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Research paper

Field evaluation of transgenic poplars expressing the constitutively active small G protein for improved biomass traits

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ABSTRACT

An *Arabidopsis* small G protein, RabG3b, has been previously characterized as a positive regulator of xylem development in *Arabidopsis*. Transgenic poplars overexpressing a constitutively active form of RabG3b (RabG3bCA) were developed, and their performance as potential biomass crops was evaluated in the field in Suwon, Korea (37°26′N, 126°98′E) from May to October 2016. RabG3bCA transgenic poplars showed increased stem growth and produced 3–4 times greater dry biomass than hybrid control poplars. Fiber length and diameter were also increased by 15–26% and 28–38%, respectively. Transgenic poplars were analyzed for enzymatic digestibility and biofuel production after chemical pretreatments. Glucose and ethanol yields were higher in pretreated transgenic poplars than hybrid control, and the pretreatment effectiveness of sodium hydroxide was better than that of the other chemicals. We propose that RabG3bCA can add value to the development of biomass crops.

1. Introduction

Advances in industrialization and economic development demand alternative renewable, non-polluting energy resources that can replace fossil fuels [1]. *Populus* is a premier bioenergy crop and its lignocellulosic biomass provides renewable resources for biofuels and biomaterials such as pulp and paper [2,3]. Poplar trees have good biomass-related traits: they grow in temperate climates with a short rotation period; they grow fast at a high density; and they are rich in cellulose, the main component for ethanol production [4,5]. While efforts have been made to develop transgenic poplars in order to improve biomass yield and biofuel production, most studies have been performed under greenhouse conditions. Therefore, their field performance needs to be verified for the development of energy crops.

Completion of the genome sequences of *Populus* has allowed genetic modifications of cell wall traits [3,6,7]. While cellulose is the major cell wall component used for biofuel production, lignin and hemicelluloses disturb enzymatic digestion [8]. Accordingly, key genes targeted for cell wall modifications are involved in the biosynthesis of cell wall components (e.g., cellulose, hemicelluloses, and lignin), lignin conjugation, and pectin degradation [9]. To increase cellulose biosynthesis, *CesA8*, which encodes a cellulose synthase, was overexpressed; however, unfortunately, it silenced the gene, reducing plant growth and biomass yield [10]. In contrast, RNA interference of *GAUT12*, which encodes a glycosyltransferase for glucuronoxylan and/or pectin biosynthesis, improved enzymatic saccharification with increased growth and biomass yield [11]. When lignin content was reduced by down-regulation of *CCR*, encoding a lignin biosynthetic cinnamoyl-CoA reductase, it enhanced enzymatic digestibility but interfered with normal growth [12]. An effort was made to modify the linkage of the lignin polymer. Expression of *FMT*, encoding a monolignol ferulate transferase, introduced a labile ester linkage into the lignin polymer and enhanced wall digestibility [13]. The resulting poplars had no defects in growth, but increased biomass yield, making this a promising approach for developing biomass crops.

Small GTP-binding proteins associate with GTP and GDP and serve as molecular switches for regulating cellular processes such as signal transduction, membrane trafficking, and cell growth and differentiation in eukaryotic cells [14]. They constitute a superfamily that is further classified into Rop, Arf, Ran, and Rab subfamilies in plants [15]. Small GTP-binding proteins in poplars are largely uncharacterized compared with those in *Arabidopsis* and other plants. A recent study showed that the small GTP-binding protein gene *PdRanBP* in *Populus deltoides* was associated with vascular development and wood formation [16]. In a

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transgenic approach, *PdRanBP* acted as a negative regulator for secondary cell wall expansion. We previously demonstrated that the small GTP-binding protein RabG3b activates tracheary element differentiation through autophagy in *Arabidopsis* [17]. Overexpression of a constitutively active RabG3b (RabG3bCA) in *Arabidopsis* enhanced xylem development. When the transgenic poplar overexpressing RabG3bCA was developed, it improved xylem development, and thus, wood formation in the greenhouse [18]. Moreover, transgenic poplars showed more glucan and higher enzymatic digestibility [19]. In this study, to further evaluate biomass traits of RabG3bCA transgenic poplars in nature, independent transgenic lines were planted in the field and analyzed for growth and biofuel production.

2. Materials and methods

2.1. Plant material and field test

A hybrid control 'Bonghwa' (*Populus alba* x *Populus tremula* var. *glandulosa* BH1) and RabG3bCA transgenic poplars that we previously generated [18] were grown in the greenhouse (28–30 °C, 12-h light/12-h dark photoperiod) for 3 months and planted in the field (National Institute of Forest Science, Suwon, Korea; 37°26′N, 126°98′E) in spring (April 29, 2016). Suwon is located 30 km south of Seoul and has a temperate climate with 4 distinct seasons. Poplar trees were planted in rows with 75 cm for the inter-row spacing and 25 cm for the inter-plant spacing over an area of approximately 120 m^2. Each row consisted of a single line. For each line, 80 trees were planted and used for growth measurements. Plants were randomly selected for gene expression and histochemical analyses. The field trial was conducted from April to October 2016.

2.2. Growth and biomass measurements

Plant growth was determined by measuring stem length and diameter, and internode and leaf numbers at elongation, dormant, and enlargement stages. Poplars were cut at the base of the tree at the indicated times, and wood was weighed fresh and dried at 65 °C until constant mass was obtained in an oven for measuring the fresh and dry weights.

2.3. Gene expression analysis

Total RNAs were isolated from poplar leaves by using TRIsure reagent (BIOLINE, USA). The samples were treated with RNase-free DNase I to remove contaminant genomic DNA. For quantitative expression analysis, total RNAs (2 μg) were added for the synthesis of first-strand cDNAs by using the PrimeScript™ RT reagent Kit (TAKARA, Japan), according to the manufacturer's protocol. PCR was performed using gene-specific primers 5′-ATG ACA GGC TTG TCA CAT TGC A-3′ and 5′-AGC ACA ACC TCC TCT TTG CTC A-3'.

2.4. Immunoblotting analysis

Western blot analysis was performed, as described previously [18]. Total proteins were extracted from poplar leaves by using the extraction buffer (50 mmol/kg Tris, pH 8.0, 2 mmol/kg EDTA, 2 mmol/kg DTT, 1 mmol/kg PMSF, protease inhibitor cocktail). The samples were separated on a 15% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membrane was incubated with anti-RabG3b antibody overnight at 4 °C. Antibody-bound proteins were detected after incubation with the secondary antibody conjugated to horseradish peroxidase by using the ECL system (Amersham Biosciences, UK).

2.5. Histochemical analysis

Histochemical analysis was performed, as described previously [18]. Poplar stems collected at 30 cm from ground level were fixed in a solution containing 0.062 mol/kg glutaraldehyde and 4% para-formaldehyde ($HO(CH_2O)nH$, Wako, Japan) in 0.1 mol/kg phosphate buffer (pH 7.4) overnight at 4 °C. After rinsing in 0.1 mol/kg phosphate buffer (pH 7.4), the samples were dehydrated and embedded in LR white resin (TED PELLA, USA). Cross-sections (15 μm) were prepared using a microtome (RM2235; LEICA, Germany), stained with 2 mol/kg toluidine blue O (TED PELLA, USA), and then observed using a light microscope (EZ4E; LEICA, Germany).

2.6. Fiber measurements

Fiber size was measured, as described previously [18]. Stem sections were obtained from the bottom of poplars and cut into pieces (2 mm × 1 mm X 2 mm). Wood pieces were soaked in Schulze's reagent (5.127 mol/kg nitric acid, 0.803 mol/kg potassium chlorate) and incubated for 1 week at room temperature. The samples were then boiled for 10 min and shaken vigorously to separate into individual cells. The macerated cells were photographed using a fluorescent microscope (Axio Imager.A2; ZEISS, Germany) and used to measure fiber length and width.

2.7. Analysis of biomass components

Compositional analysis of klason lignin was performed according to the NREL procedure [20]. Powdered stem samples of poplars were used to determine biomass components. To remove the extractives (minor components in cell walls), acetone and hot-water extractions were performed. Stems were ground, extracted in 20 cm^3 of acetone for 8 h at room temperature, and filtered through Whatman No. 2 filter paper (8 μm). The acetone-extracted samples were then extracted in boiling water for 2 h, filtered, and allowed to air-dry. The prepared extractive-free samples were solubilized in 7.056 mol/kg sulfuric acid for 2 h at 20 °C. The reaction solution was diluted in 0.294 mol/kg sulfuric acid with boiling water, and the sample solution was boiled for 3 h. The boiled sample solution was cooled to room temperature overnight to precipitate klason lignin, and filtered through a crucible filter. The residue in the filter was calculated in terms of klason lignin content. Compositional analysis of cellulose and monosaccharides was analyzed, as described previously [21]. 2nd-stage hydrolysis involved dilution by deuterium oxide (D_2O) as an NMR solvent, instead of boiling water, for ^{1}H-NMR spectroscopic analysis. Composition was calculated on the basis of the interpretation of the ^{1}H-NMR spectra.

2.8. Pretreatment and enzymatic hydrolysis

Powdered stem samples were mixed with 50 cm^3 of 0.098 mol/kg sulfuric acid, 0.096 mol/kg hydrochloric acid, 0.601 mol/kg sodium hydroxide, or 0.175 mol/kg potassium hydroxide solution in a stainless-steel cylinder reactor (WACS-1060; Daihan Scientific, Korea). The samples were incubated in an oven for 1 h at 120 °C, filtered, and washed with distilled water until a pH of 4.5–6.0 was achieved. Enzymatic hydrolysis was performed in 100 cm^3 sodium citrate buffer (pH 4.8) containing 8 g of the dried pretreated substrate and commercial enzyme Cellic C-Tec2 (Novozymes, Bagsvaerd) with 30 FPU/g cellulose. The hydrolysis mixture was incubated for 72 h at 50 °C. The sugar contents in the enzymatic hydrolysate were measured using the HPLC system (Shimadzu, Japan) with an Aminex HPX-87P column (300 mm × 7.8 mm) (Bio-Rad, USA) and reflective index detector (RID-10a; Shimadzu, Japan). The mobile phase was 50 mmol/kg magnesium sulfate, and the flow rate was 0.6 cm^3/min.

2.9. Ethanol fermentation

The hydrolysis mixture of stem samples was used for measuring glucose and ethanol production. *Kluyveromyces marxianus* was cultured