10056 J. Agric. Food Chem. 2010, 58, 10056–10063
DOI:10.1021/jf102284f

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Plant-Produced Trastuzumab Inhibits the Growth of HER2 Positive Cancer Cells

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To study the agricultural production of biosimilar antibodies, trastuzumab (Herceptin) was expressed in *Nicotiana benthamiana* using the magnICON viral-based transient expression system. Immunoblot analyses of crude plant extracts revealed that trastuzumab accumulates within plants mostly in the fully assembled tetrameric form. Purification of trastuzumab from *N. benthamiana* was achieved using a scheme that combined ammonium sulfate precipitation with affinity chromatography. Following purification, the specificity of the plant-produced trastuzumab for the HER2 receptor was compared with Herceptin and confirmed by western immunoblot. Functional assays revealed that plant-produced trastuzumab and Herceptin have similar in vitro antiproliferative effects on breast cancer cells that overexpress HER2. Results confirm that plants may be developed as an alternative to traditional antibody expression systems for the production of therapeutic mAbs.

KEYWORDS: Affinity purification; biosimilar; biopharming; HER2; plant-produced mAb; Herceptin; *Nicotiana benthamiana*; therapeutic mAbs; trastuzumab

INTRODUCTION

Antibody research over the past 30 years has led to the development of valuable biopharmaceuticals for the diagnosis and treatment of human disease (*1*). To date, the United States Food and Drug Administration (FDA) has approved 22 monoclonal antibodies (mAb) for clinical use, while hundreds of others are in clinical trials (*2*, *3*). Antibodies currently approved for clinical therapy have a wide range of applications, including the treatment of microbial infections, autoimmune diseases, and cancer (*4*, *5*). The advantage of using antibodies in therapeutic applications is their low toxicity and high specificity for a target antigen (*6*); however, to ensure the efficacy of some treatments, high antibody serum concentrations must be maintained over a period of several months (*7*). One treatment cycle for a single patient can require hundreds of milligrams to gram quantities of mAbs (*7*,*8*). Therapeutic mAbs are thus among the most lucrative products within the biopharmaceutical industry (*9*). From 2004 to 2006, market sales of the top five therapeutic mAbs (Rituxan, Remicade, Herceptin, Humira, and Avastin) increased from $6.4 billion to $11.7 billion (*3*). During 2010, the market value of these antibodies is predicted to rise to over $30 billion (*6*). In the past, such high market demands for biopharmaceuticals have led to a manufacturing bottleneck (*9*).

Therapeutic mAbs have traditionally been produced in mammalian cell systems; however, these systems are associated with high production costs and are hindered by time-consuming culturing processes (*10*, *11*). In an attempt to meet rising market demands, pharmaceutical companies are working to improve the efficiency of existing biopharmaceutical production systems (*9*,*10*) and increase the number of antibody production facilities (*9*). Following construction, these facilities must be validated under Good Manufacturing Practice (GMP), a process that can take an average of three years (*12*). Although some improvements have been made to increase antibody production, pharmaceutical companies still may not be able to meet future demands. As a result, alternative expression systems that would allow the production of biosimilar antibodies (follow-on biopharmaceuticals that have been proven to be similar to innovator drugs) are also being investigated (*9*, *10*, *13*, *14*).

Agricultural production of therapeutic proteins (biopharming) is one alternative to traditional mammalian cell expression systems for the large-scale production of therapeutic mAbs. In comparison to mammalian systems, plant bioreactors offer many advantages for the pharmaceutical industry, including lower upstream production costs, speed of manufacturing, indefinite scalability, and ease of handling (*15*, *16*). Plants also offer the advantage of biological safety, as there is no health risk from contamination with zoonotic pathogens and toxins (*17*,*18*). Plant biopharming is also beneficial for the agricultural industry since biopharmed crops can be maintained and harvested using current agricultural practices (*19*), and thus provide valuable new markets for farmers (*20*). Conversely, the limitations of plant bioreactors include higher downstream processing and purification costs, and the addition of plant-specific *N*-glycans to the recombinant antibodies. Full-length recombinant antibodies were first

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 Published on Web 08/27/2010

successfully expressed in tobacco plants in 1989 (*21*). Since then, a wide variety of transgenic plant hosts have been successfully used for recombinant antibody production (*22*), including plants that have been genetically modified to express recombinant antibodies with humanized *N*-glycan profiles (*23*). The expression of antibodies in plants has also been achieved using different expression platforms, including both stable and transient plant transformation technologies (*6*).

To achieve regulatory affirmation of plant-produced biosimilar therapeutics, researchers must be able to demonstrate that plant-produced antibodies maintain the identical structural and functional integrity as their mammalian counterparts (*5*). Plant-produced antibody preparations must also be analyzed to ensure that they are homogeneous, not adversely immunogenic, and devoid of significant contaminants (*5*). No study has been conducted to date to compare a plant-produced antibody with a clinically approved therapeutic mAb.

Trastuzumab (Herceptin, Genentech Inc., San Francisco, CA) is a humanized murine immunoglobulin G1κ antibody that is used in the treatment of metastatic breast cancer. Trastuzumab binds to the extracellular domain of human epidermal growth factor receptor 2 (HER2), a member of the ErbB family of transmembrane tyrosine kinase receptors, that is overexpressed in 20−30% of metastatic breast cancer patients (*24*−*26*). Under normal cell conditions, HER2 is directly involved in the activation of signaling pathways that mediate cell growth and differentiation (*24*, *27*, *28*). Overexpression of HER2 results in the disruption of normal signaling pathways, causing the loss of cell growth regulation and the development of resistance to apoptosis (*29*, *30*). By targeting cells that overexpress HER2, trastuzumab mediates the arrest of cell proliferation and the lysis of cancer cells by antibody-dependent cellular cytotoxicity (ADCC) (*27*, *31*, *32*). In treatment, patients with HER2-overexpressing metastatic breast cancer are administered a loading dose of 4 mg of trastuzumab/kg followed by a weekly maintenance dose of 2 mg/kg (*33*). Upon the basis of market demand, treatment of human metastatic breast cancer with trastuzumab thus requires kilogram quantities of this biopharmaceutical. An alternative expression system may therefore be required to supply future worldwide need for therapeutic antibodies such as trastuzumab.

Genetically modified plants have proven successful for the large-scale production of mAbs; however, no study has yet been conducted to characterize and compare a plant-produced antibody having a primary structure identical to a clinically approved therapeutic mAb. In this study, trastuzumab was expressed in *N. benthamiana*, a relative of tobacco, using a viral-based transient expression system (*34*). Trastuzumab expression in *N. benthamiana* plants was quantified, and plant-purified trastuzumab was characterized in comparison to Herceptin. Plant-produced and commercial trastuzumab were found to bind the same ligand and have similar *in vitro* antiproliferative effects on breast cancer cells that overexpress HER2. These results indicate that agricultural biopharming could be developed for effective use as an alternative to mammalian cell systems for the large-scale production of biosimilar antibodies.

MATERIALS AND METHODS

Cell Lines and Plasmids. All cloning procedures were performed using Top10 F′ *Escherichia coli* cells (Invitrogen, Burlington, Canada). Plasmids used in the magnICON viral-based transient expression system (pICH21595, pICH25433 pICH20111, pICH24180, and pICH14011) were obtained from Icon Genetics GmbH (Halle, Germany (*34*)). All mammary adenocarcinoma cell lines (MCF-7, SK-BR-3, and BT-474) were obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured according to ATCC specifications unless stated otherwise.

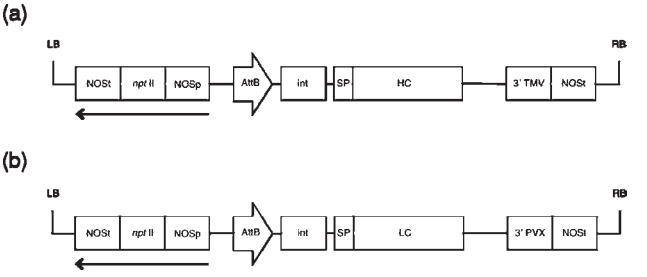

Figure 1. Schematic diagram of the constructs for the expression of trastuzumab in *N. benthamiana*; pTrasHC (**A**) and pTrasLC (**B**). Both expression constructs contain the *npt* II gene under the control of the nopaline synthase promoter (NOSp). NOSt, nopaline synthase terminator; LB and RB, left and right borders, respectively; AttB, recombination site; int, intron; SP, *Arabidopsis* basic Chitinase signal peptide; HC, coding sequence of the heavy chain of trastuzumab; LC, coding sequence of the light chain of trastuzumab; 3′TMV, 3′ untranslated region; 3′PVX, 3′ untranslated region.

Vector Construction and Plant Infiltration. The variable coding regions of the heavy (V_H) and light (V_L) chains of trastuzumab (*35*) were synthesized as gene segments by the PBI/NRC DNA/Peptide Synthesis Laboratory of the National Research Council of Canada (Saskatoon, Canada), both incorporating preferred plant codons (*36*−*38*), a 24 amino acid N-terminal murine SP (GenBank accession no. AAA38889.1) and 5′ *Xba*I and 3′ *Not*I restriction sites. The complete heavy chain coding sequence was assembled by subcloning murine SP-V_H into the *Xba*I/*Not*I sites of pMM29 (*37*), a plasmid containing the tobacco optimized coding region of a human gamma-1 heavy chain constant (C_H) domain fused to a six-Histidine and a KDEL tag. Three amino acids (Asp_{359}, Leu_{361}, and Lys_{450}) differed between the human gamma-1 heavy chain constant domain and the heavy chain of trastuzumab. Site directed mutagenesis was used to change Asp_{359} to Glu and Leu_{361} to Met and to remove the *Not*I site. The complete heavy chain coding sequence including murine SP was then amplified by PCR to remove Lys_{450}, the six-Histidine, and KDEL C-terminal tags using primers that contained *Bsa*I sites and was subcloned into pICH21595 ((*34*) Icon Genetics) to generate pMTrasHC. The complete light chain coding sequence was assembled by subcloning murine SP-V_L into the *Xba*I/*Not*I sites of pMM29 (*37*). The *Not*I site was removed by site directed mutagenesis, and the complete light chain coding sequence including murine SP was PCR amplified using primers containing *Bsa*I sites and subcloned into pICH25433 ((*34*) Icon Genetics) to generate pMTrasLC. The *Arabidopsis* basic Chitinase SP (*39*) later replaced the murine SP in both pMTrasHC and pMTrasLC, generating pTrasHC and pTrasLC, respectively (**Figure 1**). All primers used for the development of pTrasHC and pTrasLC are listed in **Tables 1** and **2**, respectively.

The TMV-based 5′ module (pICH20111), PVX-based 5′ module (pICH24180), and integrase (pICH14011) vectors ((*34*) Icon Genetics) were unaltered. All five plasmids (pICH14011, pICH20111, pICH24180, pTrasHC, and pTrasLC) were introduced into the *Agrobacterium tumefasciens* strain At542 by electroporation. *N. benthamiana* plants were vacuum infiltrated according to the protocol described in ref *40* with several modifications. Briefly, all cultures were grown at 28 °C and 220 rpm to a final optical density at 600 nm (OD_{600}) of 1.8. Equal volumes were combined and pelleted by centrifugation at 8,000 rpm for 4 min, resuspended, and diluted by 10^3 in infiltration buffer (10 mM 1-(*N*-morpholino)ethanesulphonic acid (MES) at pH 5.5 and 10 mM $MgSO_4$). The aerial parts of six-week-old *N. benthamiana* plants were submerged in a desiccator containing the *A. tumefaciens* resuspension solution under vacuum (0.5 to 0.9 bar) for 90 s followed by a slow release of the vacuum, after which plants were returned to the greenhouse for 8 days before being harvested.

SDS−PAGE and Western Blot Analyses. Fresh leaf biomass from three *N. benthamiana* plants was harvested 8 days post-infiltration (d.p.i), ground separately under liquid nitrogen, and combined with two volumes of cold extraction buffer [40 mM phosphate buffer, 50 mM ascorbic acid, and 10 mM ethylenediaminetetraacetic acid (EDTA) disodium salt

Table 1. Nucleotide Sequences of the Primers Used in the Construction of pTrasHC

name	type	nucleotide sequence
		Removal of the *Not*I Site
TrasHC-NotI	forward	5′-GTGACAGTATCAAGTGCTTCCACCAAGGGACCAAGC-3′
	reverse	5′-GCTTGGTCCCTTGGTGGAAGCACTTGATACTGTCAC-3′
		Amino Acid Modification (Asp_{359} → Glu; Leu_{361} → Met)
TrasHC-2AA	forward	5′-CACTTCCACCTTCTAGGGAAGAAATGACAAAGAACCAAGTG AGCC-3′
	reverse	5′-GGCTCACTTGGTTCTTTGTCATTTCTTCCCTAGAAGGTGGAA GTG-3′
		Subcloning into pICH21595 (Addition of *Arabidopsis* Basic Chitinase SP and Removal of Lys_{450}, 6xHis, and KDEL Tags)
TrasHC-S	forward	5′-TTTGGTCTCAAGGTATGGCTAAAACAAATCTCTTTTTATTCTT GATTTTCTCC-CTTTTACTTTCCTTAAGCTCAGCGGAAGTTCAACT TGTTGAGAGTG-3′
	reverse	5′-TTTGGTCTCAAAGCTCATTATCCTGGGCTAAGGCTAAG-3′

Table 2. Nucleotide Sequences of the Primers Used in the Construction of pTrasLC

name	type	nucleotide sequence
		Removal of the *Not*I Site
TrasLC-NotI	forward	5′-CAAAGTTGAGATCAAGAGGACCGTGGCTGCACCAAG-3′
	reverse	5′-CTTGGTGCAGCCACGGTCCTCTTGATCTCAACTTTG-3′
		Subcloning into pICH25433 (Addition of *Arabidopsis* Basic Chitinase SP)
TrasLC-S	forward	5′-TTTGGTCTCAAGGTATGGCTAAAACAAATCTCTTTTTATTCTT GATTTTCTCCC-TTTTACTTTCCTTAAGCTCAGCGGACATTCAAAT GACTCAATCCC-3′
	reverse	5′-TTTGGTCTCAAAGCTCATTAACACTCTCCTCTATTGA-3′

dihydrate, pH 7.0]. Crude extracts were clarified by centrifugation at 10,000 rpm for 30 min and then 5,000 rpm for 10 min at 4 °C. Total soluble protein (TSP) concentration was determined using the Bio-Rad Protein Assay (Mississauga, Canada). Bovine serum albumin (BSA; Thermo Scientific, Nepean, Canada) was used as the protein standard. Western immunoblots were performed as described (*36*), using a mixture of goat antihuman IgG γ- and κ-chain specific probes conjugated to alkaline phosphatase (Sigma-Aldrich, Oakville, Canada), diluted to 1:2500 in phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 (PBST).

Quantitative ELISA. Ninety-six-well microtiter plates (High-binding; Corning Inc. Life Sciences, Lowell, MA) were coated overnight at 4 °C with 0.3125 μg/mL of mouse antihuman IgG γ-chain specific antibody (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) at pH 7.4. Plates were blocked with 4% (w/v) skim milk (EMD Biosciences, Newark, NJ) dissolved in PBS for 24 h at 4 °C and then washed five times with PBST. Serial dilutions of clarified extract from *N. benthamiana* plants expressing trastuzumab were added to the plate, which was incubated at 37 °C for 1 h. Serial dilutions of human myeloma IgG1 (Athens Research & Technology Inc., Athens, GA), which is of the same antibody isotype as Herceptin, were used as a quantification standard with 10 μg of TSP from untreated *N. benthamiana* plants. The plate was washed five times with PBST before adding polyclonal rabbit antihuman IgG (H + L)-horseradish peroxidase (HRP) conjugate (Abcam, Cambridge, MA), diluted to 1 μg/mL in PBS, for 1 h at 37 °C. The plate was washed five times with PBST before development with 1-Step Turbo TMB-ELISA (Thermo Scientific). Color development was stopped with 1.8 M sulfuric acid, and optical densities were measured at 450 nm using an EnVision 2100 Multilabel microtiter plate reader (Perkin-Elmer, Woodbridge, Canada). Quantitative ELISAs to determine the expression of antibody in plants were performed in triplicate. Three independent expression experiments were performed, involving a total of 11 plants. Overall expression of the antibody is reported as the average ± standard error of the mean for all 11 plants.

Antibody Purification. Infiltrated *N. benthamiana* leaf tissue was harvested 8 d.p.i and stored at −80 °C. Frozen leaf tissue (250 g) was combined with two volumes (500 mL) of cold extraction buffer in a food processor (Morphy Richards Inc., Mexborough, South Yorkshire, United Kingdom) and disrupted for three 30 s pulses. Disrupted tissue was collected and homogenized further using a benchtop Polytron homogenizer (PT10/35, Kinematica Inc., Bohemia, NY). Large plant debris was removed from the homogenate by dead-end filtration through miracloth (Calbiochem, San Diego, CA). Ammonium sulfate was slowly added to the filtered homogenate to a final concentration of 20%. The plant homogenate was then incubated at 4 °C for 1 h with gentle stirring. Insoluble material was pelleted by centrifugation at 10,000 rpm for 30 min at 4 °C and the resulting supernatant collected. The concentration of ammonium sulfate in the resulting supernatant was subsequently increased to 60%, incubated at 4 °C for 2 hs with gentle stirring, and centrifuged at 10,000 rpm for 30 min at 4 °C. Pelleted protein was resuspended in 250 mL of 20 mM sodium phosphate, pH 7.0, and then passed through a series of filters (2.7 μm glass microfiber (GF/D), 1.2 μm glass microfiber (GF/C), 0.8 μm cellulose acetate, 0.45 μm cellulose acetate; Whatman, Piscataway, NJ). The protein solution was dialyzed and concentrated in a 250-mL Amicon ultrafiltration stirred cell (Millipore, Billerica, MA) fitted with a molecular cutoff membrane of 30 kDa (Millipore), then applied (4 mL/min) to a chromatography column (ID = 2.5 cm; Bio-Rad) containing 10 mL of protein G Sepharose 4 Fast Flow affinity media (GE Healthcare, Baie d′Urfe, Canada) pre-equilibrated with 20 mM phosphate buffer at pH 7.0. A series of washings were performed with 20 mM phosphate buffer, pH 7.0, to ensure the removal of all contaminating solutes from the protein G column. The antibody was eluted from the column with 0.1 M glycine, pH 2.2, and immediately buffered with 1 M Tris·Cl, pH 9.0. The buffered eluate was subsequently applied (2.5 mL/min) to a protein A affinity column (5 mL HiTrap protein A HP column, GE Healthcare) connected to an AKTA-FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden). To ensure the removal of all contaminating solutes from the protein A column, a series of washings were performed with 20 mM phosphate buffer, pH 7.0. The antibody was eluted from the column with 0.1 M glycine, pH 2.2, and immediately buffered with 1 M Tris·Cl, pH 9.0. The antibody eluate was dialyzed against 20 mM phosphate buffer, pH 7.0, and concentrated using polyethylene glycol 35,000. Coomassie-stained SDS−PAGE gels and western immunoblots were used to analyze the purity and structural integrity of plant-produced trastuzumab.

N-Terminal Sequence Analysis. Plant-purified trastuzumab (3 μg) was separated by reducing 12% SDS−PAGE and then transferred to a Sequi-Blot PVDF membrane (Bio-Rad) which was treated with Coomassie blue G-250. N-terminal sequencing analysis (Edman degradation) was performed at the Hospital for Sick Children's Research Institute (The Advanced Protein Technology Centre, University of Toronto, Canada).

Cell Culture. MCF-7, SK-BR-3, and BT-474 cell lysates were prepared from cell lines grown to 95% confluence. Cells were treated with a 1× trypsin-EDTA solution (0.25% trypsin, 0.1% EDTA; SAFC Biosciences, Lenexa, KS) for dissociation from the cell culture flasks, washed twice with ice-cold PBS, and lysed with NP40 cell lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, and 0.02% NaN_3; Invitrogen) supplemented with 1 mM phenylmethanesulfonyl fluoride solution (PMSF; Sigma Aldrich) and 10% protease inhibitor cocktail (4-[2-aminoethyl]benzenesulfonyl fluoride, *N*-[*trans*-epoxysuccinyl]-L-leucine 4-guanidinobutylamide, bestatin hydrochloride, leupeptin hemisulfate salt, aprotinin, and sodium EDTA; Sigma-Aldrich). TSP concentration was determined for each lysate using the BCA Protein Assay (Thermo Scientific). Through western immunoblot analysis, HER2 was detected in the cell lysate preparations using 0.1 μg/mL of either Herceptin or plant-produced trastuzumab in PBST. Antibody samples were detected using a mixture of goat antihuman IgG γ- and κ-chain specific probes conjugated to alkaline phosphatase (Sigma-Aldrich), diluted to 1:2500 in PBST.

Cell Proliferation Assay. MCF-7 and SK-BR-3 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg/mL fungizone, 1% penicillin/streptomycin (all from Invitrogen), and 10% fetal bovine serum (FBS; Sigma-Aldrich). The BT-474 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 basal medium (Invitrogen) supplemented with 1 mg/mL fungizone, 1% penicillin/streptomycin, and 10% FBS. SK-BR-3, BT-474, and MCF7 cells were seeded into 6-well plates (Corning, Lowell, MA) (5×10^4 cells/well). After allowing the cells to adhere, the cells were treated with 2 μg/mL of nonspecific plant-purified human IgG1 (negative control; human myeloma IgG1 [Athens Research and Technology] was spiked into untreated *N. benthamiana* plant extract and subsequently purified using the same scheme developed for the purification plant-produced trastuzumab), 2 μg/mL of plant-produced trastuzumab, or 2 μg/mL of Herceptin; untreated cells were also included as a control. Relative cell proliferation was determined by viable cell counts using trypan blue stain (Invitrogen). Cell counts were performed every two days for a total of eight days. Data are expressed as a percentage of the untreated control.

RESULTS

Accumulation of Trastuzumab in *N. benthamiana* Plants. Trastuzumab was expressed in *N. benthamiana* plants using the magnICON viral-based transient expression system (*34*). Six-week old *N. benthamiana* plants were vacuum-infiltrated with *A. tumefaciens* clones transformed with provectors containing the HC- and LC-coding sequences of trastuzumab. Results of preliminary experiments determined that the murine signal peptide (SP) did not allow much accumulation of trastuzumab; therefore, the murine SP was replaced by the *Arabidopsis* basic Chitinase SP on both HC- and LC-expression constructs. The assembly of trastuzumab with the *Arabidopsis* SP-containing constructs was examined 8 d.p.i on a nonreducing western immunoblot treated with a mixture of antihuman IgG γ- and κ-chain specific probes. As shown in **Figure 2**, the tetrameric form of the antibody (H_2L_2) was the most prominent band. Trastuzumab expression was also determined by nonreducing immunoblot and confirmed by quantitative ELISA through comparison with known concentrations of a human IgG1 standard. Plants expressed an average of 43.3 ± 4.7 mg of trastuzumab per kilogram of fresh leaf tissue (0.59 ± 0.08% total soluble protein; TSP).

Purification and Characterization of Plant-Produced Trastuzumab. A purification scheme was developed to facilitate the recovery of trastuzumab from *N. benthamiana* plants. Primary plant extracts were treated with 20% ammonium sulfate to remove high molecular weight contaminants, followed by 60% ammonium sulfate to enrich antibody yield through precipitation. Trastuzumab was subsequently purified by both protein G and then protein A affinity chromatography. Purified plant-produced trastuzumab was compared with Herceptin using

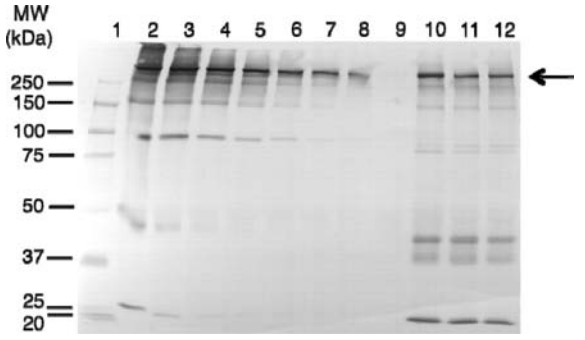

Figure 2. Quantification of trastuzumab expression in *N. benthamiana*. Trastuzumab was expressed in *N. benthamiana* using a viral-based transient expression system. Crude plant extracts were analyzed on a nonreducing immunoblot probed with a mixture of antihuman IgG γ- and κ-chain specific probes. Lane 1, protein molecular weight standard; lanes 2−8, human IgG1, 1000, 500, 250, 125, 62.5, 31.3, 15.1 ng, respectively, + 10 μg of total soluble protein (TSP) from untreated *N. benthamiana*; lane 9, 10 μg of TSP from untreated *N. benthamiana*; lane 10−12, 10 μg of TSP from three replicate *N. benthamiana* plants expressing trastuzumab. Molecular weights of protein standards are indicated on the left. The tetrameric (H_2L_2) form of plant-produced trastuzumab is indicated by the arrow on the right.

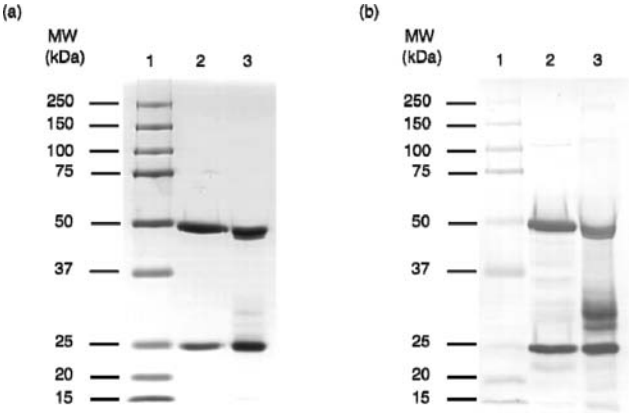

Figure 3. Analysis of the purity of plant-produced trastuzumab. Reducing, Coomassie stained SDS−PAGE (**A**) and immunoblot (**B**). Lane 1, protein molecular weight standard; lane 2, Herceptin, 1.2 μg; lane 3, plant-produced trastuzumab, 1.2 μg. The immunoblot was probed with a mixture of antihuman IgG γ- and κ-chain specific probes. Molecular weights of protein standards are indicated on the left.

SDS−PAGE under reducing conditions followed by staining with Coomassie blue. As seen in **Figure 3A**, the two major bands observed at ca. 50 kDa and 25 kDa are the heavy and light chains of trastuzumab, respectively. The heavy chain of plant-produced trastuzumab migrated slightly faster than the heavy chain of Herceptin, likely due to the differences between plant and mammalian post-translational glycosylation. As expected, there were no detectable differences in the electrophoretic mobilities of the light chains of Herceptin and plant-produced trastuzumab. In addition to the bands representing the heavy and light chains of trastuzumab, two less prominent bands were observed that migrated between the 25 and 37 kDa markers; these bands were enhanced by immunoblotting (**Figure 3B**). A series of nonreducing SDS−PAGE gels and immunoblots probed with γ- or κ-chain specific probes revealed that these were heavy chain degradation products (not shown), likely produced by protein degradation in planta since the addition of a protease inhibitor cocktail to the

extraction buffer had no effect on the antibody banding pattern in crude plant extracts (not shown).

N-terminal sequencing by Edman degradation indicated 100% cleavage of the *Arabidopsis* SP from both the heavy- and light-chains of the plant-produced trastuzumab. The N-termini of both the HC and LC polypeptides of plant-producted trastuzumab (Glu-Val-Gln-Leu-Val-Glu and Asp-Ile-Gln-Met-Thr-Gln, respectively) are identical to those of Herceptin (Drug bank accession # BTD00098). This result, in combination with the similarity in sizes between the HC and LC of plant-produced trastuzumab and Herceptin, strongly suggests that both mAbs have identical primary structures.

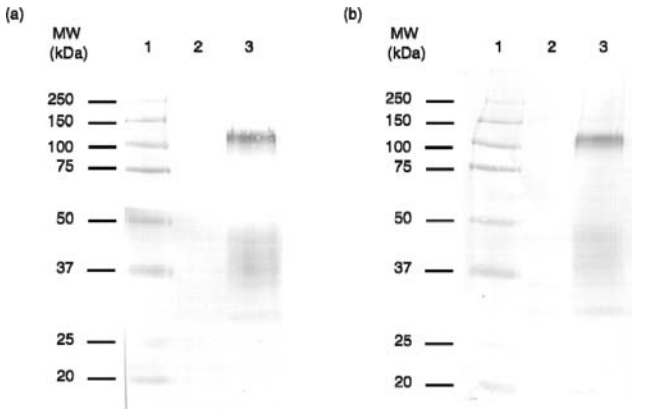

Figure 4. Qualitative analysis of the binding of plant-produced trastuzumab to HER2 ligand. MCF-7 and BT-474 cell lysates were analyzed by nonreducing immunoblots probed with Herceptin (**A**) or plant-produced trastuzumab (**B**). Lane 1, protein standard; lanes 2−3, 25 μg of TSP of the MCF-7 and the BT-474 cell lysates, respectively.

Specificity of Plant-Produced Trastuzumab. Qualitative binding analyses were performed to demonstrate the specificity of plant-produced trastuzumab for HER2. MCF-7 and BT-474 cell lysates were resolved on a western immunoblot that was subsequently probed with either plant-produced trastuzumab or Herceptin. One major band was observed between 100 and 150 kDa on both of the immunoblots probed with either mAb (**Figure 4**). The single band on both immunoblots corresponds to HER2 from the BT-474 cell lysates. A large smear and a less prominent band between 25 and 50 kDa were also observed on both immunoblots and likely represent artifacts of the cell lysate preparation procedure. No bands were observed in the lane containing the MCF-7 cells lysates, as this cell line does not overexpress HER2.

Inhibition of Tumor Cell Proliferation. The effect of plant-produced trastuzumab on the growth of breast tumor cells that overexpress HER2 was examined using a cell proliferation assay. Both HER2 overexpressing tumor cells (BT-474 and SK-BR-3) and normal HER2 expressing tumor cells (MCF-7) were treated with Herceptin or plant-produced trastuzumab. After 8 days, both plant-produced trastuzumab and Herceptin showed 52.5%

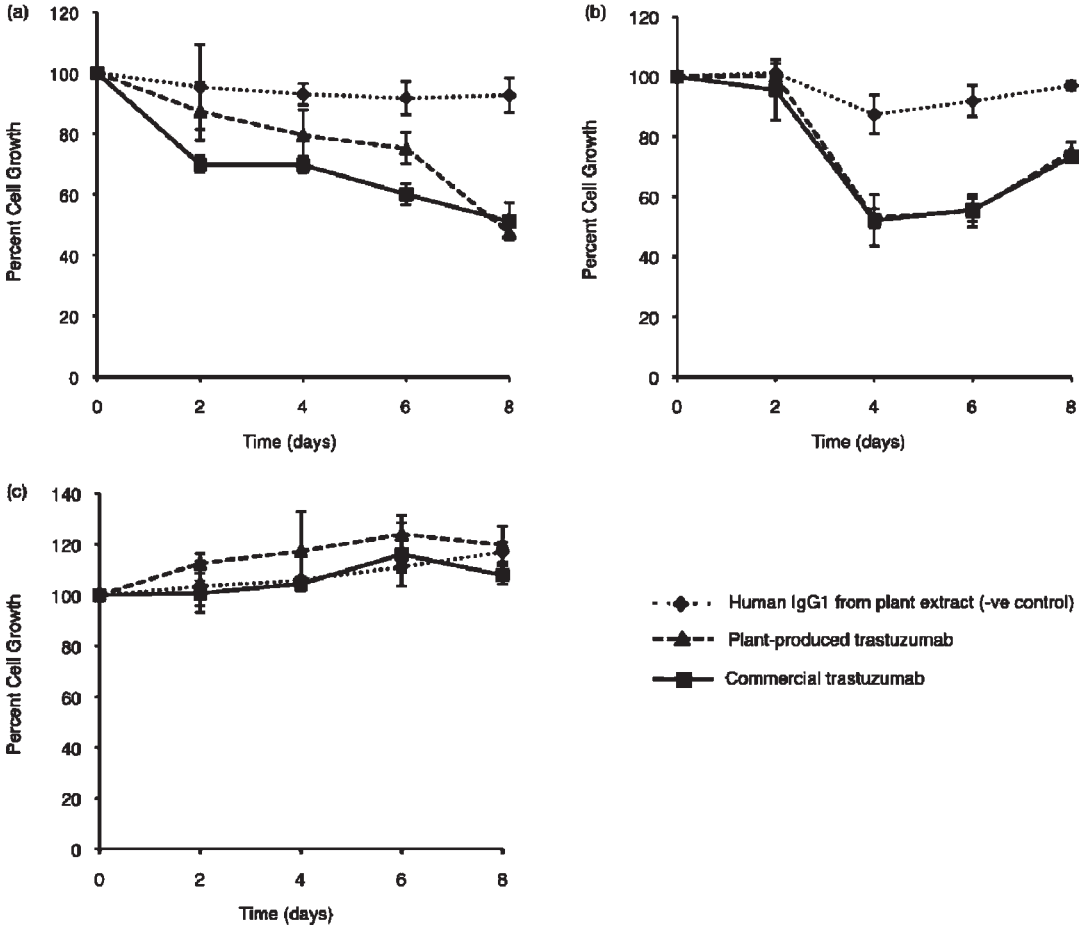

Figure 5. Effect of plant-produced trastuzumab on the proliferation of human breast tumor cells that overexpress HER2. BT-474 (**A**), SK-BR-3 (**B**), and MCF7 (**C**) cells were seeded into 6-well plates (5×10^4 cells/well) and treated with 2 μg/mL of nonspecific plant-purified human IgG1 (negative control), 2 μg/mL of plant-produced trastuzumab, or 2 μg/mL of Herceptin. Cell counts were performed every two days to determine the relative cell proliferation. Data are expressed as a percentage of untreated control and are presented as the means of triplicates ± SEM.

and 48.8% inhibition of BT-474 cell proliferation, respectively (**Figure 5A**). After 4 days, plant-produced trastuzumab and Herceptin showed 47.1% and 47.8% inhibition of SK-BR-3 cell proliferation, respectively (**Figure 5B**). The growth of SK-BR-3 cells, but not BT-474 cells, rose after 6 days of treatment with both plant-purified trastuzumab and Herceptin (**Figure 5B**). This could be explained by the fact that SK-BR-3 cells have approximately two times more HER2 on their cell surfaces than BT-474 cells (*41*). As shown in **Figure 5C**, plant-produced trastuzumab and Herceptin had no antiproliferative effect on MCF-7 cells. Thus, plant-produced trastuzumab selectively inhibits the proliferation of both BT-474 and SK-BR-3 cells. As a negative control, all breast tumor cell lines were also treated with a nonspecific plant-purified human IgG1. This nonspecific plant-purified antibody had no effect on the breast tumor cell proliferation, which demonstrates the absence of plant contaminants that could inhibit the proliferation of breast tumor cells.

DISCUSSION

Numerous researchers have shown that plant-produced mAbs retain biological activities (i.e., specificity, cytotoxicity, and neutralization activity) that are similar to parental mAbs produced in mammalian cell culture (reviewed in refs *22* and *42*); however, no study has yet been conducted to characterize and compare a plant-produced mAb to a clinically approved therapeutic antibody with the identical primary structure. TeraCIM, an antiepidermal growth factor receptor (EGF-R) antibody with conditional registry approval in Cuba, is a clinically approved mAb that has also been produced in plants (*43*). Although it was determined that the plant-produced antibody and TeraCIM have binding similar to those of A431 human-tumor-culture cells, the plant-produced antibody was modified to remove glycosylation sites and to add a KDEL ER-retention signal (*43*). Another plant-produced antibody, anti-HIV mAb 2G12, will soon enter human clinical trials (*44*, *45*), but its parent antibody has not yet had clinical approval.

Our research on the expression and purification of the anti-breast cancer antibody trastuzumab contributes further evidence that plants can be used for the production of biosimilar therapeutic mAbs, as we were able to produce trastuzumab in *N. benthamiana* with identical primary structures to those of its mammalian cell-derived counterpart. Although analysis of our primary plant extracts revealed that *N. benthamiana* plants express an average of 43.3 ± 4.7 mg of trastuzumab per kg of fresh weight (0.59 ± 0.08% TSP), optimization of this expression system should allow the production of 500 mg to 5 g per kg fresh weight (*34*, *46*). Antibody expression levels increase in a time-dependent manner, but antibody stability can also decrease over time; heavy- and light-chain polypeptide expression levels increase until 10−11 d.p.i., and antibody stability begins to decrease at 8−9 d.p.i (*34*). We chose to harvest plants 8 d.p.i. in order to obtain the most full-length IgG with the lowest amount of antibody fragments/breakdown products.

A purification scheme was developed to facilitate the recovery of trastuzumab from plants. Although analysis of plant-purified trastuzumab revealed the presence of heavy chain degradation products, these products likely result from proteolytic degradation in planta (supported by the research in ref *47*). These impurities could thus be removed using an additional chromatography step such as gel filtration or ion exchange chromatography. Most importantly, plant-produced trastuzumab was found to have specificity similar to that of Herceptin for binding to HER2 and was determined to be as effective as Herceptin in inhibiting the growth of cells overexpressing HER2.

One of the remaining major limitations of producing therapeutic mAbs in plants is the addition plant-specific *N*-glycans, which may induce an immune response in human treatment, especially with repeated immunotherapy (*48*−*50*). Antibody glycosylation is also essential for structural stability, decreased protease sensitivity, complement activation, and effector function (*37*, *48*). Several strategies are currently being developed to generate genetically modified plants with humanized *N*-glycan profiles (*12*, *51*, *52*). These strategies include knocking out the endogenous glycosyltransferases responsible for the addition of the plant-specific *N*-glycans β1,2-xylose and α1,3-fucose (*51*, *52*) as well as the expression of β1,4-galactosyltransferase (GalT) for the addition of terminal β1,4-galactose (*12*, *53*, *54*). Future research on plant-produced trastuzumab will require either the examination of the effect of plant-specific *N*-glycans on its therapeutic efficacy in vivo as well as its potential immunogenicity or the production of it in host plants modified to express proteins with mammalian-like glycosylations.

Validation of plant-produced mAbs as biosimilar therapeutics will require that they be shown to have biological properties (i.e., bioactivity and biosafety) similar to those of clinically approved parental mAbs. This article clearly shows that a plant-expression and purification system can produce a therapeutic mAb with identical primary structures and similar in vitro bioactivities to those of its clinically approved parental mAb, indicating that agricultural biopharming could be an effective alternative to mammalian cell systems for the production of biosimilar therapeutics such as mAbs.

ABBREVIATIONS USED

ADCC, antibody-dependent cellular cytotoxicity; ATCC, American Type Culture Collection; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF-R, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FDA, United States Food and Drug Administration; FPLC, fast-performance liquid chromatography; GalT, β1,4-galactosyltransferase; GMP, good manufacturing practice; HC, heavy chain; HER2, human epidermal growth factor receptor 2; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; IgG, immunoglobulin G; kDa, kilodalton; KDEL, lysine−aspartate−glutamate−leucine; LC, light chain; mAb, monoclonal antibody; MES, 1-(*N*-morpholino)ethanesulphonic acid; PBS, phosphate buffered saline; PBST, PBS Tween 20; PCR, polymerase chain reaction; PVX, potato virus x; RPMI, Roswell Park Memorial Institute; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SP; signal peptide; TMV, tobacco mosaic virus; TSP, total soluble protein.

ACKNOWLEDGMENT

We thank ICON Genetics, GmbH for the use of the magnICON expression system. We are also grateful to Fernando Olea-Popelka for designing the plant-optimized HC and LC coding sequences and to Ashley J. Meyers for her help in the laboratory, for the development of the quantitative ELISA, and for critical review of this manuscript.

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Received for review June 16, 2010. Revised manuscript received August 6, 2010. Accepted August 14, 2010. This work was funded by grants to JCH from the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), the Natural Sciences and Engineering Research Council (NSERC), the Canada Research Chairs (CRC) Program, SENTINEL Bioactive Paper Network, and PlantForm Corporation.