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Research article

Characterization of plants expressing the human β1,4-galactosyltrasferase gene

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ABSTRACT

Modification of the plant N-glycosylation pathway towards human type structures is an important strategy to implement plants as expression systems for therapeutic proteins. Nevertheless, relatively little is known about the overall impact of non-plant glycosylation enzymes in stable transformed plants. Here, we analyzed transgenic lines (*Nicotiana benthamiana* and *Arabidopsis thaliana*) that stably express a modified version of human β1,4-galactosyltransferase (STGalT). While some transgenic plants grew normally, other lines exhibited a severe phenotype associated with stunted growth and developmental retardation. The severity of the phenotype correlated with both increased STGalT mRNA and protein levels but no differences were observed between N-glycosylation profiles of plants with and without the phenotype. In contrast to non-transgenic plants, all STGalT expressing plants synthesized significant amounts of incompletely processed (largely depleted of core fucose) N-glycans with up to 40% terminally galactosylated structures. While transgenic plants showed no differences in nucleotide sugar composition and cell wall monosaccharide content, alterations in the reactivity of cell wall carbohydrate epitopes associated with arabinogalactan-proteins and pectic homogalacturonan were detected in STGalT expressing plants. Notably, plants with phenotypic alterations showed increased levels of hydrogen peroxide, most probably a consequence of hypersensitive reactions. Our data demonstrate that unfavorable phenotypical modifications may occur upon stable *in planta* expression of non-native glycosyltransferases. Such important issues need to be taken into consideration in respect to stable glycan engineering in plants.

1. Introduction

In recent years much effort has been devoted to engineering plants for the production of recombinant glycoproteins with optimized glycosylation. Accordingly, complex human glycoprotein therapeutics with defined N- and O-glycan moieties can be produced in plants (Strasser et al., 2014). These recent advances make whole plants useful alternatives to existing mammalian cell-culture based expression platforms. However, the impact of the introduced glycan modifications, the resulting alterations in Golgi protein organization and the concomitant excess/shortage of certain metabolites on overall plant physiology are less known. In particular, glyco-engineering leads to incorporation of mammalian-type sugar residues into endogenous plant glycoproteins, causes perturbations of nucleotide sugar flux and deposits additional proteins in the secretory pathway. The majority of the plant glyco-engineering approaches resulting in homogenous glycosylation rely on transient expression of heterologous glycosyltransferases and other proteins necessary for targeted glycosylation (Castilho et al., 2010). The transient expression technology is especially applicable to leaves from *Nicotiana benthamiana* plants and can be combined with powerful viral-based transient systems leading to high expression of glycoproteins with defined N-glycan structures (Jez et al., 2012). In commonly used protocols, all expression constructs are transferred simultaneously to plants by infiltration of leaves with a mixture of *Agrobacteria* and recombinant proteins are extracted 3–10 days post-infiltration. During this short time period the glyco-engineering procedure does not cause any severe morphological

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phenotype that would affect the quality and/or quantity of recombinant protein production. The stable integration of whole pathways into the plant genome and the putative effect on overall plant development and growth is poorly investigated. While no obvious phenotypical modifications were reported in transgenic plants that express human glycosyltransferases (e.g. β1,4-galactosyltransferase (GalT) and N-acetylglucosaminyltransferase III, IV and V) under standard growth conditions (Bakker et al., 2001; Rouwendal et al., 2007; Frey et al., 2009; Nagels et al., 2011, 2012) alterations on the plant development were observed upon stable expression of human Lewis fucosyltransferase and a nucleotide sugar transporter in transgenic *Nicotiana tabacum* (Joly et al., 2004; Khalil et al., 2010).

In previous studies human GalT was expressed in transgenic tobacco for several reasons: On the one hand this enzyme promotes the generation of complex N-glycans that terminate with β1,4-linked galactose, a widespread N-glycan structure in humans and the required acceptor substrate for subsequent sialylation (Castilho et al., 2010; Jez et al., 2013). On the other hand targeted to an early Golgi compartment the enzyme serves as powerful tool to eliminate plant specific core fucosylation (Bakker et al., 2006).

Here, we characterized plants (*N. benthamiana* and *Arabidopsis thaliana*) stably transformed with a chimeric human β1,4-galactosyltransferase that targets the enzyme to a late Golgi compartment.

2. Experimental procedures

2.1. Construction of binary vectors

The STGalT binary vector for *N. benthamiana* and *A. thaliana* transformation was described previously (Strasser et al., 2009). The binary vector used for transient expression of the monoclonal antibody (mAb) 4E10 (p4E10) was generated as described previously (Strasser et al., 2009).

2.2. Generation of transgenic plants

N. benthamiana wild type plants expressing STGalT (STGalT-WT) were described previously (Strasser et al., 2009). Selected STGalT-WT lines were crossed with the glycosylation mutant ΔXT/FT, lacking the plant-specific β1,2-xylose and core α1,3-fucose residues, leading to the generation of STGalT^{X}-ΔXF plants (Strasser et al., 2009). Generation of STGalT transformants in a ΔXT/FT background was also done by direct transformation of the glycosylation mutant with the STGalT binary vector carrying an additional expression cassette for hygromycin resistance (STGalT-ΔXF). Putative transformed plantlets were selected on Kanamicyn-(STGalT-WT) or hygromycin-containing media (STGalT-ΔXF).

A. thaliana ecotype Columbia (Col-0) was transformed with the STGalT construct by floral dipping (Clough and Bent, 1998). Transformed seeds were selected on kanamycin-containing media.

For all transformation events the presence of the human GalT DNA was confirmed by PCR using gene-specific primers. Endogenous proteins of selected PCR-GalT positives were analyzed for the presence of galactose by *Ricinus communis* agglutinin I lectin blot as described earlier (Bakker et al., 2001).

Plants were cultivated in a growth chamber at a constant temperature of 24 °C, 60% relative humidity, and a 16 h light/8 h dark photoperiod.

2.3. Genomic PCR amplification (gPCR)

Genomic DNA was isolated from leaf material (~8 mg) as described previously (Strasser et al., 2004b). The human GalT DNA was amplified by PCR using primers 5′-GGCAAAGCAGAACCC AAATGTGA-3′ and 5′-TCTTCTCCTCCCCAGCCCCAATAAT-3′. DNA extracts from ΔXT/FT plants and from ΔXT/FT plants transiently expressing STGalT served as negative and positive controls, respectively. For internal control the *N. benthamiana* catalase gene (NbCat) was amplified from all genomic DNA samples using specific primers (5′-CATTCGCGGTTTTGCTGTC-3′ and 5′-TGGTGGCGTGGCTATGATTT GTA-3′) (Strasser et al., 2004a).

2.4. Reverse transcription real-time PCR amplification (qPCR)

Total RNA was extracted from leaves of *N. benthamiana* using the SV Total RNA Isolation System (Promega). 500 ng of RNA were reverse transcribed at 42 °C using oligo(dT) primer and AMV reverse transcriptase (Promega). qPCR was performed with a C1000 Touch™ Thermal cycler (Biorad) with the iQ™ SYBR® GREEN Supermix (Biorad). *N. benthamiana* elongation factor1α gene (EF1α, accession number AY206004) was used as internal control. Specific primers were used to amplify a STGalT fragment (5′-CATGATCCGCCACT-CAAGAGAC-3′ and 5′-GCTCGGTGTCCCGATGTCCACT-3′) and an EF1α fragment (5′-GCTGACTGTGCTGTCCTGATTATT-3′ and 5′-TCAC GGGTCTGTCCATCCTTA-3′). Amplification occurred after an initial denaturation (7 min/95 °C) in 40 cycles (95° C/5 s—54° C/ 15 s—95° C/15 s). At the end of each run, a melting curve was recorded between 60 °C and 95 °C. To process the amplification data, the CFX Manager™ Software was used. The expression levels of hGalT were normalized in relation to ΔXT/FT plants. Values, resulting from 3 independent experiments, are given in mean ± standard error of the mean.

2.5. Preparation of total soluble protein (TSP) extracts

Leaf material (250 mg) was submerged in liquid nitrogen and ground in a swing mill (Retsch®, MM2000) for 2 min at amplitude 60. Two volumes (v/w) of 1 × PBS were added to the samples and incubated on ice for 10 min. Finally, the extracts were centrifuged (9000 g for 5 min at 4 °C) and the supernatant was stored at −20 °C for further analysis.

2.6. Immunoblot analysis of human GalT in transgenic *N. benthamiana*

Prior to separation by 12% SDS-PAGE, TSP extracts were mixed with reducing 4× Laemmli buffer and incubated for 5 min at 95 °C. Subsequently, the separated proteins were transferred to a nitrocellulose membrane and probed with protein-specific antibodies (monoclonal mouse anti-GalT, diluted 1:10,000 in TBS buffer containing 0.05% Tween-20). Detection was performed using HRP-conjugated secondary antibodies (anti-mouse IgG-peroxidase antibody diluted 1:10,000 in TBS-Tween). SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL) was used as a substrate. Total soluble proteins from a ΔXT/FT plant transiently expressing STGalT were used as a positive control. The membrane was stained with Ponceau S (Sigma Aldrich) to visualize transferred proteins.

2.7. Histochemical detection of hydrogen peroxide (H_2O_2)

H_2O_2 was detected histochemicaly in leaves using 3,3-diaminobenzidine (DAB) as substrate (Weigel and Glazebrook, 2002). Leaves from about 30 day old plants were detached and dipped either in buffer (100 mM HEPES pH 6.8) or in buffer supplemented with DAB (1 mg/mL). The solutions were delivered to plant cells by vacuum infiltration and the leaves were incubated overnight in the growth chamber with constant light. The tissues were then decolorized with 96% ethanol for 30 min at 70 °C. This treatment decolorized the tissues, except for the brown colored