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RESEARCH PAPER

Synergistic effects of 2A-mediated polyproteins on the production of lignocellulose degradation enzymes in tobacco plants

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Abstract

Cost-effective bioethanol production requires a supply of various low-cost enzymes that can hydrolyse lignocellulosic materials consisting of multiple polymers. Because plant-based enzyme expression systems offer low-cost and large-scale production, this study simultaneously expressed β-glucosidase (*BglB*), xylanase (*XylII*), exoglucanase (*E3*), and endoglucanase (*Cel5A*) in tobacco plants, which were individually fused with chloroplast-targeting transit peptides and linked via the 2A self-cleaving oligopeptideex from foot-and-mouth disease virus (FMDV) as follows: [RsBglB-2A-RaCel5A], [RsXylII-2A-RaCel5A], and [RsE3-2A-RaCel5A]. The enzymes were targeted to chloroplasts in tobacco cells and their activities were confirmed. Similarly to the results of a transient assay using *Arabidopsis thaliana* protoplasts, when *XylII* was placed upstream of the 2A sequence, the [RsXylII-2A-RaCel5A] transgenic tobacco plant had a more positive influence on expression of the protein placed downstream. The [RsBglB-2A-RaCel5A] and [RsE3-2A-RaCel5A] transgenic lines displayed higher activities towards carboxylmethylcellulose (CMC) compared to those in the [RsXylII-2A-RaCel5A] transgenic line. This higher activity was attributable to the synergistic effects of the different cellulases used. The [RsBglB-2A-RaCel5A] lines exhibited greater efficiency (35–74% increase) of CMC hydrolysis when the exoglucanase CBHII was added. Among the various exoglucanases, E3 showed higher activity with the crude extract of the [RsBglB-2A-RaCel5A] transgenic line. Transgenic expression of 2A-mediated multiple enzymes induced synergistic effects and led to more efficient hydrolysis of lignocellulosic materials for bioethanol production.

Key words: 2A-mediated polyproteins, cellulase, enzymic hydrolysis, lignocellulosic biomass, molecular farming, plant expression.

Introduction

Surging oil prices and pressing demand to reduce greenhouse gas emissions are driving technological innovations for cost-effective production of alternative energy sources such as bioethanol. Lignocellulosic biomass, the most abundant renewable resource on Earth, is an attractive raw material for producing bioethanol, as it does not compete with the food and animal feed industries and produces significantly lower greenhouse gas emissions compared to corn-based bioethanol, electricity, or hydrogen (Charles, 2009). However, the requirement for pretreating a lignocellulosic biomass to enhance enzyme accessibility to the cell-wall polymers and costly saccharification by enzymic hydrolysis are the main limiting factors for the economic production of bioenergy from this important feedstock.

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Efficient conversion of lignocellulosic biomass to fermentable sugars by enzymic hydrolysis requires three cellulose-degrading enzymes (β-1,4-endoglucanase, β-1,4-exoglucanases, and β-D-glucosidase), two hemicellulose-digesting enzymes (xylanase and xylosidase), and other enzymes involved in debranching side chains (e.g., arabinofuranosidase, ferulic acid esterase, glucuronidase, and acetyl xylan esterase) (Lee at al., 2011; Margolles-Clark, 1996; Saha *et al.*, 2003). Production of lignocellulose hydrolysis enzymes by conventional microbial fermentation methods is not cost-effective for the saccharification of lignocellulosic biomass. Hence, the feasibility of using plants as alternative bioreactors for low cost, large-scale production of recombinant proteins has been investigated (Streatfield, 2007; Jung *et al.*, 2010). Plant-based production of recombinant proteins is estimated to be achievable at 2–10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell culture systems (Twyman *et al.*, 2003; Kamenarova *et al.*, 2005). Endo-β-1,4-glucanase is recovered at levels >16% of total soluble protein (TSP) in single transgenic maize seed (Hood *et al.*, 2007) and comprises up to 26% of TSP when produced in the apoplasts of *Arabidopsis thaliana* leaves (Ziegler *et al.*, 2000). Accumulation of β-glucosidase (BglB) from *Thermotoga maritima* in chloroplasts of tobacco leaves accounts for 5.8% of TSP (Jung *et al.*, 2010).

Another strategy for cost-efficient enzymic hydrolysis is to apply enzymic synergism to the heterogeneous lignocellulosic biomass to produce fermentable sugars. Verma *et al.* (2010) demonstrated that plants can be used to efficiently produce various enzymes for preparing a cocktail solution consisting of endoglucanase, β-glucosidase, swollenin, xylanase, and acetyl xylan esterase and observed synergistic effects among the enzymes in the degradation of filter paper, pinewood, and orange peel. These results suggest that simultaneous expression of multiple enzymes in plants could increase the degradation efficiency of lignocellulosic materials, reduce enzyme production cost, and provide a more convenient enzyme harvest compared to single heterogeneous enzyme expression in plants.

The 2A self-cleaving oligopeptide from foot-and-mouth disease virus (FMDV), which comprises 18 amino acids, mediates cleavage or polyprotein dissociation at its C-terminus during the translation of multiple 2A-linked proteins in the ribosome (Donnelly et al., 2001; Samalova *et al.*, 2006). The cleaving efficiency of the 2A-mediated polyprotein system has been studied using various reporter proteins such as green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescence protein, and β-glucuronidase in various cell types (Felipe *et al.*, 2003; Samalova *et al.*, 2006), as well as targeting to various subcellular localizations in HeLa and plant cells (Amrani *et al.*, 2004; Felipe and Ryan, 2004; Samalova *et al.*, 2006). Most 2A sequence studies have used reporter genes to characterize the self-cleavage activity of 2A sequences in plant cells with few exceptions, such as applications for metabolic engineering using multiple enzymes that involve carotenoids biosynthesis in tobacco and tomato leaves (Ralley *et al.*, 2004) and in rice endosperm (Ha *et al.*, 2010).

The plant chloroplast is an attractive organelle to sequester hydrolysis enzymes to avoid deleterious effects during plant development and to increase stable accumulation in plant (Jin *et al.*, 2003; Dai *et al.*, 2005). However, a test for targeting up- and downstream proteins of 2A to chloroplasts in an *in vivo* system has not been described in detail. The protoplast system was tested to confirm whether the current study's protein expression system with the FMDV 2A sequence was coexpressed and colocalized to the targeted organelle. Polyproteins consisting of β-glucosidase (BglB) and endoglucanase (Cel5A) from *Thermotoga maritima*, xylanase (XylII) from *Trichoderma reesei*, and exoglucanase (E3) from *Thermomonospora fusca* linked to the FMDV 2A sequence were coexpressed in tobacco plant chloroplasts and shown to have the potential of hydrolysing cellulose through synergistic effects of the coexpressed enzymes. Although plants have been previously used as bioreactors, this work provides a biotechnological example of the coexpression of multiple hydrolysis enzymes for efficient biomass degradation.

Materials and methods

Construction of 2A expression cassettes

Primers for 2A sequences (sense: 5′-AGCTTCAGCTTCTGAACTTTGACCTGCTCAAGTTGGCAGGAGACGTCGAGTCCAACCCTGGGCCTG-3′; antisense: 5′-TCGACAGGCCCAGGGTTGGACTCGACGTCTCCTGCCAACTTGAGCAGGTCAAAGTTCAGAAGCTGA-3′) were used to introduce *Hind*III and *Sal*1 restriction enzyme sites into the 326 3G transient expression vector. PCR fragments of GFP and RFP were introduced into the 326 3G 2A-mediated polyprotein vector at *Bam*HI–*Hind*II or *Not*I–*Hind*III sites, respectively, after ligation into pCR 2.1 Topo vector (K4500-40, Invitrogen). 326 3G [GFP-2A-RFP] and [RFP-2A-GFP] vectors were constructed and used as backbone vectors for the following procedure. Transit peptides, small subunit of Rubisco complex (Rs), and Rubisco activase (Ra), for targeting to the chloroplast (Kim *et al.*, 2010), were fused to the N-terminal of the reporter genes, constructing the vectors 326 3G [RsGFP-2A-RaRFP] and [RsRFP-2A-RaGFP]. Xylanase (*XylII*, accession X69574) cloned from *Trichoderma reesei* was substituted for reporter genes placed at the restriction enzyme sites, *Bam*HI–*Hind*III or *Not*I–*Xho*I, constructing the vectors 326 3G [RsXylII-2A-RaGFP] and [RsGFP-2A-RaXylII]. Eventually, four transient expression vectors, [RsGFP-2A-RaRFP], [RsRFP-2A-RaGFP], [RsGFP-2A-RaXylII], and [RsXylII-2A-RaGFP], were constructed to analyse 2A self-cleavage activity and targeted to the chloroplast in protoplasts isolated from *A. thaliana* plants.

The 326 3G [RsGFP-2A-RaRFP] vector was used as a backbone vector for the construction of binary vector pCambia 2300 [RsBglB-2A-RaCel5A], [RsE3-2A-RaCel5A], and [RsXylII-2A-RaCel5A]. The genes *BglB* (AE000512, β-glucosidase from *Thermotoga maritima*), *XylII*, and *E3* (U18978, exoglucanase from *Thermomonospora fusca*) were substituted for the GFP gene located in front of the 2A sequence between the *Bam*HI and *Hind*III restriction enzyme sites in the 326 3G transient expression vector. The gene *Cel5A* (AE000512, endoglucanase from *Thermotoga maritima*) was substituted for the RFP gene placed between the *Not*I and *Xho*I restriction enzyme sites. 326 3G [RsBglB-2A-RaCel5A], [RsE3-2A-RaCel5A], and [RsXylII-2A-RaCel5A] were cut by restriction enzymes *Xba*I and *Apa*I or *Xba*I and *Kpn*I, and the fragments were introduced into binary vector pCambia 2300. The primers are presented in Table 1.

Transient expression and targeting of FMDV 2A-mediated polyproteins to the chloroplast

DNA of the 2A-mediated polyprotein vectors [RsGFP-2A-RaRFP], [RsRFP-2A-RaGFP], [RsGFP-2A-RaXylII], and [RsXylII-2A-RaGFP] were prepared using the Plasmid Maxi kit (12163, Qiagen), and protoplasts were isolated from whole *A. thaliana* seedling after growing for

Table 1. Oligonucleotide primers used in this study

Gene	Sense (5′–3′)	Antisense (5′–3′)
2A	AGCTTCAGCTTCTGAACTTTGACCTGCTCAAGTT	TCGACAGGCCCAGGGTTGGACTCGACGTCTCCT
	GGCAGGAGACGTCGAGTCCAACCCTGGGCCTG	GCCAACTTGAGCAGGTCAAAGTTCAGAAGCTGA
Rs	TCTAGAATGGCTTCCTCTATG	GGATCCTTCGGAATCGGTAAG
Ra	GTCGACATGGCCGCCGCAGTT	GCGGCCGCAATCAGAAGTGTCGTA
GFP	GGATCCATGAGTAAAGGAGAA	AAGCTTTTTGTATAGTTCATC
	GCGGCCGCATGAGTAAAGGAGAA	CTCGAGTTTGTATAGTTCATC
RFP	GGATCCATGGTGCGCTCCTCC	AAGCTTCAGGAACAGGTGGTG
	GCGGCCGCATGGTGCGCTCCTCC	CTCGAG CAGGAACAGGTGGTG
XylII	GGATCCATGGTTGCCTTTTCC	AAGCTTGTTGCTGACACTCTG
	GCGGCCGCATGGTTGCCTTTTCC	CTCGAGGTTGCTGACACTCTG
BglB	GGATCCATGGAAAGGATCGAT	AAGCTTTGGTTTGAATCTCTT
E3	GGATCCATGAGTAAAGTTCGT	AAGCTTCAGAGGCGGGTAGGC
Cel5A	GCGGCCGCATGGGTGTTGATCCT	CTCGAGTTCAATGCTATCTCC
$BglB_{Int2020}$	ATTCCTCTCAGAGAT	–
$XylII_{Int280}$	GGATCTTCTGCTCCC	–
$E3_{Int450}$	CCGTCGCCGAGCCCTTCCC	–
$Cel5A_{Int164}$	–	TTATTGGAATTCGAACA

3 weeks using enzyme solution [0.4 M mannitol, 8 mM $CaCl_2$, 5 mM MES-KOH (pH 5.6), 1% cellulase RS10 (216011, Yakult Honsha), 0.25% macerozyme R-10 (202042, Yakult Honsha)]. Each DNA (15 µg) was introduced into the protoplasts by e
polyethylene glycol (PEG)-mediated transformation (Jin *et al.*, 2001). Accumulation of GFP placed at anterior and posterior positions of 2A sequence were monitored for 12, 24, and 36 hours after transformation, and images were detected using an inverted microscope (Eclipse TE2000-U, Nikon). Protein extracts were prepared from the transformed protoplast for Western blot analysis with anti-GFP, -RFP, and -T7 primary antibodies, respectively.

Agrobacterium-*mediated transformation of tobacco leaf discs*

Leaf discs (0.5–1 cm^2) of tobacco seedlings (*Nicotiana tabacum*) grown on MS media (l^{-1}: 4.4 g MS medium including vitamin, 20 g sucrose, 8 g agar, pH 5.7) were prepared and incubated with *Agrobacterium tumefaciens* GV3013 transformed with individual 2A-mediated polyprotein vectors, pCambia 2300 [RsBglB-2A-RaCel5A], [RsE3-2A-RaCel5A], and [RsXylII-2A-RaCel5A], in co-culture media [l^{-1}: 3.2 g MS with vitamin, 0.1 mg 1-naphthalene acetic acid (NAA), 1 mg benzylaminopurine (BA), 3% sucrose, pH 5.7] for 3–5 min. The explants were placed on callus induction medium (l^{-1}: 4.4 g MS with vitamin, 0.1 mg NAA, 1 mg BA, 3% sucrose, 6 g agar, pH 5.7) for 2–3 days, and transferred onto selection medium (l^{-1}: 4.4 mg MS with vitamin, 200 mg kanamycin, 500 mg cefotaxime, 20 g sucrose, 6 g agar, pH 5.7) until the callus developed into shoots and roots. Regenerated T_0 transformants selected twice on the media were transplanted to potting soil.

Genomic DNA was extracted from the first or second leaf of young transgenic seedlings grown in the greenhouse. DNA extraction buffer contained 200 mM TRIS-HCl (pH 8.0), 250 mM NaCl, 25 mM EDTA, 0.5% SDS, and 10 mM 2-mercaptoethanol. A total of 5 µg DNA was used to confirm the introduction of the transgenes by PCR with transgene-specific primers at the 2020th bp position of *BglB*, the 280th bp of *XylII*, and the 450th bp position of *E3* (sense) and the 164th bp position of *Cel5A* (anti-sense, Table 1).

Western blot analysis

Total soluble proteins were extracted from leaves of young seedling and flowering stage of transgenic plants (extraction buffer: 50 mM TRIS-HCl, pH 8.0, 100 mM KCl, 5 mM Na_2EDTA, 5% glycerol, 14 mM β-mercaptoethanol, 20 mM $Na_2S_2O_5$, 4 mM phenylmethylsulphonyl fluoride) grown in the greenhouse. The proteins extracted were quantified by the Brad-ford assay. A total of 30 µg protein was separated by 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Immobilon-P, IPVH00010, Millipore). After incubation with Cel5A or BglB polyclonal antibody (Jung *et al.*, 2010; Kim *et al.*, 2010), enzyme expression was detected by alkaline phosphatase-conjugated goat-anti-rabbit immunoglobulin G antibody and Western Blue stabilized substrate for alkaline phosphatase (S3841, Promega). Intensity of each colorized band was analysed using the UTHSCSA Image Tool (version 2.00, University of Texas Health Science Center).

Electron microscopy and immunogold labelling

Leaf samples of wild type and [RsXylII-2A-RaCel5A] transgenic plants were fixed in 50 mM sodium cacodylate buffer (pH 7.4) containing 4.0% paraformaldehyde and 0.1% glutaraldehyde for 4 hours at room temperature. After washing with buffer and dehydrating with a graded ethanol series, the samples were embedded in London Resin White acrylic resin. Ultrathin sections (80–100 nm) were mounted on uncoated nickel grids (300 mesh) and treated with normal goat serum. The sections were incubated in rabbit anti-Cel5A polyclonal antibody diluted 1:100 in PBS buffer plus 0.05% Tween 20 for 2 days at 4 °C. The sites of antibody bonding were labelled with goat-anti-rabbit antiserum conjugated to 15 nm colloidal gold particles for 2 hours at room temperature. After staining with 4% uranyl acetate, the sections were examined with a transmission electron microscope (JEM-1400, JEOL) at an acceleration voltage of 80 kV.

Enzyme activity assay

Activity assays for β-glucosidase (BglB), endoglucanase (Cel5A), xylanase (XylII), and exoglucanase (E3) in total soluble protein (30 µg) extracted from young leaf of the transgenic lines, [RsBglB-2A-RaCel5A], [RsE3-2A-RaCel5A], and [RsXylII-2A-RaCel5A] were performed in 1 ml citrate buffer (pH 5.0) or phosphate buffer (pH 7.0) containing 5 mM *p*NPG, 1% oat spelt xylan, 1% (w/v) carboxylmethylcellulose (CMC), or 50 mg filter paper (5 mm × 5 mm) at 50 or 80 °C for 1 hour. The amount of *p*-nitrophenol (*p*NP) liberated was determined at 405 nm after addition of 500 mM Na_2CO_3. Reducing sugars liberated from oat spelt xylan, CMC, and filter paper were measured at 550 nm after boiling with DNS solution [1% (w/v) dinitrosalicylic acid, 0.05% (w/v) sodium sulphite, 2% (w/v) Rochelle salt ($C_4H_4KNaO.4H_2O$), 0.2% (w/v) phenol, 2% (w/v) NaOH)], and quantified according to standard curves for *p*NP, xylose, and glucose, respectively. All enzyme activities were measured 3–5 times for reproducibility.

Synergistic effects of transgenic plants with cellulases on CMC or filter paper

For exoglucanase preparation, *E3* (*Cel6B*) from *Thermomonospora fusca* was subcloned into pCold I Expression vector (TaKaRa) for expression in *Escherichia coli* (Lee *et al.*, 2011) and *E6* (*Cel48A*) from *Thermomonospora fusca*, and the CBHI and CBHII genes from *Aspergillus flavus* were individually introduced into the vector pPICZα A for expression in *Pichia pastoris*. The recombinant plasmids were linearized by restriction with *Pme*1 and transformed into *P. pastoris*-competent cells by electroporation. The transformants were selected on YPDZ solid media [1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 300 g ml^{-1} zeocin (R250–05, Invitrogen), 2% agar]. A single colony was inoculated in 20 ml YPG broth [1% yeast extract, 2% peptone, 1% (v/v) glycerol]. Cells were pelleted by centrifugation at 200 *g* for 5 min and transferred to 200 ml YPG broth in a 500 ml shake flask and incubated for 24–36 hours. The pelleted cells were finally incubated in 500 ml YP media (1% yeast extract, 2% peptone) with 1% methanol for induction.

Cell lysates from *E. coli* expression (E3) and enzyme supernatants from yeast expression (E6, CBHI, and CBHII) were purified through the Ni-NTA agarose column (30230, Qiagen). Exoglucanase activity was tested with 3 MM filter paper as substrate under optimal conditions: pH 7.0 and 50 °C for E3 and E6, and pH 5.0 and 50 °C for CBH1 and CBHII. One unit was defined as the amount of enzyme producing 0.07–0.08 mg reducing sugars ml^{-1} in 1 hour.

For transgenic plant preparation and synergistic effect tests, transgenic lines 2, 6, 24, 32, and 34 of [RsBglB-2A-RaCel5A] were tested with purified CBHII on CMC and 3MM filter paper. A total of 30 µg total soluble protein extracted from each line was incubated with 1 unit of CBHII in 1 ml citrate-phosphate buffer (pH 5.0) containing an individual substrate (CMC or 3MM filter paper) at 50 °C for 1 hour.

The synergistic effects of line 6 and exoglucanases (E3, E6, CBHI, and CBHII) were analysed by measuring the amount of reducing sugars released from CMC by each enzyme combination in 1 ml citrate-phosphate (pH 5) or phosphate buffer (pH 7.0) at 50 °C for 1 hour. Reducing sugars released were measured by the DNS method, as previously described.

Results

Transient expression and chloroplast targeting

Transient expression cassettes were constructed with transit peptides fused to the N-terminus of GFP, RFP, and xylanase to determine 2A self-cleavage activity and chloroplast targeting in *Arabidopsis* protoplasts (Fig. 1A). The 2A self-cleavage activity was carried out efficiently in all constructs during the posttranslational process. Self-cleavage activity was higher when GFP or XylII, but not RFP, was located in front of the 2A sequence. *XylII* seemed to have a greater influence than that of GFP on the expression of the protein located at the anterior position of the 2A sequence (Fig. 1B). GFP signals observed in chloroplasts confirmed the posttranslational chloroplast targeting of the proteins encoded at the anterior and posterior positions within 2A-mediated polyproteins (Fig. 1C).

Coexpression of cellulose degradation enzymes and detection of chloroplast targeting in tobacco plants

The genes that encode enzymes that have major roles in the hydrolysis of lignocellulosic material include *BglB*, *Cel5A*, *XylII*, and *E3*. These were used to construct a 2A-mediated coexpression system under the control of the CaMV 35S promoter (Fig. 2A). The transgenic lines of [RsBglB-2A-RaCel5A], [RsXylII-2A-RaCel5A], and [RsE3-2A-RaCel5A] were confirmed by genomic DNA PCR with gene-specific primers (Fig. 2B).

A coexpression test of individual [RsBglB-2A-RaCel5A] transgenic line was carried out by Western blotting analysis with BglB and Cel5A primary antibodies. Simultaneous expression of BglB and Cel5A was detected as discrete proteins from a single open reading frame driven by the CaMV 35S promoter in the transgenic tobacco plants. The BglB protein placed upstream of the 2A sequence was expressed at a higher level than that of Cel5A. Total soluble proteins extracted from selected transgenic lines of [RsXylII-2A-RaCel5A] and [RsE3-2A-RaCel5A] were separated by SDS-PAGE, and Western blotting was used to detect expression of Cel5A placed downstream of the 2A sequence (Fig. 3). The Cel5A protein from the [RsXylII-2A-RaCel5A] transgenic plants was expressed at a higher level than the Cel5A protein from the [RsBglB-2A-RaCel5A] and [RsE3-2A-RaCel5A] transgenic lines, suggesting that the XylII placed upstream of the 2A sequence might have positively affected the expression of downstream proteins in the transgenic tobacco plants as well as in *A. thaliana*. A full-size band (~80 kDa) corresponding to the uncleaved polyprotein was detected in younger seedlings of the [RsXylII-2A-RaCel5A] line (Supplementary Fig. S2) but was not detected or weakly detected in the protein extracts from mature transgenic plants before or after flowering (Fig. 3B). In E3-fused 2A-mediated polyprotein lines, Cel5A expression was estimated to be less than that of [RsXylII-2A-RaCel5A] and [RsBglB-2A-RaCel5A]. Yield of Cel5A accounted for up to ~8% of total soluble protein in the [RsXylII-2A-RaCel5A] line 20 (Fig. 4A).

Electron microscopy and immunogold labelling with specific polyclonal antibody to Cel5A were used to localize the chloroplast-targeted Cel5A protein. Cel5A was mainly detected in chloroplasts of the [RsXylII-2A-RaCel5A] transgenic line (Fig. 4B).

Activity of discrete hydrolysing enzymes in transgenic plants

Total soluble proteins were prepared from selected transgenic lines of [RsBglB-2A-RaCel5A], [RsXylII-2A-RaCel5A], and [RsE3-2A-RaCel5A]. TSP (30 µg) was used for the enzyme activity assays with the following substrates: *p*NPG for BglB, oat spelt xylan for XylII, CMC for Cel5A, and 3 MM filter paper for E3. Each transgenic line displayed BglB and Cel5A activity with *p*NPG and CMC, respectively, except line 24, which showed only BglB activity (Fig. 5A). Xylanase and endoglucanase activity with oat spelt xylan and CMC were also measured successfully, with TSPs extracted from the transgenic lines that coexpressed *XylII* and Cel5A (Fig. 5B). Also observed were exoglucanase and endoglucanase activities in [RsE3-2A-RaCel5A] transgenic plants (Fig. 5C). In addition, the [RsXylII-2A-RaCel5A] transgenic line showed the highest Cel5A expression level, which was about 4–8 times (and in some cases, much higher) that of the completely cleaved Cel5A of [RsBglB-2A-RaCel5A] and [RsE3-2A-RaCel5A], but exhibited the lowest cellulase activity on CMC (Fig. 6A). This result demonstrated that the interactions between Cel5A and BglB, and between E3 and Cel5A, yielded a higher degree of synergism compared to the single activity of Cel5A on CMC (Fig. 6B).

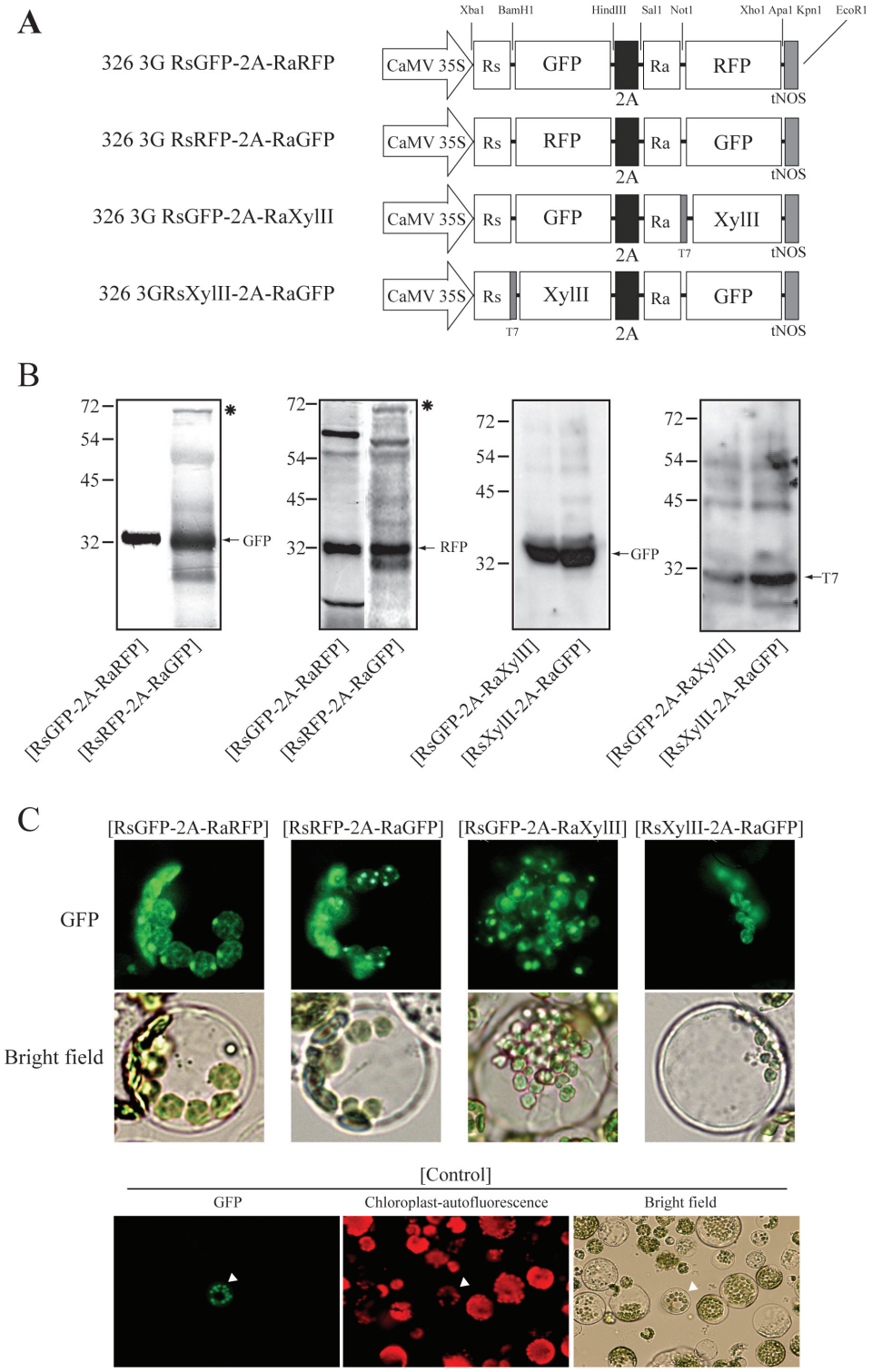

Fig. 1. Transient assay of 2A-mediated polyproteins in *Arabidopsis thaliana* protoplasts. (A) Schematic representation of the expression vectors used for protoplast transformation. (B) Analysis of expression pattern of 2A-mediated polyproteins consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), and XylII-fused with transit peptide. Transit peptides (Rs, Rubisco small subunit; Ra, Rubisco activase) were used to target chloroplasts. GFP and RFP were detected by Western blotting using anti-GFP and -RFP primary antibodies. XylII was detected by anti-T7 primary antibody. Asterisk denotes the full size of the polyprotein, and arrows indicate discrete proteins from polyproteins. (C) Targeting of chloroplasts by polyprotein-fused transit peptides was observed by GFP signals. In the control, arrowhead indicates a GFP-transformed protoplast; non-transformants were not detected. CaMV 35S, Cauliflower mosaic virus promoter; GFP, green fluorescent protein; RFP, red fluorescent protein; XylII, xylanase gene from *Trichoderma reesei*; 2A, self-cleavage sequence of foot-and-mouth disease virus; T7, T7 epitope tag; tNos, nopaline synthase terminator (this figure is available in colour at *JXB* online).

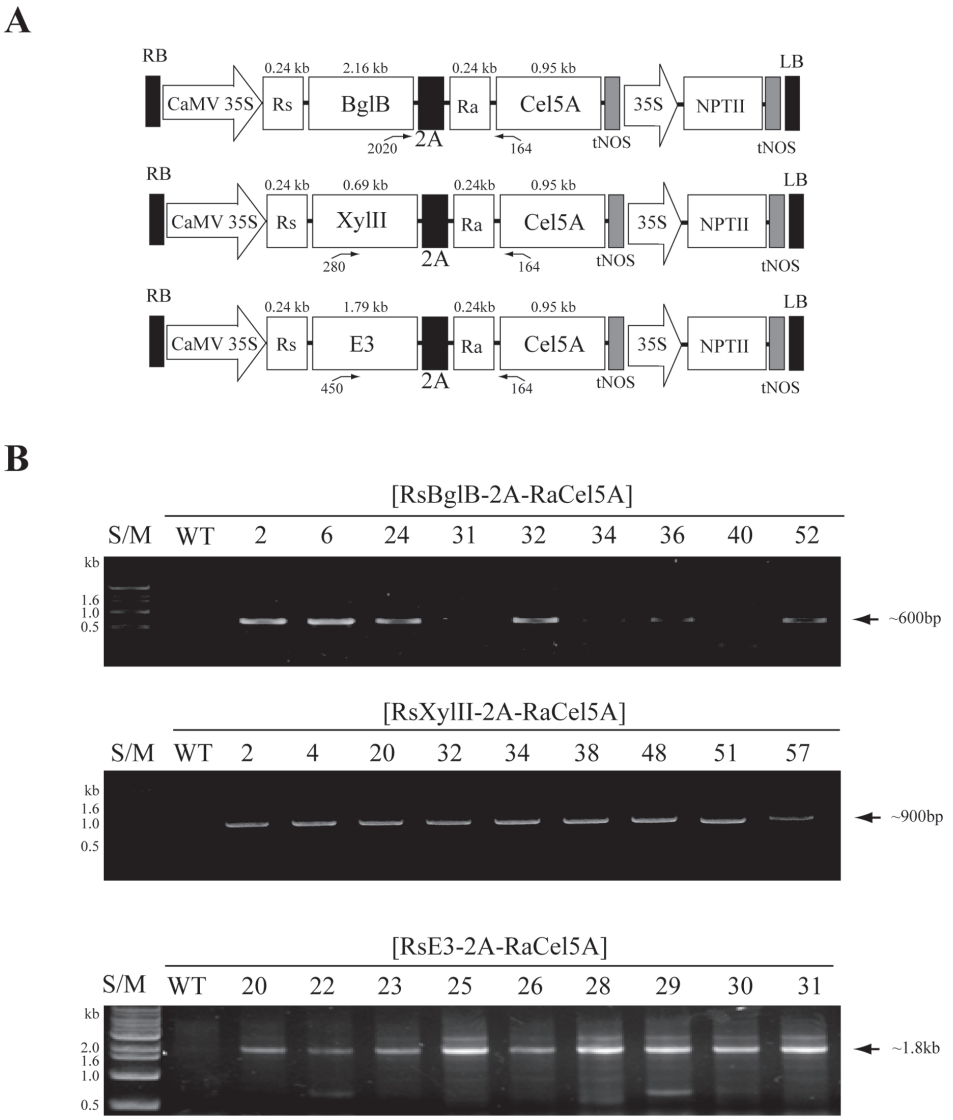

Fig. 2. The transformation of 2A-mediated polyprotein constructs consisted of cellulose hydrolysis enzymes into tobacco genomic DNA. (A) Schematic presentation of the DNA constructs containing genes for β-glucosidase (*BglB*), endoglucanase (*Cel5A*), xylanase (*XylII*), and exoglucanase (*E3*). Curved arrows denote annealing positions of specific primers on the hydrolysis genes. LB, Left border; RB, right border. (B) Genomic DNA PCR of each transgenic line was carried out with specific primers.

Synergistic effect of [RsBglB-2A-RaCel5A] transgenic lines with exoglucanase

The [RsBglB-2A-RaCel5A] transgenic lines 2, 6, 32, and 34 exhibited efficient hydrolysis activity (35–74% increase) when exoglucanase CBHII was added. All transgenic lines showed low activity when filter paper was used as the substrate (Fig. 7A).

The preparation of enzyme cocktails combining diverse cellulases requires optimal conditions. Table 2 shows the properties of each enzyme used in this study. The synergistic effects of the enzyme cocktail in combination with the transgenic line 6 protein extract and exoglucanases was higher at pH 5.0 for the CMC substrate, and in particular, E3 supplementation showed the highest activity at pH 5.0. Unexpectedly, adding CBHI to the line 6 extract reduced endoglucanase and β-glucosidase activity for digesting CMC (Fig. 7B).

Discussion

The introduction of multiple genes linked by the FMDV 2A sequence into plants is a more versatile and simpler strategy than conventional methods, such as sexual crossing, retransformation, cotransformation, or the use of directly linked transgenes (Halpin *et al.*, 2001). In the enzymic hydrolysis of lignocellulosic materials with multiple hydrolysis enzymes, 2A self-cleavage polyproteins offer an attractive expression system to achieve low-cost enzyme production and the synergistic effect of multiple enzymes expressed in one transgenic plant. Previous reports have shown that 2A cleavage activity displays wider sequence-specific variation in plants than in animal cells and is influenced by the upstream protein of the 2A sequence (Ryan *et al.*, 1999; Samalova *et al.*, 2006; Felipe *et al.*, 2010). Amrani *et al.* (2004) demonstrated that two proteins in the evolving complex of photosystem II, dissociated by activity of 2A self-cleavage, were targeted to chloroplasts

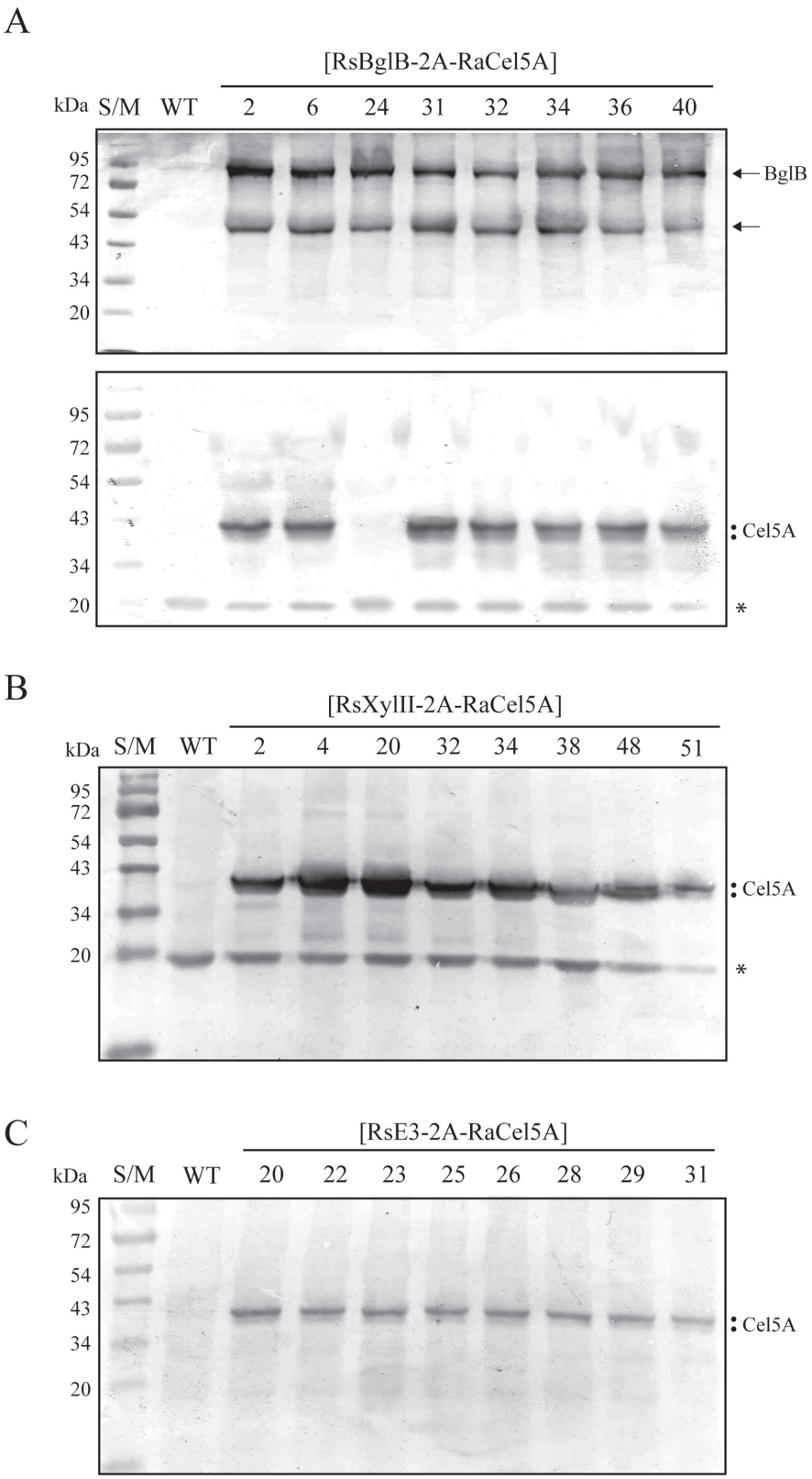

Fig. 3. Western blot analysis of 2A-mediated polyproteins transformed in tobacco plants. Total soluble protein extracted from each transgenic line was separated using SDS-PAGE and detected by individual incubation with the BglB or Cel5A primary antibodies for the [RsBglB-2A-RaCel5A] transgenic line (A) and with the Cel5A primary antibody for the transgenic lines [RsXylII-2A-RaCel5A] (B) and [RsE3-2A-RaCel5A] (C). Arrows indicate BglB (~80 kDa) and its degradation band (~48 kDa). Dots are mature (~41 kDa) and partial degradation protein (38.5 kDa) of chloroplast-targeted Cel5A. Asterisks denote nonspecific bands.

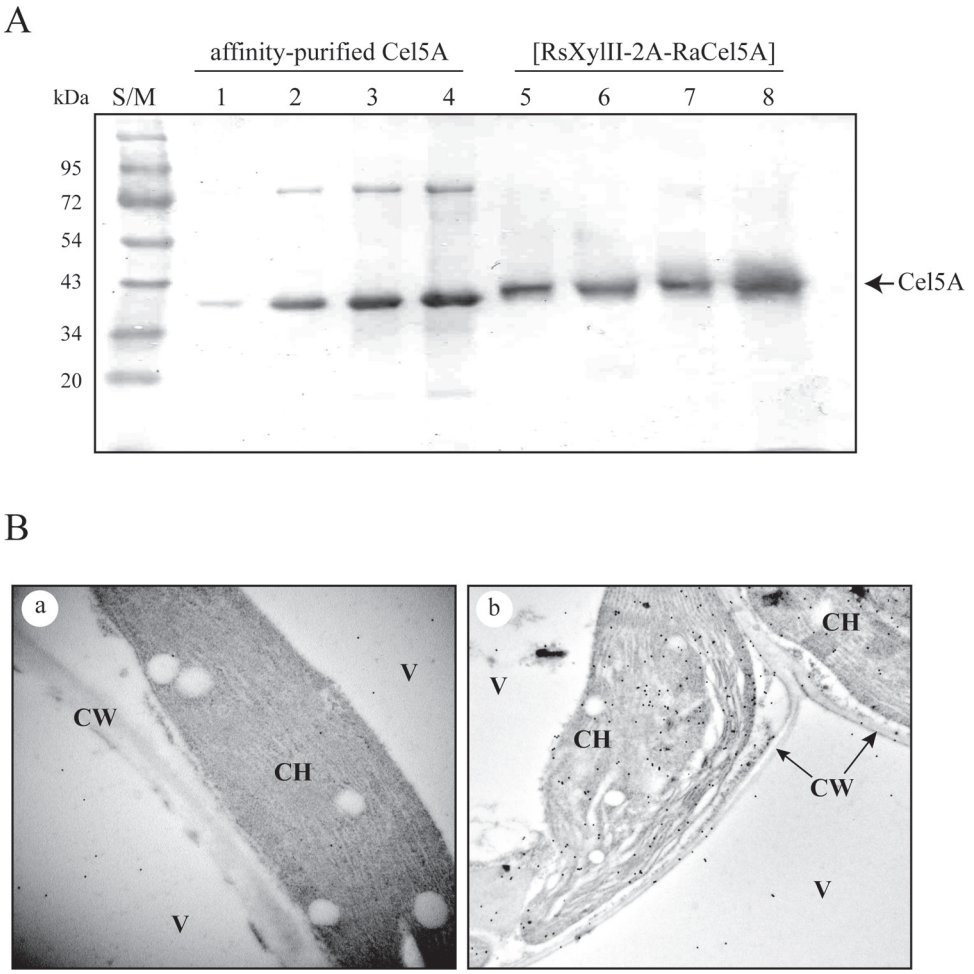

Fig. 4. Yield and immunolocalization of Cel5A expressed in the transgenic [RsXylII-2A-RaCel5A] line. (A) Quantitation of Cel5A expression levels in the transgenic tobacco plants. Affinity-purified Cel5A from bacterial expression was loaded on lanes 1–4 (0.1, 0.5, 0.8, and 1.5 μg, respectively), and total soluble proteins (TSPs) extracted from transgenic [RsXylII-2A-RaCel5A] lines were loaded on lanes 5–6 (20 and 40 μg TSP from transgenic line 2, respectively) and lanes 7–8 (10 and 20 μg TSP from the transgenic line 20, respectively). Western blotting was carried out with the Cel5A primary antibody. (B) Immunolocalization of Cel5A targeted to chloroplasts of the transgenic tobacco plant: (a) wild-type plant, (b) transgenic [RsXylII-2A-RaCel5] line 20. Labelling was carried out with the Cel5A primary antibody. Gold particles are clearly seen in chloroplasts. CH, chloroplast; CW, cell wall; V, vacuole.

in an *in vitro* system. The current study found that proteins placed downstream of 2A fused with transit peptides of Rubisco activase at the N-terminus were targeted to *Arabidopsis* and tobacco plant chloroplasts, and moreover, *XylII* placed upstream of 2A had a positive impact on the expression of second-position genes in both plants. In [RsXylII-2A-RaCel5A] transgenic plants, 2A activity showed a different pattern, depending on the developmental stage of the transgenic plants. Full-length bands (~80 kDa) of RsXylII-2A-RaCel5A were detected in young transgenic line seedlings (Supplementary Fig. S2). However, the bands almost disappeared in older transgenic lines near the flowering stage (Fig. 3B). One can assume that 2A is more active at a more mature developmental stage or that separation is more efficient by stromal processing peptidase (SPP) when the full-length peptide of [RsXylII-2A-RaCel5A] was translocated into the chloroplast of mature plants. Another construct, [RaXylII-2A-GFP], showed that GFP dominantly accumulated in the cytoplasm after being completely separated by 2A-mediated cleavage in cytosolic ribosomes (Supplementary Fig. S1). This result indicated that the discrete downstream-protein-fused transit peptide (RaCel5A) was released first into the cytoplasm after 2A self-cleavage and then targeted to chloroplasts. This suggests that 2A-cleavage activity and expression of downstream proteins can be modulated by plant developmental stage as well as by upstream proteins, as shown in an *in vitro* translation system and an animal system (Donnelly *et al.*, 2001; Felipe *et al.*, 2010).

The importance of spacer length between the transit peptide and the foreign protein to transport into the stroma and for efficient processing by SPP has been reported (Jin *et al.*, 2003). Additionally, stepwise degradation patterns of chloroplast-targeting endoglucanase (E1) in transgenic tobacco plants were reported (Dai *et al.*, 2005). The current study observed degradation bands (~48 kDa) of BglB targeted to chloroplasts, by protease in stroma. Different patterns of chloroplast-targeted Cel5A degradation were detected when compared to BglB degradation. Cel5A was fused to its N-terminus with 80 amino acids of Rubisco activase transit peptide. The upper band was the mature form (~40 kDa) cleaved by SPP at amino acid position 55 of the transit peptide when the Cel5A protein was sorted into chloroplasts. The lower band was anticipated to be formed as a

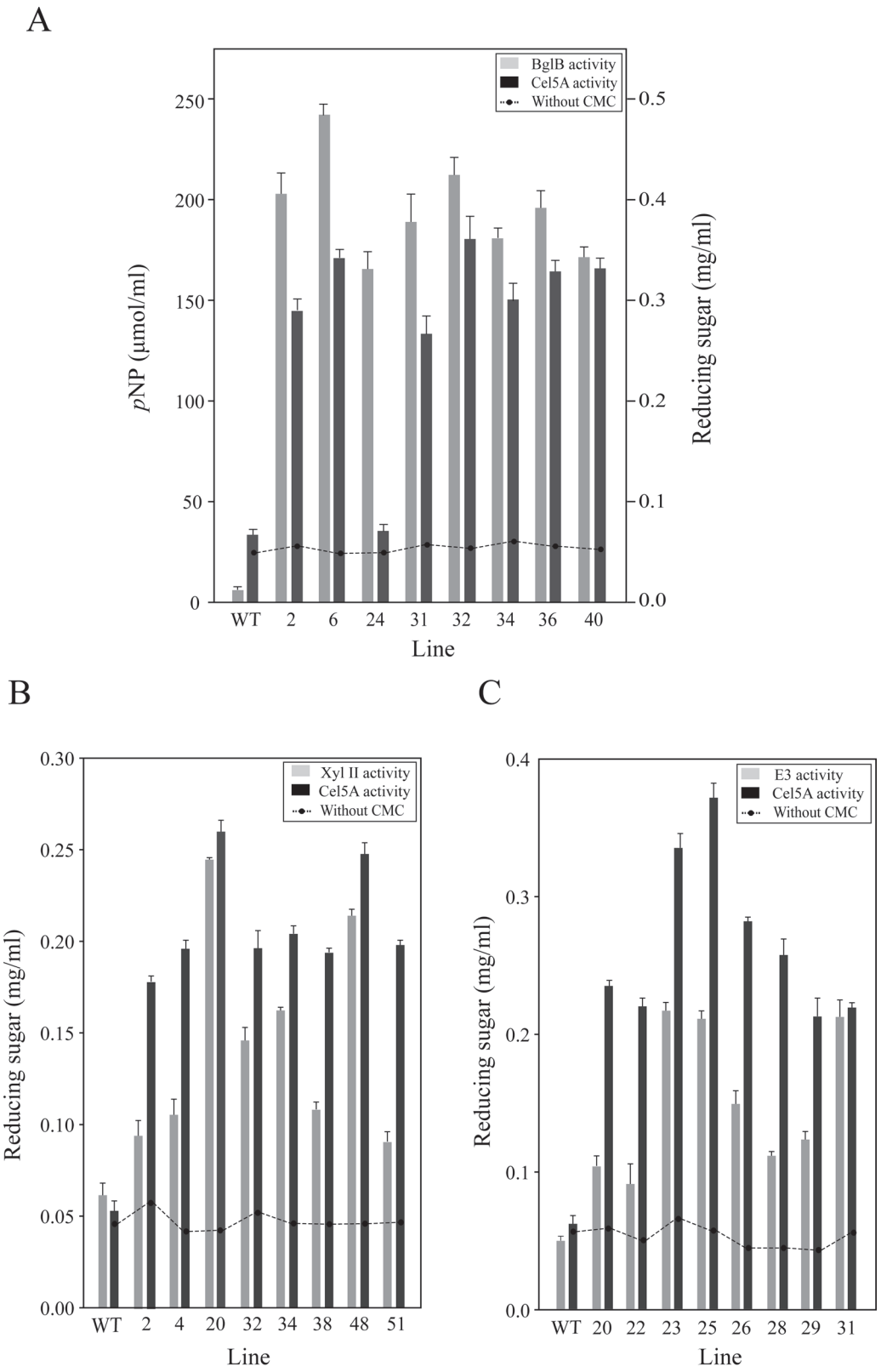

Fig. 5. Activity assays for endoglucanase (Cel5A) and β-glucosidase (BglB, A), xylanase (XylII, B), and exoglucanase (E3, C) extracted from leaves of the transgenic lines [RsBglB-2A-RaCel5A], [RsE3-2A-RaCel5A], and [RsXylII-2A-RaCel5A]. Crude extracts were incubated with *p*NPG for BglB, CMC for Cel5A, oat spelt xylan for XylII, and filter paper for E3. Controls denoted as dashed lines are the mean value of each crude extract that was incubated without substrate. WT, Wild-type plant.

partial transit peptide (25 amino acids, ~3 kDa) but remained in the mature form and was further degraded by a particular protease inside the chloroplasts.

The activity and accumulation of BglB and Cel5A with transit peptide in the chloroplasts of tobacco plants have been reported previously (Jung *et al.*, 2010; Kim *et al.*, 2010; Mahadevan

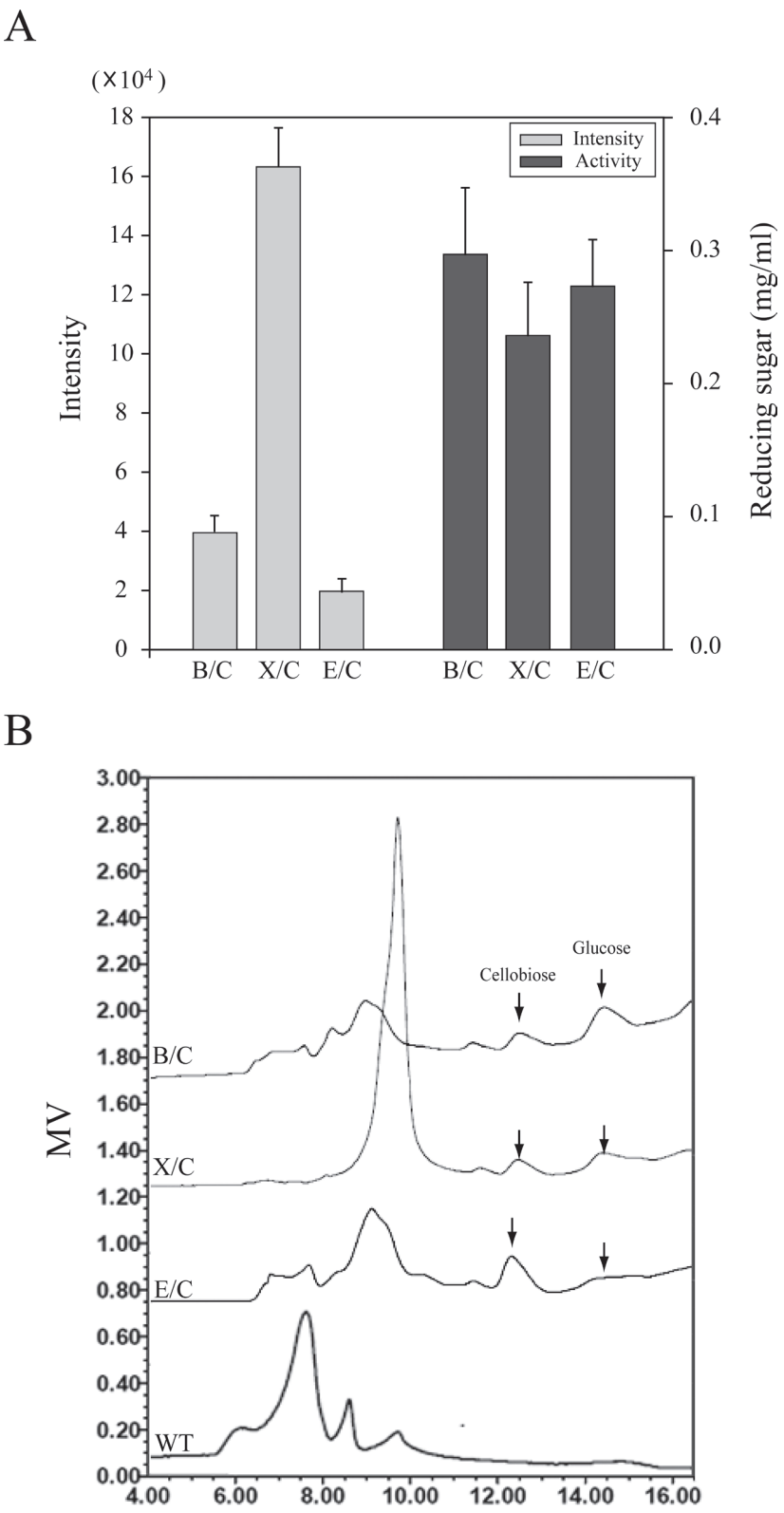

Fig. 6. Comparison of Cel5A expression levels with CMC degradation activity and HPLC analysis of reducing sugars produced by the coexpression enzymes. (A) Intensities of bands detected by Western blotting with Cel5A primary antibody measured using UTHSCSA Image Tool 2.00. The Western blot procedure was repeated five times during the growth period. (B) HPLC profiles of end products released by hydrolysis enzymes analysed after incubation with a crude extract (30 μg) of individual transgenic plants and CMC at optimum conditions for 1 hour. B/C, [RsBglB-2A-RaCel5A]; X/C, [RsXylII-2A-RaCel5A]; E/C, [RsE3-2A-RaCel5A]; WT, wild type.

et al., 2011), as has XylII accumulation via dual targeting to chloroplasts and peroxisomes in *A. thaliana* (Bae *et al.*, 2006). The current study successfully coexpressed the cellulase genes linked by the 2A self-cleavage sequence [RsBglB-2A-RaCel5A] and [RsXylII-2A-RaCel5A] and observed synergistic activity of the proteins expressed in the [RsBglB-2A-RaCel5A]

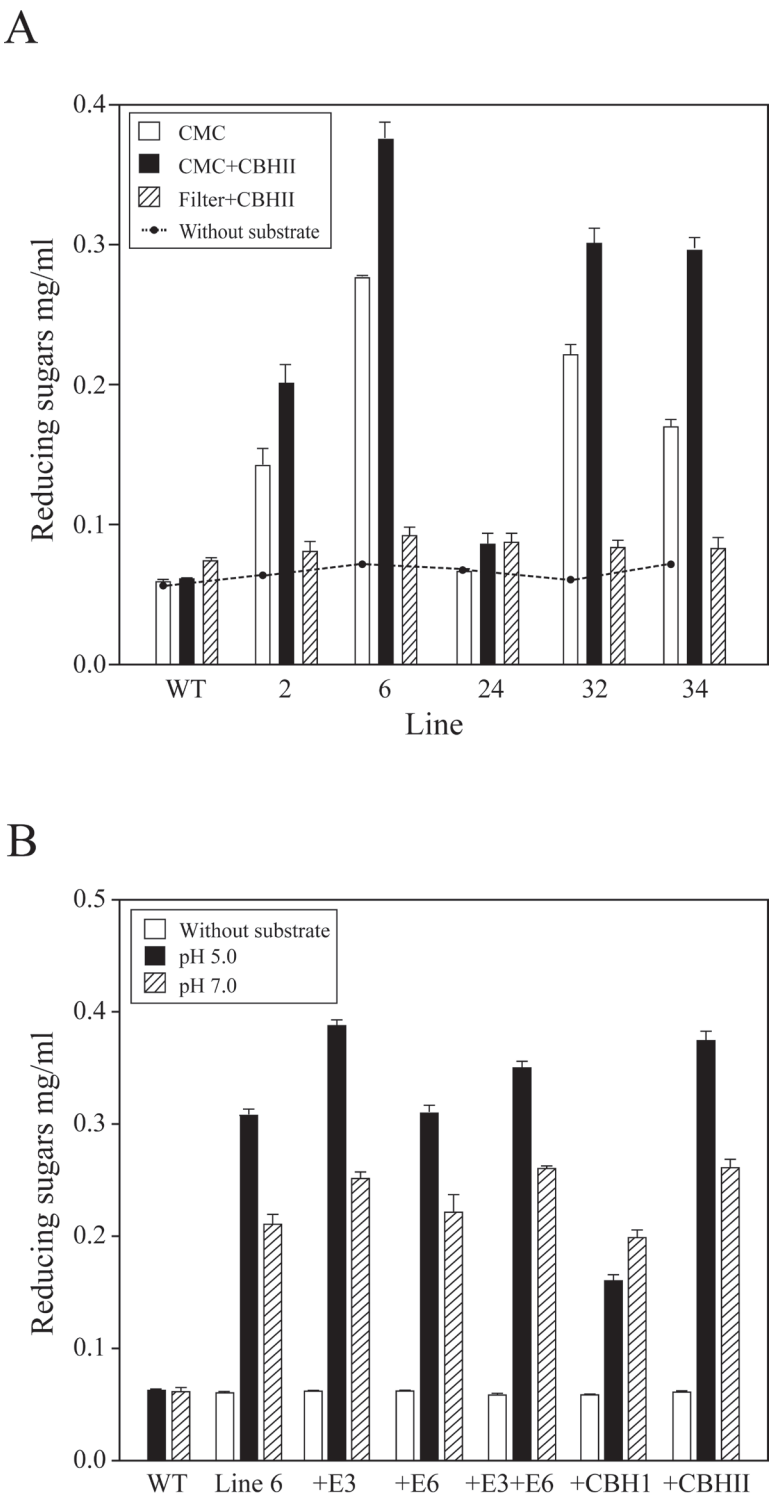

Fig. 7. Induction of synergistic effects by supplementing crude extracts from the transgenic line [RsBglB-2A-RaCel5A] with exoglucanases. (A) Crude extracts of [RsBglB-2A-RaCel5A] were incubated with CMC after adding CBHII: line 6 exhibited synergistic effects and the highest activity towards CMC. (B) Synergistic effects of various exoglucanases (E3, E6, CBHI, and CBHII) with line 6 crude extract were dependent on pH. Wild type is presented as a negative control for incubation under the same conditions.

lines and Cel5A that was ~8% of TSP in transgenic [RsXylII-2A-RaCel5A] line 20. However, the expression of exoglucanase in bacteria or plants is prone to difficulties, including a lack of expression or lower expression levels compared to the expression of endoglucanase and β-glucosidase because of gene silencing or unsuitable codon usage. This is particularly true of plants. The E3 expression level with localization in the cytosol ranges up to ~0.02% of TSP in tobacco plants and 0.001–0.002% in alfalfa (Ziegelhoffer *et al.*, 1999). However, maize has been used to optimize the first 40 amino acid codons of the CBH1 gene from *Trichoderma koningii*, which was expressed in transgenic seed at up to 3.2–9% of TSP (Hood *et al.*, 2007), but plants expressing exoglucanase have been rarely reported. Yasuda *et al.* (2005) reported that GLP-1, a

Table 2. Summary of hydrolysis enzyme properties

Enzyme	Origin	Family	M_r (kDa)	Temp (°C)	pH	Reference
TrXylII	*Trichoderma reesei*	GH 11	24.5	50	5.0	Wong and Saddler (1992) Tőrrőnen and Rouvinen (1995)
TmCel5A	*Thermotoga maritima*	GH 5	37.6	8 0	4.8	Pereira *et al.* (2010) Mahadevan *et al.* (2008)
TfE3	*Thermomonospora fusca*	GH 6	59.6	60	7.0	Zhang *et al.* (1995)
TfE6	*Thermomonospora fusca*	GH 48	104	40–50	6.0	Irwin *et al.* (2000)
AfCBHI	*Aspergillus flavus*	–	54.1	50	5.0	–
AfCBHII	*Aspergillus flavus*	–	49.6	50	5.0	–
TmBglB	*Thermotoga maritima*	GH 3	81.1	85	5.0	Goyal *et al.* (2001)

small glucagon-like peptide 1 with rice-optimized codons, as well as transcripts of endogenous *GluB1*, is not detected in transgenic rice plants due to siRNA-mediated gene silencing. However, the chimeric constructs GFP-mGLP-1 and GFP-2A-mGLP-1 were highly expressed in transgenic rice seeds because gene silencing was avoided. The current study successfully transformed the chimeric construct [RsE3-2A-RaCel5A] for exoglucanase expression into the genomic DNA of tobacco plants and detected Cel5A expression placed downstream of 2A. This result indicated that the E3 exoglucanase of [RsE3-2A-RaCel5A] was expressed in tobacco plants, although the expression level was slightly lower than that of *BglB*, as predicted, in accordance with the ratio between BglB and Cel5A in the [RsBglB-2A-RaCel5A] lines.

While comparing Cel5A expression and the activity of [RsBglB-2A-RaCel5A], [RsXylII-2A-RaCel5A], and [RsE3-2A-RaCel5A], this study found a synergistic effect between BglB and Cel5A in the [RsBglB-2A-RaCel5A] lines (Fig. 6 and Supplementary Fig. S3) and between E3 and Cel5A expressed in the [RsE3-2A-RaCel5A] lines. The enzymes had higher activities with CMC as the substrate, even though they were expressed 4–8-fold lower (even lower in some cases) than Cel5A in the transgenic line [RsXylII-2A-RaCel5A]. HPLC analysis of end products confirmed the synergistic effects of Cel5A and BglB, as well E3, which led to enhanced CMC hydrolysis into glucose and cellobiose.

Supplementing the protein extracts of transgenic [RsBglB-2A-RaCel5A] lines 2, 6, 32, and 34 with CBHII exoglucanase resulted in 35–74% increase in hydrolysis activity compared to the activity of each line without the CBHII supplement. In contrast, the synergistic effect did not occur in line 24, which showed only β-glucosidase activity due to the loss of the initial action of endoglucanase during CMC hydrolysis. Ng *et al.* (2011) reported that supplementing β-glucosidase from Taiwanese fungi into an enzyme mixture of *Trichoderma reesei*, which consisted of the main cellulase enzymes CBH1, CBHII, and EG2 (representing 60, 20, and 12% of the mixture, respectively), enhanced the efficiency of hydrolysis into fermentable sugars. Supplementation with a hemicellulase cocktail containing the side-chain degradation enzymes, endoglucanase, and β-glucosidase of a microbe-produced enzyme mixture of *Trichoderma reesei* or *Trichoderma viride* led to more efficient degradation of lignocellulosic biomass (Tabka *et al.*, 2006; Lee *et al.*, 2011). Therefore, 2A-mediated multiple enzyme expression could prove to be a powerful strategy in the enzymic hydrolysis of lignocellulosic biomass when supplemented with additional enzymes.

The preparation of enzyme cocktails containing several enzymes from different origins requires optimal conditions for their activity and synergy. As shown in Table 2, exoglucanases E3 and E6 exhibited optimal activity at pH 7.0 and 50 °C, BglB and Cel5A at pH 5.0 and 80 °C, and CBHI and CBHII at pH 5 and 50 °C. This study tested the activities of diverse combinations of the extract of the [RsBglB 2A RaCel5A] transgenic line 6 with CBHI, CBHII, E3, and E6 at pH 5 and 50 °C. As shown in Fig. 7B, synergistic effects with exoglucanases including E3 and CBHII were induced at pH 5.0 and 50 °C. Notably, E3 displayed higher synergistic activity than CBHII at pH 5.0 and 50 °C, whereas CBHI seemed to inhibit BglB and Cel5A activity. Supplemental enzyme selection can be considered to induce synergistic effects.

Low-cost enzyme production is important for bioethanol production to be economically competitive. Plant-based enzyme production may achieve this goal because of several advantages including lower scale-up cost. Enzyme production was studied in transgenic plants expressing [RsBglB-2A-RaCel5A], [RsXylII-2A-RaCel5A], and [RsE3-2A-RaCel5A], involving multiple enzyme expression systems linked to the 2A self-cleavage sequence, which produced 2A-mediated self-cleavage polyproteins with synergistic effects on substrate hydrolysis. The coexpression of lignocellulose hydrolysis enzymes linked to the 2A sequence in plants, consisting of debranching enzymes, as well as cellulase and xylanase, is a novel low-cost enzyme system that provides efficient hydrolysis of lignocellulosic materials, as does a plant-based supplemental enzyme system added to the enzyme mixture produced from fungi, such as *T. reesei.*

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Transient assay of [RsXylII-GFP] and [RaXylII-2A-GFP] in *Arabidopsis* protoplasts.

Supplementary Fig. S2. Western blotting analysis of Cel5A expressed in the [RsXylII-2A-RaCel5A] transgenic lines.

Supplementary Fig. S3. Synergistic effects of [RsBglB-2A-RaCel5A] transgenic line 24.

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