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Heterologous protein production in the moss *Physcomitrella patens*

Evelien Cools,[1,2] Patrick Van Dijck,[1,2] Nelson Avonce [1,2,3*]

[1] Department of Molecular microbiology, VIB, KasteelparkArenberg 31, 3001. Heverlee Belgium.

[2] Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, K.U. Leuven, KasteelparkArenberg 31, B-3001. Heverlee Belgium.

[3] Centro de Investigación en Dinámica Celular, Instituto de Investigación en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, México.

email: nelson.avonce@uaem.mx

Over the last decades plants have been used for the heterologous production of pharmaceuticals, industrial enzymes and edible vaccines. The moss *Physcomitrella patens* is considered as an experimental model of choice for basic molecular, cytological and developmental questions in plant biology, as well as an outstanding plant model system for heterologous protein production. Here we use *P. patens* to produce osmotin, a tobacco protein with fungicidal properties. We have generated a transgenic plant able to synthesize and secrete a biologically active osmotin protein.

Keywords: moss, bioreactor, recombinant protein, osmotin.

Introduction

The search for low cost methods to produce pharmaceutical proteins, vaccines and other industrial-related enzymes has been one of the major goals for pharmaceutical industry during the last years. Several methods such as microbial cell cultures, animal cell cultures, plant tissue cultures, transgenic animals and plants have been explored and are used today.

Plants are now considered to be an equally viable and competitive large-scale production platform for the production of pharmaceutical proteins.

Major factors in favor of plant- based systems for the production of animal- derived proteins are its potential for large-scale production, low-cost production, low risk of contamination by viruses and mycoplasmas, correct folding and the possibility to assemble multimeric proteins, often low downstream processing needed and the absence of ethical problems associated with the use of transgenic animals or animal- derived materials (1).

A drawback for the use of plants is its specific glycosylation pattern, which is different from the human one, often resulting in allergic reactions when plant produced glycosylated proteins are administered to humans.

In many cases proteins are produced in seeds (e.g. rice, corn) and the downstream processing becomes a limiting factor (2).

The moss *Physcomitrella patens* is becoming widely recognized as an experimental model of choice for basic molecular, cytological and developmental questions in plant biology (3).

This moss can be genetically engineered to knock out the genes responsible for undesirable post-translational modifications (4). The dominant phase of its life cycle is in the gametophyte phase, which is haploid. Moreover, it is feasible to target any gene of interest to a well-known naturally highly expressed locus. *P. patens* can be cultivated in many different ways including bioreactors. Production cost is reduced by the use of simple growth media of inorganic salts with airborne CO_2 as sole carbon source.

The vegetative stage of the life cycle in these bioreactors makes sure that there are no genetic instabilities and allows a stable production. For all these reasons, *P. patens* provides an ideal system for the heterologous expression of proteins and/or metabolites (4).

Here we show the production of the plant pathogen-related protein (PRP) osmotin in *P. patens*. Osmotin is a small (26 kDa) class 5 PRP protein originally isolated from tobacco plants (5).

Very low concentrations (4 µM) can kill *S. cerevisiae* (BWG1-7a strain) cells in a matter of minutes. It binds to the *S. cerevisiae* Pho36 receptor thereby inducing apoptosis in a Ras2-dependent manner.

* Profesor Investigador.

Its capacity to induce apoptosis makes osmotin an interesting antifungal protein and it has previously been proven to inhibit the growth of several other fungal species (6).

Materials and Methods

Plant Material and growth conditions

Wild type *Physcomitrella patens* Gransden strain was used for transformation experiments. Plants were propagated vegetatively as protonema homogenates on cellophane-overlaid BCD-AT(3) solid medium or as individual plants regenerated after protoplast transformation. Plants were incubated at 24°C under continuous light conditions.

Gene constructs and plant transformation

We have created an insertional construct targeted to the lambda 108 locus of *P. patens* (NCBI Accession: GQ250943). The construct contains Lambda 108 sequences on both sides, a highly expressed actin promoter, a signal peptide, a histidine tag, the tobacco osmotin gene, an alginate-binding domain, terminator sequence and a kanamycin cassette.

The plasmid pSAL-lambda 108 was created by cloning a 2kb fragment previously amplified from genomic DNA from *P. patens* by using the primers A5475 (5' GGGTGATCAGTGTTTC GTTTAAACGAG TACCATTAAAGATGTTTC 3') and A5476 (5' GGGCTGCAGGTTGAC TGTTTAAAC TGACTTGGGAAATGAAATG 3'), the amplified Lambda108 sequence was inserted in the pSAL4 backbone by using the PstI and BclI restriction sites.

The actin promoter sequence was amplified from genomic DNA of *P. patens* by using primers A5025 (5' ATATG AAGCTTTCCTT GTG ACTTTTGTGCATTATTG 3') and A5026 (5' CGGAATTCTATAGGATCCT ATTTAATACGGACCTGCACAACA 3').

The kanamycin cassette sequence was amplified by PCR with primers A5027 (5' CGGAATTC CTACTCCAAAAATGTCAAAGATACAGTC 3') and A5028 (5' CCCAAGCTTAGTTTT GATC TTGAAAGATCTTTTATCTTT 3') from plasmid DNA (pMBL5 plasmid). Both actin promoter and km cassette were cut at HindIII and EcoRI sites, and ligated to the HindIII site of the pSAL4-lambda 108 plasmid.

A synthetic construct was ordered with GENEART (Regensburg, Germany), containing in a single open reading frame, the signal peptide sequence (1-30 aa) from the xyloglucanendotransglycosylase/hydrolase precursor gene from *P. patens* (NCBI Accesion: CAH58715), a 6X histidine tag, the osmotin gene from Nicotianatabacum (NCBI Accesion: AAB23375) and the alginate binding domain (1-203 aa) of the protein P5 from *Sphingomonas Sp.* A1 (NCBI Accesion: LC043070).

This synthetic construct was cloned at the sites BamHI and EcoRI of the plasmid pSAL4-lambda 108 containing the actin promoter and the Km cassette, generating the plasmid pSal4-Osmotin P5-BD.The plasmid pSAL4-Osmotin P5-BD was cut with PmeI enzyme, the 7452 bp containing the whole construct was gel-purified and used to target it to the lambda 108 genomic region by using the protoplast transformation method as described (7).

Transgenic selection was performed by using the antibiotic G418, and the correct insertion of the construct was verified by PCR and its expression by RT-PCR. One plant clone was selected for further experiments.

Detection of the protein by placing the plant on the Hybond™ C- extra

Transgenic and Wt plants of approximately the same size were placed on a piece of Hybond™ C- extra paper. The plants were left on this Hybond paper for two days at 25°C under continuous light conditions. Afterwards, plants were removed and the membrane was incubated for 1 hr at RT in 2% skimmed milk + TBST solution (20 mMTrisHCl pH 8, 150 mMNaCl, 0.05% Tween). Primary antibody (mouse anti-His antibody, GE Healthcare) was added at 1:3000 dilution in TBST and incubated overnight at 4°C.

The membrane was washed 3X with TBST for respectively 5, 10 and 15 minutes; secondary antibody (anti-mouse antibody, GE Healthcare) was added at 1:5000 dilution and incubated 1hr at RT and washed 3X with TBST for 5 min and 1X with TBS for 5 min. 1 ml of Luminol and 1 ml of peroxide solution were added and incubated for 1-5 min, the paper was removed from the solution and visualized using the Fujifilm Las 4000 mini Chemiluminescence reader.

Evaluating biological activity of the protein

Normal BCDATG medium (7) was supplemented with 0,59 g/L of yeast complete supplement solution (Mpbio) containing essential nutrients for the yeast cells (leucine, adenine, histidine, uracyl and tryptophan) and

2% glucose. Yeast cells (*S. cerevisiae* BWG1-7a strain) were taken from a cell culture and grown overnight in 3 ml YPD at 30°C; 500 µl of this culture were centrifuged at 14,000 rpm for 10 seconds, the pellet was re-suspended in 100 µl and plated out on the supplemented BCDAT plates. Transgenic and a wild type plant tissue of approximately the same size were placed on the plates. The plates were incubated at room temperature for one week.

Results and Discussion

Detection of secreted recombinant protein

To test if recombinant osmotin protein was produced and secreted by the transgenic plant, a wild type and transgenic plant were placed on Hybond™ C- extra paper. In Figure 1 we can clearly see that the osmotin-construct containing plant showed a much higher chemiluminescence signal than the wild type plant. The wild type plant did show a low signal, probably due to non-specific binding of the antibodies to proteins produced (and secreted) by the moss. However, from this assay, we could conclude that the recombinant osmotin was present and was produced and secreted by the transgenic moss plant.

Evaluating biological activity of the protein

To test antifungal activity of the recombinant osmotin produced by the *P. patens* transgenic plant, tissue of a wild type and transgenic *Physcomitrellaplant* were placed on a thin layer of *Saccharomyces cerevisiae* BWG1-7a cells previously inoculated on BCDAT media. Growth and development of the moss was evaluated after 7 days.

As is visible in Figure 2, the wild type plant became brown after one week and completely invaded by the yeast cells whereas the transgenic plant clearly shows an antifungal activity due to the production and secretion of a functional osmotin.

Heterologous protein production in plants is becoming an increasing approach by pharmaceutical industry. Physcomitrella as a bioreactor has already shown many advantages over other plant systems (4). As a proof of concept, here we show the heterologous production of osmotin, a protein with fungicidal properties (6).

Although it is necessary to further optimize the production and purification of the protein, we have clearly shown that the selected transgenic plant is able to secrete a biologically active osmotin protein as shown by the antibiogram assay against *S. cerevisiae* BWG1-7a strain (Fig. 2).

These results demonstrate the capability of *P. patens* as a plant model system for heterologous protein production, supporting its use as bio-factory for the production of vaccines or other immunotherapeutic products.

Fig. 1. Chemiluminescence detection of secreted osmotin. A wild type and transgenic (pOsmotin+P5-BD) plant were placed on the Hybond™ C- extra paper. The histidine tag that is attached to the recombinant osmotin protein was visualized using anti- His antibodies.

Fig. 2. Antibiogram assay with the moss plants. After a week of incubation on a plate covered with BWG1-7a yeast cells, transgenic plant clearly showed antifungal activity due to the secretion of active heterologously produced osmotin while the wild type plant died.

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Producción de proteínas heterólogas en el musgo *Physcomitrella patens*

Resumen

Durante las últimas décadas las plantas han sido utilizadas para la producción heteróloga de farmacéuticos, enzimas de uso industrial y vacunas. El musgo *Physcomitrella patens* es considerado como un modelo de experimentación de elección para abordar preguntas en las áreas de biología molecular, citología y de biología del desarrollo en plantas; así como un excelente sistema modelo para la producción de proteínas heterólogas. En este trabajo se utilizó *P. patens* para la producción de osmotina, una proteína de Tabaco con propiedades fungicidas. Se generó una planta transgénica capaz de sintetizar y secretar osmotina biológicamente activa.

Palabras clave: musgo, bioreactor, proteína recombinante, osmotina.

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