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ORIGINAL PAPER

Spirodela (duckweed) as an alternative production system for pharmaceuticals: a case study, aprotinin

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Abstract Aprotinin is a small serine protease inhibitor used in human health. *Spirodela* were transformed, via *Agrobacterium*, with a synthetic gene encoding the mature aprotinin sequence and a signal peptide for secretion which was driven by the CaMV 35S promoter. A total of 25 transgenic *Spirodela* lines were generated and aprotinin production was confirmed by northern and western blot analyses. Expression levels of up to 3.7% of water soluble proteins were detected in the plant and 0.65 mg/l in the growth medium. In addition, immunoaffinity purification of the protein followed by amino acid sequencing confirmed the correct splicing of the aprotinin produced in *Spirodela* and secreted into the growth medium.

Keywords Aprotinin · Pharmaceutical protein · *Lemnaceae* · *Spirodela oligorrhiza* · Transgenic plants

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Introduction

Plants are considered as an alternative for the biomanufacturing industry to complement current production systems such as mammalian cells, yeasts and bacteria (see Fisher et al. 2004; Hood 2000; Ma et al. 2005 for review). Plants offer several advantages compared to other systems, for example reduced costs of production and no risk of animal virus contamination. *Lemnaceae* are a class of small aquatic plants presenting several interesting features compared to most other plants. They can be grown in a confined environment with the potential of secreting the proteins of interest into the growth medium, making purification easier (Edelman et al. 2003; Stomp 2005). Several proteins have already been expressed in *Lemnaceae*, including marker proteins or pharmaceuticals (Edelman et al. 1998; Gasdaska et al. 2003; Freyssinet et al. 2005; Cox et al. 2006).

Aprotinin is a natural protease inhibitor used in medical procedures to lower the systemic inflammatory response and reduce blood loss associated with cardiac surgery. It has also been administered in the

treatment of acute pancreatitis (Davies et al. 1997). This compound is also used as a manufacturing aid to prevent degradation of protein products in research and manufacturing processes. Aprotinin is a polypeptide of 58 amino acids with a molecular mass of 6511 Da. It is active as a monomer, blocking the active site of serine proteases such as trypsin, chymotrypsin, plasmin, kallikrein and lysosomal proteins (Laskowski and Kato 1980). It contains three disulfide bridges (Cys_5–Cys_{55}, Cys_{14}–Cys_{38}, Cys_{30}–Cys_{51}), the Cys_{14}–Cys_{38} bridge being readily split by reducing agents like 2-mercaptoethanol. Aprotinin was originally identified in bovine lungs, but can also be obtained from recombinant yeast (Apeler et al. 2004). In plants aprotinin has been successfully expressed in maize reaching a protein yield of only 0.07% of the water-soluble protein fraction (Zhong et al. 1999). A subsequent study performed by the same team reported a higher expression level when the aprotinin is targeted to the cell wall (Delanay et al. 2003). The highest level of expression for a single T1 seed was 8.9% TSP while the average expression was around 1.7–2.1% depending on the construct used. Since it may be difficult to purify aprotinin targeted to the cell wall, additional sources of aprotinin are worthwhile exploring.

In this work we report stable transformation and expression of aprotinin in *Spirodela*, a member of the *Lemnaceae* (duckweed) family. We used a synthetic gene consisting of the tobacco pathogenesis-related PR1a signal peptide sequence (SP) fused to the mature sequence of aprotinin, both optimised to *Spirodela* codon usage. Aprotinin was expressed at a fairly high level, 3.7% of the water-soluble proteins *in planta*. For certain lines, we observed secretion into the growth medium of up to 0.67 mg/l. The amino acid sequence of the mature peptide secreted into the growth medium demonstrated that the pre-protein is cleaved at the expected site.

Materials and methods

Synthetic gene and plasmid construction

The tobacco PR1a signal peptide (GenBank accession number No CAA29660) was fused to the mature peptide sequence of the bovine aprotinin (GenBank accession number N° CAA27063). To achieve heterologous aprotinin expression in duckweed, the amino acid sequence of this fusion was reverse-translated into a nucleotide sequence using the *Spirodela* codon usage values from the CUTG database and the Synthetic Gene Designer web program (Wu et al. 2006). Six partially overlapping 60 bp-long oligonucleotides representing gapped sense and antisense strands of the synthetic fusion gene were designed and synthesized. The forward and the reverse external primers were designed to introduce an *Xba*I site and a *Sma*I site, respectively. The chimeric gene (285 bp) was then constructed using a one-step recursive PCR where the optimal conditions were obtained using: (a) 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 2 min, 66°C for 2 min, and 72°C for 72 s; 1 cycle 72°C for 5 min, and (b) 5 nM of each internal oligonucleotide and 0.5 μM of each outermost flanking primer. The PCR product was cloned into the pPCR-Script vector (Stratagene). A mutation-free clone was isolated and sub-cloned into a vector derived from pBIN+ (van Engelen et al. 1995) using the *Xba*I and the *Sma*I site (Fig. 1). This binary vector, which contains a nos-nptII-nos gene conferring

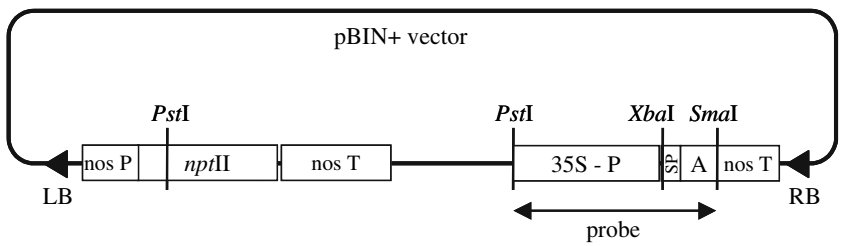

Fig. 1 Diagram of the binary vector used in this study. The arrow shows the hybridization position of the radioisotope labelled probe used for Southern experiments. SP: PR1a signal peptide; A: aprotinin synthetic gene; *npt*II: neomycin phosphotransferase; nosP: nopaline synthase promoter; nos T: nopaline synthase terminator; 35S-P: CaMV 35S promoter

kanamycin resistance, was mobilised into *Agrobacterium tumefaciens* EHA105.

Plant transformation and tissue culture

Spirodela oligorrhiza Hegelm SP was transformed and tissue culture was done as described by Edelman et al.(1998) and Li et al.(2004). Calli were co-cultivated with *A. tumefaciens* containing the vector described above. After co-cultivation, calli were regularly transferred on selective regeneration medium containing 40 mg/l of kanamycin (Li et al. 2004). Regenerating plants were then transferred on liquid medium (SH medium from Shenk and Hildebrandt 1972), containing 3 mg/l of kanamycin (SHK3 medium) for further selection. After six to seven transfers on this medium, kanamycin was removed.

Analysis of transformants

PCRs and Southern blots

Genomic DNA was extracted from the different lines according to Dellaporta et al. (1983). PCR conditions were 30 cycles of 30 s at 94°C, 30 s at 60°C and 40 s at 72°C, with 2 min at 94°C prior to the reaction and 1 min of final extension at 72°C in a Mycycler thermocycler (Biorad). Primers used for amplification of aprotinin were 5′-GTGCAAGGCGAGGAT CATC-3′ (forward) and 5′-CGCGCTCTTGAAGTT GTTCC3-′ (reverse). Primers used for amplification of *npt*II were 5′-ATGATTGAACAAGATGGATTG3-′ (forward) and 5′-TGATGGATACTTTCTCGG-CAG3-′ (reverse). These primers amplify DNA sequences 106 and 346 bp long from the coding regions of *aprotinin* and *npt*II genes, respectively.

For Southern blot analysis, DNA (10 µg) was digested with *Pstl* and separated on a 0.8% agarose gel by electrophoresis. The DNA fragments were transferred in 10x SSC to Hybond N^+ nylon membranes and hybridized overnight at 65°C according to the instructions of the manufacturer (Amersham). The most stringent wash was for 10 min in 1× SSC and 0.1% (w/v) SDS. The probe was prepared from a 1.1 kb PCR product corresponding to the CaMV 35S promoter and aprotinin sequence. Radioactive DNA probes labelled with ^{32}P-dCTP were obtained with Ready-to-go DNA labelling beads (GE-Amersham).

Northern blot analysis

RNA was extracted from *Spirodela* using the TRI REAGENT method according to instructions of the manufacturer (Molecular Research Center). For RNA gel blots, 10 µg of RNA were denatured in a formamide/formaldehyde buffer and separated on 1% (w/v) agarose gels. The RNA was then transferred in 10x SSC to Hybond N^+ nylon membranes. Aprotinin RNA was visualized by hybridization to a labelled PCR product from the aprotinin coding region (about 200 nucleotides long) at 42°C according to the instructions of the manufacturer (Amersham). Three washes in 2× SSC and 0.1% (w/v) SDS, 65°C, 10 min were performed.

Aprotinin analysis by SDS-PAGE and immunoblot

Detection of aprotinin in planta: Fresh *Spirodela* fronds, grown for two weeks on SHK3 medium, were harvested and blotted on filter paper. They were weighed (fresh weight, FW), frozen in liquid nitrogen, and then ground to a fine powder using a mortar and pestle. Fronds reduced to powder were suspended in 200 mM Tris–HCl pH 6.8, 200 mM NaCl, 10 mM EDTA (2 µL buffer/mg FW). The resulting extracts, total soluble protein (TSP) fraction were vigorously mixed (vortex) and incubated on ice for 10–15 min. The supernatant (raw extract) was collected after centrifugation at 4°C for 20 min at 18,000 g. Raw extracts were mixed 1–5 (v/v) with cold acetone and stored overnight at −20°C to allow precipitation to occur. Pellets were recovered after centrifugation at 4°C for 20 min at 18,000 g and dried in a vacuum chamber. Protein concentration in the pellets suspended in 5% SDS was determined using the Lowry method with BSA (4–40 µg in 5% SDS) as a standard. Total soluble proteins (TSP) from duckweed or commercial aprotinin (Sigma, France) were denatured with a non-reducing sample buffer (0.3 M Tris–HCl pH 6.8, 5% SDS, 50% Glycerol). Protein samples, corresponding to about 40 µg TSP, were resolved in a 15% polyacrylamide gel (non-reducing SDS-PAGE) and electroblotted onto a PVDF

membrane using the procedure described by Kyhse-Anderson (1984). Non-specific sites were blocked with 5% (w/v) skimmed milk in TBS. The proteins were probed with mouse anti-aprotinin antibody (1/1,000 dilution), supplied by Bayer CropScience, and HRP-conjugated anti-mouse IgG (1/5,000 dilution, GE Amersham, France) and detected with GE-Amersham ECL reagents according to the manufacturer's instruction. The light emission was captured using X-Ray film. For quantification, the X-Ray film was scanned and analyzed with a Molecular Dynamics Densitometer and ImageQuant software. The signals (ECL) obtained were plotted against the corresponding known aprotinin concentration (purified aprotinin from Sigma). In a defined range of concentration, the signal was linearly related to the concentration and the standard curve was built by linear regression (data not shown). The regression coefficient was used to convert the signal in unknown samples to the corresponding aprotinin concentration.

Detection of aprotinin in the growth medium: Aprotinin in the growth medium was detected by the western blot technique described above or a dot-blot technique, see below. In addition, kinetic measurements of aprotinin secretion into medium were undertaken with transformed and wild type *Spirodela* grown on 50 ml SHK3 in 200 ml Erlenmeyer flasks. The cultures were initiated using the same plant biomass. Samples corresponding to 1 ml of culture media were withdrawn at time intervals during the growth of the culture. For aprotinin detection and evaluation, samples of media (160 µl) or commercial aprotinin were diluted 4–1 (v/v) in the non-reducing sample buffer and were loaded onto a PVDF membrane fitted in a dot blot apparatus. Samples were filtered through the membrane by applying a vacuum. Wells were washed several times with anode buffer two from the discontinuous three buffer system described by Kyhse-Anderson (1984). The proteins immobilized on the membrane were submitted to the same immunoprotocol and quantification used for *in planta* detection.

Purification and characterisation of the recombinant aprotinin

To purify aprotinin, we prepared an affinity support by covalently binding the mouse anti-aprotinin to immobilized protein G. This was done using the Seize® X Protein G Immunoprecipitation Kit from Pierce, France. The procedure supplied with the kit was followed for the immobilisation of the antibody to the protein G support using the cross-linker DSS. Either plant extract or growth medium was used. Growth medium was concentrated 7-fold using an Amicon cell fitted with a 3 kDa nominal molecular weight cut off membrane. The samples were diluted 1 to 1 with Pierce Binding Buffer, and run on the affinity support. Bound aprotinin was eluted using Pierce elution buffer, then neutralised with Tris 1 M, pH 8.0. Aliquots of *in planta* and *in media* aprotinin elution fractions were run on a 15% SDS gel which was developed using immunodetection (*in planta* samples) or silver staining (*in media* samples) procedures.

Mass mapping of recombinant aprotinin was performed on protein bands purified by electrophoresis using MALDI-TOF/MS using a Voyager DE-PRO MALDI TOF mass spectrometer (Applied Biosystems, Courtaboeuf, France) and LC/MS/MS was performed on a Q-STAR XL instrument (QqTOF) (Applied Biosystems) following the parameters described by Molle et al. (2006).

Results

Production of transgenic Spirodela

Calli were produced as described by Li et al. (2004). These calli were used to generate transgenic *Spirodela.* For this, callus cultures were infected with *Agrobacterium tumefaciens* carrying *nptII* and the PR1aSP-aprotinin fusion genes. Regeneration of lines was essentially as described by Li et al. (2004). In the experiments reported here, a total of 25 lines resistant to kanamycin in liquid medium were generated. These lines are presented in Table 1. Transgene insertions in genomic DNA were verified by Southern blotting (Fig. 2, Table 1). No hybridization signal was detected with DNA coming from untransformed plants. We found that most of the transgenic lines contained several copies (2–3) of the T-DNA, whereas around 20% contained probably a single insertion, with one band visible on Southern blots (Table 1). Lines 9.9 and 9.12, which are further described below, contain 1 to 3 copies of the aprotinin gene.

Table 1 Summary of the main results obtained for the transgenic *Spirodela.*

Plant number	Southern (size of bands, kbp)		RNA northern	Protein	
	Hind III	Pst I		*In planta*	In medium
9.1	3.6	3.5	–	–	–
9.3	1.9–4.8	1.7 –2.5	+	+	–
9.4	3.8	5.4	++	++	+
9.5	2.1–3.5–4.8	2–2.5–3.5	–/+	–	+
9.7	3.5–4.8	ND	+	++	++
9.8	ND	ND	++	+++++	++++
9.9	7	4.5–7.5	++	+++++	++++
9.10	4.5–7	6	–	+	+
9.11	2.7–3.8	2.7–3.7–7	–	–/+	–
9.12	3.6–4.8–6.2	3–5	+++	+++++	+++++
9.15	3.4	3.4	–	–	–
9.17	3.6–4.8–6.2	2.8–3.6–4.5	+	++	–
9.18	3.5	2.7–3.5	–	+	+
9.19	3.6	ND	++	+++	+++
9.20	5	3	–	+	+
9.21	4.7–>10	3–6	–	+	–
9.22	4	2.5–6	+	++	–
9.23	>10	2.5–6	+	++	++
9.24	2–2.3–4.5	1.8–2.3–2.7–3.7	++	+++++	–/+
9.25	>10	2.7–5.2–6	–	+	–
9.26	3.9–4.7–10	2.4–2.7–3.7	+++	++++	+++
9.30	2.3	2.1	+++	+++++	+
9.31	>10	2.7–3–3.4 3.7–6.5	–	+	–
9.32	ND	ND	–	+	ND
9.33	3.4–4.5	9 bands	+	+++	+

Selected transgenic lines were all resistant to kanamycin in liquid medium (3 mg/l). For RNA and Protein analyses, the evaluation was done using a "– to +++++" scale based on the intensity of the band (northern and western for aprotinin *in planta*) or the dot (aprotinin in medium)

ND = not done

Aprotinin mRNA expression

Total RNA isolated from wild type and transgenic lines were used to determine aprotinin mRNA expression by northern blotting. Figure 3 reveals that aprotinin mRNA with the appropriate MW is produced in transgenic *Spirodela* lines. As can be observed in Fig. 3 and Table 1, the levels of expression vary between the transgenic lines. There was no detectable signal when using RNA from wild type plants.

In planta aprotinin production

In planta aprotinin production was determined using the western blot technique described in Materials and Methods. To be identified by western blot, aprotinin from *Spirodela* needed to be analysed under non-reduced conditions while commercial aprotinin needed to be under reduced (β-mercaptoethanol) conditions. Under these conditions, aprotinin from *Spirodela* migrates as a band at around 10 kDa (lane 9.9, non-reduced, Fig. 4) while the commercial aprotinin migrates at the expected size for this protein, i.e.: 6.5 kDa (lane aprotinin, reduced, Fig. 4). Silver staining of the electrophoresis gel showed that the commercial aprotinin under non-reduced conditions migrates with an apparent molecular weight of 10 kDa while under reduced conditions, it migrates at the expected molecular weight of 6.5 kDa (data not shown). The aprotinin band was not detectable in protein extracts from non-transformed plants (Fig. 5). Finally, we observed, on the immunoblot, a band at around 50 kDa corresponding to a non-specific band since it is present in extracts from non-transgenic lines (Figs. 4–6a).

A first estimation of the level of expression was done using the intensity of the band on western blots.

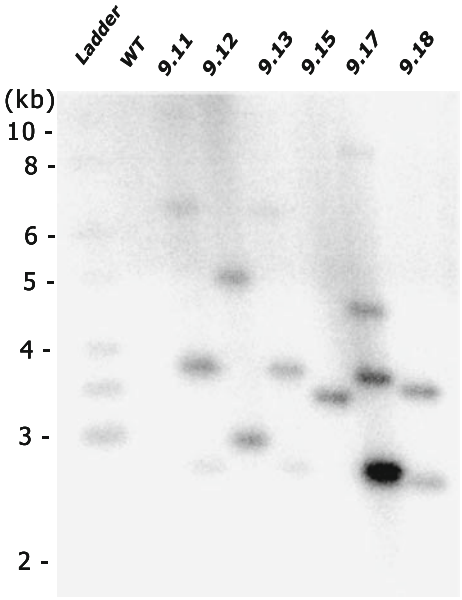

Fig. 2 Southern blot analysis of transgenic *Spirodela* lines. Genomic DNA (10 μg) was digested with *Pst*I and hybridized with a radiolabelled 1.1 kb DNA probe corresponding to the CaMV 35S promoter and aprotinin sequence (see Fig. 1). The ladder lane is GeneRuler 1 kb DNA marker (Fermentas); WT lane corresponds to DNA from non-transgenic plants; six different transgenic lines were analysed here

Results are presented in Table 1 with a "– to +++++" scale. We found that more than 90% of the transgenic lines produced aprotinin at a detectable level. In certain cases, a low level of aprotinin was observed while mRNA has not been detected. This is probably due to the fact that, under our standard conditions, the western blot is more sensitive than the northern blot. Quantification of aprotinin was done by densitometry with commercial aprotinin on selected lines showing high expression. The highest expression thus determined ranged from 1.9 to 3.7% aprotinin/TSP with the best producing lines being 9.8 and 9.9.

In order to purify aprotinin from whole plant crude extract, we prepared an affinity support using the anti-aprotinin antibody (see Materials and Methods). The TSP of line 9.9 was applied to this column and the bound proteins were eluted, run on a SDS-PAGE and detected in the same conditions. Figure 4 shows that the 9.8 kDa protein can be recovered and thus purified using this affinity column, Lane AC9.9, non-reduced, Fig. 4. One should notice that the non-specific band seen on crude extracts is not present in the AC fraction.

In media aprotinin secretion

Identification of aprotinin in growth medium was done by western or dot blots. Under western conditions, we observed, Fig. 6a, that the secreted

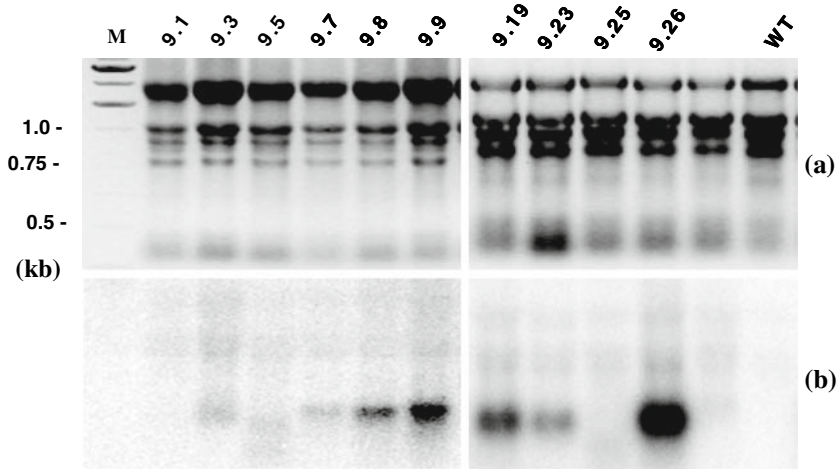

Fig. 3 Expression of aprotinin mRNA in transgenic *Spirodela* lines. Total RNA (10 μg) were denatured in a formamide/formaldehyde buffer and separated on agarose gel, then transferred to Hybond N^+ nylon membranes. Lane M is 1 kb DNA marker; WT lane corresponds to RNA from non-transgenic plants used as a negative control. The other lanes correspond to RNA from transgenic lines. (**a**) Ethidium bromide staining of ribosomal RNA shows equal loading of the gel. (**b**) The blot was hybridized with a ^{32}P-labeled 200 bp cDNA probe corresponding to the aprotinin. Exposure of the gel was for overnight

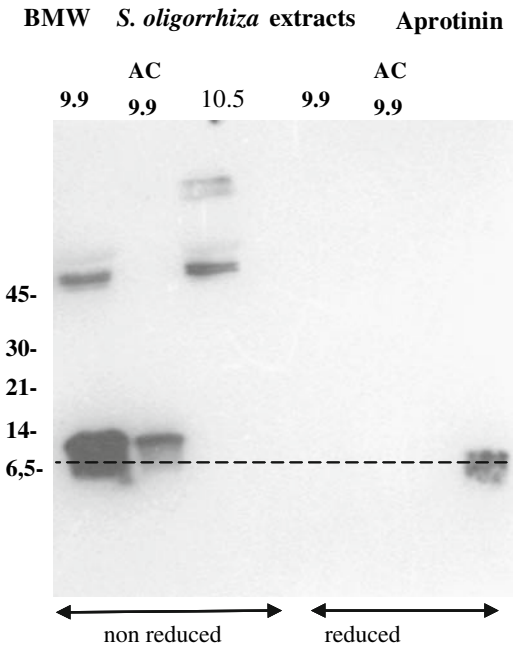

Fig. 4 X-Ray film of a western analysis of protein extracts from transgenic *Spirodela*. Extracts were from the 9.9 line. AC9.9 corresponds to the fraction obtained after purification by immunoaffinity. Proteins loaded on the gel were either non-reduced or reduced with β-mercaptoethanol. 10.5 correspond to a transgenic line not producing aprotinin. BMW corresponds to the Broad Molecular Weight markers from Amersham Biosciences; it contains aprotinin. The dotted line corresponds to the level of migration of commercial aprotinin (25 ng used in the aprotinin lane). Exposure of the film was for 45 min

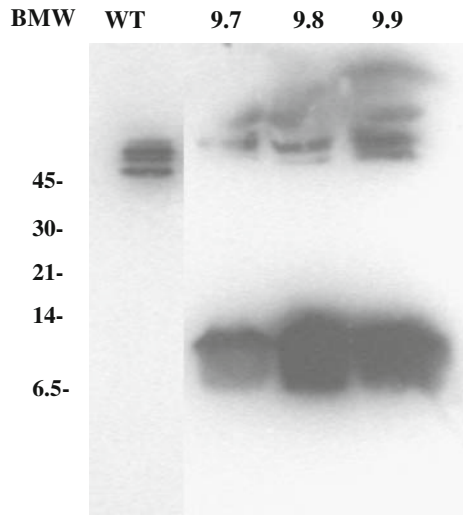

Fig. 5 X-Ray film of a western analysis of protein extracts from *Spirodela*. Extracts were from either non-transgenic *Spirodela* (WT) or transgenic lines. BMW corresponds to the molecular markers. Exposure of the film was for 45 min

aprotinin migrates at the same level as the *in planta* aprotinin, above the commercial reduced aprotinin. Aprotinin was detected in most of the growth media from the different transgenic lines (Fig. 6b). We analysed a sample of growth medium from each of the transgenic lines by dot blot and evaluated the amount of aprotinin using a "− to +++++" scale, see Table 1. For further analysis, we selected the five best secretor lines plus one with a low level of secretion, as determined with dot blot. For those lines, we started with the same quantity of plant biomass, grew the plants on SHK3 and monitored the aprotinin secretion at different times after culture initiation allowing comparison of secretion levels in those lines. The level of secreted aprotinin was hardly detectable before 11 days (Fig. 7). Then, the aprotinin gradually accumulated in the medium to reach a maximum at 21 days, when the entire surface of the flask was covered by the floating plants. We noticed the different behaviour of the lines for secretion. Despite the fact that the lines 9.9 and 9.12 have fairly similar levels of expression based on the accumulation of protein in the total soluble fraction based on the intensity of the band on western blot, secretion is much higher for line 9.12 than for line 9.9.

After 21 days, aprotinin concentration in the culture medium of line 9.12 reached 0.67 mg/l, while that of five other lines was in the range 0.20 mg/l–0.30 mg/l. This result means a higher secretion rate for line 9.12 since the five lines had similar doubling rates.

In-media purification and characterisation of the synthesised aprotinin

Based on time course results of aprotinin secretion into the media, line 9.12 was grown for 21 days on 200 ml SHK3. Medium (100 ml) was collected, concentrated and purified using the affinity column prepared above. The presence and purity of recombinant aprotinin in elution fractions E1 to E6 was checked by electrophoresis (Fig. 8a). The silver staining of the gel showed for each collected fraction a band at approximately 6.5 kDa, the predicted molecular weight of the mature aprotinin. The fractions were then pooled and concentrated to

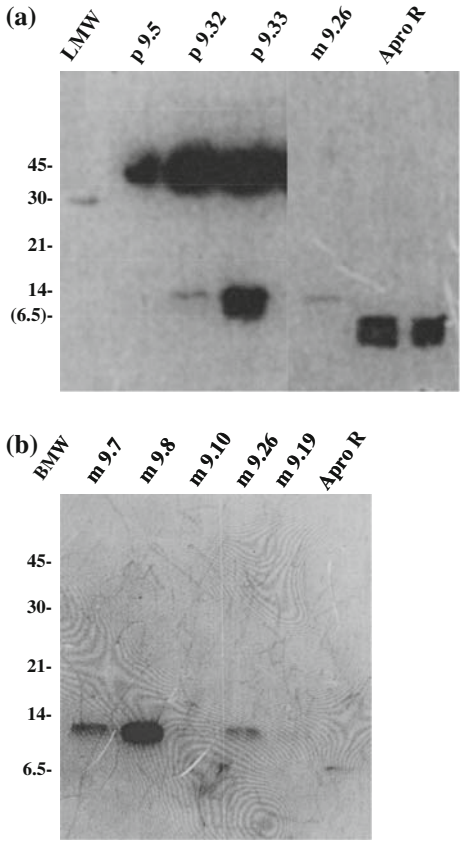

Fig. 6 X-Ray film of a western analysis of protein from transgenic *Spirodela*. (**a**) Proteins run on the gel were either protein extracts from transgenic *Spirodela* (p) or protein from growth medium (m). LMW corresponds to a Low Molecular Weight marker kit from Amersham Biosciences. This marker shows a band at 30 kDa reacting with the aprotinin antibody. It does not contain aprotinin, the position of aprotinin is indicated between brackets. (**b**) Proteins were from growth medium from different transgenic lines. BMW corresponds to the molecular markers. Numbers correspond to different lines. AproR = reduced commercial aprotinin. Exposure of the film was for 3 h

dryness using a Speed Vac. The dry sample was suspended in Laemmli sample buffer, electrophoresed on a 15% SDS gel and developed using MS-compatible silver staining (Fig. 8b). The band migrating at approximately 6.5 kDa was excised from the gel after destaining, washing and subsequent in-gel trypsin digestion, and submitted to MALDI-TOF/MS and LC/MS/MS analyses. Partial amino acid sequences are presented in Figure 9. As can be seen, the N-terminal sequence of the aprotinin obtained from the growth medium of transgenic

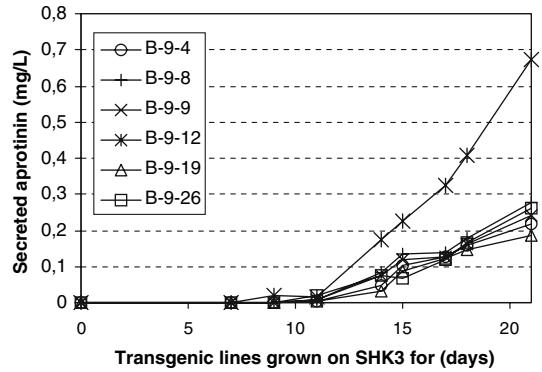

Fig. 7 Time course of aprotinin secretion in the medium. Six different transgenic *Spirodela* lines were analysed for aprotinin secretion in the medium (SHK3) during growth of the lines

Spirodela is the same as that of the commercial aprotinin. This confirms that the correct cleavage occurs at the junction of the signal peptide and the mature aprotinin peptide, as expected from its apparent migration on gel electrophoresis.

Discussion

The experiments presented here show that significant amounts of recombinant aprotinin can be produced using *Spirodela* as a plant host. The amount of expressed transgenic aprotinin is higher than what was reported previously in maize (Zhong et al. 1999; Delaney et al. 2003). This could be due to a higher accumulation of such protein in leaf tissue in our work, compared to seed tissue, as is the case for maize. On should notice that the highest expression observed in maize seeds was obtained by targeting the aprotinin to the cell wall (Delaney et al. 2003). It could also be due to a higher expression efficiency of the cassette we used, as the 35S promoter used here is known to be well expressed in leaves.

Our experiments indicate that the aprotinin which accumulated in the total soluble protein fraction of transgenic *Spirodela* is probably in a processed form. Its apparent molecular weight of around 10 kDa is due to the fact that, in order to detect the aprotinin with the antibody we had to run it on the electrophoresis gel in a non-reduced condition. It is very unlikely that this higher size is due to the presence of the signal peptide. We showed that the aprotinin from the plant and from the medium ran at the same

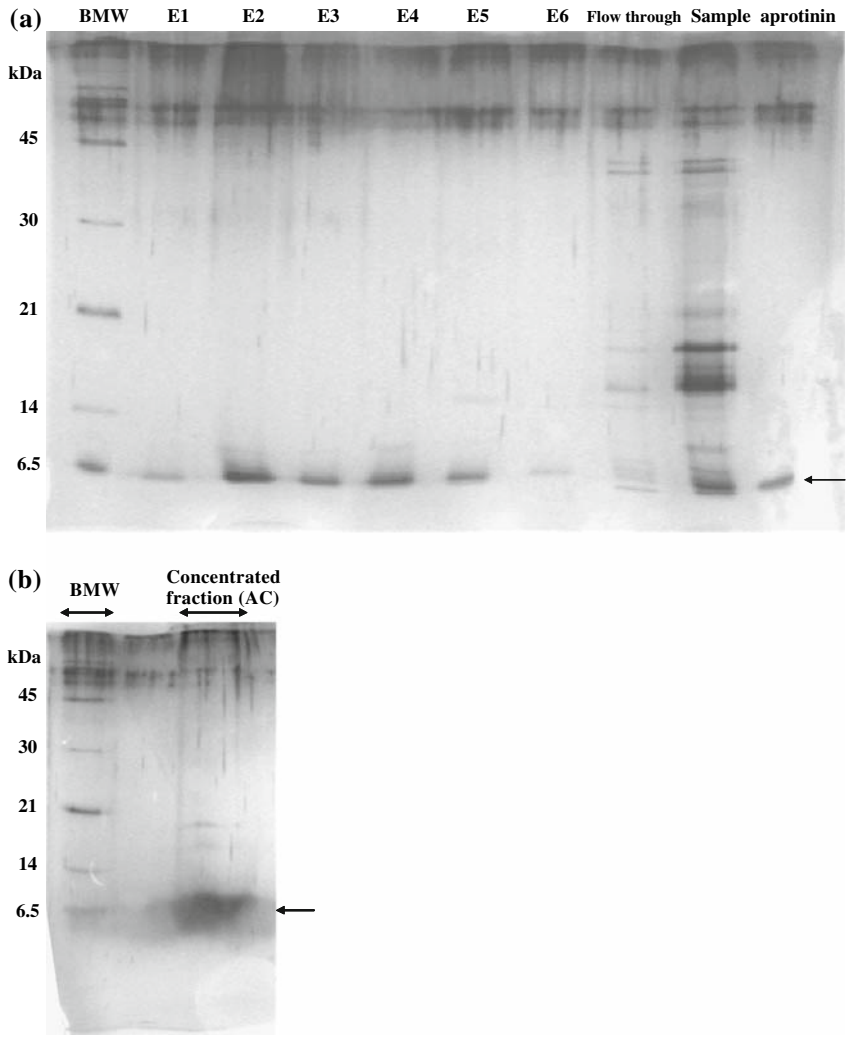

Fig. 8 Analysis of aprotinin in different fractions on a 15% polyacrylamide gel. **(a)** Sample used for purification and various fractions eluted from the affinity column. Proteins were silver stained. «BMW» corresponds to Broad Molecular Weight standard (Amersham Biosciences), while Aprotinin refers to commercial aprotinin from Sigma. «Sample» corresponds to the concentrated medium from line 9.12, while «Flow through» corresponds to the sample collected from the affinity support after the binding step. E1–E6 correspond to aliquots of the fractions eluted from the affinity support. **(b)** Analysis of the concentrated fraction. E1–E6 fractions from the gel, Fig. 6a, were pooled, dried, and resuspended in Laemmli buffer prior to being run on the present gel. Proteins were stained using MS-compatible silver. Concentrated fraction (AC) corresponds to the pool of elution fractions E1-E6 (Fig. 6a). The band at 6.5 kDa was excised from the gel and submitted to MS analyses

apparent molecular weight. PR1a has already been used and proved to work for the production of a soluble single-chain antibody in tobacco cell suspension culture (Xu et al. 2002). Other cases of transgenic protein secretion were noted in *Spirodela* using a chitinase signal sequence fused to *gfp*, with or without an HDEL motif (up to 2 mg/l of GFP excreted into the growth medium after 14 days of culture, determined by quantitative immunoblotting; Edelman et al. unpublished observation).

The concentration obtained (0.65 mg/l) could probably be further improved by adapting the plant growing conditions, since after 21 days of growth a plateau was not reached (see Fig. 5). A clear advantage of this secretion system in terms of protein production is that the protein of interest, *in casu*

Fig. 9 Amino acid sequence of aprotinin **(a)** Amino acid sequence of the protein deduced from the coding sequence introduced in *Spirodela*. The PR1a sequence is underlined. The first amino acid of the mature aprotinin is shown in bold. **(b)** Partial amino acid sequence of commercial aprotinin run on an electrophoresis gel. The band was cut from the gel and submitted to partial amino acid sequencing. **(c)** Partial amino acid sequence of aprotinin purified by immuno-affinity from growth medium of transgenic *Spirodela*. The aprotinin was purified and analysed as described in Material and methods

aprotinin, can be collected continuously by passing the growth medium through an immuno-affinity column. Alternatively, aprotinin can be concentrated from the growth medium after harvest of the plants, and then purified. In such cases, contamination from protein naturally secreted by *Spirodela* should be low since we have not been able to detect protein in the growth medium of non-transgenic *Spirodela* even after a 40-fold concentration of the medium (data not shown).

The results presented here clearly show that *Spirodela* could be a good alternative for the production of aprotinin. Aprotinin of the expected size and with an amino acid sequence identical to the original one has been produced. The level of productivity observed, in planta as well as in growth medium is compatible with an industrial production. Secretion of mature protein with the correct amino acid start is a plus for such industrial production. This productivity could probably be even increased by looking more deeply at the kinetics of secretion in the growth medium or by the use of other signal peptides. For example, since *Spirodela* is secreting an enzyme such as peroxidase (Jansen et al. 2004), fusing the peroxidase homologous signal peptide might be more efficient than an heterologous one as used in this work.

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References

Apeler H, Peters J, Schröder W, Schneider KH, Lemm G, Hinz V, Rossouw GJ, Dembowsky K (2004) Expression, purification, biochemical and pharmacological characterization of a recombinant aprotinin variant. Biotechnol Drug Res 8:483–497

Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, Black A, Passmore D, Moldovan-Loomis C, Srinivasan M, Cuison S, Cardarelli PM, Dickey LF (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. Nature Biotechnol 24:1591–1597

Davies MJ, Allen A, Kort H, Weerasena NA, Rocco D, Paul CL, Hunt BJ, Elliot MJ (1997) Prospective, randomized, double-blind study of high-dose aprotinin in paediatric cardiac operations. Ann Thoracic Surg 63:497–503

Delaney D, Jilka J, Barker D, Irwin B, Poage M, Woodard S, Horn M, Vinas A, Beifus K, Marker M, Wiggins B, Drees C, Harkey R, Nikolov Z, Hood E, Howard J, (2003) Production of aprotinin in transgenic maize seeds for the pharmaceutical and cell culture markets. In: Vasil IK (ed) Plant biotechnology 2002 and beyond. Proceedings of the 10th IAPTC&B Congress, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 393–394

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1(4):19–21

Edelman M, Perl A, Flaishman M, Blumenthal A (1998) Transgenic *Lemnaceae*. International application published under the Patent Cooperation Treaty (PCT) WO 99/19497, pp 1–56

Edelman M, Vunsh R, LI J, Hanania U, Flaishman M, Perl A (2003) Transgenic *Spirodela*: a unique, low-risk, plant biotechnology system. In plant biology 2003 program, Biotech risk assesment. Abstract 955, p 196. Ed. American Society of Plant Physiology

Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM (2004) Plant-based production of biopharmaceuticals. Current Opin Plant Biol 7:152–158

Freyssinet G, Langlais A, Rival S, Wisniewsky JP (2005) Production of vaccines and drugs in Lemnaceae. Conference on plant-made pharmaceuticals, pp 75

Gasdaska JR, Spencer D, Dickey L (2003) Advantages of therapeutic protein production in aquatic plant *Lemna*. BioProcessing J 3:50–56

Hood EE (2000) New century, new technology, new products. Transgenic Res 9:277–299

Jansen MAK, Hill LM, Thorneley RNF (2004) A novel stress-acclimation response in *Spirodela punctata* (*Lemnaceae*): 2,4,6-trichlorophenol triggers an increase in the level of an extracellular peroxidase, capable of the oxidative dechlorination of this xenobiotic pollutant. Plant Cell Environ 27:603–613

Kyhse-Anderson J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biophys Biochem Method 10:203–209

Laskowski M, Kato I (1980) Protein inhibitor of proteinases. Ann Rev Biochem 49:593–626

Li J, Jain M, Vunsh R, Vishnevetsky J, Hanania U, Flaishman M, Perl A, Edelman M, (2004) Callus induction and regeneration in *Spirodela* and *Lemna*. Plant Cell Rep 22:457–464

Ma JKC, Chikwamba R, Sparrow P, Fischer R, Mahoney R, Twyman RM (2005) Plant-derived pharmaceuticals—the road forward. Trends Plant Sci 10:580–585

Molle V, Zanella-Cleon I, Robin JP, Mallejac S, Cozzone AJ, Becchi M (2006) Characterization of the phosphorylation sites of Mycobacterium tuberculosis serine/threonine protein kinases, PknA, PknD, PknE, and PknH by mass spectrometry

Shenk RU, Hildebrandt AC (1972) Medium and technique for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Stomp AM (2005) The Duckweeds—a valuable plant for biomanufacturing. Biotechnol Ann Rev 11:69–99

Van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A, Stiekema WJ (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res 4:288–290

Wu G, Bashir-Bello N, Freeland SJ (2006) The synthetic gene designer: a flexible web platform to explore sequence manipulation for heterologous expression. Protein Expr Purif 47:441–445

Xu H, Montoya FU, Wang Z, Lee JM, Reeves R, Linthicum DS, Magnuson NS (2002) Combined used of regulatory elements within the cDNA to increase the production of a soluble mouse single-chain antibody, scFv, from tobacco cell suspension cultures. Protein Expr Purif 24:384–394

Zhong GY, Peterson D, Delaney DE, Bailey M, Witcher DR, Register JC III, Bond D, Li CP, Marshall L, Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA (1999) Commercial production of aprotinin in transgenic maize seeds. Mol Breed 5:345–356