Biotechnol Lett (2014) 36:855–858
DOI 10.1007/s10529-013-1427-8

ORIGINAL RESEARCH PAPER

Rapid induction of the triterpenoid pathway in *Arabidopsis thaliana* mesophyll protoplasts

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Received: 6 August 2013 / Accepted: 27 November 2013 / Published online: 29 December 2013

Abstract Purpose of work: The purpose of this study was to determine if *Arabidopsis* protoplast transfection could be scaled up, from the commonly used cell-based studies, to be used in triterpenoid production assays as an *in planta* alternative/complement to other expression systems.

Enzyme activities are often identified using heterologous expression systems such as yeast cells. These systems, however, may be incompatible for expressing enzymes involved in specialized (secondary) metabolism. Previous reports with long-term *in planta* expression systems show that the activity of the triterpenoid pathway can be enhanced by expressing enzymes catalyzing initial steps in the pathway. Here we show that triterpenoid production can also be enhanced in *Arabidopsis* mesophyll protoplasts after transfection. This system is designed to quantify changes in productivity of a plant metabolic pathway within 48 h and, as proof of concept, we show a significantly increased production of a triterpenoid by transiently expressing *squalene synthase 1* (*SQS1*) from 0.5 pg/protoplast in mock-transfected protoplasts to 2.7 pg/protoplast in constitutively expressing *SQS1* protoplasts.

Keywords Heterologous expression · Protoplast · Squalene epoxidase · Squalene synthase · Triterpenoids

Introduction

All basic triterpenoid structures are derived from one common primary metabolite, 2,3-oxidosqualene, through alternative cyclization reactions catalyzed by oxidosqualene cyclases. Characterization of biosynthetic enzymes within this pathway is normally achieved by expressing them in mutant yeast strains in which the native enzymes are missing. Non-plant expression systems, however, may lack plant-specific components necessary for activity. For example, in the original characterization of squalene synthase 1 (SQS1) from *Arabidopsis*, it was found that the native enzyme contained a *C*-terminal sequence that prevented activity in yeast (Kribii et al. 1997). An *in planta* system could aid in analysis if unknown regulatory components exist.

The output of the triterpenoid pathway has been increased by constitutively expressing enzymes in

Electronic supplementary material The online version of this article (doi:10.1007/s10529-013-1427-8) contains supplementary material, which is available to authorized users.

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stably-transformed cell cultures, including SQS1 (Lee et al. 2004, Mirjalili et al. 2011, Seo et al. 2005). These investigations showed an increased production of downstream products after 1 month of culture. We speculated that *Arabidopsis* mesophyll protoplasts could be used as an alternative to long term cell or tissue culture systems. They have proven to be easy to harvest from whole leaves and have long been used successfully in cell-based assays (Yoo et al. 2007).

We hypothesized that enhancing expression of *SQS* or *squalene epoxidase* (*SQE*) genes through transient expression in protoplasts would rapidly increase flux through the triterpene pathway. To determine if this is the case, *SQS1*, *SQE1*, and *SQE4* from *Arabidopsis thaliana* were each transfected into mesophyll protoplasts isolated from plants stably-expressing $35S_{pro}$*:LUP4*, previously shown to produce excess β-amyrin in cuticular wax (Buschhaus and Jetter 2012). The goals of this research were to determine if triterpenoids could be measured from preparations of protoplasts, and if their production could be measurably enhanced by expressing enzymes catalyzing the early steps in the triterpene pathway.

Materials and methods

Plasmid construction

The plasmids used for transfection were constructed using the primers listed in Supplementary Table S1, amplified by Phusion DNA polymerase (NEB) from 10 day old seedling cDNA, digested using the indicated restriction enzymes (NEB, unless otherwise noted) and ligated into the 35S-OFP1-HA plasmid, constructed from pUC19, from Wang et al. (2007) with T4 ligase (Invitrogen). A separate $35S_{pro}$*:GFP* reporter gene was included in each plasmid to allow for the quantification of transfection efficiency.

Microscopy

A Zeiss Axiovert epi-fluorescence microscope equipped with filters for GFP and Texas Red (chlorophyll autofluorescence) were used for determining protoplast transfection efficiency, which was calculated as the proportion of living cells, identified by chlorophyll autofluorescence, to transfected cells, identified with GFP fluorescence.

Protoplast transfection and analysis

Protoplasts were isolated essentially as described by Tiwari et al. (2006) with the exception that the PEG-mediated transfection was scaled-up to transfect a total of 1.2×10^6 cells in 3 ml protoplast solution using 15 μg plasmid DNA in each sample. This DNA was purified using the EZNA Endo-free plasmid mega kit (Omega Bio-Tek) according to the manufacturer's protocol.

Total lipids were extracted 16 h after transfection by three washes of hexane, the solvent was evaporated, the residue was applied to 0.5 mm TLC plates and then developed in chloroform. The zone between R_f 0.2–0.73 was scraped from the plate and extracted with 3 ml chloroform overnight. These samples were then dried down and prepared for GC-FID as described in Greer et al. (2007). Lupeol, the internal standard, was added prior to the extraction from protoplasts.

Results

Transfection efficiency was calculated 24 h after transfection by comparing the number of green fluorescent cells to the total number of living cells for each sample (Fig. 1a–c). The efficiency was found to be highly variable, from 25 to 75 % ($n \geq 98$) (Fig. 1d and other data not shown). Extracts from transfected cells were fractionated on TLC and analysed by GC–MS and GC. Production of β-amyrin after constitutive expression of *SQS1* was found to significantly increase from 0.5 pg/protoplast to 2.7 pg/protoplast (Fig. 2). The over-expression of both *SQE1* and *SQE4* increased production of β-amyrin to 2.3 pg/protoplast and 1.4 pg/protoplast, respectively ($n = 3$) but, due to the high variability, these increases were statistically insignificant. In the amyrin fractions from the transfected cell lines, no phytosterols were detected.

Discussion

We have demonstrated that *Arabidopsis* mesophyll protoplasts can be used as an effective, transient system for analysis and manipulation of metabolic pathways. Specifically, we showed that it was possible to significantly increase production of β-amyrin after expressing *SQS1*. Expression of *squalene synthase*

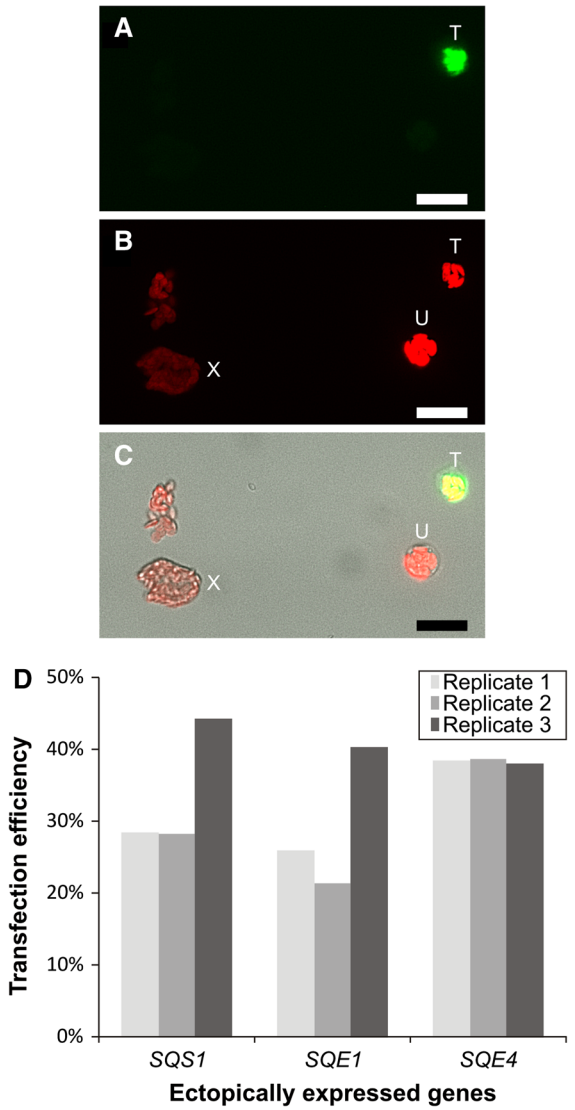

Fig. 1 Analysis of transfected protoplasts. **a–c** Representative images of transformed protoplasts in a hemocytometer immediately prior to β-amyrin quantification. **a** A transformed protoplast emitting green fluorescence. **b** The total number of protoplasts in one field of view, identified from the *red* autofluorescence emitted by the chloroplasts imaged under a Texas-*red* filter. Chloroplasts from dead cells, *X*, display lower fluorescence and can be eliminated from the quantification. **c** Overlay of **a** and **b** on bright field image. *T* indicates transformed cell, *U* indicates an untransformed cell, *X* indicates a dead cell with weak red autofluorescence. *Scale bars* 30 μm. **d** Transformation efficiency, calculated from three biological replicates used to quantify β-amyrin

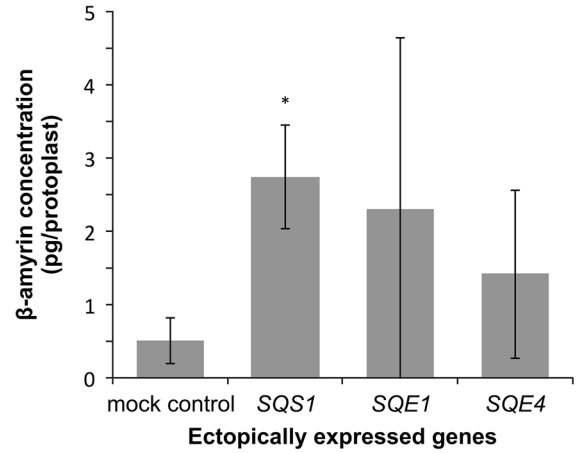

Fig. 2 Quantification of β-amyrin 48 h after expression of the indicated genes. *Asterisk* indicates p value < 0.01, $n = 3$. The protoplasts from each replicate were harvested from the same pool of leaves and each mock control underwent the same transfection procedure without plasmid DNA

from *Panax ginseng* similarly increased production of phytosterols in 4-week post-transformed root cultures of both *P. ginseng* and *Eleutherococcus senticosus*, unrelated plants that produce a number of medicinal triterpene-saponins (Lee et al. 2004, Seo et al. 2005). SQS1, the only functional SQS in *Arabidopsis* (Busquets et al. 2008), has also boosted triterpene production in 4-week post-transformed hairy root cultures of *Withania coagulans* (Mirjalili et al. 2011). Our data show that even a brief, 48 h, increase in expression can result in an increased production of triterpenes, showing that SQS1 catalyzes a rate-limiting step in the triterpene pathways. Our data also show that expression of *SQS1* alone can be manipulated to optimize a rapid *in planta* transient system for studying triterpene pathways. However, since our study utilized protoplasts from transgenic plants expressing *LUP4,* further testing in wild-type protoplasts is needed to determine the effects of *SQS1* expression on native phytosterol levels and to demonstrate the versatility of this system. Alternatively, this system could be further developed for triterpenoid assays by producing plants containing a stable-inducible *SQS1* expression construct to be transfected with unknown *oxidosqualene cyclases*.

In an attempt to characterize six of the *SQE* genes in *Arabidopsis*, Rasbery et al. (2007) expressed each gene in a yeast strain that was deficient in *ERG1,* the native SQE. Activity was only detected with SQE1, SQE2 and SQE3. Furthermore, ectopic expression of SQE4 and SQE5 was not able to rescue *sqe1-3* defects in *Arabidopsis*. Considering this, along with the fact that phylogenetic analysis places SQE4, SQE5 and SQE6 into a separate clade from true SQEs, these

proteins are considered SQE-like enzymes, and are distinct from all other characterized plant SQEs (Rasbery et al. 2007). Using our protoplast transfection system, we were unable to detect significant increases in triterpenoid production by inducing expression of either a true *SQE* (*SQE1*) or a SQE-like gene (*SQE4*).

The value of this system is the ability to express genes within a single population of plant cells to avoid the complexity of background lipids found in multiple tissue systems. Within leaves, for example, each tissue within has its own unique mixture of specialized compounds (Nuringtyas et al. 2012). A system with a similar goal was also developed for expressing biosynthetic enzymes in only plant trichomes (Wang et al. 2002) and was used to characterize CYP725A4 (Rontein et al. 2008). This is also an attractive system because trichomes can be isolated from the plant (Marks et al. 2008, Yerger et al. 1992) and, in many species, trichomes are involved in the production of specialized defensive metabolites (Schilmiller et al. 2008). However, this requires time to produce stable transformants for each ectopically expressed enzyme. This requirement can be avoided through our approach, which, assuming *Arabidopsis* leaves are available, requires only 48 h from transfection to analysis.

Acknowledgments This work was supported by NSERC Discovery Grants (nos. 298264-09 and 262461-08), the Canada Research Chairs program, and Canada Foundation for Innovation Leaders Opportunity grants to G.O.W and R. J. We thank Christopher Buschhaus for providing $35S_{pro}$::LUP4 seeds and Luke Busta for technical assistance with GC and GC–MS analyses.

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