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A novel and fully scalable *Agrobacterium* spray-based process for manufacturing cellulases and other cost-sensitive proteins in plants

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Summary

Transient transfection of plants by vacuum infiltration of *Agrobacterium* vectors represents the state of the art in plant-based protein manufacturing; however, the complexity and cost of this approach restrict it to pharmaceutical proteins. We demonstrated that simple spraying of *Nicotiana* plants with *Agrobacterium* vectors in the presence of a surfactant can substitute for vacuum inoculation. When the T-DNA of *Agrobacterium* encodes viral replicons capable of cell-to-cell movement, up to 90% of the leaf cells can be transfected and express a recombinant protein at levels up to 50% of total soluble protein. This simple, fast and indefinitely scalable process was successfully applied to produce cellulases, one of the most volume- and cost-sensitive biotechnology products. We demonstrate here for the first time that representatives of all hydrolase classes necessary for cellulosic biomass decomposition can be expressed at high levels, stored as silage without significant loss of activity and then used directly as enzyme additives. This process enables production of cellulases, and other potential high-volume products such as noncaloric sweetener thaumatin and antiviral protein griffithsin, at commodity agricultural prices and could find broad applicability in the large-scale production of many other cost-sensitive proteins.

Introduction

Transient expression of recombinant proteins in plants using *Agrobacterium* vectors has become a preferred biomanufacturing platform due to its speed, versatility, cost efficiency and industrially relevant scalability for many proteins (Giritch *et al.*, 2006; Marillonnet *et al.*, 2005; reviewed in Gleba and Giritch (2012) One major limitation of the current protocols, however, is their reliance on vacuum infiltration, a technique that requires mechanical manipulation of pot- or tray-grown plants for their submerged exposure to the inoculum in vacuum chambers (reviewed in Gleba and Giritch (2012). Such mechanical manipulation of the host plants, which is currently practised in larger scales via robotics and semi-automated systems, imparts cost and complexity to the inoculation process, and therefore, such systems lend themselves preferentially to the production of pharmaceutical proteins. Most industrial recombinant proteins such as industrial enzymes and biomaterials need to be manufactured inexpensively in high volumes; thus, the use of containerized greenhouse-grown plants is not practical or cost-effective for these applications. We therefore explored alternatives to vacuum infiltration that can be applied to plants regardless of how they are cultivated. We have found that the simple spraying of plant leaves with a dilute suspension of *Agrobacterium* vectors in the presence of a surfactant allows the effective transfection of 0.9–3.5% of leaf cells of most *Nicotiana* plants, and that the employment of T-DNA messages consisting of viral replicons capable of cell-to-cell movement increases the resultant transfection levels to up to 90% of leaf cells. This transfection procedure requires a slightly longer time to effect (10–14 days instead of the typical 4–7 days for vacuum infiltration), but the resultant expression levels are comparable to those of vacuum-infiltrated plants (Marillonnet *et al.*, 2005).

We evaluated this protocol in the manufacture of cellulases, one of today's most cost-sensitive biotechnology products that must ultimately be delivered in high volume to enable applications such as cellulosic ethanol production (Carroll and Somerville, 2009). Cellulases currently used in bioethanol production are all produced by microbial fermentation. Despite decades of research on lowering cellulase manufacturing costs, these enzymes still account for 20–40% of cellulosic ethanol production costs (Sainz, 2009). We expressed six cellulase genes of bacterial and fungal origin representing all four hydrolase classes needed for degradation of cellulose into glucose and identified several that are expressed at high levels and retain their expected enzymatic activity, even after the plant biomass was harvested and stored as silage without refrigeration for an extended period of time. The overall process is flexible and robust and consists of simple manipulations that are common to many agricultural practices. As the volumes of *Agrobacterium* inocula needed to induce cellulase biosynthesis are very small, the calculated costs of manufacturing and storing cellulases produced by this method are similar to the costs of other commodity agricultural products. The technology has been shown to work for other potential product candidates that would require high volume inexpensive production such as

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noncaloric sweetener protein thaumatin and griffithsin protein with broad antiviral activity.

Results

Plants can be transiently transfected by spraying with *Agrobacterium* vectors

We assessed the effect of direct spraying with diluted agrobacterial suspensions on transient transfection of *Nicotiana benthamiana* plants. Binary construct pICH18722 containing the TMV-based viral replicon with insertion of green fluorescent protein (GFP)-encoding gene (TMV(fsMP)-GFP) was selected for evaluating transfection efficiency (Figure S1). The viral replicon used was disabled for systemic movement by deletion of the coat protein (CP) gene; the vector also contained a frame-shift mutation in the movement protein (MP) coding sequence, resulting in loss of the cell-to-cell movement ability. Therefore, this vector expressed GFP only in cells directly transfected with T-DNA.

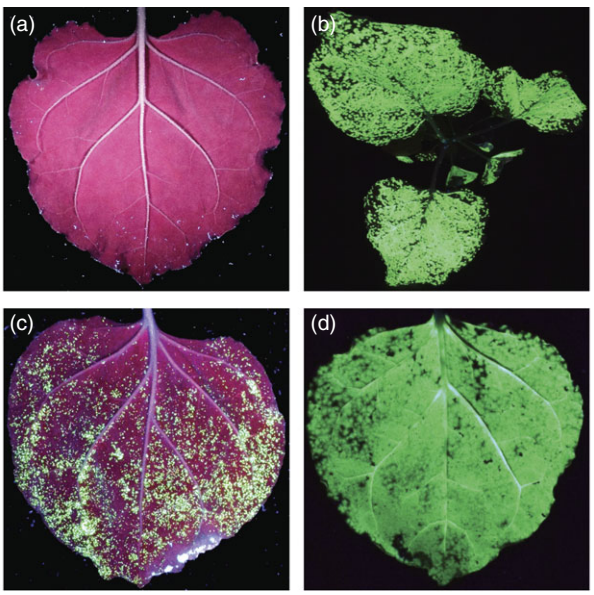
Plants were sprayed with agrobacterial suspensions diluted 10^{-2} and 10^{-3} relative to saturated overnight agrobacterial cultures (i.e. OD_{600} = 0.015, corresponding to approx. 10^7 CFU/mL and OD_{600} = 0.0015, corresponding to approx. 10^6 CFU/mL, respectively), supplemented with surfactant Silwet L-77® (GE Silicones, Inc., USA) at 0.1% (v/v), a concentration that is typically used in industrial agricultural applications (Harvey, 1998). Four days after spraying, separate foci of GFP fluorescence appeared in sprayed leaves and the same pattern was still visible for at least 2 weeks after spraying (Figure 1c). The proportion of GFP-expressing cells was counted after the isolation of leaf protoplasts. Depending on agrobacterial suspension concentration, 0.9–3.5% of total leaf cells was transfected as a result of *Agrobacterium*-mediated T-DNA transfer (Figure 1e).

Use of viral replicon-based vectors capable of cell-to-cell or systemic movement results in almost complete transfection of leaf cells

To increase the proportion of plant cells expressing the gene of interest, we then used T-DNA encoding viral replicon capable of movement in a plant. GFP-expressing TMV vector pICH18711 (TMV(MP)-GFP) is disabled for systemic movement due to the CP gene deletion, but retains cell-to-cell movement ability by the presence of MP (Figure S1). *Nicotiana benthamiana* plants were sprayed with 10^{-2} and 10^{-3} dilutions of agrobacterial cultures harbouring pICH18711 construct. Spots of GFP fluorescence appeared 3 days postspraying (dps) and enlarged over the time, merging together within approximately 12 days postinfection. Visually, complete transfection of sprayed leaves developed approximately 12 days postspraying without significant difference between 10^{-2} and 10^{-3} dilutions of agrobacterial cultures (Figure 1d). Counting of protoplasts prepared from three independent leaves sprayed with bacterial inocula revealed GFP expression in more than 90% of plant cells treated with 10^{-2} dilution of agrobacterial culture. Spraying with the 10^{-3} dilution of the inocula yielded the value of about 86% (Figure 1e). Spraying with the TMV-based vector resulted in abundant GFP expression in all sprayed leaves but not in the youngest ones newly developed after spraying (Figure 1b).

Multiple cellulases of microbial and fungal origin can be efficiently expressed in plants

We evaluated the expression of six cellulolytic enzymes of bacterial and fungal origin in *Nicotiana benthamiana* plants upon



(e)	GFP-expressing protoplasts (%)	
	10^{-2}	10^{-3}
TMV(fsMP)-GFP	3.5+/-0.5	0.9+/-0.1
TMV(MP)-GFP	92.9+/-0.9	86.0+/-0.9

Figure 1 Transient transfection of *Nicotiana benthamiana* plants 12 days after spraying with suspension of agrobacterial cells harbouring TMV-based viral vectors with or without cell-to-cell movement ability. (a) Leaf of control nontransfected plant, (b) whole *Nicotiana benthamiana* plant transfected with TMV(MP)-GFP vector capable of cell-to-cell movement ability, (c) Leaf transfected with TMV(fsMP)-GFP vector lacking cell-to-cell movement ability, (d) Leaf transfected with TMV(MP)-GFP vector with cell-to-cell movement ability. (e) The percentage of GFP-expressing cells counted after the isolation of protoplasts from the whole leaf blade (the mean value and standard deviation (SD) of three different leaves of same plant. Numerals 10^{-2} and 10^{-3} show the dilution factor of the overnight agrobacterial cultures of OD=1.5 at 600 nm. In (b–d), 10^{-3} dilution was used.

spraying agrobacterial inocula harbouring TMV viral vectors enabled for cell-to-cell movement. The enzymes included in this evaluation represent all three cellulase classes that are necessary for the conversion of cellulose to glucose: (i) endoglucanases (EC 3.2.1.4), which cleave cellulose chains internally, providing free reducing and nonreducing chain ends; (ii) cellobiohydrolases/exoglucanases (EC 3.2.1.91/EC 3.2.1.-), which operate on cellulose chain termini with specificity for reducing or nonreducing ends, respectively, releasing cellooligosaccharides, preferentially cellobiose; and iii) β-glucosidases which hydrolyse cellobiose to glucose (EC 3.2.1.21). The analysis provided here includes two endoglucanases, namely endoglucanase E1 from *Acidothermus cellulolyticus* 11B and endoglucanase Cel5A from *Thermotoga maritima*; one exoglucanase, β-1,4-exocellulase (E3) from *Thermobifida fusca* with a specificity for nonreducing chain ends; two cellobiohydrolases, exoglucanase 1 (CBHI) from *Trichoderma reesei/Hypocrea jecorina* and CBHI from *Humicola grisea*, both with reducing chain end specificity; and one β-glucosidase Bgl4 from *Humicola grisea* (Table 1).

Cellulases were screened for optimal expression using translational fusions with various signal peptides (SP) providing targeting

Table 1 Cellulolytic enzymes analysed for transient expression in *Nicotiana benthamiana*

No.	Cellulase	EC number	Source organism	Accession number	MW, kDa	pH optimum	t° optimum, °C	Yield*	Activity **
1	Endoglucanase E1	3.2.1.4	*Acidothermus cellulolyticus*	P54583.1	60.7	5–6 Hood *et al.* (2007)	81 Hood *et al.* (2007)	< 1% < 0.03	17.83 ± 3.76 62.94 ± 4.8
2	Endoglucanase Cel5A	3.2.1.4	*Thermotoga maritima*	3MMW-D	37.4	6 Kim *et al.* (2010)	80 Kim *et al.* (2010)	12.5% 0.68 ± 0.01	67.32 ± 15.6 363.22 ± 81.55
3	Exocellulase E3	3.2.1.-	*Thermobifida fusca*	AAA62211.1	63.5	7–8 Zhang *et al.* (1995)	65 Zhang *et al.* (1995)	25% 1.6 ± 0.05	0.88 ± 0.46 5.58 ± 2.74
4	Exoglucanase (CBHI)	3.2.1.91	*Trichoderma reesei*	P62694.1	54.0	5 Hood *et al.* (2007)	45–50 Hood *et al.* (2007)	12.5% 0.43 ± 0.09	8.06 ± 2.31 27.39 ± 6.94
5	Exoglucanase 1 (CBHI)	3.2.1.91	*Humicola grisea*	BAA09785.1	54.1	5 Takashima *et al.* (1996)	60 Takashima *et al.* (1996)	10% 0.23 ± 0.07	6.9 ± 1.2 15.6 ± 1.82
6	β-glucosidase Bgl4	3.2.1.21	*Humicola grisea*	BAA74958.1	54.0	6 Takashima *et al.* (1999)	55 Takashima *et al.* (1999)	50% 3.28 ± 0.4	16.27 ± 0.93 106.75 ± 14.44

*Yield of recombinant protein is expressed as a percentage of TSP (top) and mg recombinant protein/g fresh weight of plant biomass (bottom). **Enzymatic activity of recombinant protein is given in μmole pNP (T = 24 h) for Nr 1, 2, 4, and 5, in mg glucose (T = 46 h) for Nr 3 or IU for Nr 6 per mg of TSP (top) and per gram fresh weight of plant biomass (bottom). Measurements were taken on biological triplicates; the average and standard deviations are provided. Plant material was transfected for cellulase expression with 10^{-3} dilutions of *Agrobacterium* cultures and harvested 11 dpi.

into different cell compartments using assembly of 5′ and 3′ TMV pro-vector modules *in planta* (Marillonnet *et al.*, 2004) (Figure S2). Selected fusions with the highest yield of active enzyme (listed in Table 2) were subcloned into assembled TMV-based viral vectors (Figure S3), and the time-course for expression was analysed. For this purpose, *Nicotiana benthamiana* plants were sprayed with *Agrobacterium* suspensions in dilutions ranging from 10^{-2} to 10^{-3}, and the plant material was inspected for recombinant protein expression from 4 to 18 dps using SDS-PAGE with Coomassie staining. The yields and timing of expression depended on the agrobacterial culture dilution and varied among the enzymes (Figure 2 and Table 1). In most cases, optimal yields were obtained for plant material sprayed with 10^{-3} dilutions at 11–12 dps (Figure 2). To standardize transfection and production, the same *Agrobacterium* inoculum density (10^{-3} dilution) and harvest time (11 dps) were used for all enzymes.

The yields of five of the six recombinant enzymes were in the range of 10% to 50% of TSP, or 0.23 to 3.28 mg/g of fresh weight (Table 1).

Plant-expressed cellulases maintain high enzymatic activity

To evaluate the enzymatic properties of expressed recombinant cellulases, plant total soluble protein (TSP) extracts were analysed

Table 2 Storage stability of plant-made recombinant cellulases

		Protein accumulation		Activity level	
Cellulase	Storage	%age of TSP	mg recombinant protein/g fresh weight plant material	Per mg TSP	Per g fresh weight plant material
Rice amylase apoplast TP-E1	-80°C	<1%	< 0.03	17.83 ± 3.76	62.94 ± 4.8
storage 14 weeks		<1%	< 0.01	46.9 ± 1.83	46.9 ± 6.52
Cytosolic Cel5A	-80°C	12.5%	0.68 ± 0.01	67.32 ± 15.6	363.22 ± 81.55
storage 12 weeks		15%	0.16 ± 0.001	76.22 ± 11.29	81.41 ± 12.36
Chloroplast TP-His-EK-E3	-80°C	25%	1.6 ± 0.05	0.88 ± 0.46	5.58 ± 2.74
storage 15 weeks		15%	0.72 ± 0.04	3.59 ± 0.83	5.36 ± 1.51
Barley-α-amylase apoplast TP-CBHI (*Tr*)	-80°C	12.5%	0.43 ± 0.09	8.06 ± 2.31	27.39 ± 6.94
storage 14 weeks		35%	0.37 ± 0.03	17.68 ± 1.49	18.87 ± 2.89
Barley-α-amylase apoplast TP-CBHI (*Hg*)	-80°C	10%	0.23 ± 0.07	6.9 ± 1.2	15.6 ± 1.82
storage 12 weeks		20%	0.23 ± 0.02	17.08 ± 0.95	19.44 ± 1.14
Cytosolic Bgl4	-80°C	50%	3.28 ± 0.4	16.27 ± 0.93	106.75 ± 14.44
storage 16 weeks		90%	1.94 ± 0.23	26.03 ± 0.53	56.12 ± 6.65

Plant material was transfected for cellulase expression by spraying with 10^{-3} dilutions of *Agrobacterium* cultures admixed with 0.1% (w/w) Silwet L-77 surfactant, harvested 11 days postspraying and stored under different conditions. Activity level of recombinant protein is given in μmole pNP (T = 24 h) for E1, Cel5A, CBHI (*Tr*) and CBHI (*Hg*), in mg glucose (T = 46 h) for E3 or IU for Bgl4 per mg of TSP and per gram fresh weight of plant biomass. Measurements were taken on biological triplicates, with average and standard deviations provided.

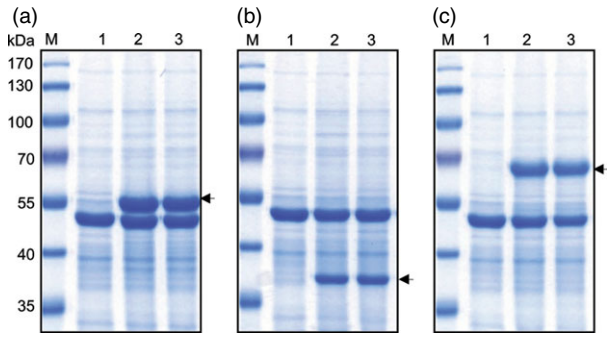


Figure 2 Expression of cellulases of microbial origin in *Nicotiana benthamiana* plants after the spraying with agrobacteria carrying TMV vectors. Coomassie-stained SDS protein gels loaded with crude extracts, corresponding to 1.0 mg fresh weight, from nontransfected leaf tissue (1) or plants transfected by *A. tumefaciens* carrying constructs encoding β-glucosidase Bgl4 (a), endoglucanase Cel5A (b) and exocellulase E3 (c) by spraying with dilutions of inoculation solutions of 10^{-2} (2), 10^{-3} (3). Harvesting time points of plant material were 7 (b, 2, 3), 11 (a, 2, 3; c, 2) and 14 (c, 3) days postinoculation. M, molecular weight marker with weights (kDa) shown on the left. Arrows indicate recombinant proteins.

for specific cellulolytic activities. Due to similar pH and temperature optima of selected enzymes and the proposed utilization of enzymes as a mixture in downstream processes, all cellulases were extracted with identical buffer, which enabled a simple pH-dependant removal of a considerable portion of nonrecombinant plant proteins by precipitation (Figure 3). All expressed cellulases were efficiently extracted in aqueous buffer and were active when incubated with the respective substrates under the same buffer and temperature conditions (Figure 3).

Plant biomass containing cellulases can be stored as silage without major loss of activity

As the harvest time of plant material expressing cellulases requires to be independent from the application of the enzymes in downstream processes, there is a need for inexpensive and stable storage of cellulase-containing plant biomass. Consequently, protein stability and activity were analysed under different storage conditions, namely, at − 80°C or as silage stored at room temperature for 12 to 16 weeks (Figure 3, Table 2). Surprisingly, all cellulases retained their specific activities upon storage as silage. Two of the six cellulases analysed (Bgl4 and CBHI) were both very stable and had high enzymatic activity in ensiled plant material. Although some cleavage was observed for Cel5A and E3 proteins, they retained reasonable enzymatic activity (Figure 3, Table 2). Moreover, the majority of nonrecombinant plant proteins were largely degraded during the storage of plant material as silage, effectively resulting in an enrichment of some recombinant cellulases as a percentage of total soluble protein and achieving, at least in the case of Bgl4, levels of up to 90% of TSP (Table 2). Storage of plant-expressed cellulases in dried plant matter was also investigated, but the results were less favourable. Together, the data presented here show that representatives of all cellulase classes necessary to catalyse the complete hydrolysis of cellulosic biomass to glucose can be manufactured in plants using this simple transfection protocol. The protocol is robust, requires only simple agricultural manipulations to put into practice, and provides high expression levels of cellulases and high activity of these important enzymes at harvest and after extended storage as silage.

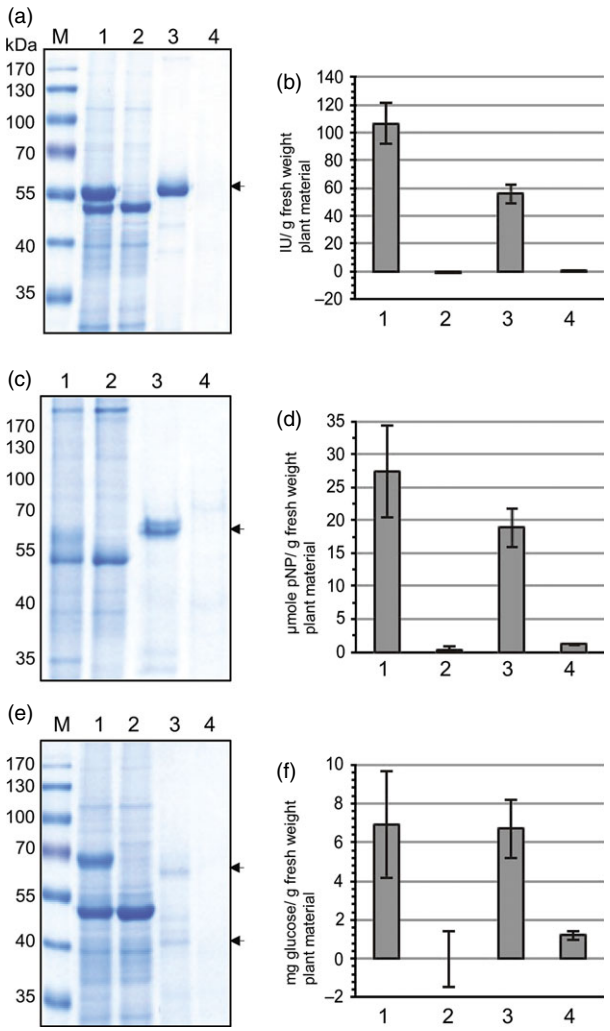


Figure 3 Storage stability of recombinant cellulases in the ensiled plant biomass: SDS-PAGE (a, c, e) and enzymatic activity analyses (b, d, f) for β-glucosidase Bgl4 (a, b), cellobiohydrolase CBHI (c, d) and exoglucanase E3 (e, f). Coomassie-stained SDS protein gels (a, c, e) are loaded with total soluble extracts (TSP, 5 µg) from *N. benthamiana* nontransfected leaf tissue (2, 4) or plants transfected by *Agrobacterium tumefaciens* carrying plasmid vectors (1, 3) pNMD1201 (a), pNMD1181 (c) or pNMD1229 (e) by spraying with 10^{-3} dilutions. Upon harvest 11 dpi, plant material was stored at −80 °C (1, 2) or as silage at ambient temperature (3, 4) for 16 weeks. M, molecular weight marker with weights (kDa) shown on the left. Arrows indicate recombinant proteins. Cellulase activities of TSP samples shown in b, d, f calculated per g of fresh weight plant material for frozen (1, 2) and ensiled (3, 4) biomass. Error bars indicate standard deviation of biological replicates, $n = 3$.

Recombinant griffithsin and thaumatin are stable upon the storage of plant biomass as silage

We tested our technology for two other cost-sensitive recombinant proteins: antiviral protein griffithsin from the red algae *Griffithsia* sp. (O'Keefe *et al.*, 2010) and the sweet protein thaumatin from the West African plant katempfe (*Thaumatococcus daniellii* Benth) (Edens *et al.*, 1982). We expressed griffithsin and thaumatin in *Nicotiana benthamiana* plants using spraying and vacuum inoculation with agrobacteria harbouring

TMV-based viral vectors pNMD2971 and pICH95397, respectively (Figure S4). Recombinant protein stability was analysed upon the storage of plant biomass either at −80 °C or as silage at room temperature for up to 16 weeks. SDS-PAGE analysis revealed nearly complete degradation of native plant proteins in ensiled plant biomass already after 4 weeks of storage (Figure 4). In contrast, both recombinant griffithsin and thaumatin remained stable and did not show any significant degradation during whole analysed periods (16 weeks for griffithsin and 5 weeks for thaumatin).

Discussion

We provide here the example of robust industrially applicable technology for production of a desired recombinant protein product based on spraying of plants with *Agrobacterium* suspensions without the need of full virus. Earlier studies with *Agrobacterium*-mediated transfection employed either vacuum infiltration or alternative methods relying on the systemic viral infection. In the first case (Marillonnet *et al.*, 2005), bacteria were forced into plant leaves by flooding the intercellular leaf spaces upon release of a vacuum, resulting in direct transfection of the majority of leaf cells. However, this process requires a vacuum chamber to transfect pot- or tray-contained plants in batches and hence cannot be applied to field-grown plants. Several authors mention *Agrobacterium* application methods that do not rely on vacuum infiltration. In Ryu *et al.* (2004), and in Azhakanandam *et al.* (2007); 'agrodrench' (drenching the soil under plants) and 'wound-and-spray/agrospray' methods, respectively, have been described. However, as the successful delivery by *Agrobacterium* results in systemic viral infection when even very rare or single events of transfection could result in detectable symptoms, the efficiency of *Agrobacterium* delivery per se remains unknown. Moreover, the presented quantitative protein expression data in these studies are far below the vacuum infiltration (Marillonnet *et al.*, 2005). The spraying or dipping used for *Arabidopsis* transformation described by Chung *et al.* (2000) is an interesting solution that is practically restricted to *Arabidopsis* and does not provide any insight into efficiency of transient delivery step in connection with transformation. Our research clearly demonstrates that upon use of proper surfactants, between 0.9 and up to 3.5% of leaf cells can be directly transfected using spray application of bacterial suspensions containing 10^{6-7} CFU/ml as inoculum. In these studies, we used the organosilicon surfactant Silwet L-77®, but good results were also obtained with other adjuvants such as Tween®20 (ICI Americas, Inc., USA) and Triton X-100™ (Union Carbide Corporation, USA). We also show that a combination of this simple and indefinitely scalable DNA delivery procedure with deconstructed viral vectors capable of cell-to-cell movement results, within 10–14 days, in efficient transfection of most leaf cells, thus providing for expression of recombinant proteins at very high levels, often reaching over 50% of the total soluble protein in leaves. Thus, even this early version of the process results in protein expression levels approaching the biological limits of plants. Although the results reported here are based on the use of *Nicotiana benthamiana* as a manufacturing host, we have good evidence that essentially the same protocol works with at least eight *Nicotiana* species, including *N. debneyi, N. excelsior*, *N. exigua, N. maritima*, *N. simulans, N. sylvestris* and some varieties of *N. tabacum* (*e. g.* Maryland Mammoth).

The process uses bacterial inocula efficiently such that treatment of 1 ton of host plant biomass could be effectively achieved with approximately 10 g of bacteria at the densities studied here. Fermentation costs for producing agrobacteria are estimated to be <5% of the total costs of the manufacturing and storage process. Undoubtedly, the procedure can be further optimized by improving the *Agrobacterium* vector and the plasmid constructs it carries, by additional selection of adjuvants and wetting agents and by screening and optimizing methods for spray delivery. In this study, an industrial *Agrobacterium tumefaciens* strain ICF320 developed by Icon Genetics GmbH (Halle, Germany) for magnICON® system (Bendandi *et al.*, 2010) was used because of biosafety reasons. ICF320 is a disarmed derivative of the strain C58 containing several auxotrophies (D*cysK*$_a$, D*cysK*$_b$, D*thiG*) that

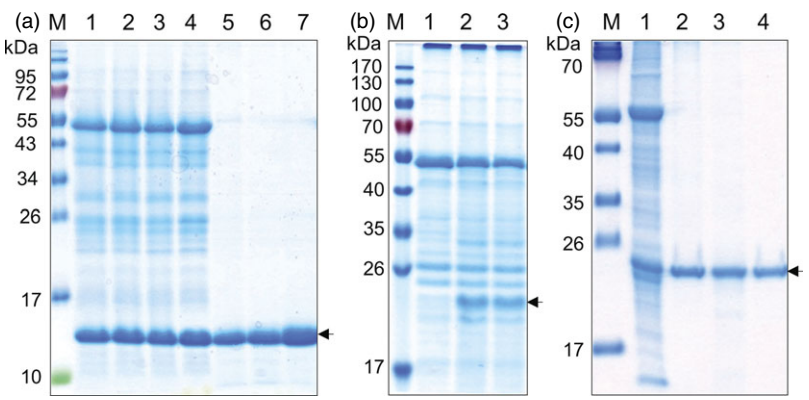


Figure 4 Expression levels and stability of recombinant griffithsin (a) and thaumatin (b, c) upon storage of plant biomass as silage. Extracts of frozen (−80 °C) and ensiled plant material resolved in a 12% (a, b) and 16.5% (c) polyacrylamide gel under reducing conditions. (a) Griffithsin-containing plant material extracted with 2 × Laemmli buffer. Lane 1, freshly harvested plant sample; lanes 2, 3 and 4, plant material after 4, 8 and 16 weeks storage at −80 °C; lanes 5, 6 and 7, ensiled plant material after 4, 8 and 16 weeks storage at ambient temperature. (b) Expression levels of thaumatin upon spray-transfection. Extracts with 2 × Laemmli buffer corresponding to 1.0 mg fresh weight. Lane 1, nontransfected plant material; lanes 2 and 3, plant material expressing thaumatin upon spraying with dilutions of inoculation solutions of 10^{-2} and 10^{-3} at 14 dpi. (c) Thaumatin-containing plant material after 5 weeks storage. Lanes 1 and 2, frozen samples extracted with 2 × Laemmli buffer and 100 mM NaCl, 50 mM acetic acid; lanes 3 and 4, ensiled plant material extracted with Laemmli buffer and 100 mM NaCl, 50 mM acetic acid, respectively. Loading corresponds to 2.0 mg fresh weight. M, molecular weight marker with weights (kDa) shown on the left. Arrows indicate recombinant proteins.

have been introduced to decrease the viability of bacteria in the environment. Several other *Agrobacterium* strains (*e.g*. standard laboratory strains AGL1, GV3101, EHA105) were also tested with our approach and provided good transfection results, too (data not shown).

Even before vector, host or procedural optimizations, we believe that this transient expression technology represents the most versatile, economical and efficient industrial process described to date for the plant-based manufacture of recombinant proteins in large scale.

The broad industrial use of this process would require release of genetically engineered agrobacteria which can be performed in a contained greenhouse environment even now. Open field application of the technology requires further studies and measures to assure the biological and environmental safety.

We chose to illustrate the potential power and utility of this transient expression-based protein manufacturing technology using cellulases, a broad class of enzymes needed in different processes but especially key to the economic conversion of cellulosic biomass to ethanol. One of the main reasons for the high cost of cellulosic ethanol today is the cost of cellulases, accounting for up to 20–40% of the cost of goods (Sainz, 2009). Relative to amylases used to produce ethanol from starch, cellulases would be needed in very large amounts because their specific activity is 10–100-times lower than that of amylases (Merino and Cherry, 2007); hence, their use rate is as high as 1% (w/w) of the cellulose treated (http://www.bioenergy.novozymes.com). As illustrated by the decades-long efforts of Novozymes, Genencor (DuPont-Danisco) and other companies and academic groups, even highly optimized microbial fermentation processes have been unable to solve the catalyst cost problem. The economics of cellulose bioconversion have also been unfavourable because of the high cost of transporting the cellulases from enzyme fermentation facilities to the ethanol manufacturing sites (Sticklen, 2008). The silage-based process described here offers a plausible lower cost option as the catalyst can be produced in immediate vicinity of feedstock production site or it could be co-transported with the feedstock.

Complete cellulose degradation requires three classes of glucanases, and in our work, we have tested six genes of bacterial and fungal origin encoding representative members all three classes. Although in these studies the expression of these enzymes was only partially optimized (e.g. codon-usage optimization and screening for best subcellular localization for each protein), we were able to express most proteins at high levels and demonstrate that all proteins expressed retained their expected enzymatic activity even when stored as silage, a traditional and inexpensive agricultural biomass preservation method. The production time and expression efficiencies of spray-based cellulase production surpass those achieved in plants stably transformed with nonreplicating expression vectors. For example, for endoglucanase Cel5A from *T. maritima*, expression levels of 4.5% and 5.2% were reported (Mahadevan *et al.*, 2011) and (Kim *et al.*, 2010), respectively) in stably transgenic tobacco plants compared to 12.5% of TSP in transiently transfected *N. benthamiana*. High accumulation of endoglucanase cel9A (about 40% of TSP) achieved by chloroplast transformation resulted in severe mutant phenotype of transgenic plants (Petersen and Bock, 2011). Expression of CBHI from *T. reesei*, E3 from *T. fusca* and Bgl4 from *H. grisea* in our system was as high as 12.5%, 25% and 50% of TSP, respectively (Table 2), compared to 4.1% for CBHI (Hood *et al.*, 2007), 3–4% (Yu *et al.*, 2007) and approximately 5% (Petersen and Bock, 2011) for exocellulase E3, or 5.8% and 9.6% for β-glucosidases (Gray *et al.*, 2011; Jung *et al.*, 2010) in stably transgenic plants. This is not surprising because in our system, cellulase production is induced at the moment of spraying with agrobacteria, and there is no negative effect of cellulases on plant growth. For all but one (E1 from *A. cellulolyticus*) of the modelled enzymes, expression levels exceeded 10% of TSP, the level which is thought to be sufficient for cell wall deconstruction without supplementation with microbially sourced enzymes (Sticklen, 2008).

The two other proteins expressed, thaumatin and griffithsin, are both good examples of cost-sensitive proteins. Thaumatin is an approved noncaloric sweet-tasting protein that is 3000 times sweeter than sucrose (on a weight basis). It can be extracted from the original source (*Thaumatococcus daniellii*) today inexpensively, but the natural plant resources are very limited (Faus and Sisniega, 2003). Griffithsin is one of the most promising antivirals, and its potential use as a preventative microbicide against HIV (and other enveloped viruses) in a form of topical ointments or suppositories would require it to be manufactured very inexpensively (O'Keefe *et al.*, 2009; Zeitlin *et al.*, 2009). Our experiments demonstrate that both proteins can be easily and very efficiently manufactured by *Agrobacterium* spay-based transient expression and that the upstream process can be efficiently separated from the downstream process by storage of the biomass as silage.

Looking ahead, the process described will also benefit from identification of new or more efficient and stable enzymes. Based on the known costs of various commodity agricultural products, we estimate that the cost of cellulases produced by this method will be significantly less than $10 per kg of active protein, thus making the economics of this process highly competitive with microbial fermentation. We believe that the process should be easy to implement and scale because it relies on existing agricultural infrastructure and practices; hence, it constitutes a simple additional step that can be integrated at any site that generates cellulosic biomass. In addition to cellulases, we have been evaluating our process with multiple enzyme and protein candidates that need to be produced inexpensively and have observed expression levels that are comparable or surpass the levels seen in plants transfected using vacuum infiltration, which has been up to now the 'gold standard' for plant-based biomanufacturing.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli DH10B cells were cultivated at 37 °C in LB medium (lysogeny broth (Bertani, 1951)). *Agrobacterium tumefaciens* ICF320 (Bendandi *et al.*, 2010) cells were cultivated at 28 °C in LBS medium (modified LB medium containing 1% soya peptone (Duchefa, Haarlem, Netherlands)).

Plasmid constructs

TMV-based assembled vectors pICH18711 and pICH18722 (Figure S1) were described in Marillonnet *et al.* (2005).

5′TMV-based pro-vector modules pICH22455, pICH20155, pICH20188, pICH22464, pICH20388, pICH20999, pICH20030 and pICH22474 (Figure S2a) used for the optimization of expression of cellulases by targeting into different subcellular compartments are described in Kalthoff *et al.* (2010).

For cloning of 3′-provector modules of TMV pro-vectors, coding sequences of genes of interest were cloned into the BsaI

sites of the pICH28575 construct (Kalthoff *et al.*, 2010). Genes of interest used in these constructs encoded β-glucosidase Bgl4 from *Humicola grisea* (GenBank: BAA74958.1; pNMD910), endoglucanase E1 from *Acidothermus cellulolyticus* 11B (Swiss-Prot: P54583.1; pNMD231), β-1,4-exocellulase (E3) from *Thermobifida fusca* (GenBank: AAA62211.1; pNMD251) and exoglucanase 1 (CBH1) from *Trichoderma reesei/Hypocrea jecorina* (Swiss-Prot: P62694.1, pNMD241) (Figure S2b). Coding sequences of all genes of interest were codon-optimized for *Nicotiana benthamiana* and synthesized by Entelechon GmbH (Bad Abbach, Germany).

The integrase vector module pICH14011 (Figure S2c) is described in Kalthoff *et al.* (2010).

Assembled TMV vectors for the expression of selected cellulase translational fusions were created on the basis of pICH18711 vector (Marillonnet *et al.*, 2005). They contain actin 2-promoter-driven TMV RdRp with 14 introns, TMV MP with 2 introns, targeting presequence, and coding sequence of gene of interest, 3′TMV nontranslated region (3′ NTR) as well as a nos terminator. The entire fragment is inserted between the T-DNA left and right borders of binary vector. pNMD1201 and pNMD3081 constructs contain coding sequences of β-glucosidase Bgl4 from *Humicola grisea* and endoglucanase Cel5A from *Thermotoga maritima*, respectively, without any fusion sequence. The pNMD1231 construct bears endoglucanase E1 from *Acidothermus cellulolyticus* fused with rice α-amylase 3A apoplast targeting presequence. pNMD1181 and pNMD3061 constructs contain exoglucanase 1 (CBHI) from *Trichoderma reesei* and exoglucanase 1 (CBHI) from *Humicola grisea*, respectively, fused with barley α-amylase apoplast targeting presequence (Figure S3).

Assembled TMV vectors for the expression of griffithsin and thaumatin were constructed similarly (Figure S4). pNMD2901 vector contained the coding sequence of griffithsin (GenBank: ACM42413.1) codon-optimized for *Nicotiana benthamiana* and synthesized by GeneArt (Life Technologies, Regensburg, Germany). pICH95397 construct contained the coding sequence of mature peptide of preprothaumatin 2 from *Thaumatococcus daniellii* (GenBank: AAA93095.1) fused with rice α-amylase 3A apoplast targeting presequence.

pICH18711, pICH18722, pICH22455, pICH20155, pICH20188, pICH22464, pICH20388, pICH20999, pICH20030, pICH22474, pICH28575, pICH95397 and pICH14011 constructs were kindly provided by Icon Genetics GmbH (Halle, Germany).

Plant material and inoculations

Nicotiana benthamiana plants were grown in the greenhouse (day and night temperatures of 19–23 °C and 17–20 °C, respectively, with 12 h light and 35–70% humidity). Six-week-old plants were used for inoculations.

For plant transfection, saturated *Agrobacterium* overnight cultures were adjusted to OD_{600} = 1.3 or 1.5 (approximately 1.2×10^9 cfu/mL) with *Agrobacterium* inoculation solution (10 mM MES pH 5.5, 10 mM $MgSO_4$), further diluted with same solution supplemented with 0.1% (v/v) Silwet L-77 (Kurt Obermeier GmbH & Co. KG, Bad Berleburg, Germany) and inoculation was carried out using a hand sprayer (Carl Roth GmbH + CO.KG, Karlsruhe, Germany).

Mesophyll protoplast isolation

Protoplasts were isolated as described in Giritch *et al.* (2006) for count of transfected GFP-expressing plant cells. For isolation of protoplasts, whole leaf blades of three different leaves of the same plant were used. For each leaf, approximately 8500 cells have been counted; the total number of cells for each treatment including three separate leaves was about 25 500. For the percentage of GFP-expressing cells, average and standard deviation (SD) values are provided.

Protein analysis

About 50 mg fresh weight *N. benthamiana* leaf material pooled from 3 leaves of different age were ground in liquid nitrogen, and crude protein extracts were prepared with 5 vol. 2× Laemmli buffer. Total soluble protein (TSP) was extracted from approximately 150 mg fresh weight plant material ground in liquid nitrogen with 5 vol. prechilled extraction buffer (50 mM sodium acetate pH 5.5, 100 mM NaCl, 10% (v/v) glycerol). The protein concentration of TSP extracts was determined by Bradford assay using Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) and BSA (Sigma-Aldrich Co., St-Louis, MO, USA) as a standard.

For analysis by 10%, 12% or 16.5% SDS-PAGE and Coomassie-staining using PageBlue™ Protein Staining Solution (Fermentas GmbH, St. Leon-Rot, Germany), protein extracts were denatured at 95 °C for 5 min. before loading. The estimation of the percentage of recombinant cellulase fusion of TSP was carried out by comparison of TSP extracts to known amounts of BSA (Sigma-Aldrich) on Coomassie-stained SDS-PAA gels.

Storage of plant material at −80 °C and as silage

Plant material sprayed with 10^{-3} dilutions of *Agrobacterium* cultures was harvested 11 dpi as a pool of plants. Midribs of leaves were removed, and leaf blades were chopped into pieces of about 5 cm^2 in size using a scalpel blade. Leaf pieces were spread out for dehydration at the room temperature for 22 h. Dehydrated leaf material (45–58% of fresh weight) was supplemented with 2% (w/dry weight) propionic acid, sodium salt and packed into plastic bags using a commercial vacuum food sealer (Food Saver V2040-I, Sunbeam Products, Inc., Boca Raton, FL) for conservation. This plant material was stored as silage at the room temperature in dark for 1–4 months. Aliquots corresponding to 150 mg fresh weight plant material were analysed in triplicates for enzymatic activity.

About 150 mg fresh weight aliquots (9 leaf discs of 1 cm in diameter pooled from 3 comparable leaves) were harvested in triplicates from identical plant material used for the preparation of silage. Samples were frozen in liquid nitrogen and stored at −80 °C.

Enzymatic activity measurements

Endoglucanase and cellobiohydrolase cellulase activity assay (Cel5A, CBHI, E1)

Enzyme activity was determined using p-nitrophenyl-β-D-cellobioside as substrate. 100 μg of plant TSP extracts was incubated in 50 mM NaAc pH 5.5, 100 mM NaCl, 5% (v/v) glycerol, supplemented with or without 5 mM p-nitrophenyl-β-D-cellobioside as enzyme blanks, respectively, in a final volume of 1 mL at 50 °C for 1–24 h. Aliquots of the reaction mixture were removed at different time points and diluted 1:10 with 0.15 M glycine pH 10.0 to terminate the reaction. The concentration of p-nitrophenol (pNP) released from p-nitrophenyl-β-D-cellobioside was determined by measurement of the absorbance at 405 nm. The commercial enzymes cellulase (endo-1,4-β-D-glucanase) from *Trichoderma* sp. (Megazyme, Bray, Ireland) and cellobiohydrolase

(CBHI) from *Trichoderma* sp. (Megazyme) served as positive controls.

β-glucosidase cellulase activity assay (Bgl4)

For determination of β-glucosidase activity, cellobiose was used as a substrate. Different protein amounts of plant TSP extracts ranging from 1 to 40 μg were incubated in 50 mM NaAc pH 5.5, 100 mM NaCl, 5% (v/v) glycerol supplemented with or without 5 mM cellobiose as enzyme blanks, respectively, in a final volume of 1 mL at 50°C for 30 min. Concentration of released glucose in the samples were determined by D-glucose (GOPOD-Format) Kit (Megazyme). The commercial enzyme cellobiase from *Aspergillus niger* (synonym: Novozyme 188; Sigma-Aldrich) served as positive control. β-glucosidase activity was calculated in cellobiase units (CBU) according to IUPAC or international units (IU) like described in Ghose (1987) from samples releasing close to 1 mg glucose.

Exoglucanase cellulase activity assay (E3)

For determination of exoglucanase activity, the insoluble microcrystalline cellulose substrate Avicel (Sigmacell cellulose type 20; Sigma-Aldrich) was used. 100 μg of plant TSP extracts was incubated in 50 mM NaAc pH 5.5, 50 mM NaCl, 5% (v/v) glycerol, 0.02% sodium azide, 1.2 mg/mL Novozyme 188 (β-glucosidase; Sigma-Aldrich), supplemented with or without 1% (w/v) Avicel as enzyme blanks, respectively, in a final volume of 3 mL at 50 °C and 90 rpm for 120 h. Aliquots of the reaction mixture were removed at different time points (2, 4, 24, 48, 72 and 120 h) for determination of the glucose concentration using D-glucose (GOPOD-Format) Kit (Megazyme). The commercial enzyme cellobiohydrolase (CBHI) from *Trichoderma* sp. (Megazyme) served as positive control.

Storage of griffithsin and thaumatin expressing plant material

Griffithsin-expressing plant material was generated, stored and analysed as described for cellulases. For thaumatin expression, plants were vacuum-inoculated with *Agrobacterium*; plant material was harvested at 9 dpi. Upon harvest, plant material (whole plants) was shredded in a table-top cutter and either frozen at −80 °C or supplemented with 2% (w/w) sodium propionate and compressed into a plastic beaker and weight down for storage as silage. For thaumatin stability analysis, crude extracts or TSP extracts were prepared from plant material corresponding to 10 g fresh weight with 3 vol. 2 × Laemmli buffer or 100 mM NaCl, 50 mM acetic acid, respectively.

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The authors declare no conflict of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Schematic representation of T-DNA regions of plasmid constructs used for the determination of transfection efficiency in spray-based *Agrobacterium* application: GFP-expressing TMV-based vector capable of cell-to-cell movement (pICH18711) and analogous vector lacking cell-to-cell movement ability (pICH18722), LB and RB, binary left and right borders, respectively; Pact2, *Arabidopsis thaliana* actin 2 promoter; Tnos, nos terminator; RdRp, RNA-dependent RNA polymerase; MP, movement protein; 3′TMV, 3′untranslated region of TMV; fs, frameshift mutation.

Figure S2 Schematic representation of T-DNA regions of plasmid constructs used for screening for optimal cellulase expression. (A) 5′TMV-based pro-vector modules; (B) 3′TMV-based pro-vector modules; (C) integrase module pICH14011 (Marillonnet *et al.*, 2004). TP, targeting presequence; His, His(6)-tag with enterokinase cleavage site; rice amylase apo TP, apoplast targeting presequence from rice α-amylase; calreticulin apo TP, apoplast targeting presequence from *Nicotiana plumbaginifolia* calreticulin (Marillonnet *et al.*, 2004); apple pectinase apo TP, apoplast targeting presequence from apple pectinase (Marillonnet *et al.*, 2004); CTP, artificial dicot chloroplast targeting presequence (Marillonnet *et al.*, 2004); Bgl4 from *Humicola grisea*, β-glucosidase Bgl4 from *Humicola grisea* (GenBank: BAA74958.1) mature chain; E1 from *Acidothermus cellulolyticus*, endoglucanase E1 from *Acidothermus cellulolyticus* 11B (Swiss-Prot: P54583.1) mature chain; E3 from *Thermobifida fusca*, β-1,4-exocellulase (E3) from *Thermobifida fusca* (GenBank: AAA62211.1) mature chain; CBHI from *Trichoderma reesei*, exoglucanase 1 (CBH1) from *Trichoderma reesei/Hypocrea* jecorina (Swiss-Prot: P62694.1) mature chain; Phsp, *Arabidopsis* heat shock protein hsp81.1 promoter; NLS, nuclear localization signal.

Figure S3 Schematic representation of T-DNA regions of assembled TMV vectors used for *Agrobacterium* spray-based expression of cellulases. Cel5A from *Thermotoga maritima*, endoglucanase Cel5A from *Thermotoga maritima* (PDB: 3MMW-D) mature chain; CBHI from *Humicola grisea*, exoglucanase 1 (CBH1) from *Humicola grisea* (GenBank: BAA09785.1) mature chain.

Figure S4 Schematic representation of T-DNA regions of assembled TMV vectors used for *Agrobacterium* spray-based expression of griffithsin and thaumatin. GRFT, coding sequence of griffithsin (GenBank: ACM42413.1); thaumatin, coding sequence of mature peptide of preprothaumatin 2 from *Thaumatococcus daniellii* (GenBank: AAA93095.1).

