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Research article

Biotechnological production of recombinant tissue plasminogen activator protein (reteplase) from transplastomic tobacco cell cultures

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ABSTRACT

Transplastomic plants are a system of choice for the mass production of biopharmaceuticals due to the polyploidy of the plastid genome and the low risk of pollen-mediated outcrossing because of maternal inheritance. However, as field-grown plants, they can suffer contamination by agrochemicals and fertilizers, as well as fluctuations in yield due to climatic changes and infections. Tissue-type plasminogen activator (tPA), a protein used to treat heart attacks, converts plasminogen into plasmine, which digests fibrin and induces the dissolution of fibrin clots. Recently, we obtained transplastomic tobacco plants carrying the K2S gene encoding truncated human tPA (reteplase) with improved biological activity, and confirmed the presence of the target protein in the transgenic plant leaves. Considering the advantages of plant cell cultures for biopharmaceutical production, we established a cell line derived from the K2S tobacco plants. The active form of reteplase was quantified in cultures grown in light or darkness, with production 3-fold higher in light.

1. Introduction

Biopharmaceuticals based on proteins, antibodies or nucleic acids are increasingly being used for disease treatment. Although only about 60 peptides have been approved by the US FDA to date, more than 140 are under clinical study and by 2020 the global sales of biopharmaceuticals are expected to be worth over $US 278.2 billion (Santos et al., 2016).

Tissue type plasminogen activator (tPA), which induces the dissolution of fibrin clots by converting the zymogen plasminogen into the serine protease plasmin, is a clinically useful thrombolytic agent (Clark, 2001) and a target for biotechnological production. tPA has five domains, N terminal finger, epidermal growth factor, serine protease, Kringle 1 and Kringle 2 (Youchun et al., 2003). The active part of tPA, the thrombolytic Kringle 2 domain, serine protease domain, two functional regions of proteasa (176–527 amino acid residues), plus the 1 to 3 amino acids of the N-terminal is known as the truncated human tissue plasminogen activator (K2S, reteplase), which has a longer plasma half-life and higher fibrinolytic activity than tPA (Nordt and Bode, 2003).

The main biotechnological systems for the production of recombinant biopharmaceuticals are based on microorganism cultures such as *Escherichia coli* and yeast at bioreactor level, while large proteins are generally produced by mammalian cell platforms (Demain and Vaishnav, 2009). Molecular farming also has been utilized for biopharmaceutical production, because transgenic plants only need water, minerals and sunlight for growth, but a drawback of the system, according to the US Agricultural Department, is a lack of guaranteed transgene contention and the risk of contamination of the human food chain if edible plant species are used as the host (Wilson and Roberts, 2012). Moreover, transgenic crops used for the production of heterologous proteins are exposed to agrochemicals and fertilizers in the field, while variable culture

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conditions and the impact of bacterial and fungal infections can lead to fluctuations in yield (Hellwig et al., 2004).

As an alternative production system, plant cell cultures share the capacity of transgenic crops for proper protein folding and can assemble complex recombinant proteins. They also have similar advantages to bioreactor systems based on microorganisms and mammalian cells, as they avoid transgene dissemination and provide controlled and sterile growth conditions, chemically defined culture media, and compliance with pharmaceutical good manufacturing practices, ensuring the biosafety and productivity of the system (Demain and Vaishnav, 2009; Santos et al., 2016). Furthermore, they allow proteins to be manufactured in days or weeks, rather than the months or years required when depending on the growth cycle of a whole plant (Doran, 2000).

Several factors affecting transgenic crops, such as climate, soil quality, season, day length and weather, are not issues for biotechnological platforms based on plant cell cultures. Additionally, the secretion of heterologous proteins into the culture medium simplifies downstream processing and protein purification (Pham et al., 2012). The first human recombinant protein approved in the US and other countries was Taliglucerase alfa, a modified glucocerebrosidase enzyme used to treat Gaucher's disease, produced by Protalix Biotherapeutics in the ProCellEx® platform based on carrot cell cultures (Tekoah et al., 2015).

Transplastomic plants have been targeted for the production of biopharmaceuticals due to the high number (approx. 100) of chloroplasts per plant cell, the high copy number (approx. 10,000) of the plastid genome, as well as the maternal mode inheritance, though low-level leakages of transgenes in pollen may occur (Bock, 2014). Transplastomic technology has enhanced field-grown plant resistance to herbicides and plagues and has been used for the production of recombinant proteins. However, the derived cell cultures have been scarcely applied for the biotechnological production of heterologous proteins (Bock, 2007). Examples of biopharmaceuticals produced in transplastomic tobacco plant cell cultures include the phage-derived endolysins, used as an antibiotic against pneumonia (Oey et al., 2009), camelid antibodies (Lentz et al., 2012), the transforming growth factor (TGFβ3), a cytokine-type protein (Gisby et al., 2011), and fragment C of tetanus toxin (TetC). The latter was accumulated up to 7 mg/L, but when the transplastomic cell suspension was cultured in a temporary immersion bioreactor (TIB), regenerated shoots achieved a TetC production of 95 mg/L (Michoux et al., 2011).

Recently, our group obtained tobacco transplastomic plants harboring the K2S gene driven by the promotor Prrn for the production of the truncated human tissue plasminogen activator (K2S, reteplase), which is one of the most important pharmaceutical recombinant proteins, widely used to break down blood clots through the conversion of plasminogen to plasmin (Abdoli-Nasab et al., 2013). The purification system of reteplase has been recently optimized, reaching a production of up to 30.6 μg/100 mg fresh weight of leaf tissue (Abdoli-Nasab et al., 2016). Taking into account the potential advantages of plant cell cultures for the production of biopharmaceuticals, in this work we demonstrate for the first time the capacity of cell suspension cultures derived from K2S transplastomic tobacco plants to produce the bioactive target peptide.

2. Material and methods

2.1. Plant material

In this work, we utilized seeds from homoplastic plants carrying the K2S gene driven by the promotor Prrn obtained as described by Abdoli-Nasab et al. (2013). Sterile seeds were germinated on solidified MS (Murashige and Skoog, 1962) medium supplemented with 500 mg/L spectinomycin, in Magenta vessels (SIGMA). The *in vitro* plantlets were cultivated in a climate chamber at 25 °C under a 16 h photoperiod and an approximate light intensity of 60 μmol m^{-2} s^{-1}.

2.2. Initiation and maintenance of the transgenic cell suspension

Leaf discs of young K2S plants were cultivated in darkness or under light conditions for callus induction in solid MS medium (Murashige and Skoog, 1962) supplemented with 2.14 mg/L of naphthalene acetic acid in combination with 0.215 mg/L of kinetin (Piñol et al., 1985) and 500 mg/L of spectinomycin (Fig. 1). After several subcultures, 30 g of friable calli were placed in 300 mL of liquid MS medium with the same hormones and antibiotic to obtain a fine cell suspension, which was subcultured every 12 days, shaken at 115 rpm and maintained at 25 °C in darkness or light conditions.

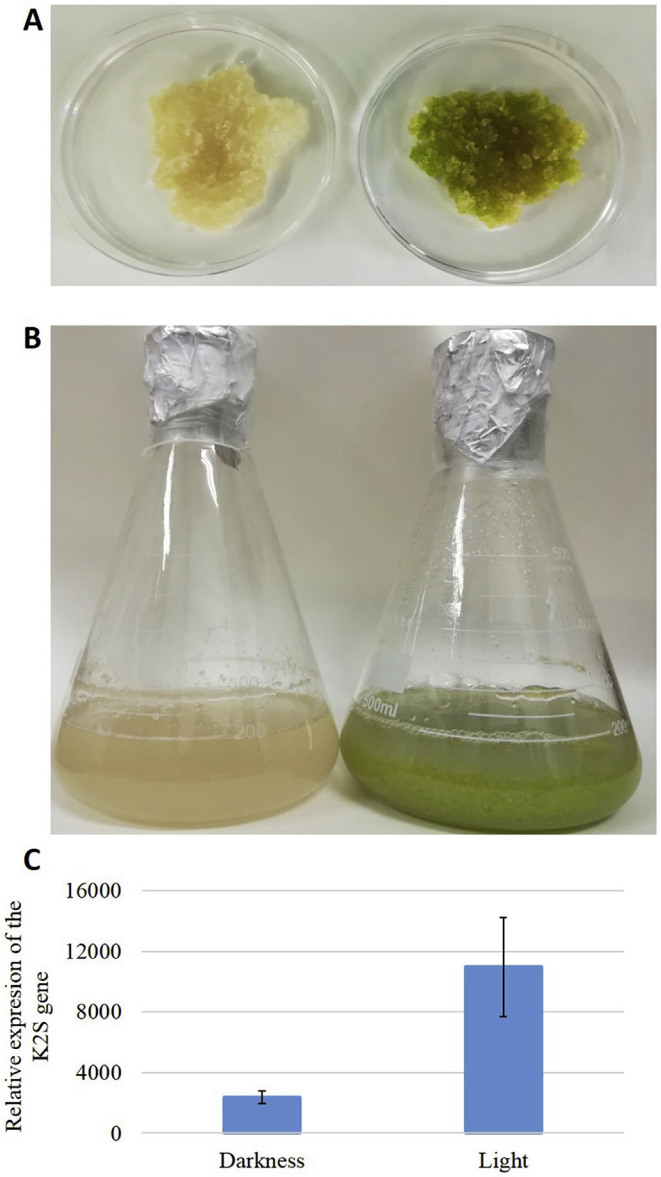

Fig. 1. Appearance of the callus cultures (A) and the derived cell suspensions (B) growing under dark and light conditions. (C) Analysis of the relative K2S gene expression normalized to Elongation Factor 1α.