

RESEARCH ARTICLE

Quantitative iTRAQ proteome and comparative transcriptome analysis of elicitor-induced Norway spruce (*Picea abies*) cells reveals elements of calcium signaling in the early conifer defense response

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Long-lived conifer trees depend on both constitutive and induced defenses for resistance against a myriad of potential pathogens and herbivores. In species of spruce (*Picea* spp.), several of the late events of pathogen-, insect-, or elicitor-induced defense responses have previously been characterized at the anatomical, biochemical, transcriptome, and proteome levels in stems and needles. However, accurately measuring the early events of induced cellular responses in a conifer is technically challenging due to limitations in the precise timing of induction and tissue sampling from intact trees following insect or fungal treatment. In the present study, we used the advantages of Norway spruce (*Picea abies*) cell suspensions combined with chitosan elicitation to investigate the early proteome response in a conifer. A combination of iTRAQ labeling and a new design of iterative sample analysis employing data-dependent exclusion lists were used for proteome analysis. This approach improved the coverage of the spruce proteome beyond that achieved in any prior study in a conifer system. Comparison of elicitor-induced proteome and transcriptome responses in Norway spruce cells consistently identified features associated with calcium-mediated signaling and response to oxidative stress that have not previously been observed in the response of intact trees to fungal attack.

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Abbreviations: CaM, calmodulin; MEP, methyl erythritol phosphate; TAIR, The *Arabidopsis* Information Resource

1 Introduction

Large outbreaks of insect pests such as epidemics of bark beetles (e.g., species in the genera *Ips* and *Dendroctonus*) and bark beetle-associated fungal pathogens (e.g., species in the genera *Ceratocystis*, *Ophiostoma*, and *Leptographium*) are some of the most important ecological risk factors of conifer forest health, causing disturbances with global impacts on carbon cycles and climate change [1]. Coniferous trees employ a variety of constitutive and induced defenses to resist attack by pathogenic fungi and insects (for recent reviews [2–6]). The co-operation of these two defensive layers serves to ward off and protect long-lived trees from the vast majority of hostile organisms to which they are exposed. Constitutive defense mechanisms limit and delay the initial invasion of these organisms, thereby enabling the tree to activate its induced defenses. Several studies in species of spruce (*Picea* spp.) have implicated jasmonates [7, 8] as well as ethylene [9–11] in the signaling of induced defenses in conifers. Many of the anatomical, biochemical, and molecular changes associated with induced defenses can be readily measured in differentiated trees as they develop during the weeks following insect or pathogen attack [7, 8, 12–15]. However, there has been limited success in measuring the early events that immediately follow within minutes or hours after perception of the attack in conifer trees. The induced responses that occur at the interface of a conifer host and invader may be initially restricted to only a few cells or cell types, and they may be difficult to observe against the molecular background of the complex bark or wood tissues of intact trees. In addition, seasonal variation in growth with long periods of dormancy and seasonal differences in the response to fungal inoculation or insect attack provide challenges for testing of the early events of conifer defense responses in intact trees.

Conifer cell suspension cultures, on the other hand, offer an alternative that circumvents some of the intractability of working with intact trees. Advantages include a more homogenous response to elicitation and the continuous availability of actively growing plant material. Prior studies have successfully applied cell cultures to the study of the molecular and biochemical processes of conifer defense. For example, jasmonate-inducible cell suspension cultures of yew trees (*Taxus* spp.) have proven to be a valuable tool for studying the biosynthesis of the diterpenoid paclitaxel, a potent anticancer drug better known under the registered trade name Taxol [16, 17]. Studies in Japanese cedar (*Cupressus lusitanica*) have examined the production of the antifungal tropolone compound β -thujaplicin and found that various elicitors (yeast elicitor, methyl jasmonate, ethylene, H_2O_2) can be used to induce β -thujaplicin production [18, 19]. Furthermore, Japanese cedar cell suspension cultures have been used to demonstrate the importance of cAMP and calcium signaling for the induction of β -thujaplicin accumulation [18, 20]. In cell suspensions of Scots pine (*Pinus sylvestris* L.), the formation of antifungal stilbenes is induced by an elicitor

derived from the fungus *Lophodermium seditiosum* [21]. More recently, cell suspension cultures of Norway spruce (*Picea abies*) have been used to investigate genes of the methyl erythritol phosphate (MEP) pathway and terpene synthases associated with induced terpenoid defenses [22]. The MEP pathway is responsible for generating most of the isoprenoid precursors in terpenoid oleoresin formation. Using Norway spruce cell suspension cultures, members of a family of 1-deoxy-D-xylulose-5-phosphate synthase genes (the first enzyme in the MEP isoprenoid pathway) were shown to be differentially induced in response to a variety of biological elicitors including chitosan and methyl jasmonate. These studies illustrated that undifferentiated conifer cell cultures respond to elicitation in a fashion that resembles, at least in part, the pathogen- or insect-induced defense responses of intact trees.

In this study, we used Norway spruce cell suspension cultures induced with chitosan, an elicitor-active glucosamine polymer of fungal cell walls, as a mimic for fungal attack on trees to identify early events of the induced proteome response which would be extremely difficult to capture in a reproducible fashion in intact trees. The changes induced by chitosan in Norway spruce cells were examined using an extensive quantitative proteomics screen based on iTRAQ labeling and iterative, data-dependent sample analysis resulting in the first large-scale, gel-free proteome analysis in a conifer, and one of the largest completed in a plant system. The iterative analysis of protein samples has not previously been reported and greatly expanded the proteome coverage that could be obtained. For a comparative analysis, transcriptome changes in response to chitosan elicitation were also measured using a cDNA microarray containing 21 843 spruce cDNAs and validated with the transcriptome response induced by the fungal pathogen *Ceratocystis polonica*.

2 Materials and methods

2.1 Reagent suppliers

Unless otherwise noted, all reagents and solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), EM Science (Darmstadt, Germany), or Invitrogen (Carlsbad, CA, USA).

2.2 Experimental terminology and definitions

For clarification, the terminology used to describe the experiments performed during this study is defined below. In this manuscript the term “sample” refers to any of the 30 mL cell suspension cultures that were used to perform the chitosan elicitation time course. The term “reaction” refers to the iTRAQ labeling and analysis of a set of four samples as shown in Fig. 1B. Each horizontal row in Fig. 1B represents a separate “reaction.” The term “iteration” describes a single

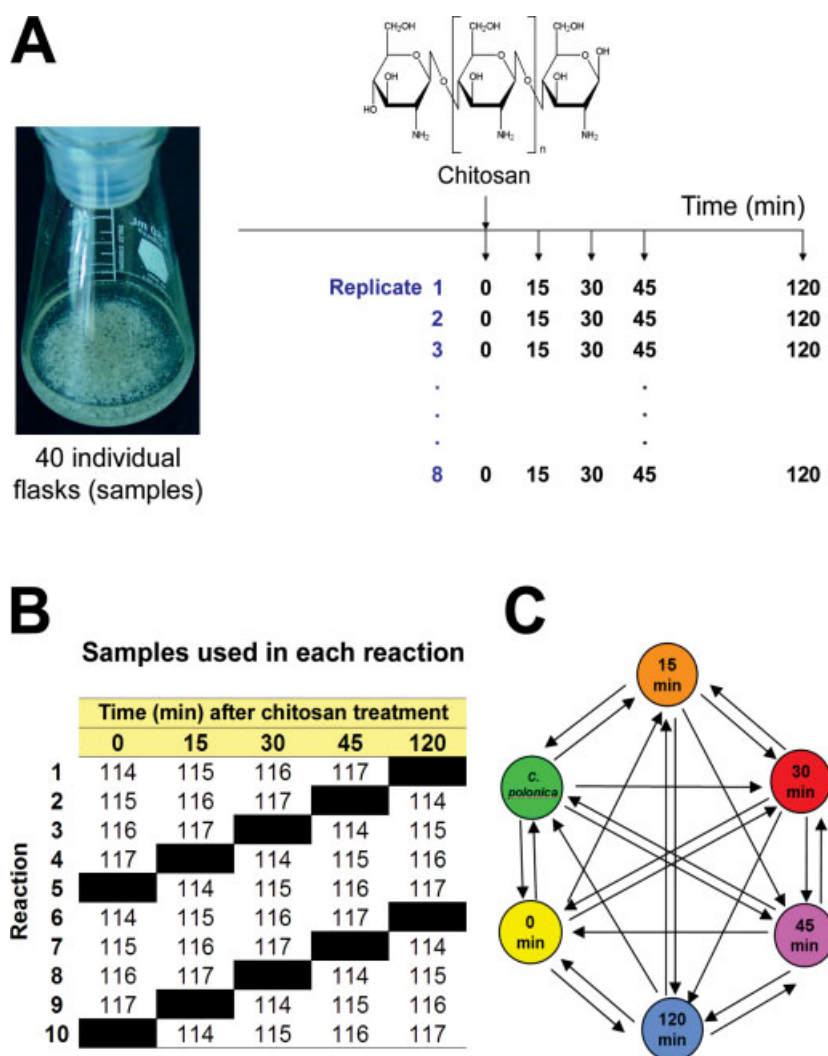


Figure 1. Design of a fully replicated global proteome analysis. (A) Chitosan was added to forty Norway spruce suspension cell cultures grown in individual flasks. Eight replicate flasks were harvested at each of five time points representing 0, 15, 30, 45, and 120 min following chitosan addition. (B) For the iTRAQ study designed to measure protein abundance, individual samples were labeled with the four iTRAQ labeling reagents in an incomplete block design as shown. Two full sets of combinations of time points and labels were used to assay all of the available samples, resulting in a fully balanced design. Each row represents a separate iTRAQ reaction with multiple reagents and the reporter mass of the iTRAQ reagents (114, 115, 116, 117) used is indicated in that reaction. Black rectangles represent the samples that were not used in each reaction. (C) The hybridization scheme used for microarray analysis is shown, including an additional treatment with *C. polonica*. Each arrow represents a single hybridization with Cy3 applied to the sample at the tail and Cy5 applied to the sample at the head. Each biological replicate was hybridized once.

round of data collection for any given reaction. Each reaction was subjected to three “iterations” of data collection as shown in Fig. 2. Finally, the term “measurement” describes the quantitative aspect of the data collected for any protein that was identified. A maximum of forty “measurements” could be made (eight biological replicates of five time points) for any single protein if it was observed in all ten of the reactions that were performed.

2.3 Cell suspension culture and elicitation

Culture conditions and chitosan elicitation of Norway spruce cell suspension culture line 186.3c (kindly donated by the Norwegian Forest Research Institute, Ås, Norway) were as previously described [22] with minor modifications. In brief, rapidly dividing embryogenic 186.3c cultures were maintained in liquid EDM6 medium [23, 24] at 24°C in the dark while shaking at 100 rpm following a 2 wk adaptation period from solid EDM6 medium. For each biological repli-

cate and each time point, a 30 mL culture in a 125 mL flask was used, and chitosan stock solution was added to a final concentration of 100 µg/mL. The chitosan stock solution was prepared by dissolving chitosan in an acidic 50 mM acetate buffer. At the appropriate time point (0, 15, 30, 45, and 120 min) after elicitation, cells were vacuum filtered for 10 s onto Whatman No. 2 filter paper until visibly dry, transferred immediately to a 15 mL sterile Falcon tube, and flash frozen in liquid nitrogen. The cells were not washed further during collection to facilitate rapid harvesting at the appointed time for all of the eight biological replicates for each time point. Separate aliquots were taken from each cell pellet for subsequent protein and RNA extraction. For *C. polonica* spore treatment, a fungal spore culture was added to the spruce culture at a ratio of 1:100 v/v with harvesting at 30 min following elicitation. The spore culture was prepared by transferring *C. polonica* mycelium (isolate 93–208/115, courtesy of the Culture Collection of the Norwegian Forest Research Institute, Ås, Norway) maintained on agar

plates containing 4% maltose w/v to agar plates (140 mm) containing 4% maltose and 0.4% yeast extract w/v. Following growth for 8 days at room temperature in complete darkness, the plates were washed with 10 mL of a 0.9% NaCl solution also containing 0.5% Tween-20.

2.4 Protein extraction

For each sample, a 0.5 g aliquot of frozen cells was ground to a fine powder under liquid nitrogen. Proteins were extracted into 3 mL of an extraction buffer containing 25 mM HEPES, 10 mM thiourea, 1% SDS, and a cocktail of six protease inhibitors (50 μ M tosyl phenylalanyl chloromethyl ketone, 50 μ M tosyl lysyl chloromethyl ketone, 2 μ M leupeptin, 2 μ M E64, 1 μ M pepstatin, 0.3 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) at a pH of 7.5. The samples were warmed to room temperature and mixed thoroughly. Insoluble material was removed by centrifugation for five minutes at room temperature at approximately $9000 \times g$. Supernatants were transferred to clean tubes and mixed with approximately 20 mL of acetone prechilled to -20°C . Proteins were precipitated overnight at -20°C . The precipitated protein was pelleted by centrifugation at $6000 \times g$ for 20 min. The acetone was decanted and pellets were washed twice with chilled acetone followed by drying under a gentle stream of nitrogen for 5 min. Proteins were resuspended in 0.5 mL of 25 mM HEPES, 0.3% SDS, 5 mM DTT and 6 M urea (pH 7.5). Protein concentration was measured using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA; samples were diluted ten-fold to overcome DTT interference). Protein preparations were stored at -80°C until required.

2.5 Protein processing and iTRAQ labeling

Sample aliquots containing 100 μ g of protein were reduced by the addition of 3 mM Tris-(2-carboxyethyl) phosphine followed by incubation at 60°C for 60 min. Methyl methane thiosulfonate was subsequently added to a final concentration of 6 mM and allowed to incubate for 10 min at room temperature to achieve cysteine alkylation. Two vials of modified trypsin (40 μ g trypsin; Promega, Madison, WI, USA) were resuspended in 250 μ L 0.1 M triethylammonium bicarbonate and 120 μ L of this solution was added to each sample followed by vortex mixing (10 μ g of trypsin added for a final ratio of 10:1 protein/enzyme w/w). Samples were allowed to digest overnight at 37°C .

Labeling of the samples with the iTRAQ reagents (Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's recommendations and as described in detail [25]. The samples were labeled as outlined in Fig. 1 using a balanced incomplete block design to accommodate the fact that there were five time points while only the four-plex iTRAQ reagents were available at the time. The pairing of the samples with the four iTRAQ reagents was arranged such that each of the time points was labeled with each of the reagents in two out of the ten reactions per-

formed. In the final design, all eight biological replicates of each time point were represented. As a result of this design, each of the time points was directly compared with each of the other time points in six different reactions.

2.6 LC and MS/MS

Each combined set of labeled samples was first fractionated *via* high-resolution cation-exchange chromatography (Poly-Sulfoethyl A Column (100 mm \times 4.6 mm), 5 μ m, 300 \AA bead (PolyLC, Columbia, MD, USA)). The column was developed with a linear gradient of KCl (0–500 mM) in 10 mM $\text{KH}_2\text{PO}_4/25\%$ ACN (pH 2.75) with the eluent being collected in 0.5 mL fractions. Absorbance at 215 nm was used to monitor the eluent and 19 peptide containing fractions were obtained from each run for further analysis. Each cation exchange fraction was lyophilized to dryness and resuspended in 120 μ L of 0.5% formic acid v/v, 2% ACN v/v. Each cation exchange fraction was split into three aliquots, with one aliquot being initially analyzed by electrospray MS/MS as described below.

Each SCX fraction was applied to a trap column (Pepmap C18, 5 mm \times 300 μ m, LC Packings, Amsterdam, The Netherlands) for desalting and concentration with a SWITCHOS II loading pump at 50 μ L/min, followed by separation on an Ultimate Nano-LC pump system (Dionex/LC Packings) with an in-house packed column (C18 RP, 15 cm \times 75 μ m, Magic C18 (100 \AA , 5 μ m) Michrom Bioresources, Auburn, CA, USA). The LC buffers consisted of 0.5% formic acid v/v, 2% ACN v/v in water (Buffer A) and 0.5% formic acid v/v in ACN (buffer B). The column was developed with a five-step 120 min LC gradient consisting of isocratic 5% B for 15 min (after which the trap column was brought in-line with the nano flow pump), 5–15% B over 45 min, 15–75% B over 40 min, 75–5% B over 10 min, and 5% B for 10 min. The LC flow rate was maintained at 300 nL/min. This system was coupled to a hybrid quadrupole-TOF tandem mass spectrometer (qStar Pulsar i, Applied Biosystems), which was used to analyze the LC eluent. Information dependent acquisition was based upon a 1 s survey scan over the m/z range of 400–1200 followed by up to two product ion scans (2.5 s/scan) over the m/z range of 100–1500. The most intense ions within the range of the survey scan with an intensity exceeding 20 counts were selected for product ion scans and were subsequently excluded for 120 s following initial selection with a 6 mass unit window.

Following MS/MS analysis of the initial set of aliquots, all of the raw data was processed through ProteinPilot (Applied Biosystems) and searched against a translated spruce EST and full length complementary DNA (FL-cDNA) database developed in the Treenomix project [15, 26, 27]. From the proteins identified, a list of peptides identified with >95% confidence was compiled and separated into 19 discreet lists based on the original fraction in which they were observed. The parent m/z values for these peptides were used to build an exclusion list for each fraction that was used during the

analysis of the second aliquot of each fraction. The exclusion list ensured that the same peptides were not selected for MS/MS during the second run. This entire process was completed a second time to allow the third aliquot of each fraction to be analyzed using an expanded exclusion list based on the first two runs. The final dataset from each reaction was exported to a tab delimited file for subsequent statistical processing and analysis. Exclusion lists were not carried over from reaction to reaction.

2.7 Statistical analysis of the iTRAQ data

In each of the ten iTRAQ reactions performed (as described above) four of the five time points were included as illustrated in Fig. 1B. Following the analysis of each reaction using ProteinPilot, the control (time 0) sample was selected as the denominator for calculating the fold change of each protein. In the reactions that did not include a control sample (reactions 5 and 10) the 15 min time point was selected as the denominator. A ratio of one was assigned to the time point chosen as the denominator in each case. In this way, a reading was obtained for each of the four time points included in every reaction. The logarithm of the data was analyzed as a random incomplete block design with reactions as blocks and time points as treatments. For each protein, an effect was estimated for each time point and a *p*-value was computed. Since the incomplete block design removes any block effect, the relative nature of the data within each block is all that is required to compute the treatment effects. The treatment effects were calculated using the least squares method of the statistical package R (version 2.5.1; <http://www.r-project.org/>).

2.8 RNA isolation and microarray analysis

Total RNA was isolated from eight biological replicate samples for each of the five time points examined following chitosan treatment. Although the samples used were the same ones that were used for protein extraction, separate aliquots were taken for RNA extraction. In addition, RNA was also isolated from cells treated for 30 min with spores of *C. polonica* as described previously [22]. RNA was isolated using the Ambion RNAqueous Midi kit according to manufacturer's instructions (Ambion, Austin, TX, USA). Total RNA was quantified and quality checked using an Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit according to manufacturer's instructions (Agilent, Santa Clara, CA, USA). A factorial hybridization design with dye balance was chosen to assess differences of transcript abundance among the treatments and time points. Each of the eight biological replicates for each treatment and time point was hybridized only once for a total of 24 hybridizations (Fig. 1C). All microarray experiments were designed to comply with MIAME guidelines [28]. Hybridizations were performed using the Genisphere Array350 kit (Genisphere, Hatfield, PA, USA) using a 21.8 K spruce cDNA microarray platform.

Thirty-five micrograms total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo d(T)₁₈ primers with a 5' unique sequence overhang specific to either the Cy3 or Cy5 labeling reactions. The RNA strand of the resulting cDNA–RNA hybrid was hydrolyzed in 0.075 M NaOH/0.0075 M EDTA at 65°C for 15 min followed by neutralization in 0.175 M Tris-HCl (pH 8.0). Following pooling of the appropriate cDNAs, samples were precipitated with linear acrylamide and resuspended in a 45 µL hybridization solution consisting of 0.25 M NaPO₄, 0.5% SDS, 1 × SSC, 2 × Denhardt's solution, 1 mM EDTA, 2.75 µL LNA d(T) blocker, 2 µg sheared salmon testes DNA (Invitrogen), and 0.3 µL of Cy5-labeled GFP cDNA (Cy5-dUTP and Ready-To-Go labeling beads, Amersham Pharmacia Biotech, Buckinghamshire, UK). Immediately prior to use, arrays were prewashed 2 × in 0.1% SDS at room temperature for 5 min each, followed by two washes in Milli-Q H₂O for 2 min each, 3 min at 95°C in Milli-Q H₂O, and dried by centrifugation (3 min at 2000 rpm in an IEC Centra CL2 centrifuge with rotor IEC 2367-00 in a 50 mL conical tube). The cDNA probe was heat denatured at 80°C for 10 min, then maintained at 65°C prior to adding to a microarray slide heated to 55°C, covered with a 22 × 60 × 1.5 mm³ glass coverslip (Fisher Scientific, Waltham, MA, USA), and incubated for 16 h at 60°C. Arrays were washed in 2 × SSC, 0.2% SDS at room temperature for 5 min to remove the coverslip, followed by 15 min at 65°C in the same solution, then three washes of 5 min in 2 × SSC at room temperature, and three washes of 5 min in 0.2 × SSC at room temperature, and dried by centrifugation. The Cy3 and Cy5 3DNA capture reagents (Genisphere) were then hybridized to the bound cDNA on the microarray in a 45 µL volume consisting of 0.25 M NaPO₄, 0.5% SDS, 1 × SSC, 2 × Denhardt's solution, 1 mM EDTA, 2.5 µL Cy3 capture reagent, and 2.5 µL Cy5 capture reagent. The 3DNA capture reagent is bound to its complementary cDNA capture sequence on the Cy3 or Cy5 oligo d(T) primers. The second hybridization was performed for 3 h at 60°C, then washed and dried as before.

Fluorescent images of hybridized arrays were acquired by using ScanArray Express (Perkin-Elmer, Foster City, CA, USA). The Cy3 and Cy5 cyanine fluors were excited at 543 and 633 nm, respectively. All scans were performed at the same laser power (90%), but with the PMT settings for the two channels adjusted such that the ratio of the mean signal intensities was ~1, and the percentage of saturated array elements was <0.5% but >0%, while minimizing background fluorescence. Fluorescent intensity data were extracted using the ImaGene 5.5 software (Biodiscovery, El Segundo, CA, USA). All scanned microarray TIF images, the gene identification file and ImaGene quantified data files are available at the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (accession numbers GSE10771, GSM271878–GSM271901).

To correct for background intensity, the lowest 10% of median foreground intensities was subtracted from the median foreground intensities. Data were then normalized by

variance stabilizing normalization to compensate for non-linearity of intensity distributions [29]. To assess the transcriptome response to elicitor treatments, a linear mixed-effects model was fitted to the normalized intensities in the Cy3 and Cy5 channels of the 24 microarray slides. The model contained an adjustment for dye effect, an array effect indicating which Cy5/Cy3 pair was on each array, and a treatment effect indicating treatment and time point. Expression variance was obtained from the eight biological replicates for each treatment and time point. Next, the ratio of the treatment minus untreated control parameter estimate to the standard error was used to calculate a *t* statistic and *p* value. The *q* value for each effect and gene was calculated for each of the two models to adjust for the false discovery rate [30]. All statistical analyses were performed within the *R* statistical package (version 2.5.1, <http://www.r-project.org/>).

3 Results

3.1 Design of a fully replicated global iTRAQ proteome analysis

Proteome analysis has come to rely heavily on shotgun methods of data capture. Unfortunately, the random nature of the data collection inherent to these methods often results in poor overlap between datasets produced from biologically replicated samples. While analyzing an increased number of replicates leads to an overall increase in the number of proteins observed, only the most abundant proteins are observed repeatedly. In contrast, the medium-to-low abundance proteins, which may be of great biological importance, are not measured frequently enough to provide statistically robust quantitative measurements. One method of compensating for this effect is to increase the number of biological replicates that are analyzed, thereby increasing the likelihood that low abundance proteins are measured with adequate frequency. Another approach is to utilize the data collected in a first pass analysis of a sample to drive subsequent iterative analyses of the same sample. In this way, nonoverlapping subsets of the proteins present within a sample are measured during repeating rounds of data collection from a given sample. In the present study, a combination of these approaches has been applied in the study of chitosan elicited Norway spruce cell suspension cultures.

The experimental design for proteome analysis employed 40 individual biological samples of cultured Norway spruce cell suspensions, allowing for the collection of eight independent biological replicates at each of the five time points assayed (Fig. 1A). The time points of 0, 15, 30, 45, and 120 min after the addition of chitosan were selected to allow the detection of the early changes to the spruce proteome induced by fungal elicitor exposure. Since there were five time points included in the study, but only four could be simultaneously compared using the four iTRAQ reagents that were available at the time of the analysis, the samples were mixed using a

balanced, incomplete block design (Fig. 1B). Protein extracts were prepared from an aliquot of each independent sample and these were analyzed using the design as illustrated. In this design, a different time point was excluded from each iTRAQ reaction as shown. In this way, the 40 independent samples were analyzed using ten reactions, which accounted for two full replicates of the block design, producing a completely balanced analysis. The labels were shuffled from reaction to reaction with the result that each reagent was used to label two of the biological replicates from each time point, thereby eliminating the possibility of any reagent-specific bias.

3.2 Iterative collection of MS/MS data leads to an improvement in proteome coverage

Proteome data were collected in an iterative fashion, employing three rounds of analysis for each sample (Fig. 2). In between rounds of data collection, the MS data were subjected to a database search against a database of translated spruce ESTs and FL-cDNAs. The result of that search was used to compile a list of peptides that were identified with a confidence level in excess of 95% as calculated by the ProteinPilot software designed for use with the iTRAQ reagents. The parent ion masses of these peptides were excluded during subsequent rounds of MS. Figure 3 shows the effect of iterative analysis on the efficiency of protein identification. Cumulative totals of the number of proteins identified with a confidence of 95% or greater (ProteinPilot score) were plotted for the ten reactions that were performed (Fig. 3A). The

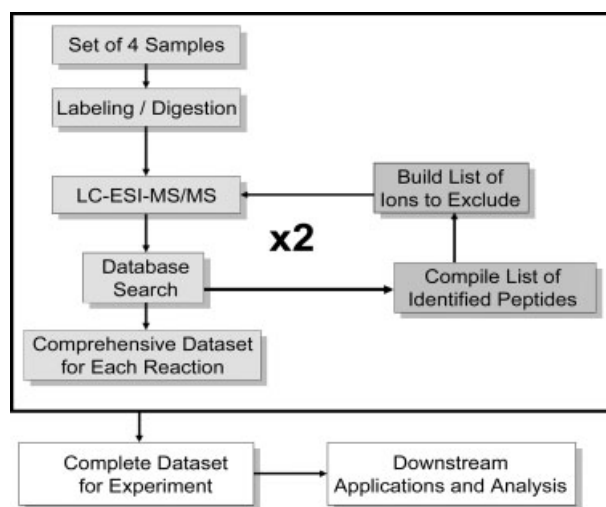


Figure 2. Schematic of iterative proteome data analysis using data-dependent exclusion lists. Data analysis for the proteome study was performed as shown in the flowchart. For each reaction, three rounds of data collection by MS were performed. Following each of the first two rounds of data collection, a complete database search was performed and the result of that search was used to develop a list of masses to be excluded from the following iteration of sample analysis. The database search performed following each round of data collection was cumulative with the previous runs.

three separate series demonstrate the improvements gained as the number of iterations was increased. A rapid rise of the number of unique proteins identified was observed during the first three reactions, after which a steady but smaller increase was observed with every additional reaction analyzed. At the end of the tenth reaction, the use of a single iteration of data collection resulted in 1597 unique proteins being identified. By completing a second and third iteration of data collection, the total number of unique proteins identified was increased to 2045 and 2296, respectively. It was interesting to note that the completion of five reactions with two iterations or three reactions with three iterations was roughly as efficient as collecting a single dataset from all ten reactions.

Similar improvements were observed in the number of proteins that were repeatedly measured during the analysis (Fig. 3B). The numbers plotted represent the total number of proteins for which at least three independent measurements were obtained at each of the five time points, which was considered to be the minimum amount of data required to perform an adequate statistical analysis of the complete time course of protein expression during the first 120 min following chitosan treatment. Due to the incomplete block design of the experiment, obtaining three replicate measures of all five time points required the collection of data from a minimum of four reactions. There was initially a rapid increase in the number of proteins that were measured with adequate frequency. This trend continued over the course of the entire experiment, but the rate of increase declined with each successive reaction. The numbers of proteins measured in triplicate (Fig. 3B) were smaller than the total number of proteins identified (Fig. 3A). On an average, 56% of the proteins detected were measured in at least three biological replicates. More specifically, after the completion of ten reactions, the number of proteins measured in triplicate was 874 for a single iteration, 1145 for two iterations, and 1347 for three iterations of data collection (Fig. 3B).

The theoretical limits predicted by the trends shown in Fig. 3 demonstrated that repeated analysis of a set of biological samples will allow the quantitative analysis of a set of low abundance proteins that cannot be observed with a single round of data collection. Initially, adding more reactions to the analysis provided a better return in terms of dataset size for the amount of raw data collected. A single iteration of ten reactions required ten LC-MS/MS runs and resulted in a total of 847 unique proteins observed, while three iterations of four reactions required 12 LC-MS/MS runs and resulted in only 487 unique proteins observed (Fig. 3B). As the number of reactions performed increased, iterative analysis resulted in a consistently greater number of proteins that could be measured, thus increasing the potential of detection of low abundance proteins.

In this type of analysis, it can generally be assumed that the proteins measured most frequently among the biological replicates are those that were more abundant in the sample, or at least were detected with the greatest signal intensity

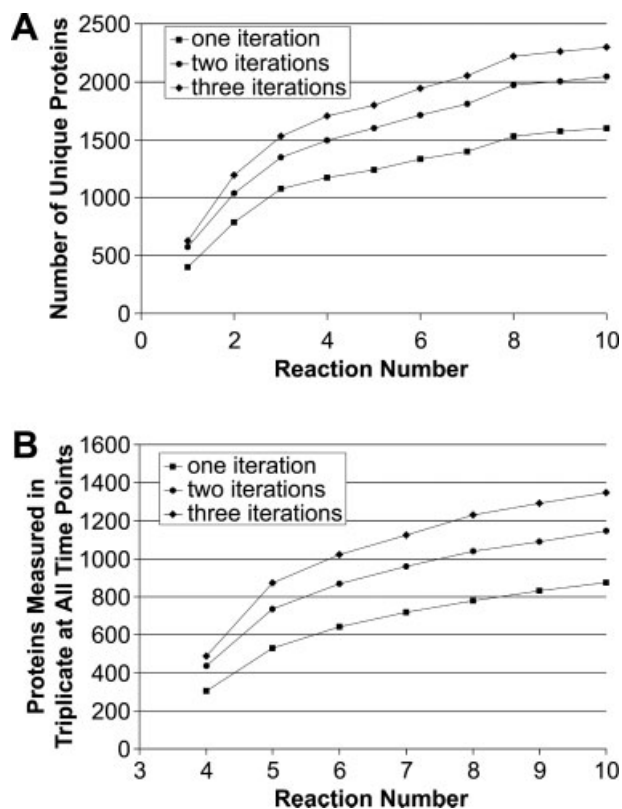


Figure 3. Effect of iterative data collection. (A) The cumulative number of unique proteins identified was plotted for the ten iTRAQ reactions that were performed. The separate plots show the extent of the improvement in proteome coverage gained by performing up to three rounds of iterative data collection using exclusion lists. The exclusion lists were applied within individual reactions and were not carried over between reactions. (B) The effect of iterative analysis on the overlap observed between datasets collected from individual reactions. Proteins were counted if they were measured at least three times at all five time points. Triplicate measurement was considered a minimum requirement for quantitative validation through statistical analysis. Note that a minimum of four reactions had to be completed to achieve threefold replication at all five time points due to the block design of the proteome analysis.

(*i.e.*, greater signal intensity increases the likelihood that a given peptide will be selected for fragmentation during LC-MS/MS analysis). The SDs for the quantitative measurements of the observed proteins are plotted as a function of the number of measurements taken (Fig. 4). There was little difference observed between the most frequently and least frequently measured proteins in terms of the SD of the quantitative measurements that were made. This observation suggests that medium-to-low abundance proteins were measured as precisely as high abundance proteins in spite of the assumed lower signal intensity observed in the raw data for the lower abundance proteins. In other words, the observed variation in measured protein abundance does not improve with increased sampling and three-fold replication

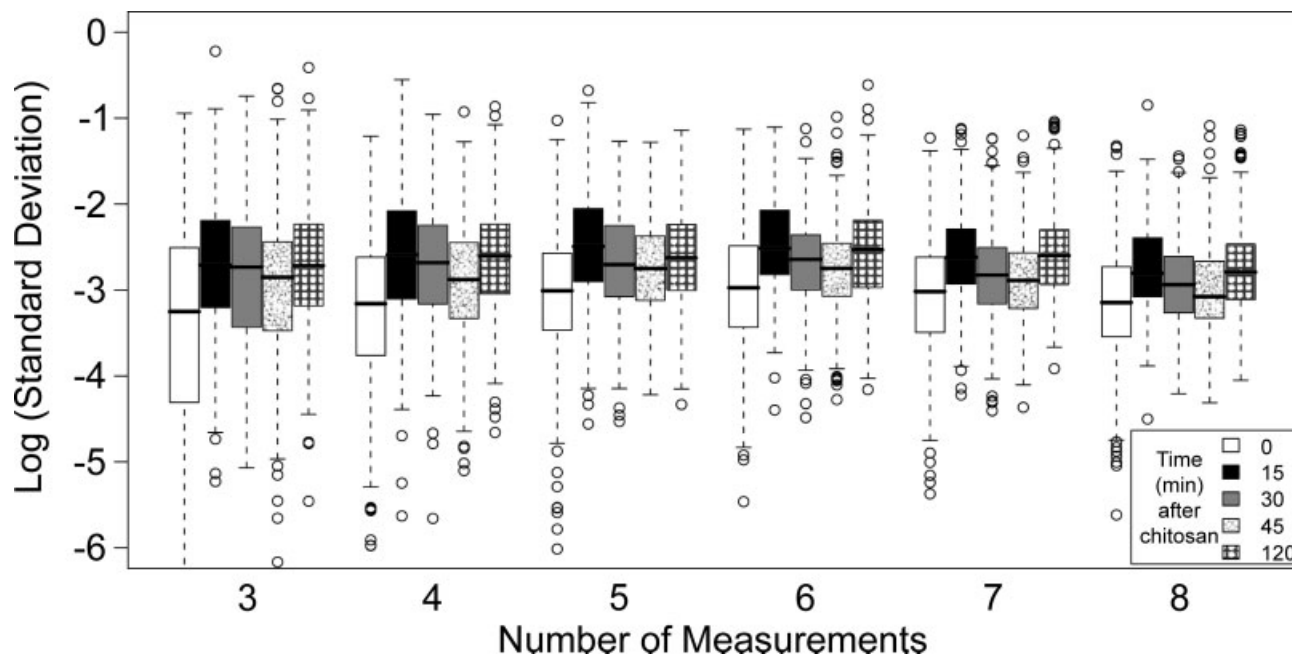


Figure 4. Effect of frequency of protein observation on the SD of the resulting quantitative protein measurements. Identified proteins were grouped on the basis of the number of replicates observed over the course of the experiment. Three measurements were required for statistical analysis, while eight measurements represents the maximum possible. For each group the population of calculated SDs were displayed using a boxplot. Shaded boxes show the median (horizontal center bar), the boundaries of the 25th and 75th percentiles (upper and lower bounds of each block) and the full range of the data (dashed lines) with individual outliers plotted as circles.

is sufficient for estimating protein abundance using an unsupervised method of data collection (*i.e.*, information-dependent acquisition). However, this analysis does not take the signal intensity of individual measurements into account. It remains possible that very low abundance signals are, in general, less accurate measures of protein abundance. Considering that peak shape degrades at very low signal intensity, it is possible that low intensity peptide signals could lead to reduced accuracy in the measurement of individual proteins. It is therefore conceivable that the exclusion of low intensity signals could lead to improvements in protein quantitation accuracy (not precision) when higher intensity signals are available for a given protein.

3.3 Characteristics of the elicitor-induced Norway spruce proteome

Statistical analysis of the proteome dataset obtained by iTRAQ analysis demonstrated that the early response of Norway spruce cells to chitosan elicitation consisted of a very narrow set of changes in protein expression. There were 1347 proteins detected for which adequate replicate quantitative measurements were obtained to allow statistical analysis (the full dataset is provided in Table 1 of Supporting Information). Only 35 proteins (2.5%) demonstrated statistically significant ($p < 0.05$) changes of at least 1.5-fold in abundance during the 2 h immediately following treatment

with chitosan. Out of these 35 proteins 17 were upregulated and 18 proteins were downregulated (Table 1). For the remaining 97.5% of the identified proteins 1136 did not meet the 1.5-fold change cutoff while 176 did not meet the 0.05 p -value cutoff (Table 1 of Supporting Information).

To investigate the overall dynamics of proteome changes in elicitor-induced Norway spruce cells, we performed a hierarchical cluster analysis of all 1347 reproducibly detected proteins. Most of the proteome response of upregulation or downregulation developed in a linear fashion, showing a continuous increase or decrease over the 120 min time course (Fig. 5A). The two clusters that are highlighted in Fig. 5A contain all 35 of the differentially expressed proteins that were observed. It is important to note that the proteome response was equally divided between proteins that were induced and proteins that were quantitatively suppressed, which is different from the overall transcriptome response (described below).

3.4 Comparison of the elicitor-induced Norway spruce proteome and transcriptome

For comparison, transcript profiles following chitosan treatment were monitored using the same set of 40 samples that were used for protein expression profiling. In the design for the microarray study (Fig. 1C) an additional sample set with eight biological replicates was included that represents elicitation with live *C. polonica* spores. *C. polonica* is a blue-stain

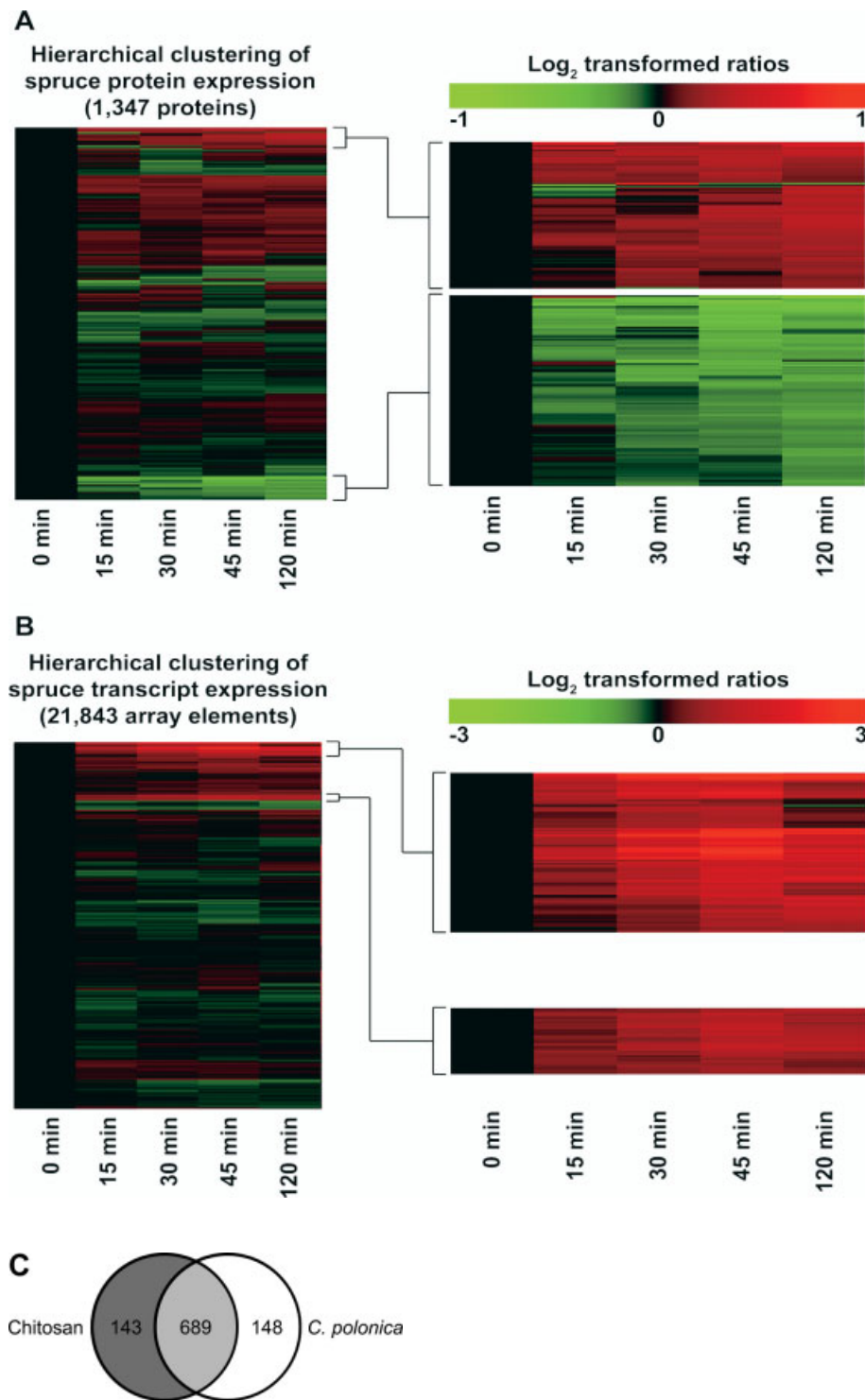


Figure 5. Heatmaps of chitosan-induced changes of Norway spruce protein and transcript abundance. (A) A heatmap was constructed using unsupervised hierarchical clustering to show the clustered expression patterns over time for all proteins detected in at least three replicates for all five time points following chitosan treatment. Abundance values were calculated as a ratio of the indicated time point and the 0 min measurement for that protein. The heatmap represents the \log_2 transformation of the geometric mean of all detected replicates for each ratio. The two clusters at the extreme top and bottom of this plot were the only clusters that contained proteins showing a 1.5-fold or greater difference in abundance when compared to the 0 min time point. (B) An identical method was used to cluster all of the transcripts measured using the 21.8 K spruce cDNA microarray. (C) Venn diagram comparison of the differentially expressed transcripts that were observed in response to chitosan elicitation and fungal spore treatment.

fungus that infects conifers across Eurasia and is associated with *Ips* bark beetles [31]. This treatment was included as an extra control to assess the relevance of chitosan as a mimic of exposure to fungus. Chitosan had previously been established as a suitable elicitor for terpenoid biosynthetic genes

in Norway spruce cells and was generally more reproducible as an elicitor than cultures of fungal spores [22]. Transcript levels were measured using a cDNA microarray containing 21 843 unique cDNA elements identified in ESTs from 12 different spruce cDNA libraries [15]. The RNA samples were

paired for hybridization as indicated in Fig. 1C. Each of the eight independent biological replicates was hybridized once, and the design allowed both direct and indirect comparisons of all time points with dye balance. The complete dataset of the transcriptome analysis is available in Table 2 of Supporting Information.

In contrast to the protein data, which showed both up- and downregulated proteins, nearly all differentially expressed transcripts increased in abundance during the 2 h following chitosan treatment. An increase in abundance of two-fold or greater with a p -value less than 0.01 was observed for 832 transcripts (Table 2 Supporting Information; Fig. 5B), while only a single transcript was detected as downregulated using these criteria. Hierarchical cluster analysis showed that most of the upregulated transcripts (813 out of 832) fell into two discrete clusters. A comparison of the response to chitosan and the response to *C. polonica* spores revealed that the two stimuli resulted in very similar responses at the level of transcript abundance with 77% overlap between the two datasets (Fig. 5C). This overlap confirms that chitosan serves as a good mimic for fungal pathogen elicitation. At 45 min after the addition of chitosan, 693 transcripts were induced by two-fold or greater ($p < 0.01$). This number decreased to 514 transcripts by 2 h postinduction, suggesting that some of the early transcriptional response had peaked already during the 120 min time course (Fig. 6). Again, this is in contrast to the proteomics data, where the response continued to become more pronounced throughout the duration of this experiment. On a percentage basis, 3.8% of the transcripts measured (832 out of 21 843) were differentially expressed as defined above, while 2.6% of the proteins measured (35 out of 1347) were differentially expressed during the period examined.

Of the 832 spruce transcripts that were induced, 455 showed sequence similarity (BLASTX; E -value $< 1 \times 10^{-5}$) to *Arabidopsis* proteins in The *Arabidopsis* Information Resource

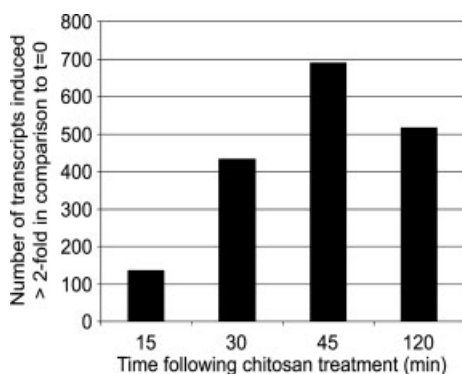


Figure 6. Chitosan induced changes of Norway spruce transcriptome over time. The largest number of differentially expressed transcripts was observed at 45 min after chitosan addition with a reduction in differentially expressed transcripts observed by two hours following elicitation. Only transcripts differing by two-fold or greater in comparison to the initial time point ($p < 0.01$) were counted.

(TAIR) database. Since some spruce microarray elements match to the same *Arabidopsis* gene, spruce transcripts matching 321 distinct *Arabidopsis* genes were identified among the differentially regulated transcripts. These genes were used to perform a gene ontology analysis and the classification for the chitosan induced transcripts was compared with the classification of the sequences represented on the entire spruce microarray to determine if any of the gene ontology categories were overrepresented in the chitosan-induced transcriptome. The result of this comparison showed that response to abiotic and biotic stimuli, response to stress, and signal transduction were all biological processes that were more frequently observed in the chitosan induced transcripts than would be predicted based on the representation of ESTs associated with these processes on the 21.8 K microarray (Fig. 7A). Similarly, receptor binding, nucleotide binding, and kinase activity were molecular functions that were overrepresented in the chitosan-induced gene set (Fig. 7B). To a lesser degree, protein binding and transcription factor activity were also overrepresented among the molecular functions.

3.5 Biological classification of differentially expressed proteins and transcripts

Table 1 identifies putative functions for most of the 35 proteins that demonstrated a statistically significant change in abundance following chitosan treatment, along with their expression levels relative to the untreated control and the p -values associated with these measurements. For comparison, the 40 most highly induced transcripts observed by microarray analysis are shown in Table 2 with functional annotations (the complete microarray dataset is provided in Table 2 of Supporting Information). Manually curated annotations were obtained *via* BLAST comparison of the respective protein and transcript sequences with either the NCBI nr database (for proteins, Table 1) or the TAIR *Arabidopsis thaliana* protein database (for transcripts, Table 2).

Twenty out of the thirty-five differentially expressed proteins were classified as proteins involved in defense or stress response based on functional characterization of similar proteins in other biological systems. These are grouped under the headings signaling, response to oxidative stress, and biotic defense (Table 1). Four proteins were classified under general metabolism and three were classified as being involved in development. Eight of the proteins that were differentially expressed were of unknown function. The signaling proteins were predominantly calcium binding proteins and arabinogalactan/proline-rich proteins. The arabinogalactan proteins appeared to be conifer-specific proteins. These Norway spruce proteins have high sequence identity (67% identity and 75% similarity, mean values) to a set of loblolly pine sequences, but arabinogalactan/proline-rich proteins from other plants were more distantly related based on sequence (41% identity and 50% similarity, mean values).

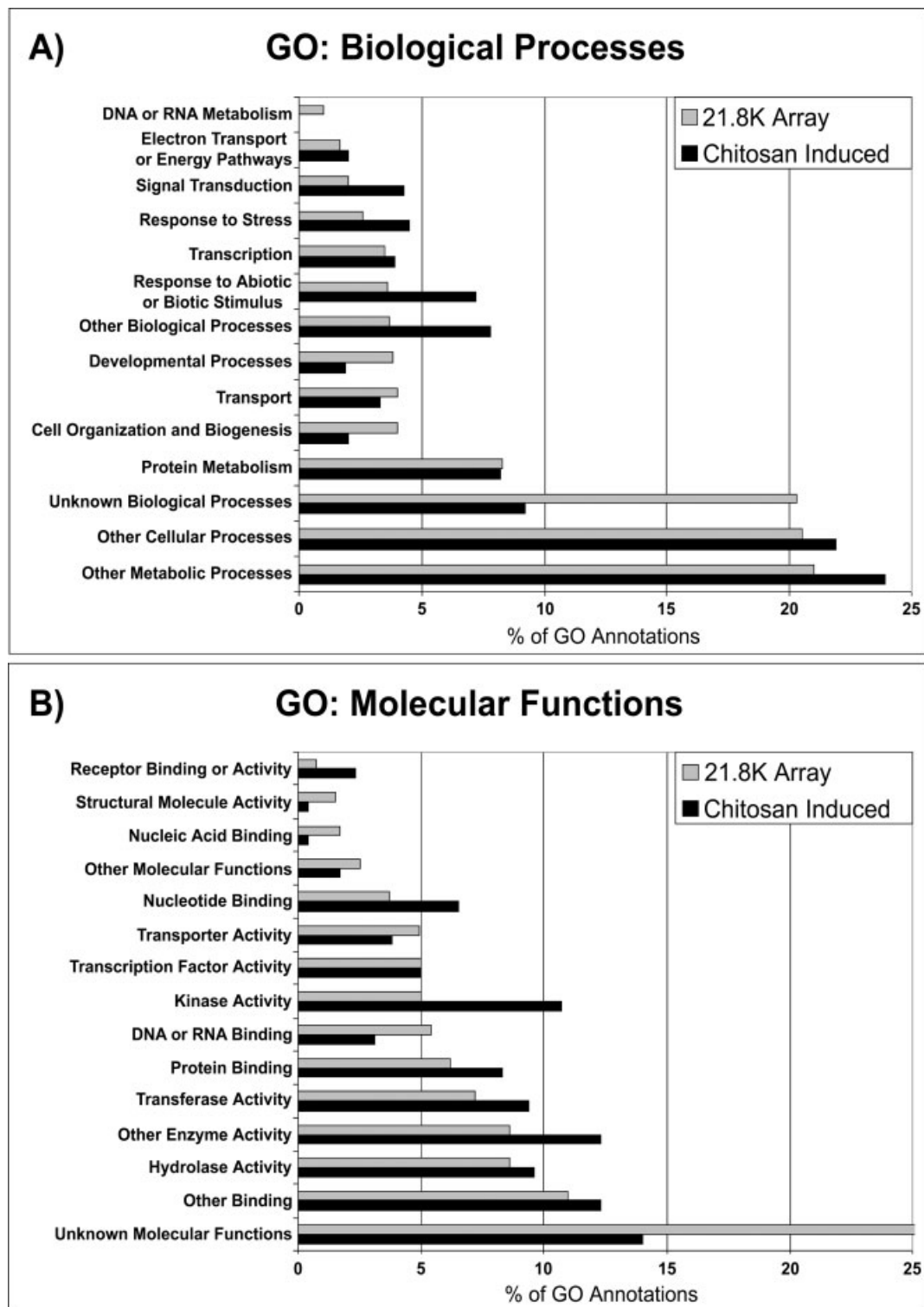


Figure 7. Gene ontology analysis of the chitosan induced transcripts observed by microarray analysis. BLASTx analysis of induced spruce transcripts against TAIR protein database was used to find the closest matching Arabidopsis homolog. The resulting list of Arabidopsis sequences was used to query the GOSlim database at the TAIR website. Gene ontology (GO) terms for this dataset of chitosan induced transcripts are compared to those for all ESTs spotted on the 21.8 K microarray to highlight biological processes (A) or molecular functions (B) that are more frequently observed in the induced dataset than would be expected by chance.

Table 1. Norway spruce proteins showing differential expression during the first 120 min following chitosan elicitation ($p < 0.05$)

Spruce accession ^{a)}	Protein name (BLASTp vs. NCBIInr)	Expect value (NCBI)	Closest match to <i>Arabidopsis</i>	Mean coverage (%) ^{b)}	Mean prot score ^{c)}	15 min vs. control		30 min vs. control		45 min vs. control		120 min vs. control	
						FC ^{d)}	<i>p</i> -val	FC ^{d)}	<i>p</i> -val	FC ^{d)}	<i>p</i> -val	FC ^{d)}	<i>p</i> -val
Signaling													
lcl 76359_1	CaM	2.90E – 64	AT1G06570	36.0	7.7	1.39	0.01	1.61	0.00	1.57	0.00	1.83	0.00
lcl 65857_3	CaM	1.30E – 64	AT3G43810	27.1	7.5	1.11	0.61	1.55	0.04	1.51	0.06	1.90	0.01
lcl 60199_1	CaM	4.40E – 72	AT3G43810	33.2	10.0	1.04	0.84	1.43	0.07	1.36	0.12	1.70	0.01
lcl 77117_2	CaM	8.00E – 55	AT1G66410	39.3	9.1	1.11	0.45	1.34	0.04	1.37	0.04	1.69	0.00
lcl 72681_2	Putative arabinogalactan/proline-rich protein	1.70E – 07	No match	14.3	4.6	1.21	0.60	1.34	0.45	1.30	0.48	2.47	0.02
lcl 158650_1	Putative arabinogalactan/proline-rich protein	1.70E – 07	No match	23.2	4.6	0.89	0.58	1.16	0.49	1.10	0.66	1.74	0.01
lcl 105044_1	Putative arabinogalactan/proline-rich protein	1.20E – 29	No match	19.3	3.4	0.77	0.35	1.13	0.65	1.17	0.63	1.59	0.03
lcl 19075_6	Putative arabinogalactan/proline-rich protein	1.20E – 29	No match	11.1	3.4	0.75	0.32	1.14	0.62	1.17	0.98	1.56	0.05
lcl 22079_5	Putative arabinogalactan/proline-rich protein	3.50E – 28	No match	13.0	4.4	0.79	0.28	0.58	0.03	0.89	0.66	0.85	0.45
lcl 23301_1	Protein kinase C substrate 80 K-H isoform 2 -like	1.30E – 69	AT3G01500	5.8	2.3	1.13	0.56	0.63	0.05	0.94	0.76	0.78	0.22
Response to oxidative stress													
lcl 21228_5	Thioredoxin dependent 2-Cys Peroxiredoxin	2.80E – 97	AT1G73740	9.8	2.5	0.91	0.45	1.09	0.49	1.19	0.16	1.66	0.00
lcl 91904_1	SAM-dependent carboxyl-methyltransferase	5.00E – 52	AT3G54660	5.6	2.4	1.09	0.80	0.82	0.62	2.96	0.02	0.69	0.36
lcl 15291_4	GST	3.20E – 57	AT3G24170	12.6	4.0	2.32	0.01	1.78	0.05	1.33	0.31	2.21	0.01
lcl 79566_1	Dehydrin 2	0.00E + 00	No match	11.1	2.0	0.96	0.82	0.88	0.49	0.87	0.47	1.80	0.01
lcl 78280_5	Putative quinone reductase	1.50E – 32	AT1G16350	24.3	6.3	0.72	0.02	0.59	0.00	0.73	0.04	0.57	0.00
lcl 108185_1	Catalase	2.90E – 214	AT2G36230	9.5	4.2	1.10	0.48	0.83	0.25	1.19	0.29	0.65	0.02
Biotic defense													
lcl 87839_1	Thaumatococcus-like protein	1.80E – 102	AT3G47800	10.1	2.6	0.74	0.29	0.45	0.01	0.57	0.07	0.64	0.11
lcl 79783_1	Thaumatococcus-like protein	0.00E + 00	AT5G17330	10.1	2.4	0.68	0.24	0.42	0.01	0.55	0.06	0.58	0.09
lcl 98432_1	Seven transmembrane receptor	2.70E – 76	AT5G64860	9.4	2.1	0.65	0.01	0.91	0.54	1.11	0.47	0.83	0.16
lcl 18547_4	Nucleotide binding	1.60E – 57	AT4G17260	15.4	2.0	0.68	0.01	0.63	0.00	0.59	0.00	0.79	0.10
General metabolism													
lcl 21042_3	Putative survival motor neuron domain containing 1	1.80E – 75	AT3G42640	7.5	2.0	1.12	0.31	1.43	0.01	1.34	0.04	1.74	0.00
lcl 14959_2	Cucumisin-like serine protease, putative or CAD	2.40E – 68	AT3G19450	10.8	2.7	1.22	0.26	1.39	0.09	1.26	0.22	1.69	0.01
lcl 21944_5	Plastid protein (<i>A. thaliana</i>)	2.80E – 67	AT2G24520	6.9	2.6	0.79	0.47	0.51	0.04	0.81	0.50	0.53	0.04
lcl 40055_5	Eukaryotic elongation factor 1A	7.10E – 97	AT4G29600	22.0	7.8	1.10	0.79	0.50	0.13	0.97	0.94	0.36	0.02
Development													
lcl 97031_1	LRR protein S/D4 [Petunia x hybrida]	3.80E – 38	AT4G29540	14.3	2.0	0.72	0.08	0.55	0.01	0.72	0.10	0.72	0.08
lcl 84864_1	Late embryogenesis abundant protein	0.00E + 00	AT5G64370	20.8	2.3	0.77	0.29	0.58	0.04	0.75	0.24	0.67	0.11
lcl 14926_4	ABI3-interacting protein 2; CnAIP2	3.20E – 09	No match	5.0	1.9	1.11	0.68	0.69	0.12	0.55	0.03	0.71	0.17
Unknown function													
lcl 100253_1	Unknown protein	2.30E – 14	No match	21.6	3.2	1.71	0.02	1.41	0.17	1.85	0.02	1.73	0.04
lcl 21720_4	Unknown protein	2.00E – 25	AT4G13780	10.0	3.4	1.80	0.01	1.37	0.15	1.36	0.19	1.57	0.05
lcl 89195_1	Unknown protein	8.00E – 07	No match	20.2	2.0	1.37	0.11	1.55	0.03	1.65	0.01	1.77	0.00
lcl 23479_1	No match	0.00E + 00	AT1G24180	11.7	3.2	0.88	0.42	0.60	0.00	0.96	0.80	0.50	0.00

Table 1. Continued

Spruce accession ^{a)}	Protein name (BLASTp vs. NCBI nr)	Expect value (NCBI)	Closest match to <i>Arabidopsis</i>	Mean coverage (%) ^{b)}	Mean prot score ^{c)}	15 min vs. control		30 min vs. control		45 min vs. control		120 min vs. control	
						FC ^{d)}	p-val	FC ^{d)}	p-val	FC ^{d)}	p-val	FC ^{d)}	p-val
lc 96821_1	Unknown protein	7.90E – 06	AT3G29430	13.3	2.8	0.55	0.05	0.83	0.56	0.52	0.07	0.65	0.21
lc 98253_1	Acireductone dioxygenase	5.80E – 74	AT1G72990	29.7	3.0	0.56	0.00	0.53	0.00	0.63	0.01	0.52	0.00
lc 20148_2	Acireductone dioxygenase	3.20E – 73	AT1G54220	14.8	2.7	0.51	0.00	0.54	0.00	0.68	0.03	0.54	0.00
lc 97264_1	Acireductone dioxygenase	3.30E – 64	AT1G72990	22.0	2.0	0.61	0.03	0.58	0.01	0.81	0.26	0.59	0.01

a) Accession refers to the entry within a translated conifer EST database, all identified protein sequences are provided in FASTA format as Supporting Information.

b) Estimate only, based on a match to an EST which may represent an incomplete sequence.

c) Represents the ProtScore obtained from Protein Pilot; ProtScore > 1.3 is equivalent to >95% confidence.

d) Mean fold change, bold numbers represent values that satisfied both statistical ($p < 0.05$) and fold change ($FC > 1.5$ or $FC < 0.66$) criteria.

The 40 transcripts that were most strongly differentially expressed fell into many of the same categories as those observed from the protein analysis (Table 2). Twenty-seven of these transcripts could be associated with defense and fell under the headings of signaling, response to oxidative stress, biotic defense, and cell wall modification. Seven transcripts were associated with development and six were of unknown function. Genes associated with signaling included calcium binding proteins and an arabinogalactan protein as was observed in the proteome data in addition to several protein phosphatases that were only observed using the microarray. It should be noted that while the arabinogalactan protein sequences (described in the previous paragraph) shared at least 75% similarity, the “arabinogalactan protein” transcript shared only 33% similarity. Although their functional annotation suggests a possible functional relationship, further experimentation would be required to confirm this relationship. Transcripts related to biotic defense included a U-box domain containing protein (a Ca^{2+} -influx induced component of the hypersensitive response), ethylene responsive elements, phenylalanine ammonia lyase, and a harpin-induced family protein. Transcripts involved in response to oxidative stress included a peroxidase and copper-binding protein (uclacyanin).

Interestingly, many of the differentially expressed proteins and the most highly induced transcripts were classified into similar functional categories based on their manually curated annotations. For example, several peptides and transcripts for calcium-binding proteins were observed in the elicitor-induced Norway spruce proteome (Table 1) and transcriptome (Table 2), respectively. A single induced transcript for an arabinogalactan protein was observed, while at the same time, several different arabinogalactan proteins were found to be upregulated. A peroxidase was induced in chito-san treated cells at the level of transcript as well as protein expression. These data clearly showed that similar biological functions were affected both at the transcriptome and proteome level in Norway spruce cells in the early response to chitosan elicitation.

4 Discussion

Elicitor inducible cell cultures have widely proven to be an effective experimental system in several angiosperm species for interrogating defensive processes such as the formation of antimicrobial secondary metabolites and associated signaling events stimulated by pathogen attack [32–39]. In the present study we used an elicitor-inducible cell suspension culture system of Norway spruce, a gymnosperm tree, to uncover early proteome and transcriptome changes induced by treatment with chitosan. The same system was previously used to demonstrate elicitor-induced terpenoid metabolism and jasmonate signaling in the response of Norway spruce cell cultures [22]. Intact trees of Norway spruce and related Sitka spruce have previously been used to establish some of the later defense responses at the molecular, biochemical, and anatomical levels [7, 8, 11, 13–15, 26].

The highly replicated experimental design that was employed in the present study facilitated the use of recursive sample analysis and data derived exclusion lists to improve proteome coverage and overlap between datasets derived from biologically replicated samples. It has been previously shown that repeating an LC-MS/MS based proteome analysis will produce two different datasets with approximately 65% overlap [40, 41]. The number of new proteins observed with each successive sample continues to drop off, and it has been estimated that 10–12 analyses would be required to obtain near complete coverage of the proteome [40]. The data collected in our study with Norway spruce cells demonstrated that the rate of discovery of new proteins was sharply reduced after only three analyses. Beyond this point, the number of new proteins discovered decreased gradually with every successive sample. The rate of new protein discovery began to plateau by the completion of the tenth reaction. However, by performing a second and third iterative analysis of each sample, the potential number of proteins that could be discovered was increased by 30 and 17%, respectively. The new proteins that were observed were of lower signal inten-

Table 2. List of transcripts induced ($p < 0.001$) following chitosan elicitation (40 most highly induced transcripts listed)

AGI	BLASTX annotation vs. TAIR <i>Arabidopsis</i> proteins	Spruce cDNA	15 min vs. control		30 min vs. control		45 min vs. control		120 min vs. control	
			FC ^{a)}	<i>p</i> -val	FC ^{a)}	<i>p</i> -val	FC ^{a)}	<i>p</i> -val	FC ^{a)}	<i>p</i> -val
Signaling										
At2g46600	Calcium-binding protein	WS00722_J06	2.72	0.000	5.29	0.000	9.26	0.000	5.41	0.000
At2g46600	Calcium-binding protein	WS00917_G15	2.47	0.000	4.27	0.000	6.76	0.000	4.22	0.000
At2g46600	Calcium-binding protein	WS01012_O12	2.10	0.000	4.33	0.000	6.49	0.000	4.63	0.000
At4g20780	Calcium-binding protein	WS00924_K15	2.30	0.000	4.72	0.000	6.85	0.000	3.50	0.000
At1g24620	Polcalcin, putative/calcium-binding pollen allergen	WS00932_J02	2.51	0.000	4.24	0.000	5.88	0.000	3.92	0.000
At2g46330	Arabinogalactan-protein (AGP16)	WS0262_H13	2.80	0.000	6.10	0.000	9.35	0.000	4.57	0.000
At2g30020	Protein phosphatase 2C	WS00111_O11	3.26	0.000	5.71	0.000	6.71	0.000	3.30	0.000
At1g07160	Protein phosphatase 2C	WS00111_H09	3.06	0.000	5.78	0.000	6.13	0.000	2.72	0.000
At2g30020	Protein phosphatase 2C	WS00824_C18	2.98	0.000	5.92	0.000	6.41	0.000	2.33	0.000
Response to oxidative stress										
At5g17820	Peroxidase 57 (PER57)	WS01027_N03	3.12	0.011	3.92	0.002	9.71	0.000	9.62	0.000
At2g32300	Uclacyanin I	WS0101_F22	2.06	0.042	5.15	0.000	10.64	0.000	8.77	0.000
At1g75000	GNS1/SUR4 membrane family protein	IS0012_A14	2.17	0.000	5.21	0.000	6.58	0.000	2.87	0.000
Biotic defense										
At1g66160	U-box domain-containing protein	WS00933_N11	4.85	0.000	10.53	0.000	13.89	0.000	4.22	0.000
At3g52450	U-box domain-containing protein	WS00913_D16	3.13	0.002	6.17	0.000	9.62	0.000	2.74	0.003
At5g64260	Phosphate-responsive protein	WS00716_F07	2.29	0.000	4.81	0.000	8.06	0.000	3.30	0.000
At5g64260	Phosphate-responsive protein	WS0045_C17	2.39	0.000	4.59	0.000	7.52	0.000	3.24	0.000
At2g37040	Phenylalanine ammonia-lyase 1 (PAL1)	WS01030_B11	1.99	0.002	3.88	0.000	5.78	0.000	5.13	0.000
At4g17500	Ethylene-responsive element-binding protein 1 (ERF1)	WS00928_B23	3.19	0.000	6.33	0.000	9.35	0.000	3.24	0.000
At3g20310	Ethylene-responsive element-binding family protein	WS00930_O13	2.16	0.000	4.69	0.000	6.33	0.000	3.39	0.000
At4g19820	Glycosyl hydrolase family 18	WS00913_A18	2.26	0.028	3.95	0.001	8.33	0.000	4.42	0.000
At5g06320	Harpin-induced family protein	WS00910_A20	2.62	0.000	4.41	0.000	7.69	0.000	4.05	0.000
Cell wall modification										
At1g13250	Glycosyl transferase family 8 protein	WS00939_I20	1.76	0.000	3.86	0.000	6.45	0.000	5.18	0.000
At4g25810	Xyloglucan:xyloglucosyl transferase	WS0038_G12	2.08	0.000	4.27	0.000	7.87	0.000	8.06	0.000
At5g57530	Xyloglucan:xyloglucosyl transferase	WS00111_G11	2.00	0.000	4.85	0.000	8.47	0.000	5.81	0.000
At4g25810	Xyloglucan:xyloglucosyl transferase	WS00836_N07	2.28	0.000	8.06	0.000	16.13	0.000	13.89	0.000
At4g30270	MERI-5 protein/endo-xyloglucan transferase	WS00912_B16	2.49	0.000	5.41	0.000	9.09	0.000	6.21	0.000
At4g30270	MERI-5 protein/endo-xyloglucan transferase	WS0264_P12	1.51	0.027	4.27	0.000	6.49	0.000	5.43	0.000
Development										
At1g02820	Late embryogenesis abundant 3 family protein	WS01012_J14	3.04	0.000	8.26	0.000	14.71	0.000	14.29	0.000
At1g56150	Auxin-responsive family protein	WS01010_E20	3.18	0.000	8.33	0.000	12.82	0.000	4.88	0.000
At4g28050	Senescence-associated protein	WS00923_G15	2.50	0.000	4.08	0.000	8.00	0.000	5.29	0.000
At1g49770	Basic helix-loop-helix (bHLH) family protein	WS00931_M02	2.00	0.000	5.15	0.000	6.99	0.000	4.63	0.000
At1g07900	Lateral organ boundaries domain protein 1	WS00938_D15	2.39	0.000	5.00	0.000	8.40	0.000	4.52	0.000
At5g01880	Zinc finger (C3HC4-type RING finger) family protein	WS00937_N23	3.52	0.000	7.04	0.000	7.63	0.000	2.47	0.000
At5g52390	Photoassimilate-responsive protein	WS01033_E03	2.36	0.000	6.49	0.000	10.53	0.000	9.17	0.000
Unknown function										
At3g03280	Expressed protein	WS01015_A14	4.29	0.000	8.33	0.000	14.49	0.000	4.05	0.000
At1g02070	Expressed protein	WS00727_N23	2.56	0.000	6.17	0.000	9.52	0.000	5.08	0.000
At3g57450	Expressed protein	WS01026_K16	2.04	0.000	4.69	0.000	7.63	0.000	4.78	0.000
At1g28190	Expressed protein	WS00925_A20	1.99	0.000	3.47	0.000	5.92	0.000	6.33	0.000
At3g03280	Expressed protein	WS00935_I07	2.88	0.000	5.52	0.000	5.71	0.000	2.32	0.000
At1g49030	Expressed protein similar to PGPS/D12	WS0101_N14	2.18	0.000	3.39	0.000	6.13	0.000	4.61	0.000

a) Mean fold change.

sity than those observed during the first iteration and likely represent proteins of lower abundance within the sample.

In total, 2.6% of the Norway spruce cell culture proteome that was measured and 3.6% of the measured transcriptome were differentially expressed within the first 120 min of chitosan elicitation. When compared to previous experiments done on bark tissue of young Sitka spruce trees induced by feeding insects, where 10% or more of the measured transcriptome and proteome were differentially expressed [15, 26], the results with Norway spruce cell cultures treated with chitosan represented a reduced level of overall response. Although the studies with intact Sitka spruce trees were done over longer time periods, even at the earliest time points (2 h) more of the identified proteins were differentially expressed than was observed in the present study. It is possible that the type of stimulus applied (insect feeding *vs.* chitosan treatment) as well as the difference in tissue complexity (bark and phloem *vs.* suspension cells) of the samples assayed contributed to the differences in the amount of differential expression that was observed. For example, the cell suspension cultures used were not photosynthetically active, while a substantial amount of differential gene expression in the green bark of Sitka spruce stems was associated with down-regulation of primary metabolic processes [15].

Both proteome and transcriptome analysis revealed a rapid response of cultured Norway spruce cells to chitosan. Previous studies in angiosperm cells have demonstrated that chitosan perception occurs within minutes and can be demonstrated by measuring changes in intracellular calcium concentration in soybean [42], or by the detection of culture medium alkalinization in tomato [43]. In Norway spruce cells, changes could be observed in both transcript and protein abundance within 15 min following chitosan addition, although changes to the proteome developed more slowly than was observed for the transcriptome response. While the early transcriptome changes peaked between 45 min and 2 h, no such peak was observed for the proteome response. Both the frequency and magnitude of protein expression changes continued to increase until the end of the 2 h period examined. Based on the methods established in the present study for spruce cell culture proteome analysis and the results obtained, it can now be tested in future work if the trend would continue beyond this time point. Similar results of staged transcript and protein responses have been observed for specific genes and proteins in other plant cell culture systems, where transcript abundance of select genes were found to be induced rapidly following elicitation with later induction of select proteins or enzyme activities [38, 44–47].

In earlier work that addressed rapid elicitor-induced responses, exposure of cultured angiosperm plant cells to pathogens or elicitors lead to an immediate increase in the production of ROS (primarily H₂O₂), accompanied by a rapid efflux of K⁺ and Cl⁻ ions, alkalinization of the growth medium and intracellular acidification [34–36]. Increased Ca²⁺ uptake has suggested that calmodulin (CaM) or proteins

containing CaM-like domains are associated with upstream signaling of this process [48, 49]. We demonstrated that early events in signaling were reflected in the proteome response observed in Norway spruce cell suspension cultures and that calcium signaling was clearly implicated. We identified several CaM isoforms and calcium binding proteins that were induced rapidly in response to the addition of chitosan to Norway spruce cells. In general, plants possess large gene families such as the one that encodes multiple CaM isoforms [50]. *Arabidopsis* has 11 genes that code for at least seven different isoforms of CaM. There is no rigorous classification system to differentiate them, and the functional significance of this redundancy is still not well understood. However, there is increasing evidence that individual gene family members are functionally distinct. For instance different CaM genes appear to be involved in disease resistance in soybean [51, 52] and *Arabidopsis* cold stress [53] where differential expression of these proteins has been observed. In addition, knockdown studies have shown that certain CaM proteins are involved in resistance to necrotrophic organisms [54]. Although the number of CaM genes in spruce is not known, four proteins from this family were induced within minutes of chitosan perception. Interestingly, calcium/CaM levels have been postulated to act downstream of jasmonic acid in the response of *Arabidopsis* plants to wounding [55]. A chitosan-induced jasmonate response with apparent *de novo* formation of *cis*-jasmonate within 30 min of elicitation of Norway spruce cells has recently been reported [22]. Transcriptome profiles also identified a number of calcium binding proteins that were rapidly induced in chitosan-treated Norway spruce, which further supports the action of calcium-mediated signaling in this system. The induction of calcium-dependent signaling is generally preceded by other signaling events. It has been shown that H₂O₂ functions as a signaling molecule in plant defense [56, 57] and it has been implicated in the activation of calcium channels in *Arabidopsis* guard cells [58]. Several of the proteins and transcripts that are differentially expressed in Norway spruce cultures following chitosan elicitation have been functionally tied to the production and action of H₂O₂. The upregulation of Norway spruce GST, 2-Cys peroxiredoxin and peroxidase may provide protection against ROS. Furthermore, cell surface components such as the arabinogalactan/proline-rich proteins can be readily crosslinked in the presence of H₂O₂. The functional significance of this process has not been conclusively determined, but it may serve as a signal in the response to wounding [59]. The analysis of the proteome of chitosan-stimulated Norway spruce cells identified several arabinogalactan/proline-rich proteins that were induced in response to elicitation. These proteins may serve as targets of ROS and could function in the signaling process employed following chitosan perception or in cell wall reinforcement.

In conclusion, chitosan-induced Norway spruce cells proved to be a useful and well-controlled system to establish the early responses of a gymnosperm tree species to elicitation in terms of protein and transcript induction. The com-

bination of iTRAQ quantitative labeling and iterative sample analysis employing data derived exclusion lists produced a more comprehensive screen of the spruce proteome than has previously been possible. A second and third iteration of data collection for each biological replicate led to increases of 31 and 23%, respectively, in the size of the final dataset. These improvements represented increases in the potential size of the dataset and presumably represented the detection of lower abundance proteins that could not be observed by performing a single iteration of sample analysis. This approach relied on the use of a biological system that was easily manipulated compared to mature trees and that is likely to provide a more homogeneous response. Some of the early events in the response to chitosan elicitation were uncovered, with quantitative changes observed in both the proteome and transcriptome within 15 min of elicitation. Both calcium-mediated signaling and oxidative stress were implicated in the response to chitosan based on the proteins and transcripts that were differentially expressed. The involvement of a class of arabinogalactan/proline-rich proteins in the response to fungal elicitors was also implicated for the first time. The proteins, transcripts and processes identified in this study, enabled by the use of elicitor-induced cell cultures, can now be tested for targeted manipulation in cell cultures and intact trees.

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6 Accession numbers

Microarray transcriptome data have also been deposited in the Gene Expression Omnibus database (accession numbers GSE10771, GSM271878-GSM271901).