Metabolic engineering of Arabidopsis for butanetriol production using bacterial genes ☆

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ABSTRACT

1,2,4-butanetriol (butanetriol) is a useful precursor for the synthesis of the energetic material butanetriol trinitrate and several pharmaceutical compounds. Bacterial synthesis of butanetriol from xylose or arabinose takes place in a pathway that requires four enzymes. To produce butanetriol in plants by expressing bacterial enzymes, we cloned native bacterial or codon optimized synthetic genes under different promoters into a binary vector and stably transformed Arabidopsis plants. Transgenic lines expressing introduced genes were analyzed for the production of butanetriol using gas chromatography coupled to mass spectrometry (GC–MS). Soil-grown transgenic plants expressing these genes produced up to 20 μg/g of butanetriol. To test if an exogenous supply of pentose sugar precursors would enhance the butanetriol level, transgenic plants were grown in a medium supplemented with either xylose or arabinose and the amount of butanetriol was quantified. Plants expressing synthetic genes in the arabinose pathway showed up to a forty-fold increase in butanetriol levels after arabinose was added to the medium. Transgenic plants expressing either bacterial or synthetic xylose pathways, or the arabinose pathway showed toxicity symptoms when xylose or arabinose was added to the medium, suggesting that a by-product in the pathway or butanetriol affected plant growth. Furthermore, the metabolite profile of plants expressing arabinose and xylose pathways was altered. Our results demonstrate that bacterial pathways that produce butanetriol can be engineered into plants to produce this chemical. This proof-of-concept study for phytoproduction of butanetriol paves the way to further manipulate metabolic pathways in plants to enhance the level of butanetriol production.

1. Introduction

Plants are increasingly used to produce novel value-added platform chemicals, polymers and non-native proteins by introducing one or more genes from microbes and animals (Bornke and Broer, 2010; Gleba et al., 2007). Bio-based approaches using plants and microbes to produce commercially important chemicals are considered as cost effective and environmentally safe alternatives to chemical-based synthesis. Plants, as autotrophic organisms, offer additional advantages over heterotrophic microbes, as they naturally synthesize precursors for many novel chemicals. By introducing microbial pathways into plants novel chemicals such as bacterial polyesters (polyhydroxyalkanoates) have been produced in plants (Poirier, 2001).

1,2,4-butanetriol (butanetriol) is an important industrial precursor for synthesizing the energetic material butanetriol trinitrate and is a useful building block for the synthesis of various drugs and other useful chemicals (Niu et al., 2003; Sato et al., 2003; Xu et al., 2004; Yamada-Onodera et al., 2007). Nitration of racemic D, L-butanetriol generates D, L-butanetriol trinitrate (BTTN). BTTN is used as a propellant and as an energetic plasticizer that is used in mining and in a variety of Department of Defense and Department of Energy applications. Compared to trinitroglycerine, which has been used in industrial and military energetic materials, BTTN is less hazardous, less shock sensitive, less volatile, more thermally stable, and has a lower melting point (Niu et al., 2003). Butanetriol is not only used as a precursor of energetic material but also in

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synthesis of various other chemicals and drugs. BTTN has potential application as a specific vasodilator for treatment of angina pectoris (Molefe, 2005; Needleman, 1976). For short-term use, trinitroglycerine is the most prescribed nitrate ester for alleviation of angina (Thatchera et al., 2004) because it is assimilated within a minute and offers instant relief. BTTN, unlike trinitroglycerine, is resistant to degradation by nitrate reductase and more stable (United States Department of Agriculture, 2008). The slow degradation of BTTN compared to trinitroglycerin offers a prolonged effect (Needleman, 1976). BTTN is also used in the synthesis of growth factors (Xu et al., 2004) and other important pharmaceutical compounds that have anticancer and antiviral applications (Sato et al., 2003; Solladie et al., 1998). Carpaine and azamine, a novel class of macrocyclic dilactones that were reported to exhibit a wide range of biological properties including antitumor activity at low concentrations, were also synthesized from butanetriol as a single source of chirality (Sato et al., 2003). D-3,4-dihyroxybutanoic acid, a precursor used for the synthesis of cholesterol-lowering drugs such as Crestor, is derived from D-3,4-dihydroxybutanal one of the intermediates in butanetriol synthesis (United States Department of Agriculture, 2008; Lau, 2007). Replacement of trinitroglycerine as an energetic plasticizer and the commercial use of butanetriol as a precursor for energetic material and drugs (Molefe, 2005) hinges on safe, cost-effective and sustainable methods for production of racemic D, L-butanetriol.

Several processes have been developed for the large-scale chemical synthesis of butanetriol (Molefe, 2005; Monteith et al., 1998). The widely used commercial method is the stoichiometric reduction of esterified malic acid (diethyl D, L-malate) using $NaBH_4$ (Ikai et al., 1999;

Monteith et al., 1998). Catalytic hydrogenation of malic acid over rubidium and carbon is another option, but these hydrogenation reactions need to run at 2900–5000 psi and elevated temperature and lead to undesirable byproducts (Niu et al., 2003). In addition, all the substrates employed for the chemical synthesis of D, L-butanetriol are derived from petroleum and require expensive metal catalysts that adversely impact the environment (Antons et al., 2002; Niu et al., 2003). Because of the adverse environmental impact of chemical synthesis of butanetriol and a move towards using sustainable resources, and to reduce reliance on petrochemical-based production of malic acid, bio-based approaches were explored for the synthesis of butanetriol enantiomers.

The synthesis of D-1,2,4-butanetriol and L-1,2,4-butanetriol from the precursors D-xylose and L-arabinose, respectively, using microorganisms has been achieved (Niu et al., 2003). The bio-conversion occurs in four steps catalyzed by four different enzymes (see Fig. 1a) (Niu et al., 2003). The last two enzymes are common for both pathways whereas the first two enzymes are substrate specific. The first oxidation step is catalyzed by D-xylose dehydrogenase (XDG) or L-arabinose dehydrogenase (ADG) and involves conversion of D-xylose or L-arabinose to D-xylonic acid or L-arabinonic acid, respectively. The second step is dehydration of the D-xylonic acid, catalyzed by D-xylonate dehydratase (XDT), or L-arabinonic acid, catalyzed by L-arabinonate dehydratase (ADT), to the corresponding D- or L-3-deoxy-glycero-pentulosonic acids, which are decarboxylated by benzylformate decarboxylase (BFD) to form D- or L-3-4 dihydroxybutanal, respectively. In the final step, these butanals are converted to the corresponding D- or L-1,2,4-butanetriol enantiomer by dehydrogenase (DH). Although the microbial synthesis of
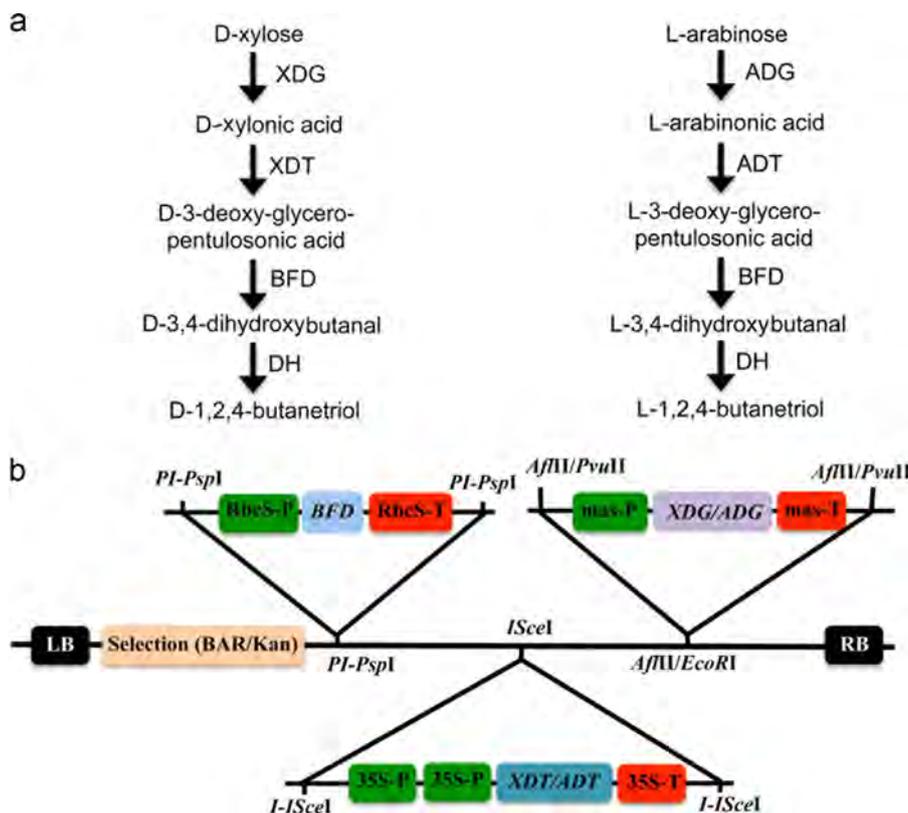
Fig. 1. Schematic diagrams of 1,2,4-butanetriol pathways and design of gene constructs for plant transformation. (a) Schematic representation of the biochemical pathways showing the synthesis of D-1,2,4-butanetriol and L-1,2,4-butanetriol from xylose and arabinose. XDG, D-xylose dehydrogenase; ADG, L-arabinose dehydrogenase; XDT, D-xylonate dehydratase; ADT, L-arabinonate dehydratase; BFD, benzoylformate decarboxylase; DH, dehydrogenase. (b) Binary vector construct containing the three genes encoding the first three enzymes used in the butanetriol synthesis pathways from xylose and arabinose. Each vector had a set of genes amplified from bacteria or codon-optimized synthetic genes for the xylose pathway or the set of codon-optimized synthetic genes for the arabinose pathway. RbcS-P and RbcS-T, Rubisco small subunit promoter and terminator, respectively; mas-P and mas-T, mannopine synthase promoter and terminator, respectively; and 35S-P and 35S-T, Cauliflower Mosaic Virus promoter and terminator, respectively. Cloning sites and selection markers are explained in the text.

butanetriol enantiomers avoids the high H_2 pressure, elevated temperature and expensive chemical catalysts, a continuous supply of expensive starting materials (D-xylose and L-arabinose) is needed.

D-xylose and L-arabinose are the major five-carbon sugars present in plant biomass hydrolysate (Carpita and McCann, 2000; Subtil and Boles, 2011) and plants contain these pentoses as building blocks for hemicellulose and pectin, major components of plant cell walls (Bindschedler et al., 2005; Carpita and McCann, 2000; Fuzfai et al., 2004; Lee et al., 1970; Loewus, 1964; Loewus et al., 1962; Rosenfield and Loewus, 1978). In addition, plants including Arabidopsis can take up D-xylose from the medium through a sugar transport protein (STP1) (Carpita et al., 1982; Haldrup et al., 1998; Sherson et al., 2003). Enzymes that convert D-xylose to D-xylulose and then to D-xylulose phosphate have been computationally predicted in several plants (http://pmn.plantcyc.org/PLANT/NEW-IMAGE?type=PATHWAY&object=XYLCAT-PWY). Arabidopsis and other plants can also take up L-arabinose from the medium using the sugar transport protein 2 (STP2) (Carpita et al., 1982; Cobbett et al., 1992; Subtil and Boles, 2011). Enzymes that convert L-arabinose to UDP-L-arabinose have been characterized in plants (Burget and Reiter, 1999; Dolezal and Cobbett, 1991), supporting the presence of the free arabinose in plant cells. The presence of sugar precursors needed for butanetriol synthesis in plants allows a green bio-based approach to produce this chemical by expressing bacterial genes involved in this pathway. Production of butanetriol in plants could reduce cost and pollution and lead to sustainable environmentally safe production of this chemical.

Here, we have generated transgenic Arabidopsis plants expressing the enzymes for butanetriol production utilizing either D-xylose or L-arabinose. Arabidopsis plants were transformed with either the D-xylose pathway genes, with either the native genes cloned from bacteria or synthesized genes modified to utilize the codons found in highly expressed genes in Arabidopsis or the L-arabinose pathway genes synthesized with the Arabidopsis preferred codons. Our results show that transgenic plants expressing bacterial genes involved in butanetriol synthesis produce this chemical and that the level of butanetriol production increases when plants are grown in a medium supplemented with the precursors (xylose or arabinose). The accumulation of butanetriol in plants expressing the arabinose pathway is 5–8 times higher than those expressing either xylose pathways.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col) was used in this study and all transgenic plants were generated in a Col background. For growing seedling under aseptic conditions, seeds were surface sterilized and plated on MS medium (Cat. #2610024, MP Biomedicals) (Murashige and Skoog, 1962) containing 0.9% phytoagar with or without sucrose, as needed. Filter sterilized D-xylose or L-arabinose solutions were added to the medium when needed, after autoclaving. The sown seeds were stratified for 3 days in the dark at 4 °C before being transferred to a growth chamber. All plants were grown under 16 h light/8 h dark and 70% humidity at 22 °C. For growing plants in soil, seeds were stratified for 3 days, planted in soil (Metromix 250) and grown in a growth chamber under long-day conditions (16 h light/8 h dark; 120 μmol/m^2/s^2 light intensity, 22 °C). Tissues from aseptically-grown and soil-grown plants were harvested, frozen in liquid nitrogen and stored at −80 °C for subsequent use.

2.2. Construction of xylose and arabinose pathways

To convert D-xylose to D-1,2,4 butanetriol and L-arabinose to L-1,2,4 butanetriol, four enzymes are needed for each pathway.

The last two enzymes, benzoylformate decarboxylase (BFD, protein id: BAN53931.1) and dehydrogenase (DH), are common whereas the first two enzymes are specific to each pathway, D-xylose dehydrogenase (XDG, protein id: ACL94329.1) and D-xylonate dehydratase (XDT, protein id: AGC85258.1) for D-xylose and L-arabinose dehydrogenase (ADG, protein id: AFJ87849.1) and L-arabinose dehydratase (ADT, protein id: ABA74965.1) for L-arabinose (Niu et al., 2003). We cloned the genes coding for the first three enzymes in each pathway in a binary vector with each gene driven by a different promoter and terminator (Fig. 1b). Each gene was cloned into a promoter vector (pSAT vectors) (Tzfira et al., 2005) and then the gene with the promoter/terminator was moved sequentially into a binary vector.

2.3. Cloning of xylose pathway constructs (XXB)

2.3.1. Cloning of xylose pathway bacterial genes (XXB$_{bact}$)

Xylonate dehydratase (XDT_{bact}) was amplified from *E. coli* using sense and antisense primers (EC-2F and EC-2R, Supplementary Table S1) and ligated into pGEM (Promega, Madison WI). XDT_{bact}-pGEM was digested with *Hind*III and *Kpn*I and the released fragment was subsequently cloned into the *Hind*III–*Kpn*I sites of the pSAT4A multiple cloning site (MCS), producing XDT_{bact}-pSAT4A. The XDT_{bact}-pSAT4A was digested with *Isce*I and the fragment containing the 2X CaMV35S (35S) promoter/terminator-*XDT* cassette was inserted into the *Isce*I site of pRCS2-ocs-bar binary vector producing XDT_{bact}-pRCS2-ocs-bar.

Benzylformate decarboxylase (BFD_{bact}) was amplified from *Pseudomonas putida* using sense and antisense primers (PP-5F and PP-5R, Supplementary Table S1). PCR product was digested with *Hind*III and *Bam*HI and ligated into the *Hind*III–*Bam*HI sites of pSAT6A MCS producing BFD_{bact}-pSAT6A. The BFD_{bact}-pSAT6A was digested with *PI-Psp*I and the RuBisCo Small Subunit promoter/terminator (RbcSP/RbcST)-BFD_{bact} cassette was inserted into the *PI-Psp*I site of XDT_{bact}-pRCS2-ocs-bar producing BFD_{bact}-XDT_{bact}-pRCS2-ocs-bar.

Xylose dehydrogenase (XDG_{bact}) was amplified from *Caulobacter vibrioides* using forward and reverse primers (CC-1F and CC-1R, Supplementary Table S1) and cloned into pGEM vector. XDG_{bact}-pGEM vector was digested with *Hind*III and *Pst*I and the released fragment was cloned into the *Hind*III–*Pst*I sites of pSAT3A MCS, producing XDG_{bact}-pSAT3A. The XDG_{bact}-pSAT3A was then digested with *Afl*II and the mannopine synthase promoter/terminator (masP/masT)-XDG_{bact} cassette was cloned in the *Afl*II site of BFD_{bact}-XDT_{bact}-pRCS2-ocs-bar producing XDG_{bact}-BFD_{bact}-XDT_{bact}-pRCS2-ocs-bar binary vector (Fig. 1b) that was used for Arabidopsis transformation.

2.3.2. Cloning of xylose pathway synthetic genes (XXB$_{syn}$)

Codon optimized genes for the xylose pathway (Supplementary Fig. S1) were synthesized at GeneArt (Life Technologies, USA). The codon usage was adapted to that of the highly expressed genes in Arabidopsis. XDT_{syn} and BFD_{syn} were obtained in pMK-RQ vector. XDT_{syn} was released from pMK-RQ with *Hind*III and *Kpn*I and ligated into pSAT4A. XDT_{syn}-pSAT4A and pRCS2-ocs-bar were digested with *Isce*I and ligated to produce XDT_{syn}-pRCS2-ocs-bar. XDG_{syn} was obtained in pMA vector and then subcloned into the *Hind*III–*Pst*I sites of pSAT3A producing XDG_{syn}-pSAT3A. XDG_{syn}-pSAT3A was digested with *Pvu*II, blunted and then inserted into the blunted end of *EcoR*I-digested XDT_{syn}-pRCR2-ocs-bar producing XDG_{syn}-XDT_{syn}-pRCR2-ocs-bar. Similarly BFD_{syn} was released from pMK-RQ using *Hind*III and *Bam*HI and ligated into pSAT6A. BFD_{syn}-pSAT6A was digested with *PI-Psp*I and the fragment containing the *BFD* coding sequence, promoter and terminator was ligated into XDG_{syn}-XDT_{syn}-pRCS2-ocs-bar

resulting in XDG_{syn}-XDT_{syn}-BFD_{syn}-pRCS2-ocs-bar (Fig. 1b) that was used for Arabidopsis transformation.

2.4. Cloning of the synthetic arabinose pathway construct (AAB_{syn})

Arabinose pathway synthetic genes (Supplementary Fig. S2) were obtained from GeneArt (Life Technologies, USA). Similar to XDG_{syn} Arabinose dehydrogenase (ADG_{syn}) is cloned in the MCS of pSAT3A. ADG_{syn}-pSAT3A was digested with AflII and inserted into AflII-digested pRCS-ocs-nptII producing ADG_{syn}-pRCS-ocs-nptII. BFD_{syn}/pMK-RQ was digested with HindIII/KpnI and inserted into pSAT6A and ADT_{syn}-pMK-RQ was digested with HindIII/BamHI and cloned into the HindIII–BamH1 sites of pSAT4A. BFD_{syn}-pSAT6A was digested with PI-PspI and ligated into ADG_{syn}-pRCS-ocs-nptII followed by ligation of ADT_{syn}-pSAT4A digested with I-SceI producing the ADG_{syn}-ADT_{syn}-BFD_{syn}-pRCS-ocs-nptII binary vector (Fig. 1b) that was used for Arabidopsis transformation.

2.5. Generation of XXB and AAB transgenic plants

To generate transgenic XXB_{bact}, XXB_{syn} and AAB_{syn} plants, plasmids were introduced into the GV3101 strain of Agrobacterium tumefaciens. Arabidopsis plants were transformed with the GV3101 strain of Agrobacterium using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected using BASTA (glufosinate ammonium, Sigma, USA) (5 μg/ml) for $XXB_{syn\ and\ bact}$ lines and kanamycin (30 μg/ml) for the AAB_{syn} lines. The homozygous T_3 transgenic lines were used for further analysis.

2.6. RT-PCR and genomic PCR analyses

Reverse transcription-PCR (RT-PCR) was performed to analyze the expression of the introduced genes. Total RNA was isolated from 3-week-old seedlings using RNAeasy plant mini kit (Omega Bio-tek). Two μg of DNase-treated RNA was used to synthesize first-strand cDNA using a cDNA synthesis kit (Invitrogen) following the manufacturer's instructions, and 1 μl of the first-strand cDNA was used for PCR in a reaction volume of 25 μl. The following PCR conditions were used for both genomic and RT-PCRs. Initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and final extension at 72 °C for 7 min. The primer sequences for genomic and RT-PCRs are listed in Supplementary Table S1. The expression of the ubiquitin 5 (UBQ5, AT3G62250) was used as a loading control in all experiments.

2.7. Quantification of free xylose and arabinose in Arabidopsis

Wild type Arabidopsis plants were grown on either MS or MS supplemented with 25 mM arabinose or 5 mM xylose for three weeks under 16 h light/8 h dark photoperiod and 70% humidity at 22 °C. To avoid contamination from cell wall polymers, protoplasts from leaves were prepared as described earlier with modifications and used for quantification of free xylose and arabinose (Yoo et al., 2007). Leaves were rinsed with distilled water before slicing and incubated in the enzyme solution. Protoplasts were washed 4 times with an excess of W5 wash buffer before measurement of the chlorophyll content. Equal amounts of cells, normalized by chlorophyll, from different treatments and equal volumes from the last washes were freeze-dried in a speed vacuum. One mL of methanol/water (70:30) was added to the protoplasts ($n=4$ replicates per treatment group), and samples were vortexed for 45 min at room temp, sonicated for 15 min, and vortexed for another 16 h at room temp. Samples were centrifuged at $3000 \times g$ for 10 min at 4 °C, 800 μL of the supernatant was transferred to a 1.5 mL

microcentrifuge tube, and dried in a speedvac. Derivatization and quantitation methods are as described in Section 2.8.2. Arabinose and xylose quantitation by GC–MS was determined by comparing peak areas ($n=2$ injections per sample) to a five point standard curve generated from commercial standards (Sigma, USA).

2.8. Determination of butanetriol content and metabolomic analysis by gas chromatography coupled to mass spectrometry (GC–MS)

2.8.1. Metabolite extraction

Plant tissues were frozen in liquid nitrogen, ground to a fine powder in a mortar and pestle, and freeze-dried in a speed vacuum. One mL of methanol/water (70:30) was added to 20 mg of plant tissue and shaken on a vortex mixer for 2 h at room temperature. Samples were centrifuged at $3000 \times g$ for 10 min at 4 °C, and 800 μL of the supernatant was transferred to a 1.5 mL microcentrifuge tube.

2.8.2. Metabolite detection and butanetriol quantitation

The metabolite extract was dried using a speed vacuum, re-suspended in 50 μL of pyridine containing 15 mg/mL of methoxyamine hydrochloride, incubated at 60 °C for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60 °C. A second derivatization step was conducted by adding 50 μL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA+1% TMCS, Thermo Scientific). Samples were incubated at 60 °C for 30 min, centrifuged at $3000 \times g$ for 5 min, cooled to room temperature, and 80 μL of the supernatant was transferred to a 150 μL glass insert. Metabolites were detected with a Trace GC Ultra coupled to a Thermo DSQ II (Thermo Scientific), which scanned 50–650 m/z at 5 scans/s in electron impact mode after separation on a 30 m TG-1MS column (Thermo Scientific, 0.25 mm i.d., 0.25 μm film thickness). Initial experiments with a TG-5 MS column resulted in co-elution of serine and butanetriol and quantitation could not be performed. Both the inlet and transfer line were set at 280 °C. One μL of sample was injected in a 1:10 split ratio twice in discrete randomized blocks with a 1.2 ml/min flow rate, and the program consisted of 80 °C for 30 s, a ramp of 15 °C per min to 330 °C, and then held for 8 min. D- and L-butanetriol standards (Aldrich, USA) were similarly prepared and used for preparation of a standard curve. Butanetriol concentrations were determined based on the mean peak areas of injection replicates for each sample.

2.8.3. Metabolomic data analysis

For each sample, a matrix of molecular features as defined by retention time and mass was generated using XCMS software (Smith et al., 2006). Samples were normalized to the total ion current (TIC), and the relative quantity of each molecular feature was determined by the mean area of the chromatographic peak among replicate injections ($n=2$). The normalized dataset was mean-centered and Pareto-scaled and principal component analysis (PCA) on molecular features was performed. For each molecular feature, differences among sample groups were determined by ANOVA with a Tukey post-test ($p < 0.01$) or Student's t-test ($p < 0.05$). GC–MS mass spectra that varied among samples were deconvoluted in AMDIS (Stein, 1999) and screened in the National Institute for Technology Standards metabolite database (www.nist.gov) for putative identifications. To validate metabolite identifications, standards for serine, aspartate, arabinolactone, arabitol, and myoinositol were purchased from Sigma-Aldrich (Sigma, USA) and were injected alongside plant extracts to verify retention times and mass spectra.

3. Results

## 3.1. Generation of transgenic lines expressing bacterial genes involved in butanetriol biosynthesis

3.1.1. Xylose pathway: bacterial (XXB_{bact}) and synthetic (XXB_{syn})

Biosynthesis of butanetriol from xylose requires four genes encoding enzymes (XDG, XDT, BFD, and DH) (Fig. 1a) catalyzing four metabolic steps. In bacteria, non-specific dehydrogenases were shown to catalyze the last step (Niu et al., 2003). In Arabidopsis, dehydrogenases (DHs) are ubiquitous and many dehydrogenases with broad substrate specificity were identified (http://www.arabidopsis.org/). We assumed that endogenous dehydrogenases in Arabidopsis could catalyze the reduction of D-dihydroxybutanal into butanetriol (fourth step). Therefore we did not include dehydrogenases in our constructs. As described in the methods section, we used bacteria containing the gene for each enzyme for the first three steps to clone these genes. Bacterial DNA was used to amplify the bacterial genes (bact) by PCR. We then constructed a single binary vector, XXB_{bact}, containing the genes encoding the first three enzymes in the pathway (XDG_{bact}, XDT_{bact}, BFD_{bact}). For efficient translation of prokaryotic genes in a eukaryotic system, we used codons found in highly expressed genes in Arabidopsis to modify the bacterial genes and constructed a single binary vector, (XXB_{syn}) containing the synthetic genes (XDG_{syn}, XDT_{syn}, BFD_{syn}). To avoid co-suppression and silencing of the introduced genes, we used three different promoters and terminators that work well in plants (Fig. 1b). Arabidopsis plants were transformed with either XXB_{bact} or XXB_{syn} and independent T_1 Basta resistant plants were tested for XXB_{syn} and XXB_{bact} gene insertion using genomic DNA PCR and for gene expression using RT-PCR (data not shown). For XXB_{bact}, 18 Basta resistant T_1 lines were selected and tested (data not shown). After two rounds of selfing, two T_3 lines were used for further analysis. Fig. 2a shows expression of the genes in these two lines. Nine independent T_1 Basta resistant plants were tested for the presence of XXB_{syn}. After two rounds of selfing, four homozygous T_3 lines were selected for further analysis. The expression of the introduced genes was confirmed by RT-PCR (Fig. 2b, left) and the amplified product was quantified by ImageJ (rsbweb.nih.gov/ij/◆) and normalized to the internal control, $UBQ5$ (Fig. 2b, right). No expression was observed in the wild type and different expression levels were observed for different genes among different lines (Fig. 2b). This is expected as we used three different promoters to drive their expression. No phenotypic differences were observed in any of the XXB_{bact} or XXB_{syn} transformed lines compared to wild type when grown on soil (Fig. 3) although the expression level of the transgenes is different (Fig. 2).

3.1.2. Arabinose pathway: synthetic (AAB_{syn})

Like the xylose pathway, biosynthesis of L-butanetriol from L-arabinose occurs in four steps catalyzed by four enzymes (Fig. 1a). We synthesized the first three genes that are codon-optimized for expression in Arabidopsis and inserted them into a binary vector (Fig. 1b) and transformed Arabidopsis. Fourteen T_1 kanamycin-resistant transgenic lines were selected and tested by genomic DNA
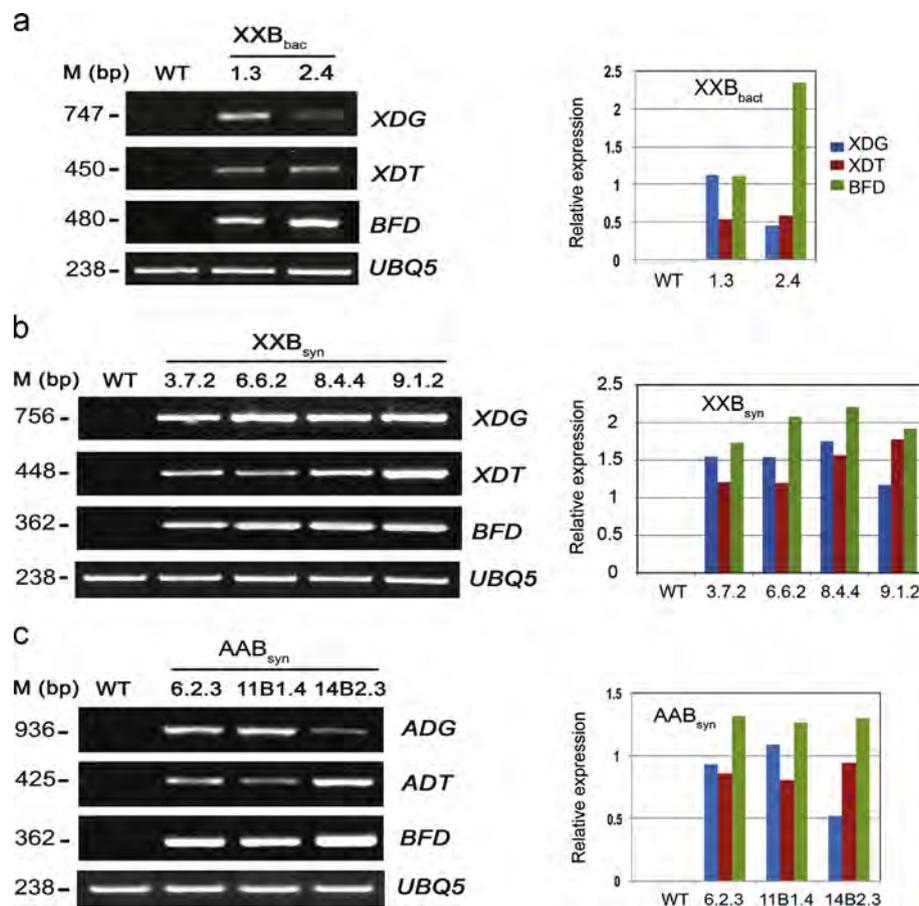
Fig. 2. Expression analyses of bacterial and synthetic genes in plants. Total RNA was isolated from wild type and transgenic lines and used for RT-PCR. (a,b,c – left panels) RT-PCR of transgenes in XXB_bact, XXB_syn, and AAB_syn lines, respectively, with a wild type control for each showed no transgene expression in the wild type and different expression levels in the transgenic lines. Benzoylformate decarboxylase (BFD) driven by the RbcS promoter showed the highest expression in all tested lines. UBQ5 was used as a control for comparison. Right panels in a, b and c show quantification of expression of bacterial and synthetic genes in wild type and transgenic lines. The intensity of RT-PCR products was quantified using ImageJ at NIH (http://rsb.info.nih.gov/ij/) and normalized to the internal control UBQ5. BFD showed the highest expression in all lines.
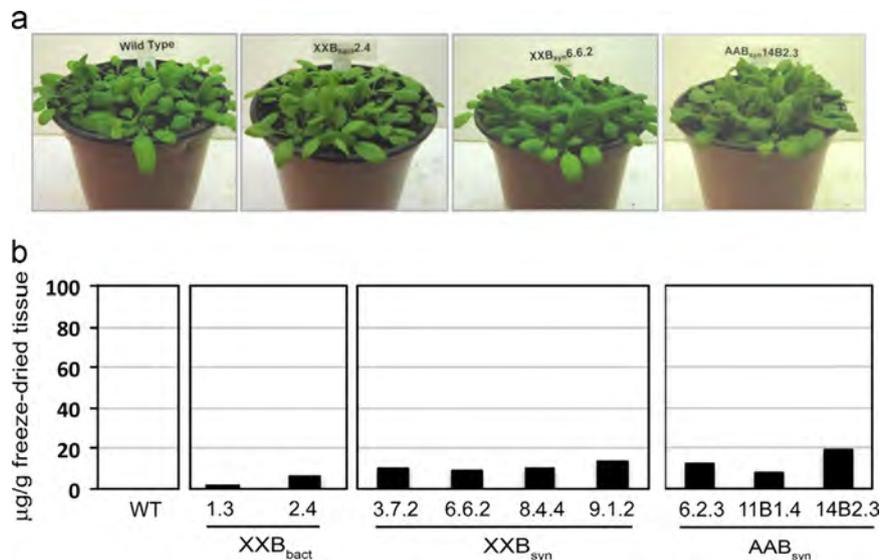
Fig. 3. Phenotypic appearance of transgenic lines and quantification of butanetriol yields in soil-grown plants. (a) Three week-old soil-grown wild type, XXB$_{bact}$2.4, XXB$_{syn}$6.6.2, and AAB$_{syn}$14B2.3 plants showed no phenotypic differences. (b) Butanetriol quantification in wild type and transgenic lines grown on soil expressed as μg butanetriol g^{-1} tissues.

PCR. Three T$_3$ lines expressing different levels of the transgenes as confirmed by RT-PCR (Fig. 2c) were propagated and used for measurement of butanetriol accumulation (see Section 3.4). Like the xylose pathway genes, no expression was observed in wild type plants and expression of introduced genes was observed in different transgenic lines (Fig. 2c).

3.2. Butanetriol accumulation in transgenic lines

To test the accumulation of butanetriol in the transgenic lines, we grew wild type, XXB$_{bact}$, XXB$_{syn}$ and AAB$_{syn}$ lines in soil for 3 weeks and ground, lyophilized tissues were used for butanetriol quantification (Fig. 3a) by GC–MS. Lyophilized ground tissues of wild type (control) and transgenic lines and a butanetriol standard (D- and L-) were dissolved in an aqueous methanol solvent and subsequently derivatized for GC–MS detection as described in section 2.8.2. D- and L-butanetriol standards eluted at 6.45 min under our GC–MS conditions (Fig. 4, panel a, arrow), however this method could not discriminate between butanetriol enantiomers. In transgenic lines such as AAB$_{syn}$6.6.2, a significantly large peak was detected at 6.45 min (Fig. 4 panel c) with a mass spectrum that matched information in both GC–MS metabolite databases and co-eluted with the butanetriol commercial standard. Butanetriol content in transgenic lines was determined based on peak area of the 103.1 m/z ion, and concentrations were determined based on a four point standard curve (Fig. 4a, inset) and subtracting the peak area of 103.1 m/z from wild type extracts. All other detected masses were evaluated for changes in transgenic lines in a metabolomics workflow (Section 3.6).

3.3. Quantification of butanetriol in xylose pathway transgenic lines

Butanetriol was detected in both synthetic and bacterial lines with slightly higher accumulation in synthetic ones, reaching the highest accumulation in XXB$_{syn}$ 9.1.2 (14 μg g^{-1}) (Fig. 3b). It was previously shown that xylose and arabinose represent a very small fraction of the monosaccharide pool (∼40–60 times less than glucose) (Carpita et al., 1982). Hence, it is possible that these pentose precursors could be limiting. To test if adding xylose affects butanetriol accumulation we grew wild type and transgenic plants on MS supplemented with different concentrations of D-xylose (Fig. 5a). We

initially omitted sucrose from the medium to force plants to take up xylose as a carbon source. A slight reduction in growth was observed among transgenic lines when grown on MS as compared to wild type (Fig. 5a). However as the xylose concentration in the medium increased, the growth of the transgenic lines (both bacterial and synthetic) was adversely affected (Fig. 5a) reaching the highest effect at 5 mM where very small brown true leaves were present in the transgenic lines while no toxicity symptoms were observed in the wild type. This suggests that a metabolite is accumulated only in the transgenic lines and its accumulation increases as the xylose concentration increases in the medium and then affects transgenic growth. To quantify the level of butanetriol in plants supplemented with xylose, we pooled tissues from plants grown at different concentrations of xylose (1.5, 2.5, and 5 mM) and quantified butanetriol using GC–MS (Fig. 5a, bottom panel). Plants grown in xylose-containing medium accumulated butanetriol 3–5 times higher than those grown without xylose. The highest accumulation was observed in XXB$_{bact}$ 2.4 grown in the presence of xylose (80 μg g^{-1}). In an earlier study, xylose as a sole carbon source appeared to be toxic for plant development and addition of sucrose was shown to decrease the toxicity (Haldrup et al., 1998). To test if adding sucrose would rescue transgenic lines and allow plants to accumulate more butanetriol, we grew wild type and transgenic lines on MS supplemented with 0.5% sucrose and 10 mM xylose. Compared to minus sucrose-grown transgenic lines (bacterial and synthetic) the plants grown on 10 mM xylose showed less toxicity symptoms in most (6.6.2 being an exception) lines (Fig. 5b). Although plants were grown at a higher precursor concentration (10 mM xylose), the extent of increase in butanetriol is not as much as in lines grown in the presence of xylose only. It is likely that in the presence of sucrose, xylose uptake is reduced resulting in no toxicity and reduced accumulation of butanetriol.

3.4. Quantification of butanetriol in arabinose pathway transgenic lines

Of the plants expressing the AAB$_{syn}$ genes the AAB$_{syn}$14B2.3 line showed the highest accumulation of butanetriol (Fig. 3b, right panel). Plants were also grown on MS with and without the precursor arabinose (Fig. 5c). There was a large increase in the production of the butanetriol when arabinose was supplied as
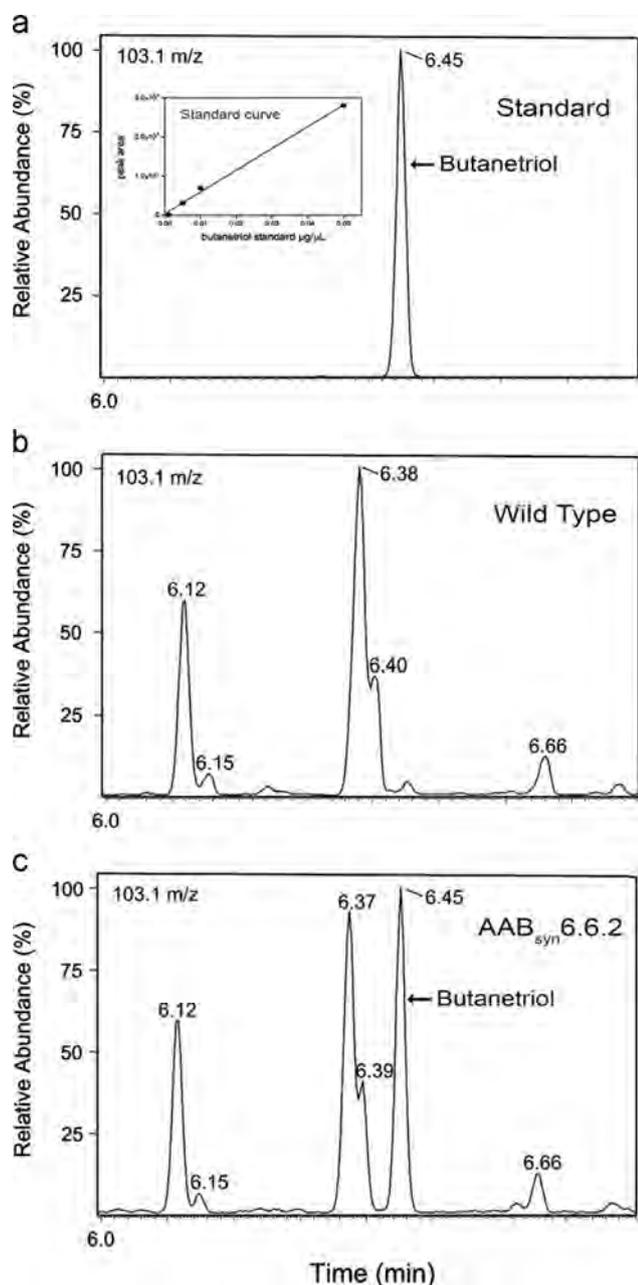
Fig. 4. Detection of 1,2,4-butanetriol in a transgenic line. Metabolites from vacuum-dried tissues of wild type and transgenic lines were extracted and detected by GC–MS. (a) Extracted ion chromatogram (EIC, 103.1 m/z) of 1,2,4-butanetriol standard solution and calibration curve. Butanetriol eluted at 6.45 min. (b) EIC of wild type. (c) EIC chromatogram of AAB$_{syn}$6.6.2 transgenic line showed large peak at 6.45 min with a mass spectrum that matches the commercial butanetriol standard.

compared to MS alone (up to $40\times$ higher in AAB$_{syn}$ 6.2.3). The transgenic lines (6.2.3 and 14B2.3) that showed the highest accumulation of butanetriol in the presence of arabinose (391 µg g^{-1} and 238 µg g^{-1}, respectively) showed toxicity symptoms as in plants expressing the XXB pathway.

### 3.5. Quantification of free xylose and arabinose in Arabidopsis

Transgenic lines grown on MS accumulated very little butanetriol; however, adding the precursors (xylose or arabinose) increased the butanetriol accumulation (Fig. 5), suggesting that free pentose precursors are limiting in Arabidopsis. To quantify

the amount of free xylose and arabinose in Arabidopsis plants grown in the presence and absence of precursors, we used protoplasts isolated from wild type plants grown on MS or on MS supplemented with 25 mM arabinose or 5 mM xylose and quantified their level by GC–MS. To avoid any contaminating xylose or arabinose coming from the cell wall lysis, the isolated protoplasts were washed 4 times with W5 wash buffer and the last wash was also tested by GC–MS. Free xylose and arabinose are found in protoplasts grown in MS medium. The level of arabinose is slightly higher than xylose, consistent with a previous study (Carpita et al., 1982). However, protoplasts from plants grown in the presence of arabinose accumulated about 10 times more arabinose than those grown without arabinose (Supplementary Table 2) suggesting that growing plants in the presence of arabinose increases the cellular content of arabinose. On the other hand, plants grown in the presence of xylose did not show an increase in the amounts of xylose in their cells (Supplementary Table 2) compared to MS-grown plants suggesting that xylose is either metabolized into other products or cells do not accumulate xylose to high levels.

3.6. Evaluation of other metabolites affected by transgenes

A modified dataset was generated by removing butanetriol-specific mass signals to identify other metabolites that vary among lines due to the introduction of the transgenes and/or the addition of precursors arabinose or xylose. The dataset consisted of relative quantities of all masses detected by GC–MS among the wild type and transgenic plant extracts. Principal Component Analysis (PCA) was conducted and the first two components explained approximately 52.8% of the variation. The PCA showed distinct clusters for arabinose and MS (-sucrose)-grown plants, suggesting the addition of arabinose into the media significantly altered the metabolite content of the wild type and AAB$_{syn}$ lines (Fig. 6a). The PC scores did not differentiate the WT and AAB$_{syn}$ lines when grown on minus sucrose MS medium; however, the addition of arabinose resulted in separation of two lines (AAB$_{syn}$14B2.3 and AAB$_{syn}$6.2.3) from WT. The transgenic line AAB$_{syn}$11B1.4 did not cluster with the other two lines (14B2.3 and 6.2.3) in the arabinose-grown samples (Fig. 6a). This agrees with the previous assessment that AAB$_{syn}$11B1.4 produced much lower quantities of butanetriol than the other lines (115 µg/g compared to 238 and 391 µg/g, respectively) (Fig. 5c). Together, these data suggest that the addition of arabinose into the media not only enhances butanetriol accumulation in the transgenic lines, but also changes the content of other metabolites.
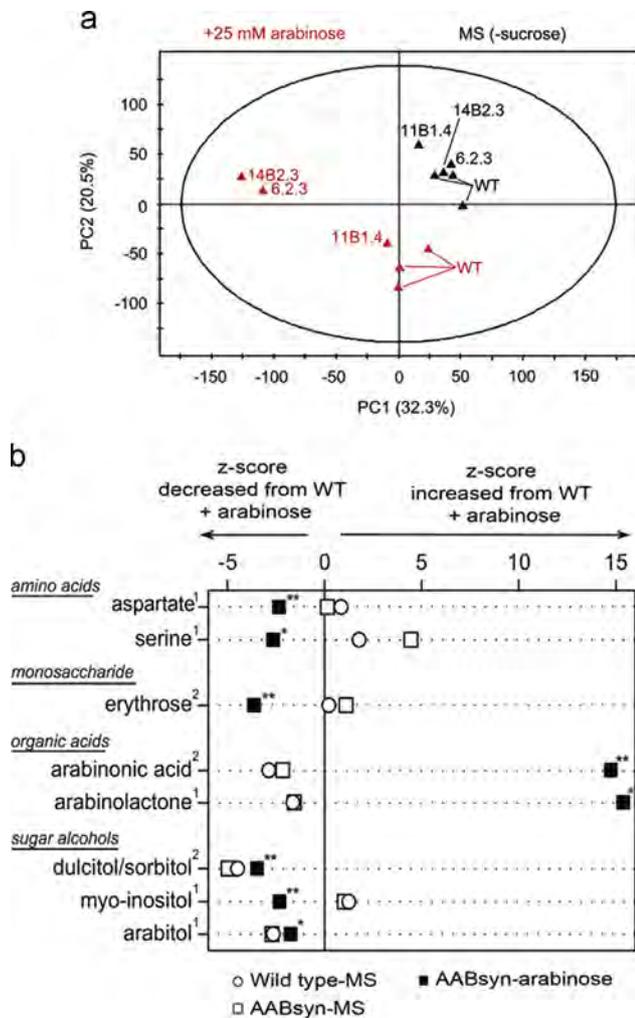
An ANOVA was applied to the dataset to determine masses that vary among wild type and AAB$_{syn}$ lines grown on MS (-sucrose) and MS+arabinose, and only m/z values that varied were annotated as metabolites as described in Section 2.8.3. The poor response of AAB$_{syn}$11B1.4 (Fig. 6a) compared to the other two lines supports the data in Fig. 5c and resulted in relatively high p-values, however wild type and transgenic plants grown in the presence of arabinose exhibited differences in eight metabolites (Fig. 6b, Student's t-test $p < 0.01$). Because the relative quantities of different metabolites highly vary, the data was normalized as z-scores based on the mean and standard deviation with wild type grown in the presence of arabinose as the control. z-Scores were determined independently for wild-type MS-, AAB$_{syn}$ MS-, and AAB$_{syn}$ arabinose-grown plants for each metabolite. z-Scores for MS-grown wild type and MS-grown AAB$_{syn}$ lines exhibited similar trends for all eight metabolites, and support the PCA data in Fig. 6a that wild type and transgenic lines do not differ in the absence of arabinose. The z-scores for MS-grown wild type and MS-grown AAB$_{syn}$ lines, therefore, represent the influence of the arabinose treatment alone on the eight metabolites. The differences between
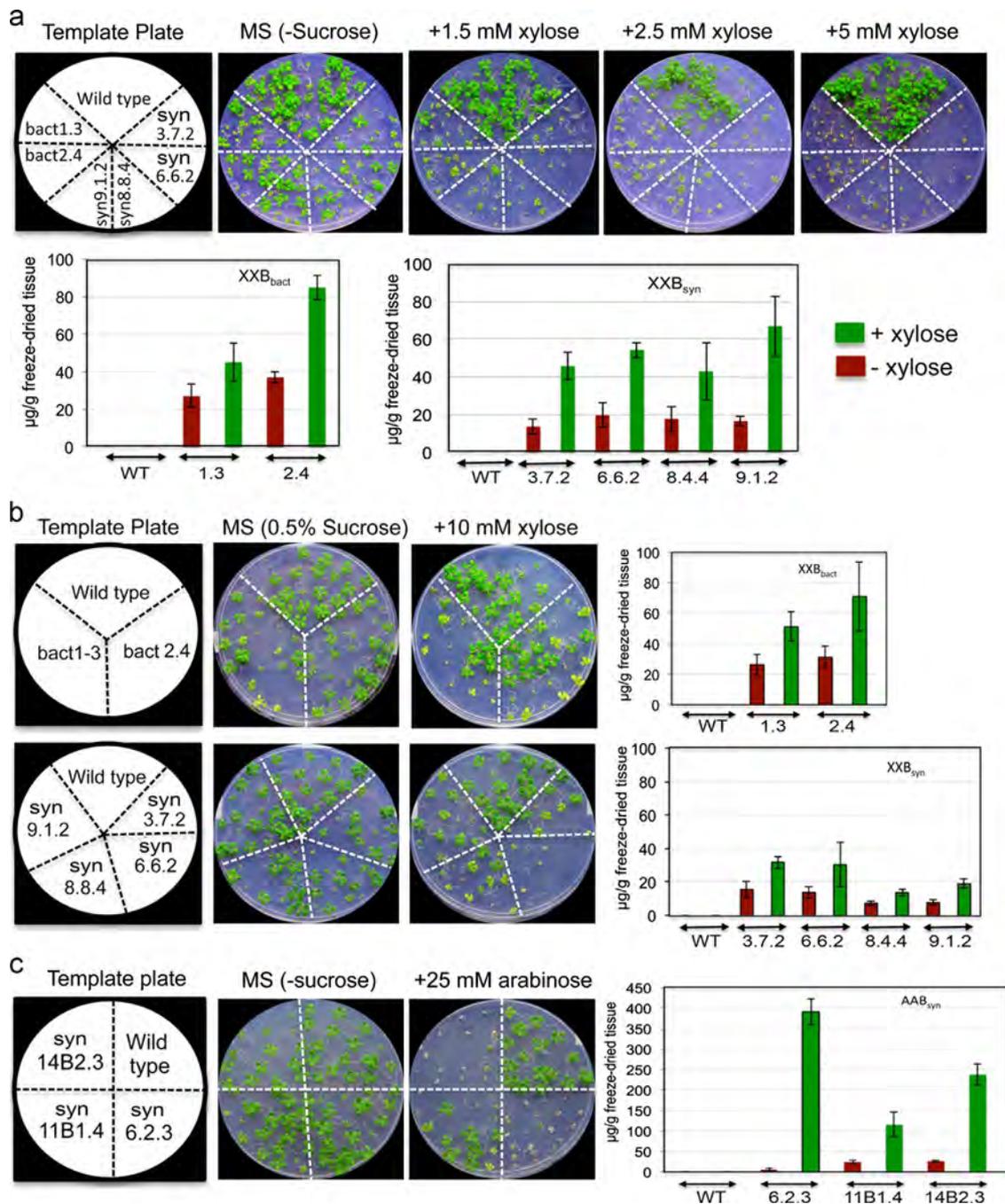
Fig. 5. Addition of precursors to plant growth medium enhanced butanetriol yield. (a) Wild type, XXB$_{bact}$, and XXB$_{syn}$ lines grown on minus–sucrose MS medium without or with increasing concentrations of xylose (upper panel). The concentration of butanetriol in the pooled plants from each plate is shown in the lower panel. (b) Plants grown on 0.5% sucrose-containing MS medium with xylose showed less toxicity symptoms. The butanetriol quantification of the pooled tissues is shown on the right. (c) Plants (left) and butanetriol quantification (right) of wild type and AAB$_{syn}$ lines grown on MS medium with or without 25 mM arabinose.

the arabinose-grown AAB$_{syn}$ z-scores and the wild-type arabinose-grown control represent changes due to the presence of the butanetriol synthetic enzymes. Compared to arabinose-grown wild type plants, arabinose-grown AAB$_{syn}$ lines had reduced levels of aspartate, serine, erythrose, dulcitol/sorbitol (could not be differentiated based on mass spectra), myo-inositol and arabitol (Fig. 6b). While in both MS-grown wild type and MS-grown AAB$_{syn}$ lines, the arabitol levels were significantly lower than those in arabinose-grown wild type ($z = -2.71$ and -2.65), the arabinose-grown AAB$_{syn}$ lines exhibit a much smaller change in arabitol ($z = -1.75$). On the other hand, as compared to wild type, the arabinose-grown AAB$_{syn}$ lines exhibited a significant increase in arabinonic acid (a component of butanetriol biochemical pathway)

and arabinolactone, which are open chain and cyclic forms of the same molecule, respectively. Of the two molecules, arabinolactone was much less abundant (data not shown), but interestingly the two metabolites increased at the same rate (z-scores = 15.38 and 14.75, respectively) suggesting an enzymatic or non-enzymatic cyclization might occur. Detection of a small amount of arabinonic acid in wild type based on our level 2 identification suggests that Arabidopsis might have an endogenous enzyme that can converts arabinose to arabinonic acid. These data support the findings shown in Fig. 6a that there is a change in metabolites present in arabinose-grown AAB$_{syn}$ lines, and that the addition of arabinose into the media was necessary to induce a change in the metabolite profile of the transgenic lines. Intermediates other than the arabinonic acid in

Fig. 7. Metabolic variation in transgenic lines grown in the presence of xylose. Principal component analysis (PCA) of the modified GC–MS dataset for wild type (WT) and xylose pathway expressing transgenic lines (XXB$_{syn}$) grown on MS medium (black) or MS medium supplemented with xylose (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. AAB$_{syn}$ lines grown in the presence of arabinose showed altered metabolite profile. (a) Principal component analysis of the modified GC–MS dataset for wild type (WT) and transgenic lines grown on MS media (black) or MS media with 25 mM arabinose (red). (b) z scores for metabolites that differ between WT-grown on arabinose and transgenic lines grown on arabinose (Student's t-test, p-values indicated as $*p < 0.1$ or $**p < 0.05$). z Scores represent the number of standard deviations a treatment group differed from WT-arabinose (set to $z=0$), whereby positive and negative values imply increased or reduced metabolite levels, respectively. Generally, z-scores greater or less than 1.96 correspond to $p < 0.05$ under a normal, Gaussian distribution. Superscripts for each metabolite correspond to metabolite identification levels as described by the Metabolomics Standardization Initiative (Sumner et al., 2007) and lower numbers indicate increased confidence in molecular identification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the arabinose pathway were not detected in this study. This might be due to (i) their presence below the level of our detection method, (ii) labile nature of these compounds and/or (iii) rapid conversion of those intermediates.

Non-targeted metabolomic analysis was also conducted for WT and XXB$_{syn}$ lines grown on either MS or MS supplemented with xylose and principal component analysis (PCA) was applied to this dataset (Fig. 7 and Table 1). PC1 (49.4% of the variation) explained a significant effect of xylose on XXB$_{syn}$ transgenic lines. Table 1 shows the metabolites that differ between WT and XXB lines grown under different conditions. Positive or negative z-scores values indicate an increase or decrease in metabolite abundance in WT/xylose vs. WT/MS, XXB/MS vs. WT/MS and XXB/xylose vs. XXB/MS (Table 1). Compared to the metabolite variation in arabinose-grown AAB$_{syn}$ lines (Fig. 6B), xylose-XXB$_{syn}$ lines exhibited a more drastic change in
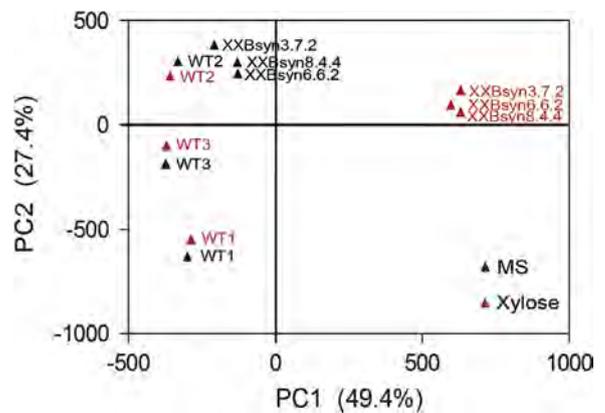
metabolism as a wide range of metabolite classes were altered (Table 1). This might be the reason for observed adverse effect of xylose on XXB lines (Fig. 5 a, b). None of the xylose pathway intermediates were identified in our analysis and this could be due to the reasons discussed above for the arabinose pathway.

4. Discussion

Biochemical pathways from microbes and non-plant systems have been introduced into plants and yeast to produce commercially important novel chemicals, polymers and non-native proteins (Bornke and Broer, 2010; Gleba et al., 2007; Toivari et al., 2012). In a recent study Arabidopsis plants were engineered with a number of different gene combinations to increase the accumulation of docosahexaenoic acid (Ruiz-Lopez et al., 2013). Previously, several transgenes involved in biosynthesis of provitamin A were introduced into rice to produce "golden rice" with higher carotenoid accumulation in the endosperm (Datta et al., 2003). Here, we demonstrate phytoproduction of butanetriol that is used in synthesis of diverse products including energetic materials and pharmaceuticals by introducing genes from bacteria or corresponding codon-optimized synthetic genes into Arabidopsis. As the current synthesis of butanetriol needs petrochemical-derived products and generates toxic by-products, a bio-based approach is preferable for the production of butanetriol. This was achieved in bacteria but required an input of the starting sugars, xylose and arabinose. As plants produce these precursors, production of butanetriol in plants could be more cost effective as the precursors would not need to be supplied.

The first three enzymes required for biological production of butanetriol (Fig. 1a) are not present in plants but are found naturally in different bacteria. The last step of the pathway requires a dehydrogenase. In bacteria, non-specific dehydrogenase(s) was shown to catalyze the last step (Niu et al., 2003). In Arabidopsis, many dehydrogenases (DHs) are present (http://www.arabidopsis.org/), suggesting that, as in bacteria, these DHs may catalyze the last step in the pathway. Working on this assumption, plants were transformed with the bacterial genes for the first three enzymes. Stacking three genes into one vector and introducing them as one cassette has advantages over generating transgenic lines with one gene at a time and crossing them to generate lines with all three genes. The latter approach would take a much longer time and also it is likely to result in variable expression of different genes depending on their insertion site.

Table 1
Principal component analysis of metabolites in WT and xylose pathway-expressing transgenic (XXB) lines in the presence and absence of xylose.

Metabolite	Class	z Score		
		WT/MS vs. WT/xylose	WT/MS vs. XXB/MS	XXB/MS vs. XXB/xylose
Agmatine	Amines/amino acids	−0.61	−0.07	11.53*
Alanine		3.80*	0.38	−1.84*
Arginine		−0.47	0.63	−6.69*
Aspartate		2.59*	−3.50*	−9.13*
Ethanolamine		0.10	−3.27*	−1.93*
Glutamate		1.45	−2.40	−1.70*
Glutamine		0.14	0.22	26.87*
Glycine		−0.31	0.88	−2.76*
L-Dopa		0.20	−1.63	−2.99*
Ornithine		−0.06	0.54	−11.53*
Pyroglutamate		−0.31	1.66	3.27*
Serine		−0.16	0.23	−3.17*
Threonine		0.12	0.50	−3.33*
Sulfuric acid	Inorganic acids	0.14	0.89	−8.33*
Palmitic acid	Lipids	−1.24	−0.43	−2.96*
Phytol		0.61	−2.22*	−3.34*
Fumaric acid	Organic acids	0.23	−0.82	−3.37*
Glyceric acid		0.29	−1.3*	−4.21*
Glycerophosphate		0.38	0.78	−9.07*
Malic acid		0.32	−1.21	−6.12*
Malonic acid		−0.75	6.80*	1.65*
Oxalic acid		−0.63	−5.64*	−8.55*
Phosphoric acid methyl ester		0.32	−0.72	−2.0*
Xylose	Sugars and related	7.87*	−0.08	14.75*
Myo-inositol		−0.31	−1.70*	1.71*
Trisaccharide		0.48	−0.51	18.74*
Sucrose		0.45	1.61*	−2.40
Threonic acid		2.08*	0.53	−19.45

* Denotes a Student's t-test $p < 0.05$. Positive or negative z-score values indicate an increase or decrease in metabolite abundance, respectively relative to the presence or absence of transgenes and/or xylose.

Such an approach has been shown to increase the production of poly-(3-hydroxybutyrate) in plants (Bornke and Broer, 2010). In plants, two copies of the same promoter can cause transcriptional inactivation of genes driven by that promoter (Brusslan et al., 1993; Park et al., 1996; Que and Jorgensen, 1998). We aimed to use three different promoters with comparable strength in driving gene expression to avoid gene silencing and to produce an adequate amount of each enzyme in the pathway. Constitutive promoters (CaMV 35S and mannopine synthase) have been widely used to overexpress several genes in plants. RbcS promoter, although light regulated, is a well characterized promoter, which is also commonly used to express introduced genes in plants (Bakhsh et al., 2011). As each gene in the cassette is driven by a different constitutive promoter and terminator we did not observe gene silencing, which is often observed if multiple genes are driven by the same promoter (Brusslan et al., 1993; Park et al., 1996; Que and Jorgensen, 1998). All three genes in both pathways are expressed well in each transgenic line. Sometimes expression of heterologous genes is reduced due to improper processing of transcripts or aberrant splicing of mRNA leading to smaller non-functional transcripts (Haseloff et al., 1997; Weising et al., 1988). In our case, a single transcript of expected size was observed from both bacterial and synthetic genes (Fig. 2). The accumulation of butanetriol in the transformed plants demonstrates that butane-triol can be produced in plants expressing the bacterial genes and that the plant dehydrogenases could catalyze the final step to produce butanetriol. For the xylose pathway, we constructed a binary vector with genes using the codons found in the naturally occurring bacterial genes and one with genes whose codons are optimized to the codon usage found in highly translated genes in Arabidopsis. Since the usage of different codons differs between bacteria and plants the expression of bacterial genes in plants

could be reduced. To overcome this, codons in bacterial genes should be optimized for proper expression in plants (Li et al., 2013). The use of optimized codons did not seem to make a difference in our study and this could be due the fact that GC content of these bacterial genes is not low like most bacterial genes. Although the production of butanetriol was less in soil grown plants transformed with the bacterial codon usage genes, one of these plants (XXBbact 2.4, Fig. 5a) had the highest amount of butanetriol among the plants with the xylose pathway.

Even though the transformed plants were able to produce butanetriol, additional strategies are needed for efficient production of butanetriol. Transgenic lines expressing D-xylose or L-arabinose pathway genes produced a maximum of about 20 μg of butanetriol per gram of soil-grown plants (Fig. 3b). However, an exogenous supply of D-xylose or L-arabinose increased the production of butanetriol considerably (up to forty-fold in the presence of arabinose) (Fig. 5c), suggesting that these precursors are limiting. In support of this, analysis of glucose, xylose and arabinose in maize coleoptiles has revealed that the later two represent a very small fraction as compared to glucose (about 40–60 times less than glucose) (Carpita and McCann, 2000). Efforts to engineer biochemical pathways to increase the amount of pentose sugar substrates could increase butanetriol production without an exogenous supply of these sugars. Xylose and arabinose are present in the cell wall and glycoproteins (Burget et al., 2003; Harper and Bar-Peled, 2002; Kotake et al., 2009). The xylose and arabinose sugars are present mainly as UDP-xylose and UDP-arabinose (Burget et al., 2003; Harper and Bar-Peled, 2002). UDP-xylose is generated from UDP-glucose via UDP-glucuronate that requires two enzymes (UDP-glucose 6-dehydrogenase and UDP-glucuronate decarboxylyase) (Bindschedler et al., 2005; Harper and Bar-Peled, 2002). UDP-arabinose is synthesized by either an epimerization of UDP-xylose

(Kotake et al., 2009; Reiter, 2008) or free arabinose is used to generate UDP-L-arabinose by arabinose kinase and UDP-L-arabinose phosphorylase (Chan and Hassid, 1975; Dolezal and Cobbett, 1991; Kotake et al., 2009; Neufeld et al., 1960; Reiter, 2008). Blocking the expression of arabinose kinase may increase the cellular amount of free arabinose. As there is an alternate pathway to produce UDP-L-arabinose from UDP-D-xylose by a UDP-D-xylose 4-epimerase (Burget et al., 2003; Fan and Feingold, 1970), blocking the production of UDP-L-arabinose from free arabinose may not have an adverse effect on plant growth. In plants, free xylose is metabolized to xylulose by xylose isomerase, which is then phosphorylated by xylulokinase (Zahnley and Axelrod, 1965). Blocking the xylose isomerase may lead to increased amount of cellular xylose. This should not affect the UDP-xylose synthesis that is important for hemicelluose synthesis as all of UDP-D-xylose is synthesized from UDP-D-glucose. Alternatively, for these sugars to be available for conversion to butanetriol, an enzyme that could catalyze the conversion of UDP-xylose or UDP-arabinose to their free states would be necessary. There are, however, pathways where free xylose and arabinose are generated by metabolism of glycoconjugates (Kotake et al., 2009) and by metabolism of myo-inositol (Loewus et al., 1962). Interestingly, the concentration of myo-inositol was decreased in arabinose-grown AAB$_{syn}$ plants as compared to MS grown or wild-type MS or arabinose-grown plants.

Transgenic lines expressing xylose pathway genes grown in the presence of xylose showed significant reduction in growth as the concentration of xylose increased to 5 mM (Fig. 5a). In contrast, wild type plants grew normally even in the presence of 5 mM xylose (Fig. 5a) suggesting that toxicity might be due to altered metabolites in the presence of xylose (Fig. 7 and Table 1). Similarly, plants expressing the arabinose pathway when grown on arabinose supplemented plates showed reduced growth but the amount of end product is considerably increased (Fig. 5c). Arabinose in the medium had no effect on wild type plants. These results suggest that the toxicity is due to either butanetriol, an intermediate compound or other chemicals generated from the intermediate or end products. To test if butanetriol is toxic to plants, we germinated and grew wild type seedlings in liquid cultures in the presence of increasing concentrations (1 μm–5 mM) of either D- or L-butanetriol. Surprisingly, even in the presence 5 mM D- or L-butanetriol no toxic effects on growth were observed (Supplementary Fig. 3), suggesting that this chemical is either not toxic to plants or plants do not take up this chemical. In addition to the end product, several other metabolites (monosaccharides, amino acids, organic acids and sugar alcohols) including some intermediate products in the pathway are altered (Fig. 6 and Table 1), which may also have toxic effects on plant growth. The first product from the arabinose pathway is arabinonic acid, and the metabolomic workflow showed that this compound was present in much higher amounts in AAB$_{syn}$ plants grown on arabinose.

Toxicity of novel chemicals and metabolites produced by metabolic engineering is a limiting factor in producing these chemicals in bacteria and plants (Bornke and Broer, 2010; Jarboe et al., 2010; Keasling, 2012; Zingaro and Papoutsakis, 2012). Further work is needed to identify the intermediate metabolites causing toxicity when transgenic lines are grown under conditions (supplemented with xylose or arabinose) that increase the production of butanetriol. Also, identification of the intermediate compounds could also help in identifying the intermediate enzymatic steps that limit butanetriol production. Once that metabolite is identified other strategies to mitigate toxic effects could be developed. The toxic metabolites could be excreted from the cell or sequestered in an organelle (Bornke and Broer, 2010). Expression of butanetriol pathway genes in photoautotrophic plant cell cultures also offers another way to produce and export it into the medium.

In summary, we show that engineered plants can synthesize butanetriol using a combination of enzymes encoded by bacterial genes and enzymes present in the plants. This proof-of-concept study for phytoproduction of butanetriol opens new avenues to further enhance and optimize production of this chemical in plants by addressing metabolic bottlenecks and mitigating toxic effects (Chou and Keasling, 2012; Keasling, 2012).

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.10.003.

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