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Inhibition of trehalose breakdown increases new carbon partitioning into cellulosic biomass in *Nicotiana tabacum*

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ABSTRACT

Validamycin A was used to inhibit in vivo trehalase activity in tobacco enabling the study of subsequent changes in new C partitioning into cellulosic biomass and lignin precursors. After 12-h exposure to treatment, plants were pulse labeled using radioactive $^{11}CO_2$, and the partitioning of isotope was traced into [^{11}C]cellulose and [^{11}C]hemicellulose, as well as into [^{11}C]phenylalanine, the precursor for lignin. Over this time course of treatment, new carbon partitioning into hemicellulose and cellulose was increased, while new carbon partitioning into phenylalanine was decreased. This trend was accompanied by a decrease in phenylalanine ammonia-lyase activity. After 4 d of exposure to validamycin A, we also measured leaf protein content and key C and N metabolite pools. Extended treatment increased foliar cellulose and starch content, decreased sucrose, and total amino acid and nitrate content, and had no effect on total protein.

1. Introduction

Higher plants fix atmospheric carbon dioxide producing sucrose, a mobile disaccharide that is distributed and used as a principal source of energy and carbon by the plant during growth and development. Trehalose, also a disaccharide, is the principal storage carbohydrate of bacteria, yeast cells, fungal spores, and certain invertebrates. Unlike sucrose, which consists of glucose and fructose units bound in an α,β-1,2 configuration, trehalose consists of two glucose units bound in an α,α-1,1 configuration. Sucrose, the more soluble of the two disaccharides, is better suited as a transport sugar within plants.[1] At sites where there is rapid growth, sucrose can be cleaved by invertase into glucose and fructose and used for energy production. Alternatively, sucrose synthase can cleave sucrose into uridine diphosphate glucose (UDP-glucose) and fructose, preserving the energy status of the cell while at the same time providing carbon skeletons for cell-wall polysaccharide synthesis. Cellulose, one of the primary cell-wall polysaccharides, relies on a steady supply of UDP-glucose for its synthesis.[2]

Higher plants possess genes that encode the enzymes trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) that are associated with trehalose biosynthesis.[3] Furthermore, trehalase, the key enzyme responsible for the hydrolysis of trehalose during degradation, is present in all organs of higher plants, with highest activities found in the flowers.[3] Even so, trehalose has been somewhat of an obscure curiosity in plants owing to the fact that it has only been observed in substantial concentrations in the resurrection plants, *Selaginella lepidophylla* and *Myrothamnus flabellifolia*.[4] This sugar is thought to accumulate in the cells of these plants enabling them to adapt to dehydration, salinity, freezing, and heat stress.[5] Typically, trehalose is almost undetectable in higher plants.[1,6,7] Even so, recent literature suggests that trehalose may play a pivotal role in cellular signaling, in coordinating metabolic partitioning and as a source-sink for the allocation of carbon resources during growth and development.[8,9] For example, up-regulation of genes associated with trehalose biosynthesis has been observed during the maturation phase of plant embryo development. This action may have relevance to cell expansion during differentiation, to increased activity of the sucrose synthase enzyme, and to altered cell-wall structure.[10] Furthermore, in yeast TPS and TPP are known to form a multimeric enzymatic complex comprised of polypeptide chains that may play important regulatory roles.[11] Using the *tps1*Δ mutant in yeast, it was demonstrated that it was unable to grow on glucose enhanced media, giving strong evidence to suggest that the TPS1

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peptide subunit may regulate cellular glucose influx.[12] The same may be true of plants. Also, there is a strong correlation between the inhibition of hexokinases in *Saccharomyces cerevisiae* by trehalose-6-phosphate (T6P), and the regulation of sugar partitioning into glycolysis, providing additional evidence to suggest that a strong tie exits between trehalose accumulation and cellular glucose utilization.[13,14]

Based on previous work demonstrating an association between trehalose biosynthesis and altered cell-wall composition in plant embryos,[8] and on the direct association between trehalase activity and trehalose accumulation in mature plants,[15] we hypothesized that inhibition of trehalose degradation in mature plants would increase metabolic partitioning of new carbon into cell-wall polysaccharides, most notably cellulose and hemicellulose, at the expense of lignin biosynthesis.

Specifically, we used the potent trehalase inhibitor, validamycin A, to investigate the association between trehalose degradation and glucose utilization for cell-wall polysaccharide biosynthesis. Our general approach made use of the radioactive isotope ^{11}C ($t_{1/2}$ 20.4 m) that was introduced into the foliar tissue of intact plants via $^{11}CO_2$ fixation,[16] and we monitored the incorporation of the tracer into cell-wall [^{11}C]cellulose and [^{11}C]hemicellulose, as well as incorporation into [^{11}C]phenylalanine, the principal substrate of the phenylpropanoid pathway and lignin biosynthesis. This approach enabled us to measure the turnover of new carbon into cellulosic biomass over timelines that were far too short to allow detection of changes in composition.

2. Materials and methods

2.1. Plant materials

Tobacco plants (*Nicotiana tabacum* L. cv Samsun) were grown from seeds in commercial potting mix with a slow-release fertilizer (Osmocote) under metal-halide lamps at 24 °C with a 16/8 h at 350 μmol m^{-2} s^{-1} photoperiod. At 3 weeks into their growth cycle, the plants were removed from their growth pots and transferred to an aerated hydroponic station. Nutrients status of the hydroponics was maintained using a modified Hoagland's solution (2.4 mM $CaNO_3$, 3.2 mM KNO_3, 0.75 mM KH_2PO_4, 1.1 mM $MgSO_4$, 7.1 μM $MnSO_4$, 3.4 μM $ZnSO_4$, 0.6 μM $CuSO_4$, 80 μM H_3BO_3, 1 μM Na_2MoO_4 and 161.8 μM FeNa–EDTA) that was changed on a 5-d cycle. Plants were used for experiments when they had seven fully expanded leaves. Tracer studies targeted leaf-3, counting down from the top where leaf-1 was the first fully expanded leaf. Biomass measurements targeted leaf-2 and leaf-3. The same growth conditions were maintained during the application of the validamycin A treatment and labeling of recent photosynthate with $^{11}CO_2$.

2.2. Treatment

Plants were treated using a solution of validamycin A that was added to the Hoagland's hydroponics solution to bring the treatment concentration to 150 μM (K_i, 10 nM). In one subset of plants, the effect of treatment after 12 h was measured relative to changes in new carbon partitioning (as ^{11}C) into cellulosic biomass of leaf-3, soluble sugars, and phenylalanine. In a second subset of plants, the effect of continued treatment over 4 d was assessed by testing for changes in foliar (leaf-2 + leaf-3) cellulose content, starch/sugar content, nitrate content, total amino acids, and soluble protein content. During the time of treatment, plants were maintained under the same photoperiod (16/8 h at 350 μmol m^{-2} s^{-1}), and fresh nutrient was introduced after 3 d. Control studies were identical to those described above with the exception that no antibiotic was added to the nutrient solution.

2.3. Radiotracer production and administration

$^{11}CO_2$ was produced via the $^{14}N(p,\alpha)^{11}C$ nuclear transformation from 20 mL volume high-purity nitrogen gas target (500 mL @ STP) using 18 MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at Brookhaven National Laboratory, and captured on a molecular sieve (4 Å). The $^{11}CO_2$ that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 400 mL min^{-1} as a discrete pulse for labeling a leaf in a 5 × 10 cm lighted (920 μmol m^{-2} s^{-1}) cell.[16] The extent of ^{11}C-fixation was correlated to leaf-level photosynthetic activity as measured by infrared gas exchange (Li-Cor Biosciences, Lincoln, NE USA; model 6162).

2.4. Analysis of radioactive cellulosic biomass

Forty-five minutes after the tracer was administered, the study leaf (leaf-3) was excised at the petiole. The 5 × 10 cm area exposed to the tracer was then cut away from the remaining leaf tissue, weighed, and then flash frozen in liquid nitrogen. Using a mortar and pestle, the tissue was powdered in liquid nitrogen, and extracted using standard procedures.[17–20] In the first step the tissue was refluxed in 6 mL of aq MeOH (50% v/v) for 10 min at 90 °C. The extract was separated by pipette, and the remaining tissue was washed twice using 6 mL of deionized water. The washings were combined with the aqueous alcohol fraction prior to counting the ^{11}C. This fraction removed small soluble compounds including radiolabeled monosaccharides and disaccharides and amino acids that were subjected to separate radio-HPLC analyses discussed below. The remaining tissue (considered cell-wall components) was subjected to an extraction using 6 mL of dilute 1 N NaOH for 10 min at 95 °C. This step separated callose (1,3-β-glucans), as well as pectins (branched chain 1,4-β-glucans) from the remaining cell-wall polymers. The extract was again separated by pipette, and the remaining tissue washed twice using 6 mL of deionized water. The washings were combined with the base portion prior to counting the ^{11}C. The remaining tissue was then subjected to acid digestion using a 3:1 mixture of dilute 1 N nitric acid: acetic acid for 30 min at 100 °C enabling hemicellulose to be solubilized, leaving behind cellulose as the undigested portion. The contents were cooled to ambient temperature and filtered onto 2-cm disks of pre-weighed glass microfiber filters (GF/A: 2.5 cm diameter; Whatman, Maidstone, UK). The collected tissue was washed 3-times during this filtration step using deionized water. Samples were immediately counted using a static NaI gamma radiation detector, decay-corrected back to a common zero time and fraction-corrected to allow correlation to total ^{11}C-activity fixed within the tissue. Once counted, filtered samples were dried under a heat lamp and re-weighed to determine the amount of cellulosic biomass.

We verified that the indigestible acid portion was cellulose by re-subjecting this material to a stronger digestion using the phenol–sulfuric acid assay.[21] This method involved refluxing the material in a 1:10 solution of phenol (5%) and concentrated sulfuric acid at 100 °C until all of the solid material was digested. Aliquots of the neutralized extract were then analyzed by HPLC for glucose levels that correlated back to the mass of cellulose digested.

2.5. Analysis of radioactive soluble sugars

A 50 μL volume of the MeOH–H_2O extract was injected onto a reversed-phase analytical HPLC column (Phenomenex, Torrance, CA, USA: Luna™ NH_2, 5 μm particle size, 250 × 4.6 mm i.d.). At injection, the mobile phase (1.5 mL m^{-1}) was sustained at 80% acetonitrile–20% H_2O. The mass levels of the soluble sugars were measured using a refractive index (RI) detector (Sonntek Inc., Upper