Exposure of *Agrobacterium tumefaciens* to agroinfiltration medium demonstrates cellular remodeling and may promote enhanced adaptability for molecular pharming

| Journal:            | Canadian Journal of Microbiology |
|---------------------|----------------------------------|
| Manuscript ID       | cjm-2020-0239.R2                 |
| Manuscript Type:    | Article                          |
| Date Submitted by the Author: | 15-Jul-2020                     |
| Complete List of Authors: | Prudhomme, Nicholas; University of Guelph Pastora, Rebecca; PlantForm Inc. Muselius, Benjamin; University of Guelph McLean, Mike; PlantForm Corporation Canada Cossar, Doug; PlantForm Corporation Canada Geddes-McAlister, Jennifer; University of Guelph, Molecular and Cellular Biology |
| Keyword:            | Agrobacterium tumefaciens, agroinfiltration medium, quantitative proteomics, molecular pharming |
| Is the invited manuscript for consideration in a Special Issue? | Not applicable (regular submission) |
Title: Exposure of *Agrobacterium tumefaciens* to agroinfiltration medium demonstrates cellular remodeling and may promote enhanced adaptability for molecular pharming.

Authors: Prudhomme, N.¹, Pastora, R.², Muselius, B.,¹ McLean, M.D.², Cossar, D.², Geddes-McAlister, J.¹*

Affiliations: ¹Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, N1G 2W1

²PlantForm Corporation Canada, Toronto, Ontario, M4S 3E2

Corresponding author: Jennifer Geddes-McAlister, Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, N1G 2W1. Phone: 519-824-4120 ext. 52129. Email: jgeddesm@uoguelph.ca
Abstract

Agroinfiltration is used to treat plants with modified strains of Agrobacterium tumefaciens for the purpose of transient in planta expression of genes transferred from the bacterium. These genes encode valuable recombinant proteins for therapeutic or industrial applications. Treatment of large quantities of plants for industrial-scale protein production exposes bacteria (harboring genes of interest) to agroinfiltration medium that is devoid of nutrients and carbon sources for prolonged periods of time (possibly upwards of 24 h). Such conditions may negatively influence bacterial viability, infectivity of plant cells, and target protein production. Here, we explored the role of timing in bacterial culture preparation for agroinfiltration using mass spectrometry-based proteomics to define changes in cellular processes. We observed distinct profiles associated with bacterial treatment conditions and exposure timing, including significant changes in proteins involved in pathogenesis, motility, and nutrient acquisition systems as the bacteria adapt to the new environment. These data suggest a progression towards increased cellular remodelling over time. In addition, we described changes in growth- and environment-specific processes over time, underscoring the interconnectivity of pathogenesis and chemotaxis-associated proteins with transport and metabolism. Overall, our results have important implications for the production of transently-expressed target protein products as prolonged exposure to agroinfiltration medium suggests remodeling of the bacterial proteins towards enhanced infection of plant cells.

Keywords: Agrobacterium tumefaciens, bioreactor, shake-flask, agroinfiltration medium, quantitative proteomics, molecular pharming
Introduction.

Plant-based production systems for biopharmaceutical proteins are attractive alternatives to mammalian cell, yeast, or bacterial systems (Chen and Davis 2016). Benefits include low cost, scalability, non-animal components, and rapid deployment of new therapies. As a genetic vector, the plant pathogen *Agrobacterium tumefaciens*, a Gram-negative bacterium isolated from soil and the causative agent of Crown Gall disease in dicotyledonous plants, is commonly used (Hughes 1996; Gohlke and Deeken 2014). The relevant properties of *A. tumefaciens* are attributed to an extrachromosomal plasmid (tumor inducing or Ti plasmid) containing the virulence (*vir*) region, which encodes proteins responsible for DNA transfer to plant cells. This includes proteins aiding in excision of the transfer DNA (T-DNA) from the Ti plasmid, a type IV secretion system for DNA movement into the plant cell, and others for the incorporation of T-DNA into the plant genome (Hughes 1996; Zhu et al. 2000; Veena et al. 2003; Aguilar et al. 2010; Nester 2015). Specifically, the T-DNA region contains DNA sequences at its ends (right and left borders) and genes of interest within these borders that are incorporated into the plant genome, resulting in transgenic plants or plant cells with desired properties.

The ability of *A. tumefaciens* to insert a portion of its DNA into the plant genome is exploited by the biotechnology industry to produce proteins for purification and downstream applications (Grohs et al. 2010; Mclean 2017). To deliver the desired transgenes into plants cells, the effective, robust, and scalable process of agroinfiltration was developed (Rivera et al. 2012; Huafang Lai and Jake Stahnke 2013; Krenek et al. 2015). Agroinfiltration, based on vacuum infiltration, involves the submergence of plant leaves into an infiltration medium that contains the *Agrobacterium* strain harboring the target recombinant DNA. The submerged plants, including the commonly used *Nicotiana benthamiana*, are subjected to negative atmospheric pressure in a
vacuum chamber to draw air out of interstitial spaces of submerged leaves that is replaced with *Agrobacterium*-containing medium when the vacuum is released (Garabagi et al. 2012). Although this process requires investment of machinery, including vacuum pumps, vacuum chambers, and large bacterial culture volumes, this approach provides scalability potential, which is instrumental in the success of plant-based production systems (Mclean 2017).

Recent studies explored the impact *A. tumefaciens* agroinfiltration on gene expression, plant defense response, and target protein production to optimize the process and improve transformation efficiency (Buyel 2015). For example, transcriptomic and proteomic analyses report dynamic reprogramming of the proteolytic machinery of *N. benthamiana* upon agroinfiltration (Grosse-Holz et al. 2018). Another study explored the role of chemical additives, heat shock, and co-expression of genes known to suppress plant stress and gene silencing or stimulate cell cycle progression to increase agroinfiltration-based transient gene expression (Norkunas et al. 2018). These studies highlight the downstream effects and importance of optimizing gene expression parameters for plant-based protein production. However, few studies have investigated the protein-level impacts of sample preparation and specifically, exposure and incubation of *A. tumefaciens* within infiltration medium prior to agroinfiltration. Such conditions may promote environmental adaptation of *A. tumefaciens* for agroinfiltration and influence the outcome of the plant transformation process.

Mass spectrometry-based proteomics explores the global impact of growth conditions and environmental parameters on cellular processes, interactions, and modifications (Aebersold and Mann 2016). It provides robust and unbiased measurements of protein abundance to define comprehensive changes to cellular proteomes and secretomes (the extracellular environment), as well as interaction and communication networks, and regulation patterns influenced by nutrient...
limitation (Geddes et al. 2015; 2016; Smits and Vermeulen 2016; Ball et al. 2019; Sukumaran et al. 2019; Muselius et al. 2020). In this study, we use state-of-the-art mass spectrometry to quantify changes in bacterial protein abundance over a time-course of exposure to agroinfiltration medium. Based on our recent work evaluating bacterial growth conditions (e.g., shake-flask vs. bioreactor) for optimal biomass production, we extend these findings to define processes of cellular remodeling specific to bacterium grown under each condition prior to agroinfiltration medium exposure and observe temporal proteome changes in a nutrient-limited environment (Prudhomme et al. In Revision). Our results identify distinct proteome profiles associated with bacterial growth conditions and exposure timing, as well as a progression towards increased cellular remodelling over time. In addition, we describe unique functional characteristics influencing pathogenesis and response to stimulus, as well as changes in bacterial nutrient acquisition and adaptability. Moreover, we validate our findings with in-depth profiling of specific proteins over time and demonstrate a >80% overlap between applications of our proteomics platform for \textit{A. tumefaciens} profiling. Overall, our results suggest that prolonged exposure of \textit{A. tumefaciens} to agroinfiltration medium may increase pathogenesis and motility, leading to enhanced infectivity of plant cells and production of the target drug.
Materials and Methods.

Strains

A modified version of the *A. tumefaciens* strain EHA105 (transformed with the T-DNA vector pPFC0058 for expression of the monoclonal antibody Trastuzumab) was used for the experiments (Hood et al. 1993; Mclean 2017). The bacteria were maintained on Lysogeny Broth (LB) agar plates at 28°C supplemented with kanamycin sulfate solution (50 µg/ml) and rifampicin solution (25 µg/ml).

Shake-flask growth conditions

An overnight culture of *A. tumefaciens* EHA105 (pPFC0058) was initiated from a single colony on LB media into 5 ml of liquid LB at 28°C and 220 rpm overnight with kanamycin (50 µg/ml) and rifampicin (25 µg/ml). Sub-culturing (1/100) was performed in 1 L shake-flasks containing 500 ml LB with kanamycin (50 µg/ml) and rifampicin (25 µg/ml) at 28°C and 170 rpm for 18 h (OD$_{600} = 1.4-1.5$).

Bioreactor growth conditions

An overnight culture of *A. tumefaciens* EHA105 (pPFC0058) was initiated from a single colony on LB media into 5 ml of liquid LB at 28°C and 220 rpm overnight with kanamycin (50 µg/ml) and rifampicin (25 µg/ml). Sub-culturing (1/100) was performed in bioreactor vessels (Sartorius) containing 500 ml of LB with kanamycin (50 µg/ml) and rifampicin (25 µg/ml) at 28°C and 600 rpm for 12 h (OD$_{600} = 1.4-1.5$).

Agroinfiltration media preparation and incubation

After sub-culturing in shake-flasks or bioreactors, cultures were diluted to OD$_{600} = 0.2$ in agroinfiltration medium (10 mM 2-N-morpholinoethanesulfonic acid, 10 mM magnesium
sulphate, pH 5.6) (Garabagi et al. 2012). Samples were subjected to static incubation at room temperature for 0, 2, and 24 h post immersion (hpi) in the dark.

**Sample collection**

Prior to collection, any settled cell material was resuspended by gentle swirling and two ml of culture was collected by centrifugation at 1,500 x g for 10 min. Supernatants were collected for further processing. Cell pellets were washed twice with cold phosphate buffered saline (PBS) and collected for protein extraction. Samples were stored on ice before processing.

**Sample preparation for proteomic analysis**

Protein extractions were performed as previously described, with modifications (Prudhomme et al. n.d.; Ball and Geddes-McAlister 2019). Briefly, bacterial cell pellets were resuspended in 100 mM Tris-HCl (pH 8.5) containing a protease inhibitor cocktail tablet. Samples were lysed by probe sonication (ThermoFisher Scientific) on ice bath for 3 cycles (30% power, 30 s on/30 s off). Two percent sodium dodecyl sulphate (SDS) and 10 mM dithiothreitol (DTT) was added, followed by incubation at 95°C for 10 min with shaking at 800 rpm. Samples were cooled and 55 mM iodoacetamide (IAA) was added followed by room temperature incubation for 20 min in the dark. Next, 100% ice cold acetone (final concentration of 80%) was added prior to storage at -20°C overnight. Samples were collected by centrifugation at 10,000 x g at 4°C for 10 min, washed twice with 80% acetone, and air dried. Pellets were resolubilized in 8M urea/40 mM HEPES and a bovine serum albumin (BSA) tryptophan assay determined protein concentrations (Wiśniewski and Gaugaz 2015). Samples were diluted in 50 mM ammonium bicarbonate and digested overnight with a mixture of LysC and trypsin proteases (Promega, protein:enzyme ratio, 50:1). Digestion was stopped with 10% v/v trifluoroacetic acid (TFA) and 50 µg of the acidified peptides were loaded onto STop And Go Extraction (STAGE) tips (consisting of three layers of...
C18) to desalt and purify according to the standard protocol (Rappsilber et al. 2007). Samples were stored as dried peptides at -20°C until measured on the mass spectrometer.

For secretome analysis, the culture supernatant was filtered through 0.22 µM syringe filters. For each sample, 500 µl of filtered supernatant was treated with DTT, IAA, followed by digestion using LysC and Trypsin. Digested peptides were desalted and purified as described above.

**Mass spectrometry**

Samples were eluted from STAGE-tips with 50 µl buffer B (80% acetonitrile (ACN) and 0.5% acetic acid), dried, and resuspended in 12 µl buffer A (0.1% TFA). Six µl of each sample was analyzed by nanoflow liquid chromatography on an Ultimate 3000 LC system (ThermoFisher Scientific) online coupled to a Fusion Lumos Tribrid mass spectrometer (ThermoFisher Scientific) through a nanoelectrospray flex-ion source (ThermoFisher Scientific). Samples were loaded onto a 5 mm µ-precolumn (ThermoFisher Scientific) with 300 µm inner diameter filled with 5 µm C18 PepMap100 beads. Peptides were separated on a 15 cm column with 75 µm inner diameter with 2 µm reverse-phase silica beads and directly electrosprayed into the mass spectrometer using a linear elution gradient from 4% to 30% ACN in 0.1% formic acid over 45 min at a constant flow of 300 nl/min. The linear gradient was followed by a washout with up to 95% ACN to clean the column followed by an equilibration stage to prepare the column for the next run. The Fusion Lumos was operated in data-dependent mode, switching automatically between one full scan and subsequent MS/MS scans of the most abundant peaks with a cycle time of 3 s. Full scan MS1s were acquired in the Orbitrap analyzer with a resolution of 120,000, scan range of 400-1600 m/z. The maximum injection time was set to 50 ms with an automatic gain control target of 4e5. The fragment ion scan was done in the Orbitrap using a Quadrupole isolation window of 1.6 m/z and HCD fragmentation
energy of 30 eV. Orbitrap resolution was set to 30,000 with a maximum ion injection time of 50 ms and an automatic gain control target set to 5e4.

**Data analysis**

For proteome data analysis, raw files were analyzed using MaxQuant software (version 1.6.0.26.) (Cox and Mann 2008). The derived peak list was searched with the built-in Andromeda search engine against the reference *A. tumefaciens* (Dec. 16, 2019; 5,344 sequences, https://www.uniprot.org/) supplemented with vector-specific sequences (Supp. Table. 1) (Cox et al. 2011). The parameters were as follows: strict trypsin specificity, allowing up to two missed cleavages, minimum peptide length was seven amino acids, carbamidomethylation of cysteine was a fixed modification, N-acetylation of proteins and oxidation of methionine were set as variable modifications. A minimum of two peptides was required for protein identification and peptide spectral matches and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%. ‘Match between runs’ was enabled with a match time window of 0.7 min and an alignment time window of 20 min (Cox et al. 2014). Relative, label-free quantification (LFQ) of proteins used the MaxLFQ algorithm integrated into MaxQuant using a minimum ratio count of one (Cox et al. 2014). The mass spectrometry proteomics data were deposited in the PRIDE partner repository for the ProteomeXchange Consortium with the data set identifier: PXD018405.

Further analysis of the MaxQuant-processed data (‘proteingroups.txt’ file) was performed using Perseus (version 1.6.2.2) (Tyanova et al. 2016). Hits to the reverse database, contaminants, and proteins only identified with modified peptides were eliminated. LFQ intensities were converted to a log scale (log₂), and only those proteins present in triplicate within at least one sample set were used for further statistical processing (valid-value filter of three in four replicates.
in at least one group). Missing values were imputed from a normal distribution (downshift of 1.8 standard deviations and a width of 0.3 standard deviations). A Student’s $t$-test identified proteins with significant changes in abundance ($p$-value $\leq 0.05$) with multiple hypothesis testing correction using the Benjamini-Hochberg FDR cut-off at 0.05, $s0 = 1$. A principal component analysis (PCA) was performed. The PCA plot was performed on the ‘category enrichment in components’ for five components with a Benjamini-Hochberg FDR = 0.05 and relative enrichment by Protein IDs. Replicate reproducibility was derived from a Pearson correlation with hierarchical clustering by Euclidean distance. For 1D annotation enrichment, Student’s $t$-test (permutation-based FDR = 0.05; $s0 = 1$) was performed followed by 1D annotation enrichment function in Perseus (Cox and Mann 2012). This analysis generates a numerical ‘score’ value, which represents the direction in which the protein LFQ intensities within a given category tend to deviate from the overall distribution of all proteins. Visualization of enrichment by Gene Ontology was performed within the RStudio platform (http://www.R-project.org/) (R Foundation for Statistical Computing. 2018). For network visualization the STRING database was used (https://string-db.org) (Szklarczyk et al. 2019).
Results.

To assess the impact of agroinfiltration medium and the effect of storage of *A. tumefaciens* cultures prior to the agroinfiltration process, we performed a quantitative proteomic analysis of the cell pellet (total proteome) and secretome (extracellular environment) (Fig. 1). With this information, we analyzed changes to *A. tumefaciens* at the protein-level in response to transformation preparation, which may require prolonged periods of bacterial storage in the agroinfiltration medium when large numbers of plants are prepared for industrial treatment. Moreover, we aimed to suggest optimal processing strategies to improve target drug production as influenced by the agroinfiltration process. Furthermore, we explored the influence of sample growth conditions (e.g., bioreactor growth vs. shake-flasks) on bacterial response to the agroinfiltration process to highlight potential protective measures demonstrated by the bacteria.

**Storage in agroinfiltration medium alters the cellular proteome of *A. tumefaciens***

In total, we identified 2,992 proteins (>55% of open reading frames) in the cellular proteome and secretome samples (before valid value filtering) and pursued further analysis of 2,640 proteins (Fig. 2A). Proteins identified in the supernatant did not meet the valid value filtering criteria, therefore we focused our analysis on the cellular proteome results. Biological replicate reproducibility was > 97% for all samples (Fig. 2B). A principal component analysis (PCA) plot demonstrated distinct clustering based on bacterial growth conditions (e.g., bioreactor vs. shake-flasks) (component 1, 33%), and a second component of separation corresponding to time stored in agroinfiltration medium (component 2, 20%) (Fig. 2C). Notably, the early storage time points (0 and 2 h) clustered more closely together with separation of the 24 h time point evident.

**Duration of exposure to agroinfiltration medium influences bacterial cell remodeling**
Given the distinction between storage time and bacterial growth conditions, we identified proteins with significant changes in abundance under the different parameters. For shake-flasks, we observed the smallest protein-level changes at early immersion (0 h) compared to the later time points. Specifically, 10 proteins were significantly increased in abundance and 10 proteins showed a significant decrease in abundance at 2 h (Fig. 3A; Supp. Table 2). Of these, we observed an increase in pathogenesis-related protein Atu3295, methyl-accepting chemotaxis protein McpC, and an outer membrane heme receptor Atu2287. Conversely, we observed a decrease in abundance of ABC transport substrate binding proteins (Atu4123, Atu3253) and a transcriptional regulator (OxyR). A comparison of the intermediate stage response (24 h vs. 2 h) to agroinfiltration medium revealed a significant change in abundance of 77 proteins, including 58 proteins with increased abundance and 19 proteins with decreased abundance (Fig. 3B; Supp. Table 3). Here, we observed an increase in two nitric oxidase-associated proteins (NirK, NorQ), a flagellin (FlaA), ABC transporter iron binding protein AfuA, and ferrienterobactin-like protein Atu4400. Decreased abundance was evident for several biosynthetic enzymes, including soluble lytic transglycosylase Atu1022 and mannosylfructose-phosphate synthase MfpsA. Finally, comparison of late-stage samples (24 h vs. 0 h) identified 204 proteins with significant changes in abundance, including 144 proteins with increased and 60 proteins with reduced abundance (Fig. 3C; Supp. Table 4). These results support the intermediate time point observation with similar patterns of abundance for nitric oxidase-associated proteins (NirK, NorQ) and FlaA, as well as biosynthetic enzymes Atu1022 and MfpsA.

We also investigated the presence of common and unique proteins produced at each of the time points and report time-specific responses at each stage of incubation, and to a lesser extent, common responses across the comparisons; however, no proteins were detected as significantly
different at all three stages (Fig. 3D). Three proteins were significantly different at 0 and 24 h but did not demonstrate a change in abundance at 2h. Although this may be due to biological variability or a fluctuation in cell protein responses over the time course of incubation, taken together, our quantitative proteomic profiling of changes to the cellular proteome of *A. tumefaciens* grown in shake-flask and exposed to agroinfiltration medium at early (0 h), intermediate (2 h), and late (24 h) stages indicates a progression of cellular remodeling over time.

Next, we explored temporal differences in exposure to agroinfiltration medium of bacterial cultures grown in a bioreactor. Again, we observed the lowest number of altered protein profiles at 0 h with seven proteins displaying significant changes in abundance: five proteins increased, and two proteins decreased (Fig. 3E; Supp. Table 5). All proteins with increased abundance were uncharacterized (Atu3295, Atu8200, Atu1155, Atu2160, Atu5516) and proteins with decreased abundance included a glucose dehydrogenase (Gcd) and uncharacterized protein Atu3752. For the intermediate stage (24 h vs. 2 h), 256 proteins were significantly different with 202 proteins of increased abundance and 54 proteins of decreased abundance (Fig. 3F; Supp. Table 6). Here, two biosynthetic enzymes (SoxA, PurU) and two ABC transporters (Atu3165, Atu3372) were higher in abundance, whereas a protease (Atu4397), ABC transporter Atu0893, and chemotaxis protein McpA were lower in abundance. At the late stage (24 h vs. 0 h) of exposure in agroinfiltration medium, bacterial cultures grown under bioreactor conditions identified 376 significantly different proteins, including 288 with increased and 88 with decreased abundance (Fig. 3G; Supp. Table 7). These observations support those at the intermediate time point and we note no apparent loss in bacterial adaptation over the 2 to 24 h time period.

Very similar to the observation of common and unique proteins produced at each of the time points during shake-flask growth, time-specific responses were present at each stage of
immersion, and to a lesser extent, we detected common responses between 0 and 2 h, as well as 2 and 24 h (Fig. 3H). Again, we did not detect any significantly different proteins at all three stages of incubation. With this information, we can begin to define the impact of agroinfiltration medium on bacterial cultures over the time course of bacterial processing and we can distinguish differences in response to exposure, dependent on bacterial growth conditions.

**Unique functional alternations upon exposure to agroinfiltration medium**

Given our identification of significant changes in protein abundance, suggesting cellular remodeling in the presence of agroinfiltration medium during 24 h exposure, we hypothesized that differences in functional groups of proteins may change over time. To explore this idea, we performed a 1D annotation enrichment analysis based on Gene Ontology Biological Processes (GOBP) (Ashburner et al. 2000; Cox and Mann 2012). For shake-flask samples, we saw a significant positive enrichment (based on GOBP) of proteins associated with multi-organism process (e.g., pathogenesis), response to stimulus (e.g., chemotaxis, locomotion, stress responses), and carbohydrate metabolic process (e.g., β-xylanase) as immersion time in agroinfiltration medium increased (Fig. 4A). Conversely, we saw a negative enrichment of proteins associated with metabolism, biosynthetic processes, and translation over time. To define networks of proteins altered over time in agroinfiltration medium, we used the STRING database to analyse proteins showing significant increases in abundance at 24 h from shake-flask samples (Fig. 4B). This analysis highlights four clusters of proteins: i) flagellin, motility, and chemotaxis, ii) metabolism (enzymatic activity), iii) siderophore biosynthesis, and iv) heme- and iron-association.

We performed a 1D annotation analysis for bioreactor samples by GOBP and observed a similar positive enrichment of response to stimulus proteins over time and a negative enrichment of proteins associated with metabolic and biosynthetic processes, translation, aminoacylation, and
amino acid activation (Fig. 4C). An investigation into protein networks altered during incubation with agroinfiltration medium from bioreactor samples highlighted increased abundance at 24 h of proteins associated with: i) flagellin, motility, and chemotaxis, ii) metabolism (enzymatic activity), and iii) transport (Fig. 4D). Taken together, our functional analysis of the impact of agroinfiltration medium on *A. tumefaciens* demonstrated growth- and environment-specific processes over time and underscores the interconnectivity of pathogenesis and chemotaxis-associated proteins with transport and metabolism. Furthermore, we observed a consistent reduction in metabolic and biosynthetic processes, as well as ribosome activity and translation, which suggest remodelling of cellular processes over processing of the bacterial culture.

**Changes to pathogenesis- and motility-associated proteins highlights bacterial adaptability**

Given our observations of diverse biological categories altered during the time course of sample preparation for agroinfiltration, we hypothesized that specific proteins influence these cellular processes and confer a balance between bacterial protection and activation over time. First, based on a positive enrichment of multi-organism process from shake-flask samples, we profiled LFQ differences in all pathogenesis-associated proteins over time. Although no significant difference in abundance was observed among the group of proteins, we detected a significant increase in production of pathogenesis-associated protein Atu3295 by 24 h in shake-flask samples (Fig. 5A; Supp. Fig. 1A). Moreover, given our observation of the importance of response to stimulus, chemotaxis, locomotion, and transport at 24 h, we explored changes in protein abundance for all flagellin-associated proteins. We noted a significant increase in flagellar protein abundance over time; Flagellin A (FlaA) showed the greatest increase in abundance (Fig. 5B; Supp. Fig. 1B). We observed similar protein response profiles for bioreactor samples over time, despite a reduced
emphasis on pathogenesis but a similar response on bacterial motility (Fig. 5C, D; Supp. Fig 1C, D).

Finally, we compared our proteomic profiling from the agroinfiltration experiments performed in this study to our recent investigation into the protein-level impact of bacterial growth conditions (e.g., shake-flask vs bioreactor) (Prudhomme et al. n.d.). Of the 2,743 proteins identified between the studies, 80% were common, supporting consistency in our protein extraction, sample preparation, and mass spectrometry methods (Fig. 5E). Moreover, our approaches enabled detection of protein-level changes distinct to each study. For example, 104 proteins were specific to our investigation of shake-flask vs. bioreactor growth conditions and 456 proteins are unique to this agroinfiltration study. Moreover, this comparison allowed us to tease apart growth- vs. environment-specific bacterial responses. For example, shake-flask growth impacted metabolic and biosynthetic pathways, whereas during analysis of temporal agroinfiltration preparation, we observed broad-spectrum changes to pathogenesis, motility, and nutrient acquisition. For bioreactor samples, growth condition analysis emphasized differences in transport and locomotion, which also drive the observations associated with agroinfiltration. Taken together, our quantitative proteomic profiling provided a reliable analysis of cellular remodelling and changes in biological processes associated with multiple steps in the agroinfiltration-mediated transient expression process and highlighted distinct responses that may influence bacterial survival and adaptability.
Discussion.

By profiling the total proteome of *A. tumefaciens* in response to prolonged exposure to agroinfiltration medium as a preparatory step for plant transformation, we report time-dependent cellular responses and dynamic remodelling of biological processes. Specifically, we observed significant changes in proteins involved in pathogenesis, motility, and nutrient acquisition systems as the bacteria adapt to the new environment. In general, these responses are specific to the exposure time and were not defined by bacterial growth conditions (e.g., shake-flask vs. bioreactor). As expected, shorter periods of exposure to agroinfiltration medium cause fewer protein-level changes to the bacterial cultures. However, analysis of exposure periods of 2 h, which is an accurate representation of the time lag between medium preparation and plant treatment, shows remodeling of cellular processes have begun and, by 24 h, we observed dynamic remodeling. Overall, these results suggested enhanced bacterial adaptability to environmental conditions during the agroinfiltration process, which may promote efficient bacterial invasion upon transformation.

Previous proteome profiling of *A. tumefaciens* response to plant tissue (e.g., tomato roots) showed induction of 30 proteins, including known virulence factors, transporters, metal-associated enzymes, as well as biosynthetic and metabolic enzymes, supporting their production during the invasion process (Rosen et al. 2003). We observed similar patterns of protein induction over the time course of exposure to agroinfiltration medium, suggesting the incubation period may serve as a ‘primer’ to activate the bacteria in preparation for host cell invasion. For example, following shake-flask growth, we observed an increase in abundance of proteins associated with pathogenesis, including uncharacterized protein Atu3295 (BA14-like protein associated with immune reactivity) during the time course of exposure (Chirhart-Gilleland et al. 1998). Previous
reports demonstrated the induction of virulence-associated genes to improve transformation frequencies. Specifically, the inclusion of acetylsyringone, a plant-secreted phenolic that induces Agrobacterium virulence gene expression through induction of a small heat shock protein involved in VirB accumulation and promoting virB/D4-mediated DNA transfer, is often used for co-cultivation in agroinfiltration media (Stachel et al. 1986; Hiei et al. 1994; Lai et al. 2006; Wydro et al. 2006; Tsai et al. 2009; Norkunas et al. 2018). Not surprisingly, given the absence of the host, we did not observe VirB in our dataset, which may indicate low protein production with the tested parameters. Taken together, these findings suggest the production of pathogenesis-associated proteins in response to additives or prolonged exposure to agroinfiltration medium may ‘prime’ the bacteria for improved host invasion efficiency.

The physical attachment of bacteria to the host is critical for infection and horizontal gene transfer to the plant (Thompson et al. 2018). There are several structures that function for surface attachment by A. tumefaciens, including flagella, several different forms of pili, and multiple complex polysaccharides (Gotz et al. 1982). The general function of flagella is to propel bacteria through an environment. Flagellar propulsion can enable bacteria to move towards conditions that are favourable, such as high nutrients, and avoid conditions that inhibit growth or damage cells. However, in some cases, flagella play an important role in surface attachment during infection (Merritt et al. 2007). Here, we observed an increase in abundance of proteins associated with motility and chemotaxis, specifically, flagellar protein production (FlaA, the primary flagellin, required for motility) during the course of exposure to agroinfiltration medium (Deakin et al. 1997). Based on the role of flagellin in motility, the increased production may be associated with searching for nutrients in a nutrient-limited environment or it may support preparation of the bacteria for host invasion. A role in nutrient sensing is corroborated by our observation of activated
ABC transporters, specifically, those associated with metal-ion uptake (e.g., iron, heme, zinc) which are commonly produced in response to nutrient-limited environments when encountered by bacteria (Heindl et al. 2016; Tanaka et al. 2018). Conversely, a connection between FlaA and bacterial virulence associated with surface attachment has been explored in the interaction between *A. tumefaciens* and host, demonstrating an influence of light-induction on gene expression (Oberpichler et al. 2008). Overall, our conserved observation of changes in motility during prolonged exposure to agroinfiltration medium, regardless of bacterial growth conditions (shake-flask vs. bioreactor), supports cell remodeling for purposes of nutrient acquisition and perhaps enhanced surface attachment efficiency upon transformation.

Despite our observations of increased production of bacterial proteins connected with pathogenesis, motility, and transportation during the course of incubation in agroinfiltration medium, suggesting adaptation of the bacteria to the surrounding environment and enhanced preparedness for transformation, we also observed a reduction in metabolism and biosynthetic processes, as well as mRNA translation over time. The absence of nutrients (starvation) can induce metabolic stress, DNA damage, and polymerase pathway activation to induce cell death as an adaptation and survival mechanism (Rodríguez-Vargas and Oliver 2016). Moreover, sufficient nutrient supply is essential for optimal mRNA translation and nutrient deprivation can inhibit global protein synthesis, supporting our observation of reduced translation in agroinfiltration medium (Gameiro and Struhl 2018). Given the reduced cellular response to a nutrient-limited environment (i.e., agroinfiltration medium), a balance between bacterial survivability (e.g., increased production of acquisition-associated transporters) and adaptability (e.g., production of pathogenesis and motility proteins) is needed to promote efficient transformation. Furthermore, exploring the relationship between prolonged periods of exposure to agroinfiltration medium and
cell viability will highlight important relationships between bacterial priming, survivability, and infectivity.

Notably, proteins detected in the supernatant were low in numbers and inconsistently identified (i.e., present in less than three of four replicates), resulting in their removal from further analysis. An important factor to consider when profiling the supernatant of bacterial cells, is the medium used for initial growth. For example, in these experiments, we culture the bacterium in LB medium, which may vary between lots and for optimal manufacturing procedures, one lot should be used for all experiments. Additionally, the use of alternative medium (e.g., yeast extract beef broth) for initial culturing prior to exposure to infiltration medium, may impact the proteins secreted. Further investigation into precise medium components and their impact on bacterial growth may enable a deeper profiling of the *A. tumefaciens* secretome. In addition, a lack of proteins identified in the supernatant may be due to low abundance of released or secreted bacterial proteins during the exposure to agroinfiltration medium, supporting low occurrence of cell lysis during the incubation period. Alternatively, the supernatant may be dilute and investigation of proteins in the extracellular environment of a concentrated supernatant or following protein precipitation methodology may provide more protein identifications (Caldwell and Lattemann 2004; Chevallet et al. 2007).
Conclusion.

Our results define dynamic remodeling of *A. tumefaciens* as changes in the cellular proteome over a time course of exposure to agroinfiltration medium as an adaptation to the nutrient-limited environment. We hypothesize that this remodeling promotes virulence, nutrient acquisition, and motility of the bacterium, which will enhance host infectivity and therefore, the transformation pathway may benefit from prolonged exposure periods. To support this hypothesis, future studies will explore target protein production following transformation of plants with *A. tumefaciens* cultures collected at early (0 h), intermediate (2 h), and late (24 h) stages of exposure in agroinfiltration medium, as well as assess cellular viability throughout the exposure time course. Moreover, we aim to knock out and overexpress the pathogenicity gene (Atu3295) and FlaA and assess bacterial infectivity and potential changes to target protein production. Based on our current findings, and previous reports of *A. tumefaciens* infectivity, we hypothesize that increased pathogenicity and motility will support hypervirulence, improve infectivity, and increase production of the target protein, which we will explore in future studies. However, increased bacterial virulence may initiate an enhanced plant defense response and therefore, we will monitor changes from both the host and pathogen perspectives simultaneously. Industry-scale production of transiently expressed proteins for pharmaceutical use requires an efficient process strategy for speed and purity. Recent research has explored animal component-free media to meet requirements of good manufacturing processes (GMP), and ready-to-use *A. tumefaciens* stocks to de-couple bacterial growth and infiltration stages (Houdelet et al. 2017; Spiegel et al. 2019). Here we propose the benefits of long-term exposure to infiltration medium to promote a process for efficient bacterial invasion and transformation.
Conflict of Interest
N.P., B.M., & J.G.-M. declare that the research is funded, in part, by PlantForm Corporation. R.S. is employed by PlantForm Corporation. M.D.M. & D.C. are original founders of PlantForm Corporation and have a financial interest in the company.

Author Contributions
M.D.M., D.C. & J.G-M. devised the project; N.P., M.D.M., D.C., & J.G-M. planned experiments; N.P. & R.S. performed experiments; N.P., B.M., M.D.M., D.C. & J.G-M. performed data analysis and interpretation; B.M. performed statistical analysis; N.P. & J.G.-M. generated figures; N.P., M.D.M., D.C. & J.G-M. wrote and edited the manuscript.

Funding
This work was supported, in part, by NSERC CRD (CRDPJ 539389 - 19), the University of Guelph, Canadian Foundation of Innovation (JELF 38798), and financial contribution from PlantForm for J.G.-M.

Acknowledgments
The authors wish to thank Dr. Jonathan Krieger of Bioinformatics Solutions Inc. for operating the mass spectrometer and members of the Geddes-McAlister lab and PlantForm for their critical reading and insightful comments during manuscript preparation. Support from members of Dr. Emma Allen-Vercoe’s lab at the University of Guelph, including Chris Ambrose, Caroline Ganobis, and Jacob Wilde for operation and training on the bioreactor system.
Data Availability Statement

The mass spectrometry proteomics data have been deposited in the PRIDE partner repository for the ProteomeXchange Consortium with the data set identifier: PXD018405

Reviewer account username: reviewer33379@ebi.ac.uk
Password: F6aHyNf7
References.

Aebersold, R., and Mann, M. 2016. Mass-spectrometric exploration of proteome structure and function. Nature 537(7620): 347–355. doi:10.1038/nature19949.

Aguilar, J., Zupan, J., Cameron, T.A., and Zambryski, P.C. 2010. *Agrobacterium* type IV secretion system and its substrates form helical arrays around the circumference of virulence -induced cells. Proc. Natl. Acad. Sci. doi:10.1073/pnas.0914940107.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., and Sherlock, G. 2000. Gene ontology: Tool for the unification of biology. doi:10.1038/75556.

Ball, B., Bermas, A., Carruthers-Lay, D., and Geddes-McAlister, J. 2019. Mass Spectrometry-Based Proteomics of Fungal Pathogenesis, Host–Fungal Interactions, and Antifungal Development. J. Fungi. doi:10.3390/jof5020052.

Ball, B., and Geddes-McAlister, J. 2019. Quantitative Proteomic Profiling of *Cryptococcus neoformans*. Curr. Protoc. Microbiol. doi:10.1002/cpmc.94.

Buyel, J.F. 2015. Controlling the interplay between *Agrobacterium tumefaciens* and plants during the transient expression of proteins. Bioengineered. doi:10.1080/21655979.2015.1052920.

Caldwell, R.B., and Lattemann, C.T. 2004. Simple and Reliable Method to Precipitate Proteins from Bacterial Culture Supernatant. Appl. Environ. Microbiol. doi:10.1128/AEM.70.1.610-612.2004.
Chen, Q., and Davis, K.R. 2016. The potential of plants as a system for the development and production of human biologics. F1000Research. doi:10.12688/f1000research.8010.1.

Chevallet, M., Diemer, H., Van Dorssealer, A., Villiers, C., and Rabilloud, T. 2007. Toward a better analysis of secreted proteins: The example of the myeloid cells secretome. Proteomics. doi:10.1002/pmic.200601024.

Chirhart-Gilleland, R.L., Kovach, M.E., Elzer, P.H., Jennings, S.R., and Roop, R.M. 1998. Identification and characterization of a 14-kilodalton Brucella abortus protein reactive with antibodies from naturally and experimentally infected hosts and T lymphocytes from experimentally infected BALB/c mice. Infect. Immun. doi:10.1128/iai.66.8.4000-4003.1998.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., and Mann, M. 2014. Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction, Termed MaxLFQ. Mol. Cell. Proteomics 13(9): 2513–2526. doi:10.1074/mcp.M113.031591.

Cox, J., and Mann, M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26(12): 1367–1372. doi:10.1038/nbt.1511.

Cox, J., and Mann, M. 2012. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. BMC Bioinformatics. doi:10.1186/1471-2105-13-S16-S12.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J. V., and Mann, M. 2011. Andromeda: A peptide search engine integrated into the MaxQuant environment. J.
Proteome Res. doi:10.1021/pr101065j.

Deakin, W.J., Furniss, C.S.M., Parker, V.E., and Shaw, C.H. 1997. Isolation and characterisation of a linked cluster of genes from Agrobacterium tumefaciens encoding proteins involved in flagellar basal-body structure. Gene. doi:10.1016/S0378-1119(96)00780-9.

Gameiro, P.A., and Struhl, K. 2018. Nutrient Deprivation Elicits a Transcriptional and Translational Inflammatory Response Coupled to Decreased Protein Synthesis. Cell Rep. doi:10.1016/j.celrep.2018.07.021.

Garabagi, F., McLean, M.D., and Hall, J.C. 2012. Transient and stable expression of antibodies in nicotiana species. Methods Mol. Biol. doi:10.1007/978-1-61779-974-7_23.

Geddes, J.M.H., Caza, M., Croll, D., Stoynov, N., Foster, L.J., and Kronstad, J.W. 2016. Analysis of the protein kinase a-regulated proteome of Cryptococcus neoformans identifies a role for the ubiquitin-proteasome pathway in capsule formation. MBio 7(1): 1–15. doi:10.1128/mBio.01862-15.

Geddes, J.M.H., Croll, D., Caza, M., Stoynov, N., Foster, L.J., and Kronstad, J.W. 2015. Secretome profiling of Cryptococcus neoformans reveals regulation of a subset of virulence-associated proteins and potential biomarkers by protein kinase A. BMC Microbiol. doi:10.1186/s12866-015-0532-3.

Gohlke, J., and Deeken, R. 2014. Plant responses to Agrobacterium tumefaciens and crown gall development. doi:10.3389/fpls.2014.00155.

Gotz, R., Limmer, N., Ober, K., and Schmitt, R. 1982. Motility and chemotaxis in two strains of Rhizobium with complex flagella. J. Gen. Microbiol. doi:10.1099/00221287-128-4-789.
Grohs, B.M., Niu, Y., Veldhuis, L.J., Trabelsi, S., Garabagi, F., Hassell, J.A., McLean, M.D., and Hall, J.C. 2010. Plant-produced trastuzumab inhibits the growth of HER2 positive cancer cells. J. Agric. Food Chem. doi:10.1021/jf102284f.

Grosse-Holz, F., Kelly, S., Blaskowski, S., Kaschani, F., Kaiser, M., and van der Hoorn, R.A.L. 2018. The transcriptome, extracellular proteome and active secretome of agroinfiltrated Nicotiana benthamiana uncover a large, diverse protease repertoire. Plant Biotechnol. J. doi:10.1111/pbi.12852.

Heindl, J.E., Hibbing, M.E., Xu, J., Natarajan, R., Buechlein, A.M., and Fuqua, C. 2016. Discrete responses to limitation for iron and manganese in Agrobacterium tumefaciens: Influence on attachment and biofilm formation. J. Bacteriol. doi:10.1128/JB.00668-15.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. 1994. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. doi:10.1046/j.1365-313X.1994.6020271.x.

Hood, E.E., Gelvin, S.B., Melchers, L.S., and Hoekema, A. 1993. New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res. doi:10.1007/BF01977351.

Houdelet, M., Galinski, A., Holland, T., Wenzel, K., Schillberg, S., and Buyel, J.F. 2017. Animal component-free Agrobacterium tumefaciens cultivation media for better GMP-compliance increases biomass yield and pharmaceutical protein expression in Nicotiana benthamiana. Biotechnol. J. doi:10.1002/biot.201600721.

Huafang Lai, Q.C., and Jake Stahnke, J.H. 2013. Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. Adv. Tech. Biol. Med. doi:10.4172/atbm.1000103.
Hughes, M.A. 1996. Plant Molecular Genetics. In 1st edition. Harlow (Essex): Addison Wesley Longman Limited.

Krenek, P., Samajova, O., Luptovciak, I., Doskocilova, A., Komis, G., and Samaj, J. 2015. Transient plant transformation mediated by Agrobacterium tumefaciens: Principles, methods and applications. doi:10.1016/j.biotechadv.2015.03.012.

Lai, E.M., Shih, H.W., Wen, S.R., Cheng, M.W., Hwang, H.H., and Chiu, S.H. 2006. Proteomic analysis of Agrobacterium tumefaciens response to the vir gene inducer acetosyringone. Proteomics. doi:10.1002/pmic.200600254.

McLean, M.D. 2017. Journal of Drug Design and Research Cite this article: McLean MD (2017) Trastuzumab Made in Plants Using vivoXPRESS ® Platform Technology. In J Drug Des Res.

Merritt, P.M., Danhorn, T., and Fuqua, C. 2007. Motility and chemotaxis in Agrobacterium tumefaciens surface attachment and biofilm formation. In Journal of Bacteriology. doi:10.1128/JB.00566-07.

Muselius, B., Sukumaran, A., Yeung, J., and Geddes-McAlister, J. 2020. Iron limitation in Klebsiella pneumoniae defines new roles for Lon protease in homeostasis and degradation by quantitative proteomics. Front. Microbiol. 11:546. doi: 10.3389/fmicb.2020.00546

Nester, E.W. 2015. Agrobacterium: Nature’s Genetic Engineer. Front. Plant Sci. doi:10.3389/fpls.2014.00730.

Norkunas, K., Harding, R., Dale, J., and Dugdale, B. 2018. Improving agroinfiltration-based transient gene expression in Nicotiana benthamiana. Plant Methods. doi:10.1186/s13007-
Oberpichler, I., Rosen, R., Rasouly, A., Vugman, M., Ron, E.Z., and Lamparter, T. 2008. Light affects motility and infectivity of *Agrobacterium tumefaciens*. Environ. Microbiol. doi:10.1111/j.1462-2920.2008.01618.x.

Prudhomme, N., Gianetto-Hill, C., Pastora, R., Cheung, W.-F., Allen-Vercoe, E., McLean, M.D., Cossar, D., and Geddes-McAlister, J. (n.d.). Growth-specific responses of *Agrobacterium tumefaciens* correlated with quantitative proteomic profiling defines new opportunities for improving target protein yields in molecular pharming. Can J Microbiol.

R Foundation for Statistical Computing. 2018. R: a Language and Environment for Statistical Computing. *In* http://www.R-project.org/.

Rappsilber, J., Mann, M., and Ishihama, Y. 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2(8): 1896–1906. doi:10.1038/nprot.2007.261.

Rivera, A.L., Gómez-Lim, M., Fernández, F., and Loske, A.M. 2012. Physical methods for genetic plant transformation. doi:10.1016/j.plrev.2012.06.002.

Rodríguez-Vargas, J.M., and Oliver, F.J. 2016. Role of Poly(ADP-Ribose) in Catalyzing Starvation-Induced Autophagy. *In* Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. doi:10.1016/b978-0-12-805421-5.00003-3.

Rosen, R., Matthysse, A.G., Becher, D., Biran, D., Yura, T., Hecker, M., and Ron, E.Z. 2003. Proteome analysis of plant-induced proteins of *Agrobacterium tumefaciens*. FEMS Microbiol. Ecol. doi:10.1016/S0168-6496(03)00077-1.
Smits, A.H., and Vermeulen, M. 2016. Characterizing Protein–Protein Interactions Using Mass Spectrometry: Challenges and Opportunities. doi:10.1016/j.tibtech.2016.02.014.

Spiegel, H., Boes, A., Perales Morales, C., Rademacher, T., and Buyel, J.F. 2019. Ready-to-Use Stocks of Agrobacterium tumefaciens Can Simplify Process Development for the Production of Recombinant Proteins by Transient Expression in Plants. Biotechnol. J. doi:10.1002/biot.201900113.

Stachel, S.E., Nester, E.W., and Zambryski, P.C. 1986. A plant cell factor induces Agrobacterium tumefaciens vir gene expression. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.83.2.379.

Sukumaran, A., Coish, J.M., Yeung, J., Muselius, B., Gadjeva, M., MacNeil, A.J., and Geddes-McAlister, J. 2019. Decoding communication patterns of the innate immune system by quantitative proteomics. doi:10.1002/JLB.2RI0919-302R.

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., Jensen, L.J., and Von Mering, C. 2019. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. doi:10.1093/nar/gky1131.

Tanaka, K.J., Song, S., Mason, K., and Pinkett, H.W. 2018. Selective substrate uptake: The role of ATP-binding cassette (ABC) importers in pathogenesis. doi:10.1016/j.bbamem.2017.08.011.

Thompson, M.A., Onyeziri, M.C., and Fuqua, C. 2018. Function and regulation of Agrobacterium tumefaciens cell surface structures that promote attachment. In Current
Topics in Microbiology and Immunology. doi:10.1007/82_2018_96.

Tsai, Y.L., Wang, M.H., Gao, C., Klüsener, S., Baron, C., Narberhaus, F., and Lai, E.M. 2009. Small heat-shock protein HspL is induced by VirB protein(s) and promotes VirB/D4-mediated DNA transfer in Agrobacterium tumefaciens. Microbiology. doi:10.1099/mic.0.030676-0.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., and Cox, J. 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. doi:10.1038/nmeth.3901.

Veena, Jiang, H., Doerge, R.W., and Gelvin, S.B. 2003. Transfer of T-DNA and Vir proteins to plant cells by Agrobacterium tumefaciens induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. Plant J. doi:10.1046/j.1365-313X.2003.01796.x.

Wiśniewski, J.R., and Gaugaz, F.Z. 2015. Fast and sensitive total protein and peptide assays for proteomic analysis. Anal. Chem. doi:10.1021/ac504689z.

Wydro, M., Kozubek, E., and Lehmann, P. 2006. Optimization of transient Agrobacterium-mediated gene expression system in leaves of Nicotiana benthamiana. Acta Biochim. Pol. doi:10.18388/abp.2006_3341.

Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K., and Winans, S.C. 2000. The bases of crown gall tumorigenesis. doi:10.1128/JB.182.14.3885-3895.2000.
Figure legends

**Figure 1: Experimental design and mass spectrometry-based proteomics workflow.** *A. tumefaciens* cultures were grown in shake-flasks or bioreactors, diluted in agroinfiltration medium and exposed at room temperature for 0, 2, and 24 h. Total proteome (cell pellet) and secretome (supernatant) were collected and extracted followed by purification, electrospray ionisation (ESI) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Data processing, analysis, and visualization performed with MaxQuant and Perseus platforms.

**Figure 2: Overview of proteomics profiling of *A. tumefaciens* exposed to agroinfiltration medium.** A) Venn diagram of total proteins identified by proteomic profiling of cell pellet (grey; 2,986 proteins) and supernatant (burgundy; 4 proteins). B) Heat map of replicate reproducibility by Pearson correlation and hierarchical clustering by Euclidean distance. C) Principal component analysis (bioreactor = square data points, shake-flask = circle data points; 0 h exposure labeled in black, 2 h exposure labeled in green, and 24 h exposure labeled in purple). Experiment performed in biological quadruplicate.

**Figure 3: Time-dependent cellular remodeling of *A. tumefaciens*.** A) Volcano plot of shake-flask samples exposed for 2 h (green) vs. 0 h (black) in agroinfiltration medium. B) Volcano plot of shake-flask samples exposed for 24 h (purple) vs. 2 h (green) in agroinfiltration medium. C) Volcano plot of shake-flask samples exposed for 24 h (purple) vs. 0 h (black) in agroinfiltration medium. D) Venn diagram of common and unique significantly different proteins across the time points from shake-flask samples (0 h = black, 2 h = green, 24 h = purple). E) Volcano plot of bioreactor samples exposed for 2 h (green) vs. 0 h (black) in agroinfiltration medium. F) Volcano
plot of bioreactor samples exposed for 24 h (purple) vs. 2 h (green) in agroinfiltration medium. **G)** Volcano plot of bioreactor samples exposed for at 24 h (purple) vs. 0 h (black) in agroinfiltration medium. **H)** Venn diagram of common and unique significantly different proteins across the time points from bioreactor samples (0 h = black, 2 h = green, 24 h = purple). For volcano plots, Student’s *t*-test *p*-value ≤ 0.05; FDR = 0.05; s0 = 1. Experiment performed in biological quadruplicate.

**Figure 4: Functional analysis of protein-level changes.** **A)** 1D annotation enrichment of Gene Ontology Biological Processes for shake-flask samples (0, 2, 24 h). **B)** STRING network analysis of significantly different proteins with increased abundance at 24 h for shake-flask samples. **C)** 1D annotation enrichment of Gene Ontology Biological Processes for bioreactor samples (0, 2, 24 h). **D)** STRING network analysis of significantly different proteins with increased abundance at 24 h for bioreactor samples. For 1D annotation enrichment, Student’s *t*-test *p*-value ≤ 0.05; FDR = 0.05; score >-0.5 < 0.5. 0 h = comparison between 2 and 0 h; 2h = comparison between 24 and 2 h; 24 h = comparison between 24 and 0 h.

**Figure 5: Protein abundance profiles.** **A)** Protein abundance profile by LFQ values of pathogenesis-related protein Atu3295 over the time course of exposure to agroinfiltration medium from shake-flask samples. **B)** Protein abundance profile by LFQ values of FlaA over the time course of exposure to agroinfiltration medium from shake-flask samples. **C)** Protein abundance profile by LFQ values of pathogenesis-related protein Atu3295 over the time course of exposure to agroinfiltration medium from bioreactor samples. **D)** Protein abundance profile by LFQ values of FlaA over the time course of exposure to agroinfiltration medium from bioreactor samples. **E)** Venn diagram of quantitative proteomics results demonstrating overlap of proteins identified in
this study and proteins identified in recent study (Prudhomme et al. n.d.). The majority of proteins (>80%) are common between the datasets, supporting consistency in the proteomics platforms, while also identifies proteins unique to each condition: purple = 104 proteins only identified in previous study comparing bioreactor vs. shake-flask growth (Prudhomme et al. n.d.); blue = 456 proteins only identified in this study. Student’s t-test: *p-value ≤ 0.05; **p-value ≤ 0.001. Error bars = standard deviation.
