotiana tabacum	***K****	IV***L	ISI****S*S
Lycopersicon esculentum	***K****	T****L	I******D*G
Hordeum vulgare	***K*REA	-L***I	I*A****QGG

	Peak 4	Peak 5	Peak 6
Cichorium "474"	FGIFSPNQQPQ	IY?PYQATLQALK	GDTLPSQQDVVSLYR
Arabidopsis thaliana	W*L*H*DR*SK	L*G*DPGA*A**R	TTKH*ANA-------
Nicotiana plumbaginifolia	**L****K**K	L*D*NHGA*****	*NN**NHWE*IQ**K
Nicotiana tabacum	**L*Y**RTAK	**NSDTNIFKS*N	ANN***E***IN**K
Lycopersicon esculentum	**L*K*D*R*K	**F*ETNVFN***	ANN***D***IK**N
Hordeum vulgare	**L*N*DKSPA	**FADGQA*S**R	*NN***RS***Q***

Fig. 3. Comparison of the six tryptic-peptide amino-acid sequences of the 38-kDa band with plant β-1,3-endoglucanase sequences of *Arabidopsis thaliana* (Uknes et al. 1992), *Nicotiana plumbaginifolia* (Castresana et al. 1990), *Nicotiana tabacum* (Ori et al. 1990), *Lycopersicon esculentum* (Van Kan et al. 1992) and *Hordeum vulgare* (Wang et al. 1992). Common amino acids are indicated with *asterisks*

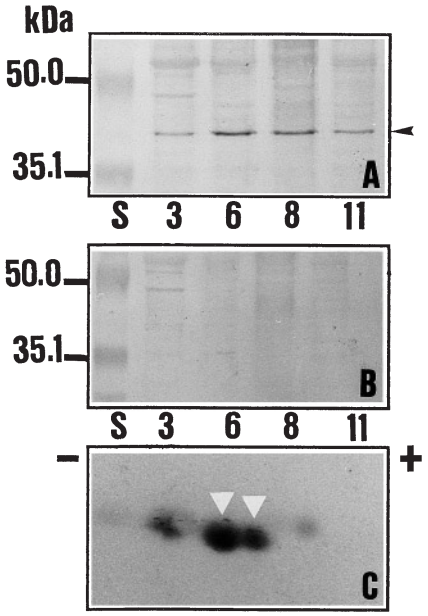

Fig. 5A–C. Detection of the 38-kDa SER-proteins in leaf-tissue explants. **A,B** Immunoblot of SDS-PAGE gel showing proteins from 3-d-old induced tissues (*lane 2*), 4-d-old induced tissues transferred 2 d (*lane 3*), 4 d (*lane 4*) and 7 d (*lane 5*) to expression medium, using the P38-SH serum (**A**) or pre-immune serum (**B**). *Lane 1*, prestained low-range molecular-weight marker proteins: ovalbumin (50 kDa) and carbonic anhydrase (35.1 kDa). **C** Immunoblot after 2D-PAGE of proteins extracted from 4-d-old induced tissues transferred for 2 d to expression medium

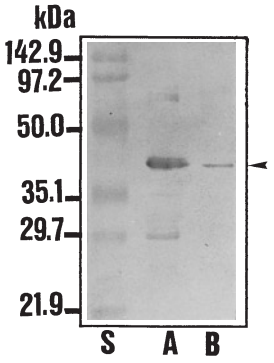

Fig. 6. Differential accumulation of 38-kDa extracellular proteins in embryogenic (*lane A*) and non-embryogenic (*lane B*) conditioned culture media. Immunoblot, using the tobacco PR2a antiserum, of conditioned-medium proteins from 7 d after transfer of 4-d-old induced tissues to expression medium. *Lane S*, prestained low-range molecular-weight marker proteins: phosphorylase B (142.9 kDa), serum albumin (97.2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35.1 kDa), trypsin inhibitor (29.7 kDa) and lysozyme (21.9 kDa)

the SER-38 proteins present in leaf tissues previously described (Hilbert et al. 1992; Helleboid et al. 1995). As shown in Fig. 5A, a detectable amount of a 38-kDa protein band was observed after 3 d of induction and 2, 4 or 7 d after transfer to expression medium. Moreover, no band was detected using pre-immune serum as a control (Fig. 5B). In the same way, 2D-PAGE immunoblots of induced leaf tissues, using PR2a antiserum, displayed at least two 38-kDa proteins (Fig. 5C). These results demonstrate that the 38-kDa extracellular proteins are immunologically related to the 38-kDa SER proteins previously detected in induced leaf-tissue explants (Helleboid et al. 1995) and strongly suggest that they are the same. Moreover, the 2D-PAGE PR2a immunoblot of induced leaf tissues (Fig. 5C) suggests, as previously, that β-1,3-glucanase activity in leaf tissues culture medium was supported by at least three independent proteins.

Comparative analysis of embryogenic and non-embryogenic conditioned media. In order to determine the presence or accumulation of 38-kDa proteins in a non-embryogenic responsive line, proteins from 11-d culture media of embryogenic and non-embryogenic lines were subjected to immunoblotting using P38-SH serum. The antiserum recognized a 38-kDa protein band in both lines (Fig. 6), but the signal obtained for the embryogenic line (Fig. 6A) was much higher than for the non-embryogenic line (Fig. 6B), suggesting that the amount of 38-kDa glucanases was much higher in the culture medium of the embryogenic line compared with the non-embryogenic line. A time-course of β-1,3-glucanase activity in the culture media of the embryogenic and non-embryogenic lines was followed (Fig. 7). For the embryogenic line '474', β-1,3-glucanase activity was present in the induction medium and remained at the

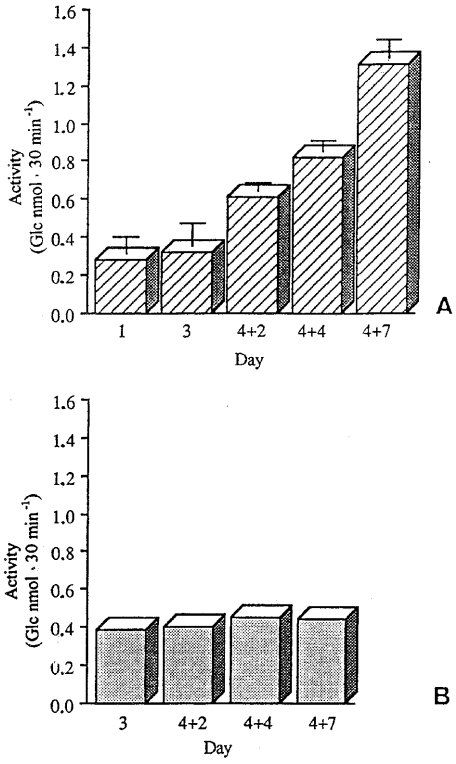

Fig. 7A,B. Changes in β-1,3-glucanase activity in conditioned medium of the embryogenic line '474' (**A**) and the non-embryogenic line (**B**). Glucanase activity was determined by using laminarin as substrate at 1 d (*1*) and 3 d (*3*) after induction, and 2 (*4 + 2*), 4 (*4 + 4*) and 7 (*4 + 7*) d after transfer of 4-d-old induced tissues to expression medium

same level (about 0.3 nmol Glc in 30 min). Upon transfer to expression medium, glucanase activity started to increase from 0.6 nmol Glc in 30 min at day 6 to 1.3 nmol Glc in 30 min at day 11 (Fig. 7A). For the non-embryogenic line, glucanase activity in induction and expression media remained at about the same level (0.4 nmol Glc in 30 min) observed in embryogenic-line induction medium (Fig. 7B). These results indicate that in expression medium of the embryogenic line '474' there is considerable accumulation of glucanase activity. Furthermore, this increase in glucanase activity in the expression medium seems to be specifically related to somatic embryogenesis since in the culture medium of a non-embryogenic line, glucanase activity stayed at the same level. Based on the fact that at day 0 the 38-kDa proteins were not detected, these results also suggest that the increase in glucanase activity observed for the embryogenic line is caused by the accumulation of the 38-kDa proteins in the medium.

Discussion

Cichorium clone '474' is an attractive model system for investigating somatic embryogenesis, because of the speed of embryo formation and the abundance of embryos produced, even more so, since induction and expression steps can be separated by including glycerol in the embryo-culture medium during the induction step (Robatche-Claive et al. 1992).

During somatic embryogenesis, extracellular proteins accumulate in the conditioned embryo-culture medium (Helleboid et al. 1995). Among the major proteins that accumulate in the medium, we have identified and characterised the 38-kDa proteins while a 30-kDa protein has been identified as chitinase (data not shown). Based on the protein sequence homology, immunological cross-reactivity of the tobacco PR2a antiserum, and enzymatic activity, we have shown that the extracellular 38-kDa proteins are acidic β-1,3-glucanases. The presence of at least three different isoforms cannot be explained by different glycosylation patterns of a single protein because different β-1,3-glucanase clones have been identified by reverse transcription-polymerase chain reaction approaches (data not shown). Using PAS and AP-Hz coloration, we revealed that the 38-kDa glucanases were glycosylated. Furthermore, it was shown that antibodies raised against the extracellular 38-kDa proteins also recognized previously identified 38-kDa SER proteins (SER38), suggesting that they are either homologous or even identical.

The β-1,3-glucanases are abundant hydrolytic enzymes found in response to most stress processes in higher plants (PR proteins). They can act with other plant hydrolases (e.g. chitinases) in general defence mechanisms against pathogen infection (Mauch et al. 1988a; Meins et al. 1992). It has been suggested that plant β-1,3-glucanases participate in the partial breakdown of the β-1,3-glucans present in the cell walls of phytopathogenic fungi (Mauch et al. 1988b). Moreover the expression of β-1,3-glucanase genes can be regulated by exogenous hormones (Felix and Meins 1986) and detected in response to different stresses, e.g. salicylic acid (Shab and Klessig 1996), ozone treatment (Ernst et al. 1992), UV light and wounding (Brederode et al. 1991). However, as recently described for a specific chitinase which allowed the development of somatic embryos at a non-permissive temperature in a temperature-sensitive carrot variant cell line (De Jong et al. 1992), plant β-1,3-glucanases may have other, as yet unidentified, functions. Non-pathogenic inducible glucanase activity has been found in different plant structures during natural development, such as during flower formation (Neale et al. 1990), spring reactivation in deciduous trees (Krabel et al. 1993), seed germination (Vögeli-Lange et al. 1994), and tetrad dissolution in anthers (Buciaglia and Smith 1994). In the latter case, during meiosis the tetrads were surrounded by a callose wall and were released into the anther locule only after hydrolysis of the callose, and this degradation was essential to obtain fertile pollen (Worrall et al. 1992). The high accumulation of β-1,3-glucanases in the culture medium during *Cichorium* somatic embryogenesis is not likely to be the result of stressful conditions caused by temperature, hormones and osmoticum, in view of their absence when using a non-embryogenic line under the same culture conditions. In a previous study, Dubois et al. (1991) demonstrated the presence of a callose sheath surrounding the *Cichorium* induced embryogenic cells before the first division. They suggested that to undergo somatic embryogenesis one of the probable roles of this callose "wall" was to isolate the single induced cells from the surrounding ones. After transfer to expression medium, the first division of the induced cells occurred and this callose sheath was no longer detected around cells of globular somatic embryos. The catalytic activity of β-1,3-endoglucanases consists of the hydrolysis of β-1,3-glucosidic linkages in β-1,3-glucans, such as laminarin or callose. We found glucanase activity in the conditioned medium in which somatic embryogenesis occurred. While callose was detected in the first reactivated cells at day 3 in synchronised induction conditions, 38-kDa extracellular proteins were detected in very low amounts in the induction medium (Helleboid et al. 1995). But after transfer to the expression medium, while most of the reactivated cells were dividing to produce somatic embryogenesis and no callose was detected on globular embryos, the 38-kDa proteins were found to be more abundantly present in the medium. As described for pollen formation in tobacco (Worrall et al. 1992), during *Cichorium* somatic embryogenesis, it may be that the presence of the callose sheath is necessary for induction or initiation, whereas its degradation is a prerequisite for the first division of the induced cells. Both 38-kDa proteins and β-1,3-glucanase activity were detected in a *Cichorium* non-embryogenic line. But, in this latter case, the level of glucanase activity was much lower than in the embryogenic line '474' and remained fairly steady during the entire culture period. In contrast, for the embryogenic line '474', the level of glucanase activity, stable during the induction step, increased considerably in the medium

after transfer of the induced leaf tissues to the expression medium. The time course of callose degradation and the increase in the level of glucanase activity suggests that the increase in glucanase activity could be responsible for the degradation of the callose.

We thank Bernard Fritig (IBMP, Strasbourg, France) for a generous gift of antisera against tobacco β-1,3-glucanases. Special thanks are due to Jean-Pierre Decotignies for the production of P38-SH antiserum. Sacco de Vries (University of Wageningen, The Netherlands) and Dirk Inzé (University of Gent, Belgium) are thanked for helpful discussions and suggestions during the course of this work. Many thanks go to Theo Hendriks (Laboratoire de Physiologie Cellulaire et Morphogenése Végétales, USTL/INRA Villeneuve d'Ascq) for critically reading the manuscript and for valuable suggestions for improving the contents. This research was supported by a grant "Contrat Plan-Etat-Région" to the Laboratoire de Physiologie Cellulaire et Morphogenése Végétales and by a doctoral fellowship from the Ministére de la Recherche et de l'Espace to Stéphane Helleboid.

References

Bauw G, Van Damme J, Puype M, Vandekerckhove J, Gesser B, Ratz GP, Lauridsen JB, Celis JE (1989) Protein-electroblotting and -microsequencing strategies in generating protein data bases from two-dimensional gels. Proc Natl Acad Sci USA 86: 7701–7705

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99

Boyer C, Hilbert JL, Vasseur J (1993) Embryogenesis related protein synthesis and accumulation during early acquisition for somatic embryogenesis competence in *Cichorium*. Plant Sci 93: 41–53

Brederode FJ, Linthorst HJ, Bol JF (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol 17: 1117–1125

Bucciaglia PA, Smith AG (1994) Cloning and characterization of *Tag1*, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Mol Biol 24: 903–914

Castresana C, de Carvahlo F, Gheysen G, Habets M, Inzé D, Van Montagu M (1990) Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene. Plant Cell 2: 1131–1143

Coutos-Thevenot P, Moes O, Jouenne T, Mauro MC, Boulay M, Delaire A, Guern J (1992) Extracellular protein patterns of grapevine cell suspensions in embryogenic and non-embryogenic situations. Plant Sci 86: 137–145

Damerval C, Devienne D, Zivy M, Thiellement H (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat seedlings proteins. Electrophoresis 7: 52–54

De Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, Van Kammen A, De Vries SC (1992) A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425–433

De Vries SC, Booij H, Janssens R, Vogels R, Saris L, LoSchiavo F, Terzi M, Van Kammen A (1988) Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins. Genes Devel 2: 462–476

Domon JM, Meyer Y, Faye L, David A, David H (1994) Extracellular (glyco)proteins in embryogenic and non-embryogenic cell lines of Caribbean pine. Comparison between phenotypes on stage one somatic embryos. Plant Physiol Biochem 32: 137–147

Dubois T, Dubois J, Guedira M, Vasseur J (1988) Embryogenèse somatique directe sur les styles de *Cichorium*: effets de la température et origine des embryo. C R Acad Sci Paris 307 Ser III: 669–675

Dubois T, Guedira M, Dubois J, Vasseur J (1990) Direct somatic embryogenesis in roots of *Cichorium*. Is callose an early marker? Ann Bot 65: 539–545

Dubois T, Guedira M, Dubois J, Vasseur J (1991) Direct somatic embryogenesis in leaves of *Cichorium*. A histological and SEM study of early stages. Protoplasma 162: 120–127

Dubray G, Bezard G (1982) A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal Biochem 119: 325–329

Ernst D, Schraudner M, Langebartels C, Sandermann HJR (1992) Ozone-induced changes of mRNA levels of β-1,3-glucanases, chitinases and 'pathogenesis-related' protein 1b in tobacco plants. Plant Mol Biol 20: 673–682

Felix G, Meins F Jr (1986) Developmental and hormonal regulation of β-1,3-glucanase in tobacco. Planta 167: 206–211

Gavish H, Vardi A, Fluhr R (1992) Suppression of somatic embryogenesis in *Citrus* cell cultures by extracellular proteins. Planta 186: 511–517

Gershoni JM, Bayer EA, Wilchek M (1985) Blot analyses of glycoconjugates: enzyme-hydrazine – a novel reagent for the detection of aldehydes. Anal Biochem 146: 59–63

Guedira M, Dubois-Tylski T, Vasseur J, Dubois J (1989) Embryogenèse somatique directe à partir de cultures d'anthères du *Cichorium* (Asteraceae). Can J Bot 67: 970–976

Helleboid S, Couillerot JP, Hilbert JL, Vasseur J (1995) Effects of α-difluoromethylarginine on embryogenesis, polyamine content and protein patterns in a *Cichorium* hybrid. Planta 196: 571–576

Hilbert JL, Dubois T, Vasseur J (1992) Detection of embryogenesis-related proteins during somatic embryo formation in *Cichorium*. Plant Physiol Biochem 30: 733–741

Kalix S, Buchenauer H (1995) Direct detection of β-1,3-glucanase in plant extracts by polyacrylamide gel electrophoresis. Electrophoresis 16: 1016–1018

Kauffmann S, Legrand M, Geoffroy P, Fritig P (1987) Biological fraction of "pathogenesis-related" proteins: four PR-proteins of tobacco have β-1,3-glucanase activity. EMBO J 6: 3208–3212

Krabel D, Eschrich W, Wirth S, Wolf G (1993) Callase-(1,3-β-D-glucanase) activity during spring reactivation in deciduous trees. Plant Sci 93: 19–23

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Mauch F, Hadwiger LA, Boller T (1988a) Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol 87: 325–333

Mauch F, Mauch-Mani B, Boller T (1988b) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinases and β-1,3-glucanases. Plant Physiol 88: 936–942

Meins F Jr, Neuhaus JM, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis related glucanohydrolases and their genes. In: Boller T, Meins F Jr (eds) Genes involved in plant defense. Springer, Berlin, pp. 245–282

Neale AD, Wahleithner JA, Lund M, Bonnett HJ, Kelly A, Meeks-Wagner DR, Peacock WJ, Dennis ES (1990) Chitinase, beta-1,3-glucanase, osmotin and extensin are expressed in tobacco explants during flower formation. Plant Cell 2: 673–684

Ori N, Sessa G, Lotan T, Himmelhoch S, Fluhr R (1990) A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO J 9: 3429–3426

Reinert J (1958) Morphogenese und ihre Kontrolle an Gewebekulturen aus Karotten. Naturwissenschaften 45: 344–345

Robatche-Claive AS, Couillerot JP, Dubois J, Dubois T, Vasseur J (1992) Embryogenèse somatique directe dans les feuilles du *Cichorium* hybride "474": synchronisation de l'induction. C R Acad Sci Paris 314 Ser III: 371–377

Shab J, Klessig F (1996) Identification of salicylic acid-responsive element in the promoter of tobacco pathogenesis-related beta-1,3-glucanase gene, PR-2d. Plant J 10: 1089–1101

Stewart FC, Mapes MO, Smith J (1958) Growth and organised development of cultured cells. II Organisation in cultures grown from freely suspended cells. Am J Bot 45: 693–703

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward ER, Ryals JA (1992) Acquired resistance in *Arabidopsis thaliana*. Plant Cell 4: 645–656

Van Kan JAL, Joosten MHAJ, Wagenmakers CAM, van den Berg-Velthuisn GCM, de Wit PJGM (1992) Differential accumulation of mRNA encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol 20: 513–527

Vögeli-Lange R, Fründt C, Hart CM, Beffa R, Nagy F, Meins F Jr (1994) Evidence for a role of β-1,3-glucanase in dicot seed germination. Plant J 5: 273–278

Wang J, Xu P, Fincher GB (1992) Purification, characterization and gene structure of (1–3)-β-glucanase isoenzyme GIII from barley (*Hordeum vulgare*). Eur J Biochem 209: 103–109

Worral D, Hird DL, Hodge R, Paul W, Draper J, Scott R (1992) Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. Plant Cell 4: 759–771