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Proteome changes following biotic and abiotic stress in forest trees

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[5] Vâlcu CM, Lalanne C, Müller-Starck G, Plomion C, Schlink K. Protein polymorphism between two *Picea abies* populations revealed by two-dimensional electrophoresis and tandem mass spectrometry. *submitted*
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[6] Vâlcu CM, *et al*. Local and systemic changes in European beech (*Fagus sylvatica*) proteome following infection with *Phytophthora citricola*. *in preparation*
Contribution: experiment design, two-dimensional electrophoresis, image and data analysis, preparing protein spots for mass spectrometry, analysis of mass spectrometry results, writing the manuscript

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List of abbreviations

2-DE	two dimensional electrophoresis
2-ME	2-mercaptoethanol
A	anode
AA	acrylamide
ASB 14	tetradecanoylamido propyl dimethylammonio propanesulphonate
BPB	bromophenolblue
C	cathode
C7BzO	3-(4-heptyl) phenyl 3-hydroxypropyl dimethylammonio propanesulphonate
C8Ø	4-octyl benzoyl amidopropyl dimethylammonio propanesulphonate
CA	carrier ampholyte
DM	dodecyl maltoside
DTE	dithioerythritol
ET	ethylene
Glm	general linear model
HED	hydroxyethyldisulphide
HR	hypersensitive response
HS	heat shock
HSP	heat shock proteins
HSR	heat shock response
IAA	iodoacetamide
IEF	isoelectric focusing
IPG	immobilised pH gradient
ISR	induced systemic resistence
JA	jasmonic acid
NDSB	non detergent sulphobetaines
NDSB 256	N-phenyl-methyl-N,N-dimethyl ammonio propanesulphonate
NP-40	Nonidet P-40
OG	n-octyl β-D-glucopiranoside
PAA	polyacrylamide
PTM	post-translational modification
SA	salicylic acid
SAR	systemic acquired resistence
sHSP	small heat shock proteins
SB 3-10	decyl dimethylammonio propanesulphonate
TBP	tributyl phosphine
TCEP	Tris (2-carboxyethyl) phosphine
VOC	volatile organic compounds

1 INTRODUCTION

1.1 Plant stress in the context of climate change

The stress concept in plants

The "stress" concept was founded by Hans Selye in 1936 [7], subsequently adapted for plant species and formally defined as "any unfavourable condition or substance that affects or blocks a plant's metabolism, growth or development" [8]. Stress factors induce a sequence of reactions grouped in three phases: response, resistance and exhaustion, followed by a recovery phase after the removal of the stressor [8].

The distinctive feature that renders plants particularly vulnerable to changes in environmental conditions is the fact that they are sessile. Since they cannot avoid non-optimal environmental conditions or escape environmental extremes through migration, as most animal species, plants have evolved a wide range of stress specific and unspecific mechanisms of resistance and tolerance. Plants have also developed great acclimation and adaptation capacities involving morphologic, physiologic and metabolic adjustments in response to changes of environmental conditions, and stress response should not be confused for these normal mechanisms of "tuning" to the surrounding environment [9]. Environmental factors act as stressors when they rise above certain intensity and/or duration thresholds. Mild stressors can be coped with by physiological and metabolic adjustment and in certain circumstances can even stimulate plant development (eu-stress) while prolonged/strong stress conditions which exceed plants' compensatory mechanisms (dis-stress) have negative effects on plant development and can cause damage or even death [8].

Plant stress in a changing and challenging environment

Currently the most important stress sources for natural or managed plant populations are directly or indirectly related to the rapid climatic changes registered over the last years and predicted for the following period. The increase in the mean temperature and the corresponding intensifications of the hydrological cycle as well as the increased frequency of extreme climatic events (extreme temperatures and irregular spatial and temporal distribution of precipitations) are more severe in continental areas at middle and high latitudes and at high altitudes [10,11].

Effects of climate change upon natural populations of a wide variety of species have already been registered. Latitudinal and altitudinal shifts in the range of species distribution have been observed in several geographic regions[12]. A meta-analysis performed by Root et al.[13] on 143 selected studies detected a statistically significant shift of species-characteristic spring events towards earlier dates with about 5 days per average decade within the last 50 years. Notable exceptions were tree species for which the estimated average phenological shift was of only 3 days[13].

Stress particularities in tree species

Life cycle related particularities of tree species constitute disadvantages in the context of rapid climatic changes [14]. Trees are exposed during their long lifespan to wide ranges of environmental conditions and stressors. The long generation times and high ages of reproductive maturity[12] and the consequently slow rate of genetic evolution by mutation accumulation also delay their response to selective pressure [15]. These drawbacks can be partly compensated by the unusually high level of genetic variability maintained within tree populations [14,16], the large tolerance of tree populations for genetic loads [17] and their high phenotypic plasticity [18]. Maintaining a high level of genetic variation of traits relevant for adaptation to different environmental conditions will facilitate a rapid adaptation to the new climatic conditions [19] while a high phenotypic plasticity will improve a species chances to resist and recover from extreme climatic events.

It is of both scientific and practical interest to investigate the mechanisms behind tree species adaptation to environmental stressors and to identify genes with potential adaptive significance in the context of climate change.

1.2 Abiotic stress in forest trees: elevated temperature

Heat stress in tree populations

Although the effects of climate change are manifold, the most direct and significant are the increase of mean global temperature and the increase in the frequency of extreme temperature events. The level of tolerance to temperature variation varies greatly between tree species and among populations.

Tree populations cover large micro-environmental heterogeneity and therefore the opportunity for micro-environmental selection is also very high [17,20]. Genes with potential adaptive significance in the context of climate change are likely to be under divergent selective pressure in populations adapted to different local conditions and to exhibit differential expression in these populations. Although the actual mechanisms behind the differential expression might differ (genetic differentiation or environmental effects), such traits potentially confer adaptive advantage under the given environmental conditions [21].

Temperature is the major selection factor along latitudinal and altitudinal gradients [22] and heat tolerance is known to vary with altitude in wild species [23]. Trees in particular, have been shown to exhibit high levels of adaptive plasticity (through parental effects) resulting in phenotypic clinal variation of traits significant for the adaptation to the temperature regime [24]. Micro-geographical adaptive genetic differentiation in response to differential environmental pressure has been described for several coniferous species [12], including clinal variations of isozymes allele frequency along latitudinal [25] or altitudinal [26-30] gradients.

Heat shock response in different tree ecotypes

At molecular level, heat stress causes the inhibition of normal protein synthesis and the induction of specific proteins (heat shock proteins, HSP) required for plant thermotolerance. The amplitude and kinetics of the heat shock response (HSR) largely varies between species and populations, and correlates with the level of thermotolerance of the respective species / population [31-35].

Inter-species correlation of HSP expression with environmental temperatures is often hindered by confounding species-specific morphological and physiological characteristics relevant to heat stress avoidance. For example, expression of chloroplast small HSP compared between eight congeneric woody plant species did not correlate with the mean environmental temperatures but rather negatively correlated with the specific leaf area (adaptation to stressful microclimates) suggesting that HSR is shaped during the life history of the species [36]. Comparing gene expression patterns between populations of the same species can overcome the inherent difficulties of comparison across species and ease the detection of meaningful differences in their reaction to heat stress. Since the highest increase in average temperature due to climate change is expected to occur at high elevations, conifer species are likely to be most affected. Tree ecotypes adapted to the local climatic conditions characteristic to contrasting elevations are therefore excellent model systems for the investigation of putative differences in the HSR.

Long term adaptation of tree populations to specific temperature regimes can be assessed by the comparison of gene expression patterns under normal conditions between ecotypes from different elevations. Adaptation to temperature variation and the capacity to survive extreme temperatures can be also assessed in such ecotypes by comparing their gene expression profiles under high temperature stress. However, plants survival to high temperatures does not depend only on its capacity to cope during the heat stress event, but also on its capacity to activate a large array of defence mechanisms during the recovery phase [37]. Following gene expression profiles of tree ecotypes adapted to contrasting elevations during the heat stress period and during their recovery from hest stress would allow the identification of expression patterns possibly responsible for differences in their capacity to withstand and recover from extreme temperatures.

1.3 Biotic stress in forest trees: pathogen and herbivore stress

Biotic stress in tree populations under climate change conditions

Apart from the direct effects of factors associated to climate change like temperature raise, enrichment of atmospheric CO_2, drought and flooding which are known to have a significant impact on tree growth, natural regeneration and competitiveness (reviewed for *Fagus sylvatica* in [38]), the indirect implications of these changes are as important although more difficult to estimate. For example, shifts in the seasonal distribution of precipitation caused by climate change cause an increased frequency of flooding events during the growing season [39], to which many tree species (e.g. *F. sylvatica* [38]) are particularly sensitive. Tree species are generally more susceptible to pathogen attack during the period of maximum growth [40-42] and prolonged water-logging is likely to increase this susceptibility both through the direct stress on the plants and indirectly by facilitating pathogen growth and mobility (reviewed in [11]). Such increased environmental pressure can render even factors of generally low risk to be very harmful.

Another indirect consequence of climate change regards the spread and dynamics of herbivorous insect populations. Apart from resources (i.e. host plant), temperature is the main limiting factor of insect herbivores development, survival, range and abundance [43]. Insect species have specific requirements for temperature at specific life stages, and the effects of temperature on insect herbivores is strongly dependent on other environmental factors like for example elevated CO_2 (through its influence upon plant content in non-structural carbohydrates and phenolics, and leaf toughness) [44], therefore the impact of climate change on insect populations is often difficult to predict [45]. However, higher temperatures are generally likely to extent insect areals towards higher latitudes/altitudes due to higher survival over winter [11], and to restrict them at lower latitudes/altitudes due to extreme temperature and drought over the summer [43,45,46]. Such shifts of areals and disturbances in insect population dynamics can result in exposure of plant communities to new and/or high densities of insect herbivores [47].

Trees' defence against pathogens

Phytophthora species are generally considered to be weak competitors in natural environments. However, there is growing evidence for their involvement in the European beech (*Fagus sylvatica*) decline [48], oak (*Quercus* sp.) decline syndrome [49-53] and Ink Disease of sweet chestnut (*Castanea sativa*) [54]. All these diseases are controlled by a complex of biotic and abiotic factors among which *Phytophthora* species can act as predisposing factors contributing to the decline of trees already subjected to other biotic and/or abiotic stressors [50,54]. For example, the incidence of oak decline is higher when infection is combined with fluctuating water tables, prolonged heavy rain or drought, heavy defoliation or secondary pathogens [48]. Under the changing and challenging environmental conditions associated with climate change, *Phytophthora* species could become important selective factors in forest ecosystems [11].

Among *Phytophthora* species, *Phytophthora citricola* is a common root pathogen with a broad host range including more than 20 tree genera [6]. Like most species of the genus, *Phytophthora citricola* exhibits specific preferences for site characteristics, i.e. for wet soils close to ponds or riverbanks [40].
Fagus sylvatica, the most abundant and dominant broad leafed tree species in Europe, exhibits high sensitivity to infection with *Phytophthora citricola*, particularly at young stages [55,56]. Although the effects of *Phytophthora citricola* infection on *Fagus sylvatica* have been investigated morphologically [57,58] and physiologically [56-58], the knowledge on the tree's intimate defence mechanisms at molecular level is still limited.

Plant-pathogen interactions depend on the specific recognition between species at molecular level [59-61]. Plants detect microbe-associated molecular patterns (MAMPs) or microbe / wound/herbivory-

induced molecular patterns (MIMP/WHIMPs) [62] and subsequently activate general and/or specific defence mechanisms in order to attain different levels of tolerance or resistance. Although signalling and defence pathways common to several species have been described, plant response to pathogens is largely pathosystem specific [63].

Several types of compounds can act as recognition factors in *Phytophthora* species [61] among which the most often encountered are cell wall fragments and elicitins (10kDa holoproteins involved in the recovery of sterol molecules from host membranes [64-66]). The array of elicitors is species-specific, as is host's capacity of recognition and the defence mechanisms activated in response [64,67]. In most of the cases, recognition of the elicitor results in hypersensitive response (HR) and subsequent establishment of systemic acquired resistance (SAR) and the associated salicylic acid (SA)-dependent defences [68-70]. Some elicitors, however, also induce SA-independent defence mechanisms [68,69,71]. Although an elicitin (citricolin) was isolated from *Phytophthora citricola* which causes necrosis and decreased gas exchange of tobacco leaves as well as oxidative burst in tobacco cell suspension culture [72], no effects have been observed on *Fagus sylvatica*. The defence pathways activated by *Fagus sylvatica* following infection with *Phytophthora citricola* are mostly unknown.

Trees' defence against herbivores

Plant reaction to herbivore attack encompasses the defence against the herbivore itself as well as against opportunistic pathogens [73]. Plant's response is elicited both by the mechanical wounding of the tissues as well as by insect-specific elicitors introduced together with saliva in the plant tissues and the defence mechanisms involved are mediated by jasmonic acid (JA) and ethylene (ET) [74].

Following both herbivore attack and wounding, plants activate mechanisms that confer them direct resistance against herbivores [75-77]. Additionally, indirect defence mechanisms are activated, consisting mainly in secretion of volatile organic compounds (VOC) that act as attractants for the parasitoids or predators of the herbivores [78-80]. VOCs [81,82], including methyl jasmonate [83,84], emitted by wounded plants can induce similar effects in neighbouring unharmed plants [85]. The increased resistance to herbivores induced through air borne volatiles was confirmed both in laboratory [82] and field experiments [85-87].

Trees' defence network against biotic stressors

Although plant-pathogen recognition is often pathosystem specific, the defence mechanisms activated by the plant are at least partly effective against a wide array of invaders (non-specific disease resistance) including herbivores [88]. Induction of plant defence against an insect herbivore confers protection against other herbivore species but also against pathogens [89]. Wounding alone can also induce resistance to pathogens [90]. Cross protection is due to the crosstalk between signalling and defence pathways that target different types of aggressors. The same crosstalk can also lead to reciprocal inhibition of the two pathways and such antagonistic effects have also been described [91].

Defence against most types of biotic stressors (e.g. insects, fungal, bacterial or viral pathogens) involves SA, JA or ET signalling; therefore the responses are often redundant. For example gene expression profiles induced by pathogens and herbivores exhibit overlaps [92,93], although they are activated through different signalling pathways. Defence pathways mediated by SA and JA or ET are inter-regulated at multiple levels: SA and JA have been shown to act as mutual antagonists [73] and the pathways share common regulators such as NPR1 (non-expresser of PR genes 1) [94] or fatty acids [95].

In order to gain a deeper understanding of the inter-regulation and redundancy of signalling and defence pathways activated against different biotic stressors (e.g. infection with the root pathogen *Phytophthora citricola* and wounding as elicitor of the herbivore response), these responses need to be investigated in comparable experimental conditions that would eliminate any confounding variable.

1.4 Proteomics technologies for the study of stress response in plant species

Although the vast amount of genomic information generated in the last decades has transformed the very essence of biological research, a satisfactory accuracy of gene prediction and of biological function assignment is still difficult to achieve even when complete genome sequence data are available and the most advanced bioinformatics tools are applied [96,97]. On the one hand, not all open reading frames (ORF) identified at genome level code for functional genes [96], and on the other hand one gene can correspond to several different modified proteins. Moreover, the putative function and sub-cellular localisation of gene products can only be inferred from sequence data with disappointing inaccuracy. The rather poor correspondence found between protein expression and mRNA level [98-100] limits the use of the large amount of data ensuing from transcriptome analysis for the description of protein expression patterns. Other factors determining protein function like post-translational modification (PTM), non-enzymatic modifications or interactions with other proteins and metabolites as well as expression level and turn-over rate can only be detected at protein level. All these point to the fact that the only valid approach for deciphering gene function is to focus on the gene products, which are directly involved in all biochemical processes within the cells. Verification of structure, function, modification, sub-cellular localisation and integration into functional and regulatory networks of gene products becomes thus an obligatory step in genome annotation [97,101] and the fast developing field of proteomics provides the array of tools necessary to accomplish these tasks [96,102]. Complementary approaches like transcriptomics, proteomics and metabolomics [103] have to be integrated in order to fill the information gap between genotype and phenotype.

The vast complexity of the proteome (up to at least 10000 proteins species are estimated to be simultaneously expressed in eukaryotic cells [104]) and the large dynamic range of protein concentrations [105] (estimated to more than 10^5-10^6 for eukaryotes [98]) pose specific challenges to proteomic studies. Additionally, the high level of structural and physicochemical heterogeneity characterising the proteins as compared to nucleic acids causes difficulties in their isolation, separation and identification [103]. Although for plant tissues these technical challenges are particularly severe [103,106], proteomic technologies have been so far successfully used to address a wide array of scientific questions in the field of plant sciences [107,108]: proteome maps have been established for several plant tissues [109-114] and sub-cellular compartments [115-120] as well as for specific physiological states [121-124]; proteome changes during plant development [125-127] or symbiotic interactions [117,128,129] and in response to various biotic [130-136] and abiotic [137-146] stressors have been described for many species; genetic variability has been characterised within and between populations, and phylogenetic relationships have been established based on comparisons at [147-153] or below species level (between genotypes, cultivars, ecotypes, lines or geographic origins) [154-161].

Despite the immense advances of non-gel based proteomic techniques in the last decades, the technique mostly preferred for the comparison with a high resolving power of protein expression between large number of complex samples remains two dimensional gel electrophoresis coupled with the mass spectrometric identification of separated proteins [104].

Two dimensional gel electrophoresis

The strength of two dimensional gel electrophoresis (2-DE) as a protein separation technique mainly resides in the fact that it uses two orthogonal parameters: net charge and molecular weight. 2-DE patterns reflect protein expression level, isoforms and PTM [104]. The pioneering work of O'Farrell [162] and Klose [163] significantly increased the resolving power of the 2-DE technology but was still far from achieving an optimal resolution of separation [164,165]. Subsequent developments, of which introduction of immobilised pH gradients (IPGs) [166] is probably the most significant, allowed for major improvement of resolution and reproducibility [167] of 2-DE separations.

Sample preparation protocols have been optimised for a large variety of challenging samples (e.g. [168-176]). New detergents, reducing agents and chaotropes have been developed for a more efficient solubilisation of proteins (reviewed in [177-179]), including hydrophobic proteins. Various pre-fractionation techniques (reviewed in [104,180,181]), narrow overlapping IPGs and IPGs with extended separation distances have been designed to minimise spot overlapping and to maximise the number of proteins detected by 2-DE and allow the detection of low-abundance proteins [182,183]. Very promising perspectives for the enrichment of samples in low abundance proteins are also offered by the more recently developed protein Equalizer™ technology [184]. Separation ranges have been extended to high pH values [185] and non-linear gradients allow for a more even display of protein spots over wide separation gradients. Loading capacities as high as 5 mg protein can be reached for micro-preparative 2-DE with narrow range, 24 cm long IPG strips [104], if samples are applied by in-gel rehydration [186]. Special IPG strips can also be designed to match particular experimental needs [187].

Silver and fluorescent staining and radio labelling lowered the detection limit to ng levels [188]. Fluorescent staining ensures a comparatively higher reproducibility and wider linear dynamic range allowing the quantification of changes in protein expression [167,188]. Currently up to 5000 proteins can be simultaneously resolved on 2-DE gels [104] using the above mentioned developments. The CyDye™ DIGE Fluors for pre-labelling of proteins prior to 2-DE allow for sample multiplexing and the use of internal standards, ensuring highly accurate and reproducible quantitative assessments of differential protein expression [188]. Several specific staining methods have also been developed for the detection of PTMs (reviewed in [104,188]).

A wide array of image analysis software has been developed for a more rapid, accurate and reproducible analysis of 2-DE patterns, and important steps have been taken towards automation of 2-DE procedure and achievement of a higher throughput 2-DE analysis ([104,189]) including commercial availability of IPG dry strips and of precasted gels.

Despite all these important advances, 2-DE remains rather labour-intensive and therefore time-consuming and hampered by a number of limitations related to: sample availability; need for optimisation of protocols for specific types of samples; difficulties in the extraction, separation and detection of very low abundant and very hydrophobic proteins [190]; dependence of 2-DE pattern on the sample preparation protocol and staining method; limitation to certain protein size and pI ranges [101]; co-migration of proteins [191]; time-consuming image analysis, more often than desirable prone to user-induced errors; still limited availability of sequence information necessary for the identification of proteins of most species.

1.5 Objectives of the present investigations

The general aim of the present study was to investigate some of the intimate mechanisms of the response of tree species to stress factors likely to intensify as a result of the current global environmental changes. Tree species expected to be particularly affected by the considered stressors were selected as study species.

The specific aims of the present study were as follows:

- Establishing protein extraction protocols and two dimensional electrophoresis technique for the optimal extraction and separation of proteins from woody plant samples (*Picea abies* needles and roots, *Fagus sylvatica* leaves and roots) [1-3].

- Identifying differences in the protein expression patterns of *Picea abies* ecotypes adapted to environmental conditions characteristic to contrasting elevations, under normal temperature conditions as well as during their exposure to and recovery from heat stress [4,5].

- Identifying changes in the local and systemic protein expression patterns of *Fagus sylvatica*, following infection with the root pathogen *Phytophthora citricola* [6].

2 GENERAL METHODOLOGY

2.1 Optimisation of 2-DE separations

Standard protocols described for 2-DE using IPGs [104,165,167] need to be adapted for particular types of tissues [104] in order to achieve optimal resolution and reproducibility of protein separation. Optimisation of sample preparation, IEF and SDS-PAGE were carried out for four types of samples: *Picea abies* needles and roots and *Fagus sylvatica* leaves and roots respectively.

Sample preparation for 2-DE from woody plants poses specific problems [1,104,190] and was therefore given special attention [1,2]. Acetone precipitation procedure and the composition of extraction and rehydration buffers were optimised as described in [1,2]. The selection of IPGs was limited both for optimisations and stress response experiments by sample availability and time constrains to medium pH ranges (4-7 and 6-9 or 6-11). Optimal focusing conditions were first empirically established by double loading of samples at the cathode and anode and than by comparison of 2-DE patterns with this "reference" pattern. Throughout the optimisation steps, IPG strips were silver stained after the second dimension along with 2-DE gels, to verify protein transfer between the two dimensions. Silver staining was selected for protein detection based on its good sensitivity and cost-effectiveness.

Limitations imposed by the silver staining on data analysis are discussed in the discussions section (4). The optimisation steps and the corresponding results are summarised in Table 2.1-1.

According to these optimisation steps, the protocols used during the stress response experiments are summarised in Figure 2.1-1 and Table 3.1-1.

Image and data analysis

2-DE image analysis was performed with ImageMaster 2D Platinum [4,5] or Progenesis SameSpots [6]. Raw data were exported from the image analysis software and analysed within the frame of general linear modelling (glm) with R2.2.1 [4,5] or R4.2.0 [6].

The reproducibility of 2-DE separations was assessed between biological replicates and at different technical replication levels in order to estimate the variance associated to each step in the separation procedure[3].

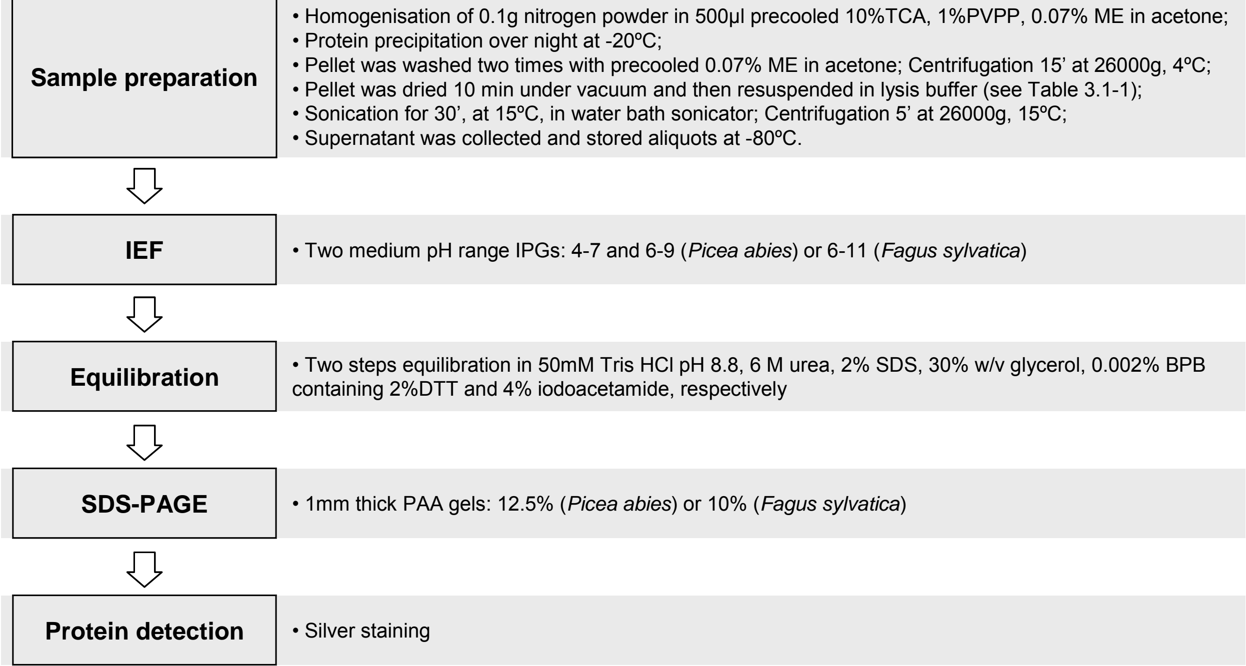


Figure 2.1-1 2-DE general protocol

Table 2.1-1 Optimisation of 2-DE protocols for *Picea abies* needles and roots and *Fagus sylvatica* leaves and roots. Last column presents the optimisation results.

Optimised step		Optimised parameters	Variants tested	Result / Optimised protocol
Sample preparation	Protein extraction	Extraction procedure	Grinding in liquid nitrogen and homogenising with sample buffer Grinding in liquid nitrogen followed by acetone precipitation and solubilisation in sample buffer Grinding in liquid nitrogen followed by acetone precipitation and sonication in sample buffer	Grinding in liquid nitrogen followed by acetone precipitation and sonication in sample buffer (higher protein yield, better 2DE separation)
		Acetone precipitation		
		Precipitation temperature	-20°C; -76°C	No significant differences / -20°C
		Precipitation duration	2h; over night	Over night (more efficient extraction)
		Composition of precipitation solution	2-mercaptoethanol, DTT, TCEP, TBP	Inconsistent / DTT or 2-mercaptoethanol
		Composition of washing buffer	acetone; 20mM DTT in acetone; 10% water in acetone; 20mM DTT, 10% water in acetone	No significant differences / 20mM DTT in acetone
		Number of washing steps	1, 2 or 3 washing steps	2 washing steps (more efficient extraction, better 2DE separation)
	Sample buffer	Reducing/Oxidising agent	DTT; DTE; TCEP; TBP; alone or in different combinations; HED	100mM DTT (spruce needles and beech leaves) 2mM TCEP + 50mM DTT (spruce and beech roots)
		Chaotropes	urea, thiourea	7M urea + 2M thiourea (more efficient extraction, better 2DE separation)
		Detergents	Triton X-100; Igepal CA-630; CHAPS; C7BzO; C8Ø; ASB 14; OG; DM; SB3-10; NDSB 256, alone or in different combinations	2% CHAPS + 2% SB3-10 (spruce needles) 2% OG (beech leaves) 2% DM (spruce and beech roots)

Table 2.1-1 continued

Optimised step		Optimised parameters	Variants tested	Result / Optimised protocol
Isoelectric focusing	Sample loading		Passive rehydration Active rehydration Active rehydration + IEF in Manifold Cup loading in Manifold (at C for acidic IPGs, at A for basic IPGs)	Cup loading in Manifold (at C for acidic IPGs, at A for basic IPGs) (better 2DE separation); For acidic IPGs, similar 2DE pattern quality achieved with active rehydration + IEF in Manifold
	Rehydration buffer	Reducing/Oxidising agent	DTT; DTE; TCEP; TBP; alone or in different combinations; HED	100mM HED (basic proteins from all samples, acidic proteins from spruce needles and beech leaves) 2mM TCEP + 50mM DTT (acidic proteins from spruce and beech roots)
		Chaotrope	urea, thiourea, alone or in combination	7M urea + 2M thiourea (better 2DE separation)
		Detergent	Triton X-100; Igepal CA-630; CHAPS; C7BzO; C8Ø; ASB 14; OG; DM; SB3-10; NDSB 256, alone or in different combinations	2% CHAPS + 2% SB3-10 (spruce needles) 2% OG (beech leaves) 2% DM (acidic proteins from spruce roots) 2% CHAPS (beech roots and basic proteins from spruce roots)
		Other components	10% isopropanol; 5% glycerol, alone or in combination	Addition of 10%isopropanol for the basic gradient (better 2DE separation)
Protein alkylation	Alkylation protocol	Time of alkylation	Before/after IEF; single-step/two-steps equilibration	Alkylation with 20mM or 100mM AA before IEF or with 15mM IAA after IEF (two step equilibration) ensured complete alkylation of proteins / alkylation with 15mM IAA after IEF
		Alkylation agent	15mM or 100mM IAA, 20mM or 100mM AA	
SDS-PAGE	SDS-PAGE gel	Gel composition	± glycerol	Glycerol containing gels (better 2DE separation)
		Gel concentration	10%; 12.5%; 15%	10% for optimal display of all proteins (beech) 12.5% for optimal display of small HSP (spruce)
Staining	Staining method		Coomassie (Anderson [192]; Kang [193]) Silver staining (Heukeshoven and Dernick [194]) Coomassie (Anderson [192]) + Silver staining	Silver staining (higher sensitivity, lower amount of sample necessary) Coomassie staining or MS-compatible silver staining and higher protein loads for MS analysis

2.2 Experimental setup for the study of proteome changes following abiotic and biotic stress

2.2.1 Abiotic stress: *Picea abies* response to and recovery from heat stress [4,5]

Detailed experimental setup and protocols are described in [4,5]. A brief outline of experiments is presented below and in Fig. 2.2.1-1.

Seeds from two *Picea abies* ecotypes adapted to different environmental conditions (i.e. contrasting altitudes) were germinated and grown under standardised conditions. To compare protein expression patterns under normal growth conditions [5], five replicate samples were used, each consisting of equal amounts of biological material pooled from 30-40 individuals. The results were validated by comparison with a set of other 15 pooled biological replicates.

To compare the response of the two ecotypes to and during the recovery from heat stress[4], a heat shock treatment was applied following the profile described in Fig. 2.2.1-2. Needles and roots were harvested immediately after the heat stress treatment, as well as after 5h, 10h and 30h of recovery. Randomly chosen individuals were pooled as for previous experiments to obtain three biological replicates for each experimental time point. Control samples were harvested simultaneously with heat shock plants, for each of the time points.

Proteins were extracted and 2DE was performed following previously optimised protocols [1,2], as described in Fig. 2.1-1[4]. Gels were stained with silver staining [194]. Image analysis was performed with ImageMaster 2D Platinum and R2.2.1 was used for statistic analysis.

Spots were cut from Coomassie [192] or MS compatible silver stained preparative gels, destained and dehydrated. Tandem MS identification of proteins was performed at the Functional Genomic Platform (Bordeaux). Peptide identification was performed with SEQUEST using all *Picea* ESTs available at dbEST.

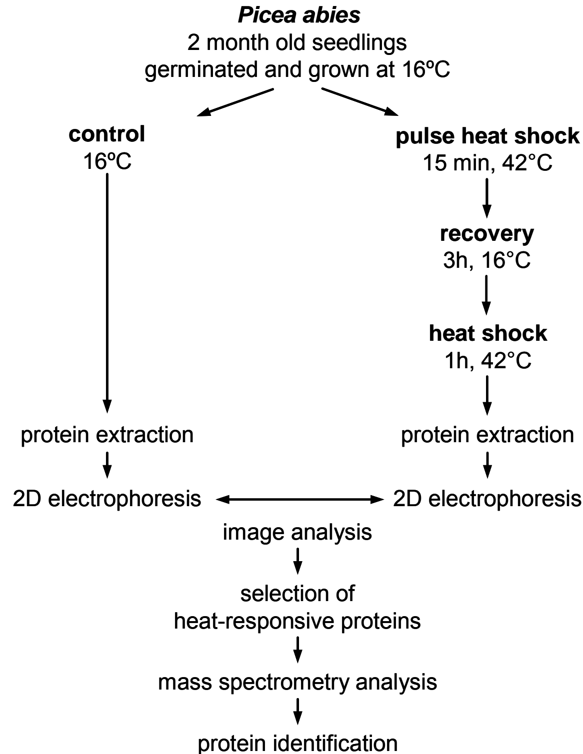


Figure 2.2.1-1 Response to and recovery from heat stress of *Picea abies* seedlings: experimental design.

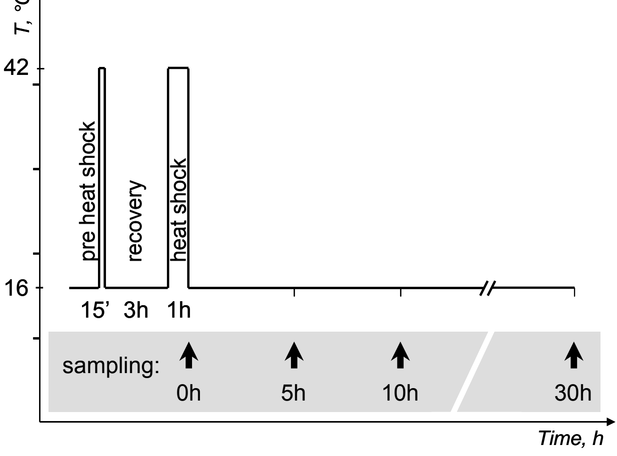


Figure 2.2.1-2 Temperature profile and sampling time points.

2.2.2 Biotic stress: *Fagus sylvatica* defence response to pathogen attack and wounding [6]

Detailed experimental setup and protocols are described in[6]. A brief outline of experiments is presented below and in Fig. 2.2.2-1 and 2.2.2-2.

After germination, *Fagus sylvatica* seedlings were grown in sterile water for three months or in peat-Vermiculite mixture until the age of three months or one year. Wound experiments were performed as described in Fig. 2.2.2-1 and Table 2.2.2-1. Changes in protein expression patterns were investigated at local and systemic level following the wounding of leaves (Wound experiment A) or roots (Wound experiment C). In a third experiment (Wound experiment B), the reception of wounding signals through air by un-wounded plants was investigated in addition to the local and systemic wound responses.

Infection experiments with the root pathogen *Phytophthora citricola* followed the general plan presented in Fig. 2.2.2-2 and Table 2.2.2-1. Local and systemic reaction of *Fagus sylvatica* seedlings was investigated under growth conditions close to natural ones (Infection experiment 1) as well as in liquid system, which allowed an easy access to the roots without the risk of wounding (Infection experiment 2). The reaction to pathogen attack was investigated during different stages of root infection in Infection experiment 2.

Infection and wounding experiments were performed under identical conditions to facilitate the direct comparison of plant responses to pathogen attack and wounding and the identification of both specific and shared patterns of protein regulation. Protein extraction and 2DE followed optimised protocols [1,2] described in Fig. 2.1-1 and [6]. Proteins with low solubility were extracted from the remaining pellets and separated by SDS-PAGE. 2DE and 1DE gels were silver stained [6]. 2DE image analysis was performed with Progenesis SameSpots while 1DE gels were analysed with ImageQuant 5.2. For statistic analysis R4.2.0 was used. Proteins regulated following biotic stress will be identified by *de novo* sequencing in a follow-up project.

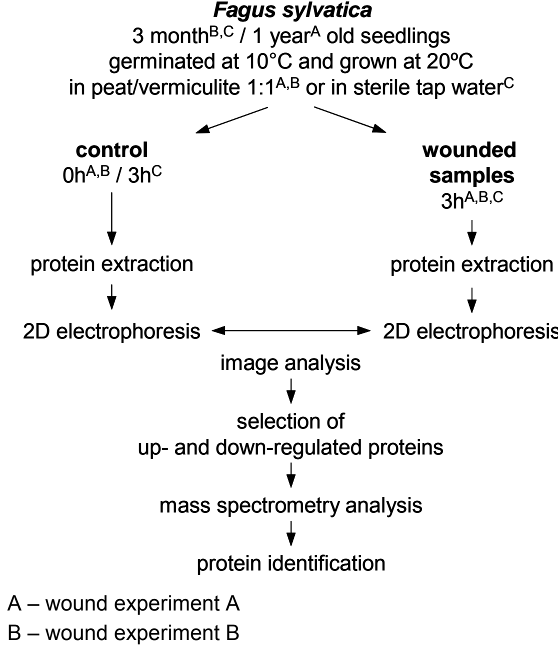


Figure 2.2.2-1 Reaction of *Fagus sylvatica* seedlings to wounding: experimental design.

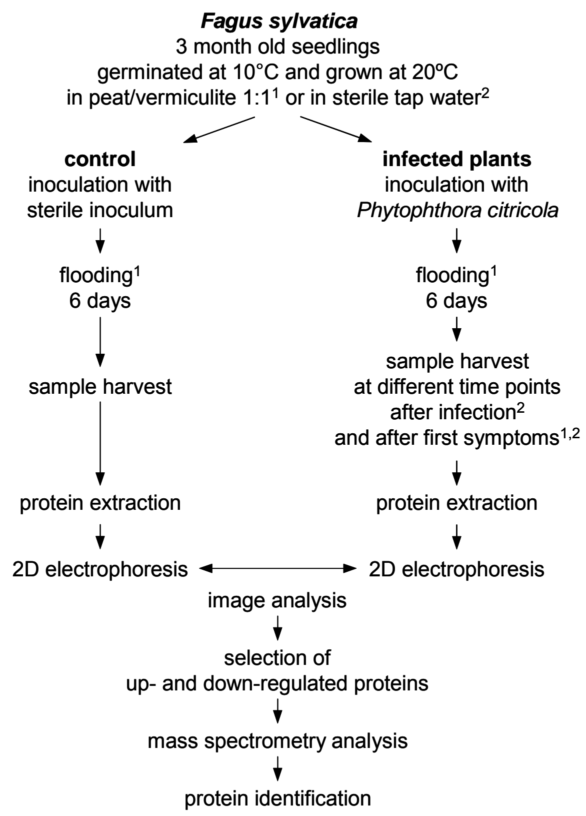


Figure 2.2.2-2 Reaction of *Fagus sylvatica* seedlings to infection with *Phytophthora citricola*: experimental design.

Table 2.2.2-1 Overview of wound and infection experiments (*Fagus sylvatica*)

	Wound experiment A	**Wound experiment B**	**Wound experiment C**	**Infection experiment 1**	**Infection experiment 2**
Experiment design					
Plant age	1 year	3 months	3 months	3 months	3 months
Medium	Peat/Vermiculite	Peat/Vermiculite	Sterile tap water	Peat/Vermiculite	Sterile tap water
Special treatments			Accommodated for 2 days in 2l containers	Inoculated with 5 ml *Phytophthora citricola* inoculum (sterile inoculum for control plants) / plant Flooded 1-2 cm above soil level for 6 days	Accommodated for 2 days in 2l containers Inoculated with 1 pluck *Phytophthora citricola* culture (sterile medium for control plants) / plant
Sampling time	3h after wounding (control at moment 0)	3h after wounding (control at moment 0)	3h after wounding	At first symptoms (7 days)	3h, 12h, 2 days post infection, after clear symptoms (4-6 days)
Samples	Control (leaves) Local response (leaves) Systemic response (leaves): “up” and “down”	Control (leaves) Local response (leaves) Systemic response (leaves) Neighbouring trees (leaves)	Control (roots, leaves) Local response (roots) Systemic response (leaves)	Control (leaves) Systemic response (leaves)	Control (roots, leaves) Local response (roots) Systemic response (leaves)
Sample size (pooled samples)	C, L, S: 2 pools, 24 and 33 plants respectively	C, L, S: 2 x 20 plants N: 2 x 15 plants	C, L, S: 2 x 10 plants	C: 2 x 35 plants S: 6 x 35 plants	C, L, S (3h, 2 days): 15 plants C, L, S (12h, 6 days): 2 x 15 plants

3 RESULTS

3.1 Optimised 2-DE separations of woody plant proteins [1-3]

The results of each of the optimisation steps are summarised in Table 2.1-1.

The sample preparation protocol strongly influenced the quality of the 2-DE separations. Different extraction and focusing conditions were found to ensure optimal separation of proteins from different types of samples as well as of proteins from different pI ranges (Table 3.1-1 and [1,2]). For some types of sample, the resolution of 2-DE patterns was also improved using sample and focusing buffers of different composition with regard to the reducing agent and/or the detergent (Table 3.1-1 and [1,2]).

The protocol used during the stress response experiments following the optimisation steps described in chapter 2.1, Table 2.1-1 and [1,2] is summarised in Figure 2.1-1; the optimised compositions of the extraction and focusing buffers are presented in Table 3.1-1.

Protein expression was examined among biological and technical replicates in order to establish the appropriate statistical tools for the identification of differential expression of proteins [3,6]. Integrated spot volume was found to be normally distributed among biological pooled samples [3]. Since all the samples used in all experiments consisted of independent pools (equal amounts of material pooled from different individuals), this permitted the use of parametric statistics for the analysis of all 2-DE expression data. The predictors of spot volume variance were identified for the different data sets and the identification of differential protein expression was performed based on these parameters [6].

Table 3.1-1 Optimised composition of extraction and focusing buffers.

Species	Tissue	Buffer	Chaotrope	Reducing / Oxidising agent	Detergent	Other components*
Picea abies	needles	extraction	5M urea 2M thiourea	100mM DTT	2% CHAPS 2% SB3-10	0.5% Pharmalyte 3-10
		IEF 4-7	5M urea 2M thiourea	100mM HED	2% CHAPS 2% SB3-10	0.5% Pharmalyte 3-10
		IEF 6-9	5M urea 2M thiourea	100mM HED	2% CHAPS 2% SB3-10	0.5% Pharmalyte 3-10 10% isopropanol
	roots	extraction	7M urea 2M thiourea	2mM TCEP 50mM DTT	2% DM	0.5% Pharmalyte 3-10
		IEF 4-7	7M urea 2M thiourea	2mM TCEP 50mM DTT	2% DM	0.5% Pharmalyte 3-10
		IEF 6-9	7M urea 2M thiourea	100mM HED	2% CHAPS	0.5% Pharmalyte 3-10 10% isopropanol
Fagus sylvatica	leaves	extraction	7M urea 2M thiourea	100mM DTT	2% OG	0.5% Pharmalyte 3-10
		IEF 4-7	7M urea 2M thiourea	100mM HED	2% OG	0.5% Pharmalyte 3-10
		IEF 6-9	7M urea 2M thiourea	100mM HED	2% OG	0.5% Pharmalyte 3-10 10% isopropanol
	roots	extraction	7M urea 2M thiourea	2mM TCEP 50mM DTT	2% DM	0.5% Pharmalyte 3-10
		IEF 4-7	7M urea 2M thiourea	2mM TCEP 50mM DTT	2% CHAPS	0.5% Pharmalyte 3-10
		IEF 6-9	7M urea 2M thiourea	100mM HED	2% CHAPS	0.5% Pharmalyte 3-10 10% isopropanol

* all rehydration buffers contained 0.002% BPB

3.2 Abiotic stress: *Picea abies* response to and recovery from heat stress [4,5]

An autochthonous *Picea abies* ecotype adapted to high altitudes (above 1300m a.s.l.) and the best adapted German ecotype to low altitude conditions (300m a.s.l.) were compared with respect to protein expression patterns under normal conditions as well as in response to and during the recovery from heat shock. Needle and root 2DE patterns were compared between two months old seedlings germinated and grown in controlled conditions from seeds originating from stands of the two *Picea abies* provenances.

A number of 19 constitutively expressed proteins exhibited significant qualitative and quantitative inter-population polymorphism[5]. Seven proteins were over-expressed in the roots of seedlings from the low altitude ecotype, including a tracheary element differentiation protein (TED2), a voltage-dependent anion-selective channel (Porin) and two isoforms of a NAD(P)-binding non-metallo dehydrogenase. The high altitude ecotype expressed two specific proteins (one of them a paralog of the rice r40c1 protein) and ten proteins over-expressed including glycine cleavage system T protein and protochlorophyllide reductase in the needles and glyoxysomal malate synthase in the roots.

A total of 78 protein spots were reproducibly induced following the heat shock treatment[4]. 37% of the heat responsive proteins were organ-specific: 15% for the needles and 22% for the roots. The kinetics of heat regulated proteins was characterised during the recovery from heat shock by means of linear models with the integrated spot volume as dependent variable and time as predictor (for full description of the general linear models, glm, see[4]). Two major types of kinetics were identified and denoted as “early heat responsive proteins” and “late accumulating proteins” respectively. The first class was induced during the heat shock treatment or at the beginning of the recovery phase while the second class accumulated throughout the recovery phase following different accumulation curves. A minority of proteins exhibited transient expression during the recovery phase.

Protein expression patterns and the temporal dynamics of the heat responsive proteins expression were compared between the two *Picea abies* ecotypes by means of glm (see[4] for details). Two protein spots exhibited ecotype-specific kinetics of expression: a precursor of HSP21 accumulated at higher levels during the recovery from heat shock of needles from the lower elevation ecotype while monodehydroascorbate reductase (MDHAR) was specifically up-regulated during the heat shock in the needles and roots of the high elevation plants. Three more spots were unambiguously found to be ecotype specific. A member of the HSP 20 family was highly induced during the recovery from heat shock in seedlings from the low elevation ecotype while glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and an unknown protein accumulated only in high elevation plants.

Expression levels and temporal expression dynamics of the tissue non-specific heat responsive proteins were also compared between needles and roots (linear models described in[4]). 47% of the tissue non-specific proteins showed very significant differences in the temporal pattern of expression between the two tissues in at least one ecotype. Late accumulating proteins exhibited higher levels of expression in needles, while early heat responsive proteins were stronger expressed in roots. Roots also exhibited a delay in the accumulation of proteins during the recovery phase. A group of 7 neighbouring protein spots, possibly isoforms of γ-tocopherol methyltransferase (γ-TMT), were only detected in roots. They were all classified as early responsive proteins, but their expression was maintained for a longer period into the recovery phase by the roots of the high elevation ecotype. Two sHSPs from the HSP 20 family, two glycine-rich RNA-binding protein isoforms (GRP1A) and a probable mannitol dehydrogenase MTD also showed differential expression between needles and roots.

3.3 Biotic stress: *Fagus sylvatica* defence response to pathogen attack and wounding [6]

A set of five experiments (Fig. 2.2.2-1, 2.2.2-2 and Table 2.2.2-1) was designed for the investigation and comparison of local and systemic proteome changes in *Fagus sylvatica* seedlings following infection with the root pathogen *Phytophthora citricola* and wounding as elicitor of plant response to herbivore attack. Patterns of protein expression were characterised for leaves and roots of infected plants and of plants with wounded leaves or roots.

Infected plants exhibited root tip necrosis after 4-6 days (Fig. 3.3-2) and leaf wilting (Fig. 3.3-1) after 7 days from inoculation. All plants infected in soil system but one died within 7 weeks from inoculation (infection experiment 1). Only two control plants died, with symptoms different from the ones of infected plants.

Plants infected in liquid system (infection experiment 2) responded by the up-regulation of 53 proteins (22 local and 31 systemic) and the down-regulation of 29 proteins (12 local and 17 systemic). Early and late stages of infection only shared the regulation of four proteins, all of them locally expressed. Comparatively more proteins were identified as stress-regulated in the leaves of plants infected in soil system (infection experiment 1): 102 proteins, of which 30 shared with the plants from the experiment in liquid system.

31 proteins responded to root wounding (16 local and 15 systemic) and 26 to leaf wounding (2 local, 14 systemic and 10 both local and systemic) with only three proteins in common. Only limited evidence supported an asymmetrical vertical transmission of the systemic wounding signal (Annex 6: Supplementary material 2) [6].

Unharmed seedlings "witnessing" the wounding of neighbouring plants exhibited changes in the expression of 22 protein spots, of which 19 also regulated in the infected or wounded plants. Overall, 188 stress-responsive proteins were detected [6], of which 134 in response to pathogen attack, 36 in response to wounding and 18 shared between the two types of stress response (Annex 6: Table 1) [6].

Clustering of stress-regulated proteins on the basis of their expression patterns in different experimental conditions revealed different classes of proteins (Annex 6: Fig. 2 and 3) [6].
Most of the proteins (90.4%) were regulated by a single type of stress. However, overlapping response was observed between pathogen infected and wounded plants at both local and systemic level.

Although the majority of proteins were consistently up- or down-regulated in different experimental variants under the same stressor or under the two different stressors, a number of three proteins exhibited opposite directions of regulation following infection in liquid and soil system respectively. Another 13 proteins were oppositely regulated in response to infection and wounding.

a. Control

b. 7 days post infection

Figure 3.3-1 Infection experiment 1: symptoms of infection with *Phytophthora citricola* in *Fagus sylvatica* leaves.

a. Control

b. 6 days post infection

Figure 3.3-2 Infection experiment 2: symptoms of infection with *Phytophthora citricola* in *Fagus sylvatica* roots.

4 DISCUSSIONS

4.1 Proteomics technologies for differential display experiments [1-3]

Differential display 2-DE experiments can generate valuable scientific knowledge, provided that the limitations of the technology involved are known and considered during the interpretation of results. This starts with acknowledging that the term *differentially expressed proteins* actually denotes observed changes in protein abundance that can originate not only from differences in protein synthesis rates but can also result e.g. from differences in protein stability under different experimental conditions or protein modifications causing shifts in the spot's position on the 2-DE gel.

A prerequisite for obtaining reproducible 2-DE separations of proteins is the use of optimised protocols in each step of the technique [104]. The influence of the protein extraction protocol on the quality of the 2-DE pattern is very significant [190], which explains the extensive literature proposing ever improved sample preparation protocols [1,2,168-176] as well as new detergents (reviewed in [177-179]) or other reagents [195] meant to enhance extraction efficiency and the purity of the extracts from a large array of sample types. The IEF has also been continuously improved [182,183,185,186] and adapted [187] to particular experimental needs, while recent improvements in staining and labelling methods have opened new opportunities for the design of complex and standardized experiments [188,196]. Carefully optimised 2-DE technology [1,2] can yield highly reproducible results even in the case of troublesome samples like plant tissues [3].

The sensitivity and the linear range of the staining method determine the range of protein concentration for which estimation of protein abundance and of protein fold regulation under given experimental conditions can be accurately performed. There is an apparent asymmetry in the probability of detection and in the precision of fold-regulation measurement associated to up-regulation and down-regulation events for low abundant vs. high abundant proteins. Assuming equal probabilities of up- and down- regulation for proteins exhibiting different levels of expression, the up-regulation of protein expression is more likely to be identified as compared to down-regulation, particularly for low-abundant proteins. Low-abundant proteins are characterised by relatively higher variances of integrated spot volumes on 2-DE gels [3] (Fig. 4.1-1). Following up-regulation, detection and quantitation become more consistent and increase the likelihood of the statistical identification of the change in expression, while the opposite can be expected following protein down-regulation. The probability of detection on the 2-DE gel will increase following up-regulation even for proteins with levels of expression below the limit of detection. In such cases up-regulation of proteins constitutively expressed at low levels might be mistakenly interpreted as *de novo* synthesis; thus assertion of as *de novo* synthesis vs. up-regulation of low-abundant proteins based on their expression levels on 2-DE gels should be done with care. Previous investigations on microarray data have also suggested that weakly expressed genes are frequently associated with higher false-positive rate in differential expression analysis [197].

On the same basis, the accuracy of the estimations of fold regulation largely depends on the constitutive and post-treatment levels of each protein's expression relative to the lower detection limit and the saturation limit of the staining method. For low expressed proteins, the fold of regulation is often estimated with higher accuracy if the protein is up-regulated while the opposite is true for highly expressed proteins, close to the saturation limit of the staining method.

The probability to detect changes in protein expression levels also depends on the protein turn-over relative to the duration of treatment. Down-regulation of proteins is more likely detected for proteins with high turn-over rates, while lower turn-over rates facilitate the detection of protein up-regulation.

Another important factor is the statistical approach undertaken for the identification of

differential protein expression. Differences in spot volumes measured on 2-DE gels are typically identified using t-tests. Performing large numbers of such tests can, however, result in the false positive identification of some proteins' regulation i.e. statistically significant differences can be obtained by chance alone. The results of such investigations have therefore to be adjusted using a multiple testing correction [198]. A wide array of statistical methods for multiple testing correction have been developed covering an as wide range of stringencies, and the choice of the method is somewhat arbitrary. Moreover, multiple testing corrections are solely based on the p-values associated to protein pairs based on the statistical tests, but do not take into account the actual features of the data set and the way these features influence the test results.

A more recently statistical approach developed primarily for transcriptome analysis using microarrays, is Significance Analysis of Microarrays (SAM) which aims to control the false discovery rate (FDR) [199]. This approach has also been successfully applied in proteomic studies [200]. However, other authors suggest that although appropriate to high-throughput experiments like transcriptome analysis based on microarrays (with output in the range of thousands of transcripts), SAM might not be an appropriate statistical approach for proteomic data derived from 2DE electrophoresis [201], typically in the range of several hundreds of spots. Although the method considers each spot in the context of the whole data set, as in the case of multiple testing corrections, it does not take into account that proteins have specific levels of natural variation in the study population.

When several biological replicates are available, a good alternative approach for a more reliable identification of differential protein regulation is to directly assess the level of natural variability of each protein in the population and, based on this information to set spot-specific criteria imposed in addition to the statistical testing. Spot features identified to have a significant influence on the integrated spot volume variance (Fig. 4.1-1) [3,6] can be thus used to estimate spot-specific thresholds of variation above which up- or down-regulation can be assumed to be biologically meaningful [6].

Last but not least, the importance of experimental design is widely recognised in the field of 2-DE based proteomics [198,202,203]. Representativeness is ensured by the random sampling of biological replicates and by samples sizes that cover the natural variation in the study population [203]. Inter-individual phenotypic variation imposes the use of very large sample sizes, which are often impractical in 2-DE. The need of large sample sizes is even higher in the case of tree species, known to maintain unusually high levels of variation within populations [14,16]. When the scientific interest lies in processes relevant at the population scale rather than at individual level, and 2-DE patterns need not be correlated with other individual-specific physiologic, genetic or etiologic factors, a possible solution is the use of pooled samples [3,5]. In some cases the pooling can also be imposed by the low amount of sample available per individual [203]. The loss of information that accompanies sample pooling is compensated by a gain in relevance [5,204], provided that enough randomly chosen individuals are pooled and results are interpreted accordingly. This approach has been successfully used in both transcriptome [205,206] and proteome analysis [152,207-212].

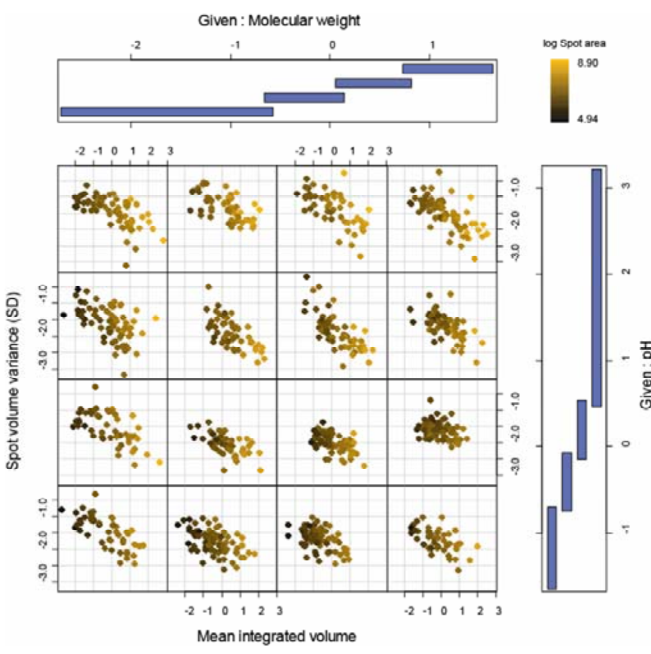


Figure 4.1-1 Spot parameters with significant influence on spot volume variance [3]. All variables have been Box-Cox [213] transformed for normality.

4.2 Abiotic stress: *Picea abies* response to and recovery from heat stress [4,5]

Protein expression patterns under normal growth conditions as well as in response to and during the recovery from heat shock were compared between needles and roots of *Picea abies* seedlings originating from ecotypes adapted to contrasting elevations.

All proteins exhibiting ecotype-specific expression were organ-specific and the level of between-population polymorphism was organ dependent (over-expressed in roots). This observation parallels previous findings from other species [214,215] and probably reflects either a lower selective pressure in comparison with "housekeeping" proteins expressed in all organs [216] or a more complex expression regulation of organ-specific proteins [217]. Quantitative between-population polymorphism prevailed over qualitative polymorphism. This might reflect differences in the proportion of individuals that express the given protein, differences in the actual level of protein expression at individual level (rates of synthesis and degradation, stability) or both. Among the proteins exhibiting ecotype-specific expression we identified proteins involved in photosynthesis, photorespiration, root tracheary element differentiation and trans-mitochondrial membrane transport. Irrespective of the mechanisms underlying their polymorphism (genetic differentiation of the populations or environmental influence upon gene expression through imprinting), these proteins are potentially under selective pressure and might constitute the material for subsequent population divergence.

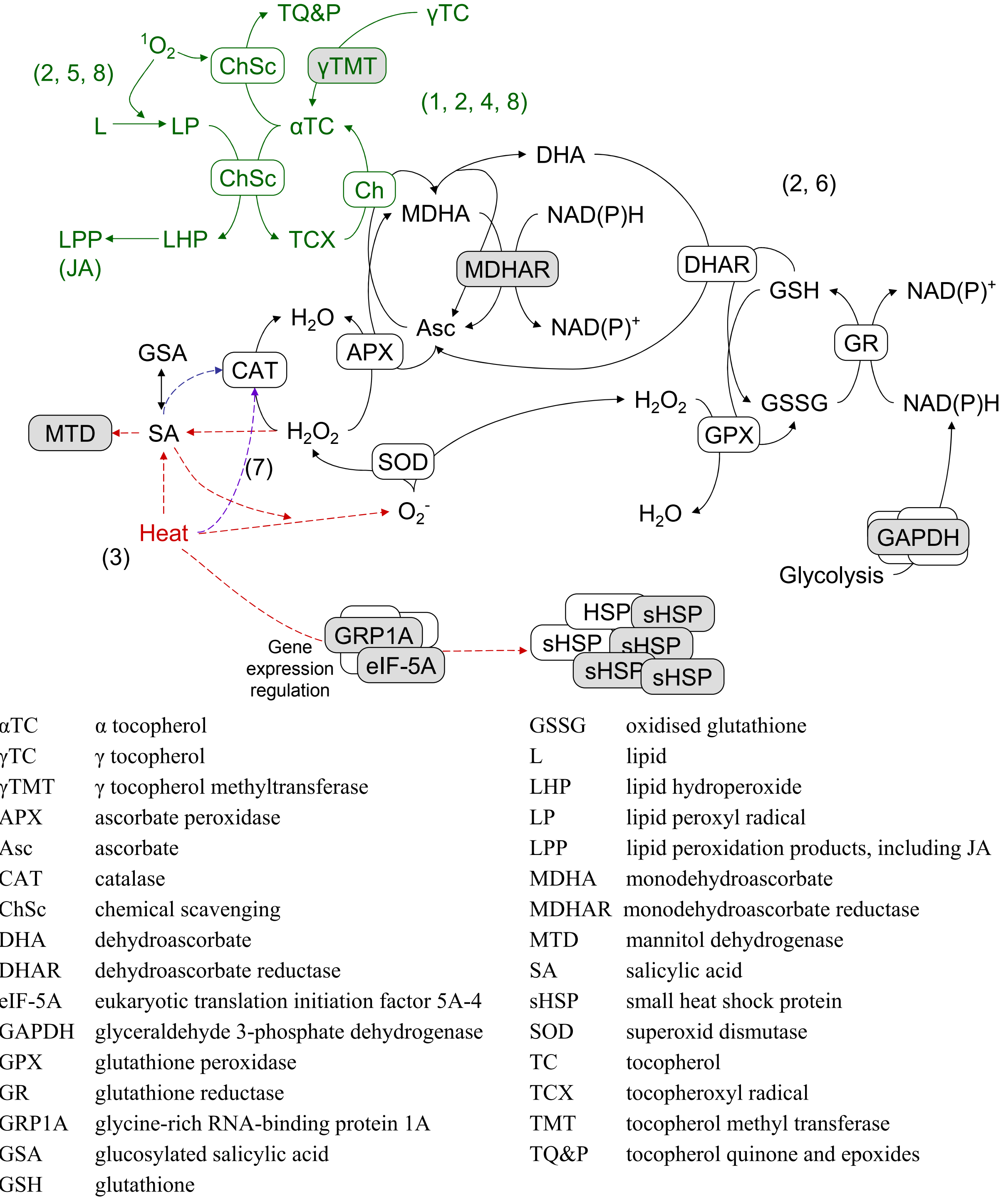
Under the experimental heat stress conditions presented in section 2.2.1, the overall modifications of 2DE patterns were similar for the two ecotypes. Several individual proteins (most of them stress related proteins) exhibited nevertheless tissue or ecotype specific expression and regulation. We found evidence that *Picea abies* seedlings exposed to heat stress develop during the heat treatment and during their recovery from stress a state of heat induced oxidative stress. In the case of the high elevation seedlings this condition appears to be more drastic and extends longer into the recovery phase.

Production of reactive oxygen species (ROS) is a secondary effect of most environmental stresses in plants [37,218-220]. The stress tolerance of plants largely depends on their ability to scavenge ROS [37,218,220], and there are several mechanisms through which ROS can be degraded within the plant cells. The ascorbate-glutathione cycle is one of them, and the enzymes of this pathway are frequently considered indicators for oxidative stress [221]. Other mechanisms involve liposoluble antioxidants like carotenoids or vitamin E. Enzymes part of both mechanisms were found to be regulated by heat stress in our experiments.

Heat is known to induce oxidative stress in plants [37,220,222] and the responses to heat and oxidative stress are closely linked [37,223]. Plants activate antioxidant defence mechanisms in response to heat [222], and recent studies have shown that external factors which reduce these defences (like airborne ethylene or methyl salicylate) also reduce thermotolerance [222,224]. Development of thermotolerance seems to involve an increase in the ability of plants to prevent or recover from oxidative stress [37] and compounds that induce oxidative burst can also induce thermotolerance [225]. Heat tolerant tomato cultivars have enhanced ROS scavenging capacities compared to heat sensitive ones [226]. Moreover, oxidative stress can induce the accumulation of some HSPs [37], while total antioxidant activity and sHSP expression exhibit similar seasonal patterns [227].

Heat induced oxidative stress is not restricted to the heating period but extends to the recovery phase as well. Experiments on *Arabidopsis* seedlings showed that heat stress causes oxidative stress mediated damage during up to 3 days of the recovery phase [37]. The level of lipid peroxidation increased more that 3 fold after 2 days and more that 6 fold after 3 days. A heat pretreatment prevented lipid peroxidation during the recovery phase, but the seedlings survival rate was only improved by 20%. Our observations suggest that *Picea abies* seedlings experience a state of oxidative stress during the heat treatment and at the beginning of the recovery phase.

The seedlings were thus found to activate under heat stress conditions multiple antioxidant protection mechanisms in addition to the heat protection machinery (HSPs), in a complex system of interdependent and inter-regulated pathways summarised in Fig. 4.2-1. The differences observed in the level of oxidative stress experienced under heat stress conditions by ecotypes adapted to different elevations and in their ability to activate effective heat and oxidative stress protective mechanisms have putative adaptive significance under the pressure of changing environmental conditions.



αTC	α tocopherol	GSSG	oxidised glutathione
γTC	γ tocopherol	L	lipid
γTMT	γ tocopherol methyltransferase	LHP	lipid hydroperoxide
APX	ascorbate peroxidase	LP	lipid peroxyl radical
Asc	ascorbate	LPP	lipid peroxidation products, including JA
CAT	catalase	MDHA	monodehydroascorbate
ChSc	chemical scavenging	MDHAR	monodehydroascorbate reductase
DHA	dehydroascorbate	MTD	mannitol dehydrogenase
DHAR	dehydroascorbate reductase	SA	salicylic acid
eIF-5A	eukaryotic translation initiation factor 5A-4	sHSP	small heat shock protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	SOD	superoxid dismutase
GPX	glutathione peroxidase	TC	tocopherol
GR	glutathione reductase	TCX	tocopheroxyl radical
GRP1A	glycine-rich RNA-binding protein 1A	TMT	tocopherol methyl transferase
GSA	glucosylated salicylic acid	TQ&P	tocopherol quinone and epoxides
GSH	glutathione		

Figure 4.2-1 Overview of plant reaction to heat induced oxidative stress based on (1) [228], (2) [229], (3) [225], (4) [226], (5) [230], (6) [231], (7) – activation [226,232-235]/ inhibition [225,236,237], (8) [238].
Shaded proteins were found to be up-regulated following heat shock in *Picea abies*; Continuous lines: metabolic conversions; Dashed lines: possible regulatory interactions (– – – positive; – – – negative; – – – regulation depends on species and experimental conditions); —— reactions described at the level of the thylakoid membrane.

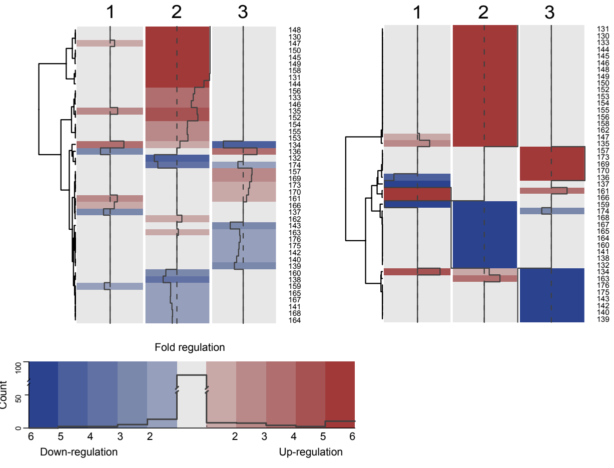
4.3 Biotic stress: *Fagus sylvatica* defence response to pathogen attack and wounding [6]

Protein expression patterns were investigated at local and systemic level in *Fagus sylvatica* seedlings following infection with the root pathogen *Phytophthora citricola* and following leaf or root wounding. The latter treatment was used as elicitor of plant response to herbivores both in the treated plants and, through volatile signals, in neighbouring plants [74].

Fagus sylvatica plants inoculated either in soil or in liquid system with *Phytophthora citricola* exhibited typical infection symptoms and were highly sensitive to the pathogen, paralleling previous observations on young seedlings [56].

The infection with *Phytophthora citricola* caused ample changes in protein expression at both local and systemic levels. Leaf and root wounding treatments also resulted in local and systemic regulation of protein expression and the response patterns exhibited partial overlap. A significant number of regulated proteins were shared between the tree's defence responses against the pathogen and wounding, consisting of both co-regulated and differentially regulated proteins (Annex 6: Fig. 3) [6].

In order to identify protein expression patterns across experimental treatments, cluster analysis was performed based on the fold regulation of proteins induced by each treatment. Hierarchical clustering based on a similarity matrix calculated using Pearson correlation coefficient and the corresponding heat map is presented in Fig. 4.3-1 and Fig. 4.3-2. Clustering following this method takes into account the level of protein up- or down-regulation as well as patterns of protein expression among different treatments. However, since the differences of the fold regulation of different proteins is rather high, this criterion over-weights the patterns of expression, so that results are difficult to interpret and patterns fail to reveal themselves. In order to obtain a classification of proteins based on their expression patterns in different experimental conditions, the information concerning the fold regulation can be discarded by the standardisation of proteins on a comparable scale of expression [100]. The intensities of tiles corresponding to different proteins in the heat map will no longer represent the same levels of protein regulation in this case, but rather regulation levels relative to the maximum expression recorded for each protein in any of the experimental variants (Fig. 4.3-1 and Fig. 4.3-2). The two different clustering methods reveal complementary information and structure the data set according to different criteria. Patterns of protein expression identified by means of clustering are schematically presented in (Annex 6: Fig. 3) [6] and expose obvious overlaps between *Fagus sylvatica* response to pathogen and wounding. The significance of this overlaps can be better understood in the context of signalling and defence pathways involved as illustrated in Fig. 4.3-3.

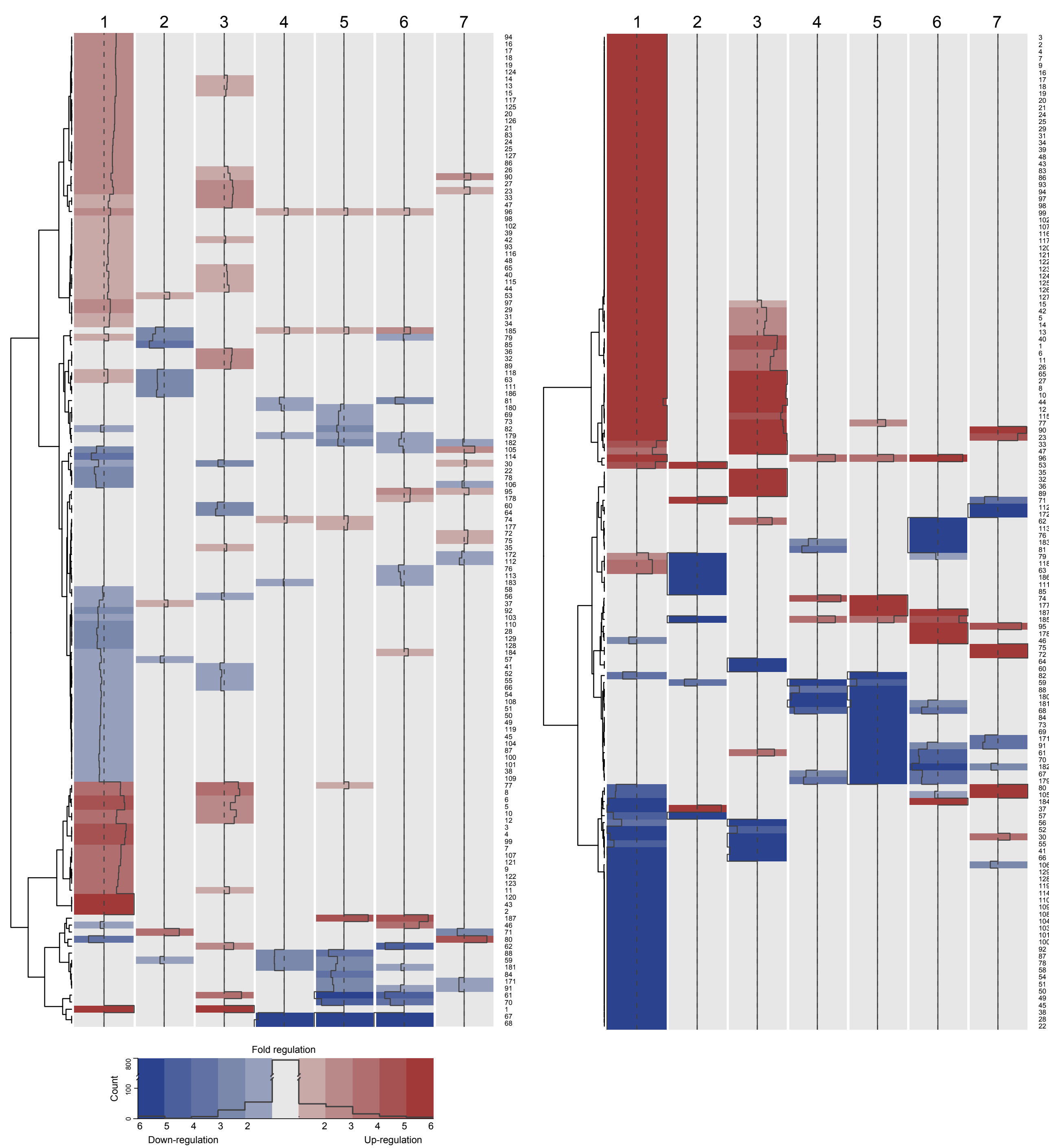


1 - Early local response to infection; 2 - Late local response to infection; 3 - Local response to root wounding

Figure 4.3-1 Cluster analysis of *Fagus sylvatica* root proteins regulated following root infection with *Phytophthora citricola* and/or wounding. Clustering has been done using non-standardised (left panel) or standardised (right panel) fold regulation data.

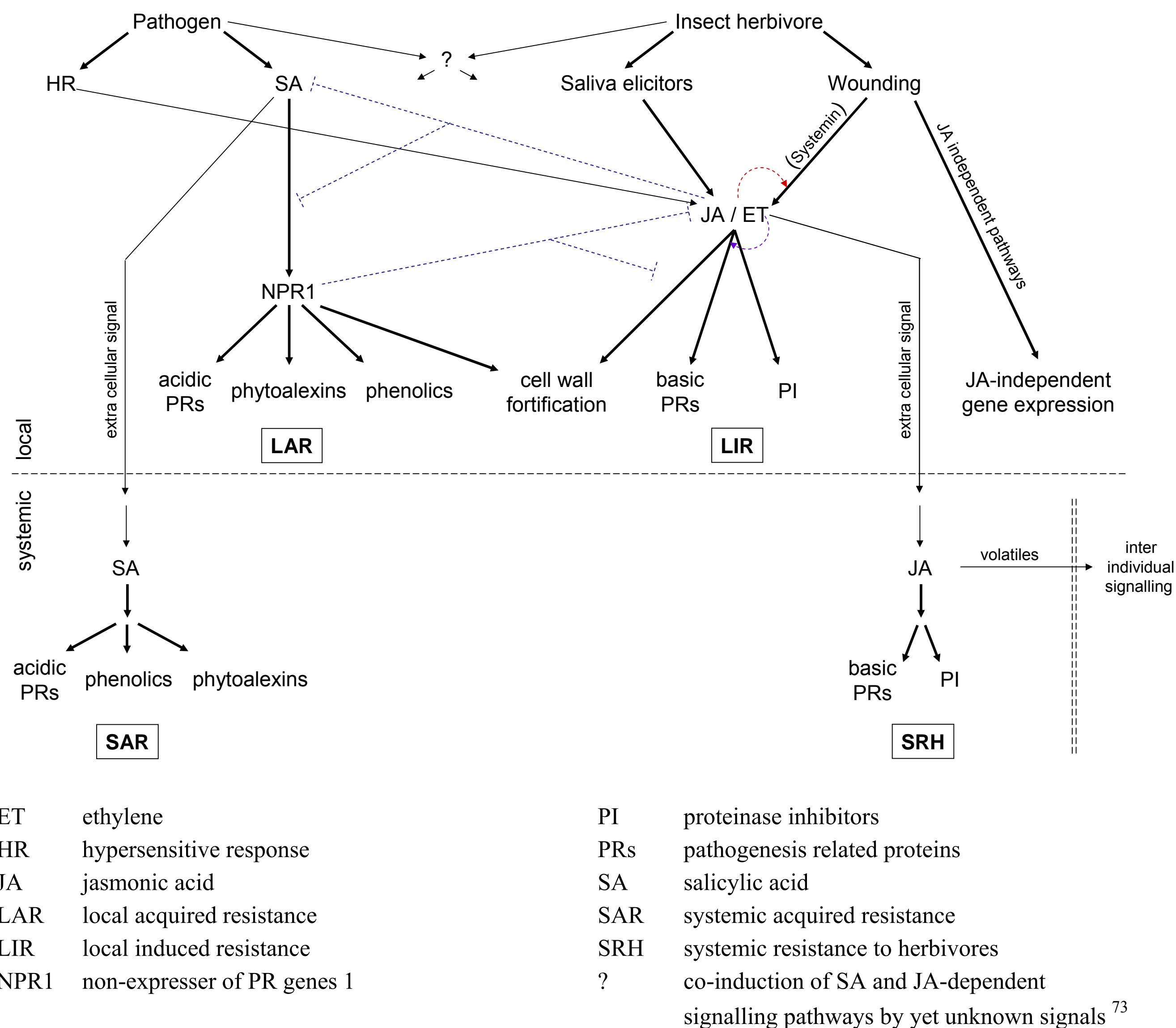
Incompatible interactions of *Phytophthora* species with different hosts have been widely investigated and shown to often involve pathosystem specific recognition [61] and defence mechanisms [64,67]. Except for the SA-dependent defence mechanisms widely recognised as pathogen induced, *Phytophthora* was also shown to elicit SA-independent responses usually

activated by herbivore attack or wounding [68,69,71,93,239,240]. Although less studied as compared to incompatible interactions, compatible interactions involving *Phytophthora* species also revealed overlaps between plants' defences against pathogens and herbivores [241]. Herbivore attack is equally able to elicit plant defence mechanisms against pathogens [90] which supports the hypothesis of a crosstalk between the two defence pathways.



1 - Late systemic response to infection in soil system; 2 - Early systemic response to infection in liquid system; 3 - Late systemic response to infection in liquid system; 4 - Local response to leaf wounding; 5 - Systemic response to leaf wounding; 6 - „Listening trees"; 7 - Systemic response to root wounding

Figure 4.3-2 Cluster analysis of *Fagus sylvatica* leaf proteins regulated following root infection with *Phytophthora citricola* and/or wounding. Clustering has been done using non-standardised (left panel) or standardised (right panel) fold regulation data.



ET	ethylene	PI	proteinase inhibitors
HR	hypersensitive response	PRs	pathogenesis related proteins
JA	jasmonic acid	SA	salicylic acid
LAR	local acquired resistance	SAR	systemic acquired resistance
LIR	local induced resistance	SRH	systemic resistance to herbivores
NPR1	non-expresser of PR genes 1	?	co-induction of SA and JA-dependent signalling pathways by yet unknown signals [73]

Figure 4.3-3 Simplified work model showing the main interactions between plant response to pathogen attack and wounding based on [73,94,242-245]. Dashed lines: possible regulatory interactions (– – – positive; – – – negative; – – – synergistic and antagonistic regulation of different genes). Inter-regulation of pathways also occurs at systemic level.

A close inter-regulation of defence pathways against different biotic stressors does however not necessarily imply that the effect is a synergistic one. Although such effects have been described for some plant-pathogen-herbivore systems [88,90,246,247], neutral[248] and antagonistic [91] effects have also been observed. Asymmetric relationships have been described for some species, where infection with a leaf pathogen decreases plants' susceptibility to an insect herbivore, while herbivore attack previous to inoculation with the fungal pathogen does not influence pathogen's growth neither locally nor systemically [88].

The protein expression patterns observed in *Fagus sylvatica* leaves and roots following infection and wounding confirm the existence of overlaps and possible inter-regulation between pathogen and herbivore defence pathways. The existence of several proteins exhibiting opposite directions of regulation following the two types of biotic stress suggests a likely antagonistic effect between the elicited defence pathways. Identification of regulated proteins will clarify if this is a valid explanation for the shared response regulation.

5 CONCLUSIONS

Plants are generally vulnerable to changes of environmental conditions due to their distinctive sessile phenotype. Tree species are even more exposed to a wide range of stressful conditions over the extensive geographical ranges and during their long life cycles. Coping with multiple simultaneous types of biotic and abiotic stressors requires complex mechanisms of resistance and/or tolerance which in turn require a high availability of resources. Under the strong selective pressure of limited resources, inducible defences have evolved as effective alternative or complementary mechanisms to the constitutive defence machinery. The capacity to tolerate extreme environmental conditions and to effectively defend against biotic stressors like pathogens or insect herbivores is essential for the survival of tree species, particularly under the conditions of the current climatic changes.

Plant stress responses have been the focus of innumerable studies involving a vast array of technologies. Among the techniques developed for the study at molecular level of plant defence mechanisms, proteomics has emerged as a set of very effective tools of investigation.

In the present study, two-dimensional gel electrophoresis differential display experiments were successfully used to identify changes in protein expression patterns following abiotic [4,5] and biotic [6] stress in forest trees. Protein extraction and separation protocols were optimised for all types of tissues analysed [1,2] and ensured the high quality and high reproducibility of the two-dimensional electrophoresis patterns [3].

Protein expression patterns were compared between *Picea abies* ecotypes adapted to different climatic conditions (i.e. different elevations) under normal environment [5] as well as in response to and during the recovery from heat stress [4]. Several proteins exhibited differential expression between the two ecotypes and even ecotype specificity. Such differences in the constitutive expression of proteins among ecotypes can represent the result of divergent selective pressure and might therefore carry adaptive significance.

The investigation of the response to heat stress revealed further qualitative and quantitative differences in the reaction of the two ecotypes to heat stress and heat induced oxidative stress [4]. These differences indicate different capacities of the ecotypes to activate effective heat and oxidative stress protective mechanisms and implicitly different capacities to withstand and recover from elevated temperatures. Such phenotype segregation between ecotypes adapted to different elevation is extremely relevant for the survival of tree populations under ever increased temperature regimes.

Pathogen and herbivore defence pathways have been widely studied in many plant species, including some tree species and were frequently proven to be species or even genotype specific. The crosstalk between the signalling pathways elicited by the two types of stressors was also shown to be largely species specific. Although in-depth information regarding several plant-pathogen and plant-herbivore systems exists, it cannot easily be extrapolated to other systems. The most efficient strategy for the investigation of such complex defence networks involves multifactorial experiments where plant responses to different stressors can be directly compared.

Proteome changes induced in forest tress by fungal pathogens and insect herbivores were investigated at both local and systemic levels [6]. Wounding was used as elicitor of the herbivore response and the protein expression patterns induced by wounding of *Fagus sylvatica* leaves and roots were compared with expression patterns induced following infection with the root pathogen *Phytophthora citricola*. The overlap observed between protein expression patterns induced by the two types of biotic stressors suggest that signalling and defence pathways activated in response to different aggressors are inter-regulated at different levels forming complex networks designed to ensure optimal defence reactions with minimal resource investment. Several proteins exhibited opposite expression regulation in response to pathogen attack and wounding, suggesting the possible existence of trade-offs between these defence mechanisms.

6 SUMMARY

A proteomic approach was undertaken for the study of forest trees responses to biotic and abiotic stress factors. Protein extraction and two-dimensional gel electrophoresis protocols were optimised for all types of samples analysed, and the reproducibility of protein separation was investigated for different levels of biological and technical replication. The high resolution and reproducibility of protein expression patterns based on the optimised protocols guaranteed a high reliability of results. Differential display experiments were employed for the study of changes in protein expression patterns under stress conditions.

In Europe, *Picea abies* and *Fagus sylvatica* are considered representative species for coniferous and broad leafed tree species respectively. These species were therefore selected as experimental models for the study of trees response to abiotic and biotic stressors.

Protein expression was investigated in two *Picea abies* ecotypes adapted to different elevations. Direct comparison of protein expression in the needles and roots of the two ecotypes under normal growth conditions revealed differences in the constitutive expression of proteins with potential adaptive significance. The analysis of protein expression in response to and during the recovery from heat stress revealed differences in the activation of heat and oxidative stress protective mechanisms that can affect the capacity of the two ecotypes to withstand and recover from elevated temperatures.

Mechanisms involved in the defence against pathogens and herbivores were investigated at local and systemic level. *Fagus sylvatica - Phytophthora citricola* pathosystem was selected as model system and wounding was used as elicitor of the response against herbivores. Local and systemic protein expression patterns in response to pathogen attack and root or leaf wounding exhibited overlaps consisting of co-regulated as well as differentially expressed proteins. Shared protein expression patterns indicate inter-regulation of signalling and defence pathways elicited by pathogens and herbivores while the existence of proteins exhibiting opposite regulation after infection and root wounding suggests antagonistic connections between the pathways.

Overlapping and/or mutually antagonistic responses to different stress conditions indicate a complex inter-regulation of the signalling and defence pathways activated by different stressors. These pathways specifically interact to maximise chances for plant survival under different environmental conditions while minimising resource investment.

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Annexes:

Annex 1
Vâlcu CM, Schlink K. Reduction of proteins during sample preparation and two-dimensional electrophoresis of woody plant samples. Proteomics 2006, 6: 1599-1605. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Abstract reproduced with permission.
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Annex 2
Vâlcu CM, Schlink K. Efficient extraction of proteins from woody plant samples for two-dimensional electrophoresis. Proteomics 2006, 6: 4166-4175. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Abstract reproduced with permission.
Original article available at:
http://www3.interscience.wiley.com/cgi-bin/abstract/112658801/ABSTRACT

Annex 3
Vâlcu CM, Vâlcu M. Reproducibility of two-dimensional gel electrophoresis at different replication levels. Journal of Proteome Research, in press. Unpublished work copyright 2007 American Chemical Society. Abstract and figures reproduced with permission.
Original article available at:
http://pubs.acs.org/cgi-bin/abstract.cgi/jprobs/2007/6/i12/abs/pr070396e.html

Annex 4
Vâlcu CM, Lalanne C, Plomion C, Schlink K. Protein expression during the recovery from heat stress of Norway spruce (Picea abies) provenances from contrasting elevations. Submitted manuscript

Annex 5
Vâlcu CM, Lalanne C, Müller-Starck G, Plomion C, Schlink K. Protein polymorphism between two Picea abies populations revealed by two-dimensional electrophoresis and tandem mass spectrometry. Submitted manuscript

Annex 6
Vâlcu CM, et al. Local and systemic changes in European beech (Fagus sylvatica) proteome following infection with Phytophthora citricola. Manuscript in preparation

SHORT COMMUNICATION

Reduction of proteins during sample preparation and two-dimensional gel electrophoresis of woody plant samples

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Protein extraction procedure and the reducing agent content (DTT, dithioerythritol, tributyl phosphine and tris (2-carboxyethyl) phosphine (TCEP)) of the sample and rehydration buffers were optimised for European beech leaves and roots and Norway spruce needles. Optimal extraction was achieved with 100 mM DTT for leaves and needles and a mixture of 2 mM TCEP and 50 mM DTT for roots. Performing IEF in buffers containing hydroxyethyldisulphide significantly enhanced the quality of separation for all proteins except for acidic root proteins, which were optimally focused in the same buffer as extracted.

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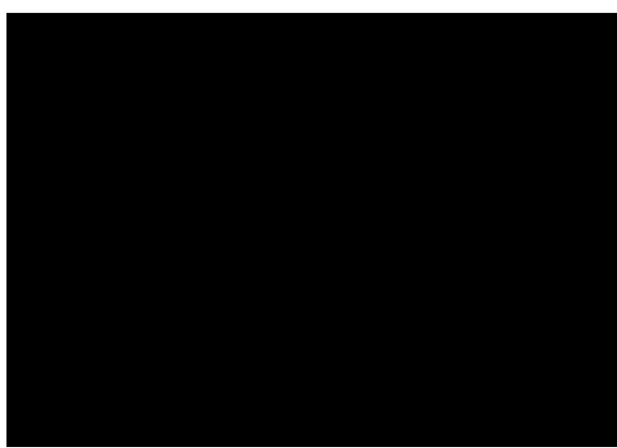
Reducing agent / Sample preparation / Two-dimensional gel electrophoresis / Woody plant



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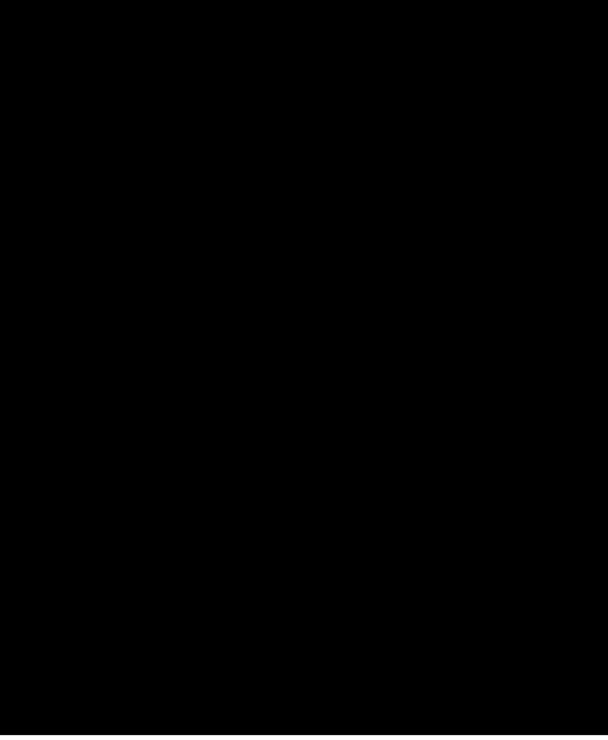
Abbreviations: **AA**, acrylamide; **BPB**, bromophenolblue; **DTE**, dithioerythritol; **HED**, hydroxyethyldisulphide; **IAA**, iodoacetamide; **2-ME**, 2-mercaptoethanol; **TBP**, tributyl phosphine; **TCEP**, tris (2-carboxyethyl) phosphine



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RESEARCH ARTICLE

Efficient extraction of proteins from woody plant samples for two-dimensional electrophoresis

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Protein extraction from plant samples is usually challenging due to the low protein content and high level of contaminants. Therefore, the 2-DE pattern resolution is strongly influenced by the procedure of sample preparation. Efficient solubilization of proteins strictly depends on the chaotrope and detergent in the extraction buffer. Despite the large number of detergents that have been developed for the use in protein extraction and IEF, there is no single compound able to efficiently extract proteins from any source. Hence, optimization has to be performed for each type of sample. We tested several chaotrope/detergent combinations to achieve optimal solubilization and separation of proteins from Norway spruce [*Picea abies* (L.) H. Karst.] needles and European beech (*Fagus sylvatica* L.) leaves and roots. The same chaotrope mixture (7 M urea, 2 M thiourea) was found to be suitable for the extraction and separation of proteins from all samples. Nonetheless, the efficiency of the surfactants tested varied between samples so that optimal extraction and separation was achieved with different detergents or combination of detergents for each sample. The 2-DE separation of spruce needle proteins was optimal in a mixture of two zwitterionic detergents (2% CHAPS and 2% decyl dimethylammonio propanesulfonate). Beech proteins were best separated in buffers containing sugar-based detergents (2% n-octyl β-D-glucopiranoside in the case of leaf samples and 2% dodecyl maltoside for the root samples). IEF was performed in buffers with the same composition as the extraction buffer except for the root proteins that were better focused in a buffer containing 2% CHAPS.

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Abbreviations: **ASB 14**, tetradecanoylamido propyl dimethyl ammonio propanesulfonate; **C8Ø**, 4-octyl benzoyl amidopropyl dimethylammonio propanesulfonate; **C7BzO**, 3-(4-heptyl) phenyl 3-hydroxypropyl dimethylammonio propanesulfonate; **DM**, dodecyl maltoside; **NDSB**, non detergent sulfobetaines; **NDSB 256**, N-phenyl-methyl-N,N-dimethylammonio propanesulfonate; **OG**, n-octyl β-D-glucopiranoside; **SB 3–10**, decyl dimethylammonio propanesulfonate

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Reproducibility of two-dimensional gel electrophoresis at different replication levels

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Running title: Reproducibility of two-dimensional gel electrophoresis

Abstract

Reliability of two-dimensional electrophoresis differential display experiments depends on the reproducibility of the separations. The contribution of biological and technical variation to the overall variance of the two-dimensional patterns was estimated based on the factors found to influence spot volume variance. The second dimension and the staining were responsible for most of the spot volume variance, while using pooled samples lowered biological variation to the level of technical variation.

Keywords: biological variation, proteomics, reproducibility, technical variation, two-dimensional electrophoresis.

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Figure 1.

Spot volume distribution and variation within and between pooled samples.

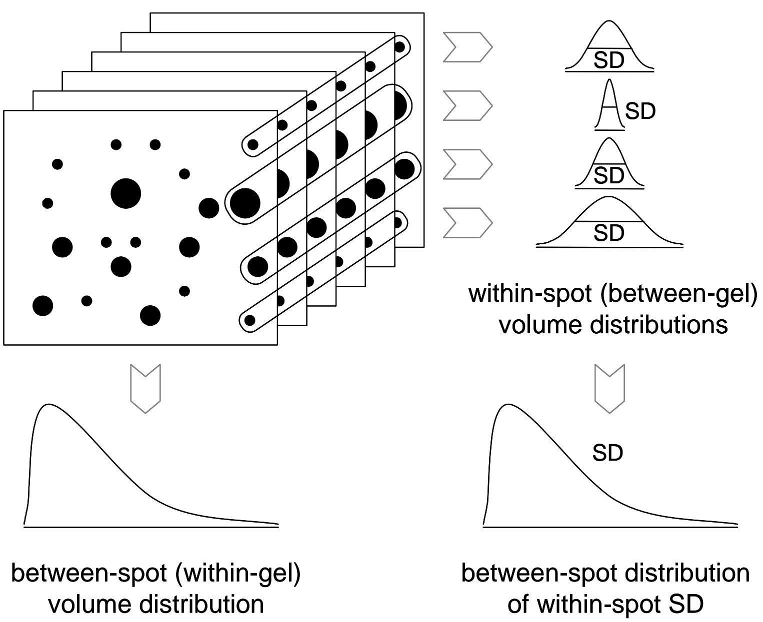

Figure 2.

Effect displays plots26 for the high-order terms of spot volume variance predictors. x and y coordinates were z-score transformed and spot volume SD was Box-Cox transformed for normality. Minimal general linear model: spot volume SD = -1.665 - 15.409*mean normalised volume - 3.013*mean normalised volume2 + 8.874*log area + 2.792* log area2 - 0.781*x + 1.314*x2 + 0.082*y

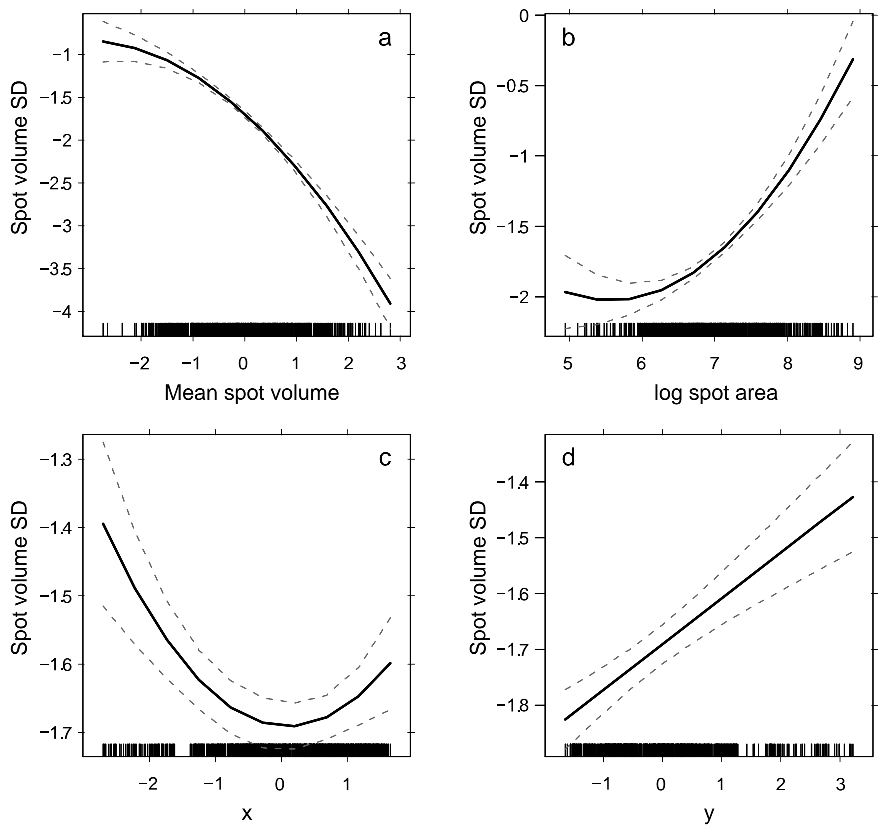

Figure 3.

Variance of spot volume (mean SD ±95% confidence intervals of mean) for different levels of biological and technical replication.

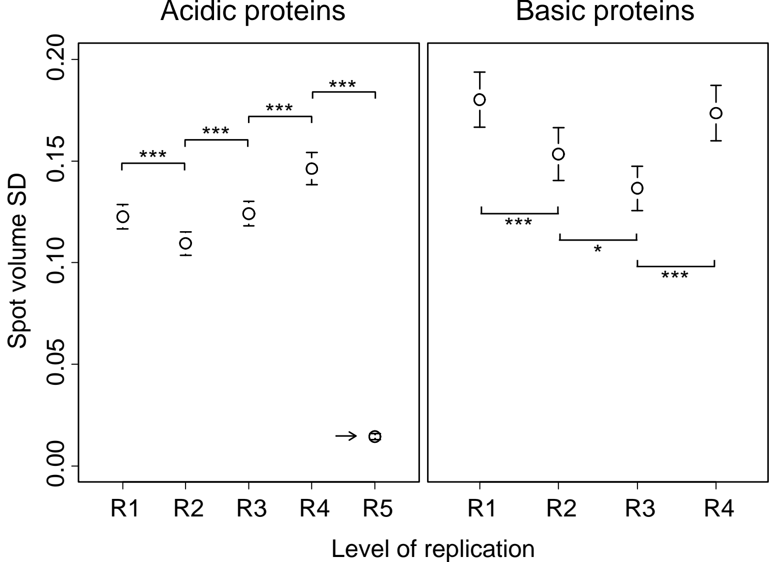

Table 1.

Experimental setup for the comparison of spot volume variance at different replication levels.

Level of replication	Description	Biological sample	Protein extraction	2DE run	2DE gel
Biological replicates					
R1	three different biological samples (pools) subjected to independent extraction and separated within the same 2DE run	≠	≠	≡	≠
Technical replicates					
R2	three independent extractions of the same biological sample (pool) separated within the same 2DE run	≡	≠	≡	≠
R3	three aliquots of the same extract, separated within the same 2DE run	≡	≡	≡	≠
R4	three aliquots of the same extract, separated in different 2DE runs	≡	≡	≠	≠
R5	the same gel scanned and analyses three times	≡	≡	≡	≡

Note: ≡ denotes identical samples/extractions/gels or samples run together, within the same 2DE run; ≠ denotes different samples/extractions/gels or samples run in different 2DE runs.

Heat stress response in Norway spruce (*Picea abies*) provenances from contrasting elevations

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Running title: Heat stress response in spruce provenances

Abstract

Plants adapted to different environmental conditions vary in their capacity to withstand and recover from heat stress. Heat induced damage is not restricted to the high temperature periods experienced by the plants, but extends to the recovery phase. The amplitude of the defence mechanisms activated during this period determine plants capacities to recover from stress. We compared protein expression patterns following exposure to severe heat stress in two *Picea abies* provenances from contrasting elevations, by means of two dimensional electrophoresis. Several proteins exhibiting provenance and tissue specific expression were identified by tandem mass spectrometry. Up-regulation of proteins involved in protection from oxidative stress suggested that both provenances experienced a state of heat induced oxidative stress during the high temperature treatment. In the case of the high elevation provenance the oxidative stress extended well into the recovery period. Qualitative and quantitative differences in the expression of small heat shock proteins during heat stress and the recovery phase also indicate a better adaptation of plants from the lower elevation provenance to high temperatures. Changes of protein expression patterns in response to heat stress suggest that *Picea abies* seedlings adapted to different elevations differ in their capacities to avoid and recover from heat induced oxidative stress.

Keyword index: heat stress, oxidative stress, small heat shock proteins, *Picea abies*, provenance, two dimensional electrophoresis

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1. Introduction

The unprecedented rate of increase in global average temperatures registered over the past century is predicted to further increase in the future, accompanied by an increase in the frequency of extreme climatic events (Jones et al., 2001). Such rapid changes are likely to represent strong selective forces acting upon natural populations (Sorensen et al., 2003; Jump and Penuelas, 2005). As predicted by Bradshaw (Bradshaw, 1972) this selective pressure is stronger in the case of sessile organisms in general and plants in particular as compared to mobile organisms. Plants have to tolerate broader ranges of environmental conditions and usually exhibit higher phenotypic plasticity and differentiation along environmental gradients (Bradshaw, 1972; Huey et al., 2002). Extreme temperatures are considered major selective factors that cause such differentiation along latitudinal and altitudinal gradients (Saxe et al., 2001). Furthermore, they determine species preferences for different microhabitats and implicitly their geographical distribution (Hamerlynck and Knapp, 1996).

Trees have been shown to respond to climatic selective pressure (for example temperature and/or water availability) by micro-geographical adaptive differentiation and between year genetic segregation (e.g. preferential establishment of certain genotypes in warm or cold years) (reviewed in (Jump and Penuelas, 2005)). In coniferous species, genetic differentiation occurs not only across large geographic distances, but also on smaller scales along altitudinal gradients (Bergmann, 1978; Lundkvist, 1979; Mitton et al., 1980; Puglisi et al., 1999) and between stands on slopes with different exposition or water regimes (reviewed in (Bush and Smouse, 1992)). Provenance-specific heat tolerance has been described (i.e. in *Pinus caribea* (Bedi, 1993)) but the response of tree populations under different climatic selective pressure to heat stress has not yet been compared at proteome scale.

Plants respond to heat stress with a complex set of reactions known as the heat shock response (HSR). HSR includes inhibition of the expression of normal proteins and induction of a specific set of proteins (a large part of which is represented by heat shock proteins - HSP) resulting in the induction of thermotolerance. A gradual increase of the temperature or short periods of very high sublethal temperatures followed by a recovery period at normal growth temperature induce in a great variety of species a state of acquired thermotolerance to temperatures otherwise lethal (Koppenaal et al., 1991; Howarth and Ougham, 1993). Although HSR shows general characteristics common to all plant species so far investigated, its amplitude and kinetics are species specific and depend not only on the intensity and duration of the heat treatment but also on the individual history of the plant (Howarth and Ougham, 1993). In nature, trees are likely to experience only short periods of heat stress during the day. However, heat induced damages like the oxidative stress mediated ones develop over periods of days after the heat stress event (Larkindale and Knight, 2002) and plants activate a large array of defence mechanisms during this recovery phase. The immediate reaction of the plant during the heat stress is essential for the plant's survival. The amplitude and kinetics of the plant's reaction after the heat stress period is as important, because it determines the plant's capacity to recover from stress.

In this study we investigated the response to heat stress and during the recovery from heat stress of plants potentially adapted to different environmental conditions. For this we compared protein expression patterns of two Norway spruce (*Picea abies*) provenances located at different altitudes, by means of two dimensional electrophoresis (2DE). For trees, as for many other plant species, seedlings have lower thermotolerance than adult plants (Helgerson, 1990), due to a less effective cooling through transpiration (Howarth, 1989; Howarth and Ougham, 1993), so they are likely to be the most vulnerable stage during the lifetime of a plant. To avoid any previous stress we used two months old *Picea abies* seedlings germinated and grown under identical controlled conditions.

2. Materials and methods

2.1. Biological material

Seed samples from *Picea abies* stands originating from two different German regions of provenance were used. The first one, designated as low elevation (L), is located near Westerhof at 300m a.s.l. (sub-montane region of provenance 84008) and the second one, designated as high elevation (H), is situated close to Füssen at 1300-1600m a.s.l. (sub-alpine region of provenance 84030). The Westerhof stands are well adapted to low elevations and recommended for forest sites up to 400m a.s.l., while the Füssen stands are registered as autochthonous populations. The two locations have average annual temperatures (1961-1990) of 8.5°C and 4.5°C and average annual precipitations of approximately 800mm and 1750mm respectively (Climate data available at www.klimadiagramme.de)(Enders, 1996). The seed lots were supplied by Staatliche Samenklenge Laufen, Germany.

2.2. Heat shock experiments

Vernalised seeds were soaked over night in sterile water, surface sterilised with 30% hydrogen peroxide, extensively washed with sterile water and than sown in sterile Phytatray boxes (Sigma-Aldrich) on Vermiculite soaked with sterile nutritive solution (Ingestad, 1979). Plants grown at different temperatures may exhibit different levels of heat tolerance. In order to distinguish differences in plants' basic reaction to heat stress from differences in their HSR derived from accommodation related mechanisms, we grew all plant material in controlled, identical conditions. Seeds were germinated and seedlings were grown in growth chambers at 16°C, 100% humidity and 16h photoperiod (30 $\mu molm^{-2}s^{-1}$; Osram True light T8, 5500 Kelvin).

Heat shock experiments were performed on 2 months old seedlings. Plants were moved for heat shock in another growth chamber at 42°C, under identical light conditions. A 15 min pulse heat shock at 42°C was followed by 3h recovery at 16°C and then 1h heat shock treatment at 42°C (after (Nover et al., 1983), modified). Control and heat shocked plants were harvested immediately after heat shock and at 5h, 10h and 30h following heat shock. Needles and roots were harvested, frozen in liquid nitrogen within 30 seconds and stored at -76°C until extraction.

2.3. Protein extraction

Three replicate control and heat-shocked pooled samples (each consisting of 30 to 40 plants) were extracted for each time point. Protein extraction was previously optimised (Vâlcu and Schlink, 2006b; Vâlcu and Schlink, 2006a). Briefly, samples were ground in liquid nitrogen and proteins precipitated over night at -20°C in 10% TCA, 1% PVPP, 0.07% 2-mercaptoethanol in acetone. After centrifugation for 30 min at 26000g (4°C) and two washing steps with 0.07% 2-mercaptoethanol in acetone (each 1 h at -20°C), the pellet was dried for 10 min under vacuum, resuspended in extraction buffer (1.5ml for the needle samples and 1ml for the root samples) and sonicated for 30 min in a water-bath sonicator, at 15-20°C. Needle proteins were extracted in buffer containing 5M urea, 2M thiourea, 100mM DTT, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 2% decyl dimethylammonio propanesulphonate and 0.5% Pharmalyte 3-10. Extraction of root proteins was performed in buffer containing 7M urea, 2M thiourea, 2mM Tris(2-carboxyethyl) phosphine, 50mM DTT, 2% dodecyl maltoside and 0.5% Pharmalyte 3-10. The extract was centrifuged for 30 min at 26000g (15°C) and the supernatant was collected and stored at -76°C until isoelectric focusing (IEF). Protein concentration was measured using the RC-DC Protein Assay (Bio-Rad).

2.4. Two-dimensional electrophoresis

Immobilised pH gradient (IPG) strips 24cm long (GE Healthcare) were rehydrated over night and samples were applied by cup-loading near the cathode for the 4-7 IPGs and near the anode for the 6-9 IPGs. The reducing agents were replaced in the rehydration solution with 100mM

hydroxyethyldisulphyde (HED), except for the acidic root proteins which were focused in the same buffer as extracted. 10% isopropanol was added to the rehydration buffer for the basic IPG strips. Focusing was performed in an Ettan IPGPhor Cup Loading Manifold (GE Healthcare) at 20°C and 50mA/IPG gel strip as follows: 300V/0.9KVh, 600V (gradient)/1.5KVh, 1000V (gradient)/2.4KVh, 8000V (gradient)/13.5KVh, 8000V/36.7KVh for acid IPGs and 41.7KVh for basic gradients, respectively. Focused IPG strips were stored at -76°C until the second dimension.

Prior to the second dimension strips were equilibrated two times for 15 min under gentle shaking at room temperature in 10 ml equilibration solution (50mM Tris HCl pH 8.8, 6M urea, 2% SDS, 30% (w/v) glycerol, 0.002% bromophenolblue) containing 2% DTT and 4% iodoacetamide, respectively. Equilibrated strips were sealed on top of the second dimension gel with 0.5% low melting agarose solution. The second dimension was performed over night in an Ettan Dalt6 chamber (GE Healthcare), using Laemmli's buffer system and 1mm thick polyacrylamide gels. For optimal display of proteins with small molecular sizes (the MW range of small heat shock proteins), 12.5% polyacrylamide gels were used. Gels were silver stained according to Heukeshoven and Dernick (Heukeshoven and Dernick, 1988), with the acetic acid in the stop solution substituted for 1% glycine.

2.5. Image and data analysis

Gels were scanned with ImageScanner (GE Healthcare) at 300dpi, under blue light. Spot identification, quantitation and matching were performed with ImageMaster 2D Platinum (GE Healthcare).

All spots with MW smaller than 50kDa were identified and manual editing was performed using a WACOM digital plate for better accuracy. Thirty landmarks were placed on each gel before matching. For each time point a synthetic reference gel was created based on the heat-shock gel with the highest number of spots and all unmatched spots were subsequently added to this reference gel. Mismatches were manually corrected. Relative volumes (i.e. normalised per gel) were used in order to compensate for small between-gels variations caused by differences in protein load and/or staining. Only spots that appeared on at least two out of the three biological replicates were considered for analysis.

In order to identify heat up-regulated proteins, t-tests were performed between the normalised volumes of spots on the heat-shock and the control triplicate gels for each of the time points. Protein spots displaying very significant ($p < 0.01$) differences between the expression on control and heat-shock gels were selected for further analyses. The selected spots were tracked throughout the time scale for both provenances and if possible, for both tissues.

For the comparison of protein expression patterns between provenances and tissues, linear models and stepwise simplification of the models were performed with R2.2.1(R-Development-Core-Team, 2005). Benjamini and Hochberg False Discovery Rate (Benjamini and Hochberg, 1995) was applied as multiple testing correction. In order to avoid type I errors, only very significant differences were considered ($p < 0.01$). To further avoid errors due to a lower technical reproducibility close to the limit of detection, a minimal spot intensity of 0.4 was imposed for the selection of provenance or tissues-specific spots.

Silver staining was used for the detection of proteins on 2DE gels due to its high sensitivity. However, this method has a poor linear dynamic range and does not allow for a very precise measurement of the differences in protein expression. Therefore the differences in protein expression were given (in Tables 1 and 2) only as ranges of fold up-regulation.

Protein spots that displayed very significant differences in their level of expression or expression kinetics between the two provenances as well as some of the spots showing such differences between roots and needles or with atypical behaviour were selected for mass spectrometry analysis.

2.6. Mass spectrometry

For the preparative electrophoresis, 500 to 800 µg of protein were applied by paper bridge loading (Sabounchi-Schutt et al., 2000). Acidic and basic IPGs were focused for a total of 65kVhr and 72kVhr respectively. Gels were stained either with colloidal Coomassie G-250 according to (Anderson et al.,

1991) for 6 days and then destained in water, or with MS-compatible silver staining (with glutaraldehyde and formaldehyde omitted from the sensitising and silver solution, respectively). Spots were cut from gels with a scalpel and destained in 40% ethanol / 50mM ammonium bicarbonate for the Coomassie stained gels and in a 30mM potassium hexacyanoferrate / 100mM sodium thiosulphate 1:1 mixture for the silver stained gels (Gharahdaghi et al., 1999). After dehydration with acetonitril spots were dried for 30 min under vacuum and stored at -20°C until analysis. Proteins were in-gel digested with trypsine, over night at 37°C. Triptic digests were analysed with a nanospray LCQ ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Peptide identification was performed with SEQUEST using all *Picea* ESTs available at dbEST with Xcorr higher than 1.90 for +1 peptides, 2.20 for +2 peptides and 3.75 for +3 peptides, and Delta CN higher than 0.1.

3. Results

The aim of this study was to compare plantlets from a high altitude and a low altitude provenance of *Picea abies* with respect to their protein expression patterns during heat stress and their recovery from heat stress. 2D electrophoresis profiles were compared between stressed plants and control plants harvested at the same moments. The number of protein spots separated per gel under the threshold of 50kDa ranged from 286±11% for root samples and 385±14% for needle samples on the basic gradient, to 707±11% for the root samples and 744±6% for the needle samples on the acidic gradient.
A total of 78 protein spots were found to be reproducibly up-regulated or induced following heat shock (Tables 1 and 2, Supplementary material). 45 of them appeared to be *de novo* synthesised (induced) or their constitutive expression level was below the sensitivity of the method. 49 of the spots were identified in both needles and roots, while 12 spots (5 constitutively expressed and 7 induced post heat shock) were found only in needles and 17 spots (3 constitutively expressed and 14 induced post heat shock) only in roots.
Several protein spots with provenance or tissue specific expression or expression kinetics were identified by tandem mass spectrometry (Table 3 and Supplementary material). All identifications but three (spots 24, 50 and 78) were based on 2 to 5 peptides. Protein identification based on single peptides should generally be considered with caution. However, the high XCorr values indicate good identification (identifications with XCorr values higher than 4.5 are usually considered to be certain). Additionally, the identity of spot no. 50 was indirectly confirmed by the identities of spots no. 54, 56 and 58 with which it shares the identified peptide, apparently all isoforms of the same protein, with closely similar MW, pI and expression kinetics. For spots no. 24 and 78 all protein hits with e-values smaller than 1e-04 in BLAST searches (short, nearly exact matches option) within GenBank using the peptide sequence identified through the *Picea glauca* clones listed in Table 3 (see Supplementary material for peptide sequences) were further investigated. In case of spot no. 24, all hits were small heat shock proteins from *Pseudotsuga menziesii* with a calculated MW of about 18.2 kDa. In case of spot no. 78, all hits were monodehydroascorbate reductases from various plant species. None of the alignments had any gaps. The alignments with the best e-values (4e-04 for spot 24 and 8e-07 for spot 78) had 100% identities while the alignments with the lowest e-values (2e-04 for both spots) had 92% identities (100% positives) and 82% identities (94% positives) respectively. The identification of spots 24 and 78 can therefore be considered reliable although it was based on single peptides.

3.1. Kinetics of heat regulated proteins during the recovery from heat shock

We characterised the kinetics of protein spots up-regulated in response to a 1h heat shock and during the recovery from the stress, based on their expression immediately after heat shock and at three subsequent time points (5h, 10h, and 30h). For each spot time series, a linear model with integrated spot volume as dependent variable and time as predictor was fitted. In order to identify potential non-linear kinetics, the full model included time as a second order polynomial (Grafen and Hails, 2002). For constitutively

induced proteins the average volume of the control gels was also included in the full model. Models, with the lowest AIC (Akaike Information Criterion (Crawley, 2002)), were selected from the candidate models by the automated stepwise simplification procedure. Model parameters of the minimal adequate models were used to describe the kinetics of the heat up-regulated proteins.

Two major types of kinetics were identified (representative examples are presented in Fig. 1). Proteins up-regulated or induced during the high temperature treatment or at the beginning of the recovery phase, whose expression decreased during the recovery phase (hereafter denoted "early heat responsive proteins") had negative significantly different from zero slopes of time or of the second order polynomial of time (Fig. 1a). Some of these spots were identified as γ-tocopherol methyltransferase (γ-TMT) precursors (spots 50, 54, 56 and 58), a eukaryotic translation initiation factor (eIF) (spot 26) and monodehydroascorbate reductase (MDHAR) (spot 78) (Table 3).

A large set of proteins (Fig. 1b), with positive significantly different from zero slopes of time or of the second order polynomial of time, accumulated in time due to a continuous steady or increasing expression respectively (hereafter denoted "late accumulating proteins"). This category of proteins accumulated at much higher levels than the early heat responsive proteins (compare Fig. 1a and 1b). Some of these protein spots were identified as four members of the HSP 20 family, two glycine-rich RNA-binding proteins (GRPs) and a probable mannitol dehydrogenase (MTD) (Table 3).

Proteins with both slopes of time and of second order polynomial of time null or with opposite signs formed a third group of proteins, whose temporal dynamics could not be characterised and consisting mostly of proteins expressed at only one or two time points.

Exceptions from these patterns were 4 spots, which accumulated to a maximum of expression at 5 or 10h of recovery and then declined (Table 1 and 2): spots 40 and 76 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were up-regulated in both tissues, while spots 66 and 75 were specifically induced in roots.

3.2. Comparison of protein expression patterns between provenances

We first compared the global expression of heat-responsive proteins at the end of the heat treatment and during the recovery period for the two provenances. We found no significant differences between the amplitude of the heat induced response (calculated as the maximum difference between the average spot volumes in heat stressed and control samples; t-test for the maximum expression of proteins, $t_{1,262}$ = 0.011, p=0.992). Also, there were no differences in the timing (Wilcoxon rang test with continuity correction, W = 6079, p = 0.945, n = 264) or the duration (Wilcoxon test, W = 8652.5, P = 0.92, n = 264) of the reaction.

Further we compared the temporal dynamics of the heat up-regulated proteins expression during the seedlings' recovery from heat shock. For this, linear models were fitted with the log transformed normalised spot volumes as dependent variable and the interaction between provenance (high elevation vs. low elevation) and time and second order polynomial of time as predictors, controlling for the total intensity of the gel to which each spot belonged (to compensate for small differences in staining). In the case of constitutively expressed proteins the average logarithm of the three biological replicate controls was forced in the model. All spots for which the total spot intensity of the gels was retained in the model and accounted significantly for the variation of the relative spot volumes were eliminated from analysis.

Except for two cases, all differences recorded in the expression level and kinetics of the two tissues were not provenance-specific. Two spots (spots 73 and 78) were regulated in a provenance-specific manner: both were constitutively expressed at a very low level in both provenances but exhibited very significant differences between the provenances in their expression level and kinetics during the recovery phase (Table 2, Fig. 2). Spot 73, a precursor of HSP21 (a HSP 20 family member), accumulated at a higher level during the recovery from heat stress in the needles of the lower elevation seedlings (approximately 35 fold increase, in comparison with less than 2 fold increase in the high elevation plants). Spot 78, MDHAR, was specifically up-regulated during the 1h heat stress period in the needles and roots of the

high elevation seedlings. In roots, the expression level increased during the first 5h of recovery, remained 3 times higher compared to the control plants for up to 10h following heat shock and than declined. In needles, its expression maintained a lower level of up-regulation (approximately 2 fold) throughout the recovery period.

Six other spots were present in only one of the provenances (Table 2). Three of them (spots 72, 74 and 77, Table 2) had a low level of expression so we cannot rule out that the appearance of qualitative differences might be artefactual due to quantitative differences close to the detection limit. The remaining three (spots 71, 75 and 76) had nevertheless undoubtedly provenance-specific expression (Fig. 2). One of the spots (spot 71) was highly induced during the recovery from heat stress of the low elevation seedlings and was identified as a member of the HSP 20 family (Table 3), while it was only faintly visible on overloaded high elevation 2DE gels, under very high contrast. The other two proteins accumulated only in the high elevation seedlings. Out of the latter proteins, spot 76 (GAPDH) reached a maximum expression at 5h post heat shock and than declined, while spot 75 (unidentified) was transiently induced after 10h of recovery from stress only in roots.

3.3. Comparison of protein expression patterns between tissues

Among the proteins identified both in needles and roots, 18 were constitutively expressed in both tissues and 24 were induced in both tissues in the heat stressed plants (Table 1). A number of 7 proteins were constitutively expressed only in one of the tissues (6 in needles and 1 in roots).

To compare protein expression and potentially different temporal dynamics of heat responsive proteins between needles and roots, linear models with log transformed integrated spot volumes (to allow for comparisons between the two types of tissue) as dependent variable and the interaction between type of tissue (roots vs. needles) and time and second order polynomial of time as predictors were fitted. A significant interaction between time (or the second order polynomial of time) and tissue denoted differences in the temporal dynamics of the protein's expression between tissues. For proteins constitutively expressed in both tissues, the average logarithm of the integrated spot volume for the three biological replicate controls was forced in the model.

23 protein spots exhibited very significant differences in the temporal pattern of expression between needles and roots in at least one of the provenances (of which 11 were constitutively expressed and 12 were *de novo* synthesised following heat shock, see Table 1 and 2). Interestingly, the late accumulating proteins (relevant for the recovery phase) exhibited higher expression in needles while the early heat responsive proteins (possibly more relevant for the reaction to heat stress) were stronger expressed in roots (Fisher Exact Test, $p = 0.002$). We also observed a delay in the accumulation of the heat responsive proteins that could be matched between tissues, in the roots of both provenances (paired Wilcoxon test, low elevation provenance: $W = 15$, $p < 0.001$, $n = 37$; high elevation provenance: $W = 47.5$, $p = 0.004$, $n = 35$). Late accumulated proteins accounted for this delay (all delayed proteins were late accumulated and represented 44% of the proteins matched between tissues).

A group of 7 protein spots induced during the heat stress period (pH = 4.7-4.8, MW = 35.3-38 kDa), possibly isoforms of the same protein, specifically appeared in the root tissue. Three of them and a neighbouring protein constitutively expressed but also up-regulated in roots, have been identified as γ-TMT precursors (spots 50, 54, 56 and 58). All of them declined during the recovery phase to reach control levels of expression before the end of our measurements (30h post heat shock). To test whether plants from the two provenances maintained the expression level of this group of proteins at different levels or for different periods, we used a linear model with spot normalised volume as dependent variable and provenance, time and their interaction as predictors, while controlling for the total intensity of the gel to which each spot belonged. The interaction provenance:time was highly significant (ANCOVA, $F_{1,85} = 16.998$, $p < 0.001$). The expression of these proteins rapidly decreased in the roots of low elevation plants, but was maintained until after 10h in roots of the high elevation plants, as indicated by the negative difference between the slopes of the regression lines for the low and high elevation provenances

(difference in slopes ± SE, -0.00153 ± -0.00037). The differences in the expression kinetics of these proteins were not detectable using the single protein targeted approach used previously, but became evident when the comparison was performed at the level of the group of spots, most probably of γ-TMT precursors isoforms.

Five other protein spots exhibiting differential expression between the two tissues were identified as sHSPs belonging to the HSP 20 family (spot 24 and 39), GRP1A (spots 1 and 8) and a probable MTD (spot 67) (Table 3).

One of the sHSPs (spot 24) was induced late during the recovery from heat stress in roots, but was absent from the needles. Spot 39, a precursor of HSP22 (a HSP 20 family protein), had very low expression detectable in some of the needle samples but was below the level of detection in roots.

Of the two spots identified as GRPs, spot 8 was identified in the needles immediately after the heat shock (time 0 of recovery period) and induced in roots only after 5h of recovery. Its expression level continued to rise until 30h post heat shock. Spot 1 was constitutively expressed in needles and only slightly increased in this tissue, but was induced in roots after 30h of recovery to a rather high level of expression.

A similar pattern was found for spot 67, a probable MTD, which was constitutively expressed in needles, up-regulated approximately 2 fold after 30h of recovery, and induced in roots only at the end of our measurements.

4. Discussion

The overall changes in the expression patterns during the recovery from heat stress were similar for the two provenances. More than 70 spots were up-regulated or induced in each of the provenances and there were no significant differences in the amplitude or the timing of their expression. This is probably explained by the severe heat treatment applied (26°C above growing temperature following 15min of preconditioning) that triggered a strong HSR in both provenances. More differences in the expression kinetics of heat regulated proteins might become apparent under milder heat stress conditions. However, under these experimental conditions, we identified several proteins with tissue or provenance specific expression. Most of them were stress related proteins (HSPs or oxidative stress proteins).

Further, we will discuss the general expression patterns registered after heat shock and during the recovery phase, as well as the specific differences observed between protein expression in seedlings from the two provenances and between the needles and roots.

4.1. Kinetics of heat up-regulated proteins during the recovery from heat shock

Two types of temporal dynamics characterised most of the heat up-regulated proteins: an early increase in the level of expression followed by a decline; or a gradual accumulation of the protein to a maximum level at the end of the experiment (30h after the heat stress treatment).

Among the early regulated proteins we identified a translation initiation factor eIF-5A. eIF-5A is a relatively abundant cytoplasmic protein conserved in all eukaryotes. Several isoforms of eIF-5A with tissue specific expression and specific response to environmental conditions have been described (Chou et al., 2004). eIF-5A is not essential for global protein synthesis, but is required for the translation of specific mRNAs (Xu et al., 2004) most probably associated with senescence and cell death following environmental stresses (Wang et al., 2001; Chou et al., 2004) and probably plays a role in mRNA turnover (Zuk and Jacobson, 1998). Transcripts from two eIF-5A genes accumulate in plants following salt and copper stress (Chou et al., 2004), drought, chilling injury and osmotic stress (Wang et al., 2001). In our experiments, eIF-5A appeared to be up-regulated during the heat treatment and had a higher level of expression in needles compared to the roots. Its expression rapidly decreased in both tissues after shifting the plants to their normal growth temperature, suggesting that its role is restricted to the heat stress period.

MDHAR and several protein spots identified as γ-TMT precursors were also early regulated in roots, suggesting that plants experienced heat induced oxidative stress during the heat treatment and at the beginning of the recovery period.

Among the late accumulating heat up-regulated proteins we identified several sHSPs as well as two GRPs and a probable MTD.
The accumulation of sHSPs until the end of our measurements and possibly even later reflects the high stability of these proteins (HSPs have half-lives longer than 30h at both high and normal growing temperatures (Chen et al., 1990; DeRocher et al., 1991; Wood et al., 1998)) but also their continuous synthesis long after the end of the heat shock treatment. In soybean seedlings transcription of HS genes continues for few hours after a short severe heat shock even if the high temperature is not maintained (Kimpel et al., 1990). Moreover, sHSPs are synthesised for longer periods post severe heat shock than following mild heat shock (Howarth, 1991). In *Pisum sativum* for example, sHSPs persist at steady levels until 1 to 3 days of recovery from severe heat shocks (40°C) but decline two fold within 24h of recovery from a milder heat shock (38°C) (DeRocher et al., 1991). Hence the severity of the heat treatment applied here probably explains why sHSP were still being synthesised after 30h recovery in our *Picea abies* seedlings.

4.2. Differences in protein expression patterns between provenances

Although the general pattern of gene expression during the recovery from heat stress of the two spruce provenances did not differ, we identified several individual protein spots exhibiting provenance-specific expression or regulation. Four such proteins that were identified by means of mass spectrometry, point to differences in the intensity of the stress experienced by seedlings from the two provenances. Two spots with specific or stronger up-regulation in the low elevation seedlings, belong to the HSP 20 family. Out of the two proteins specifically regulated in the high elevation seedlings, one is a marker for oxidative stress (MDHAR) and one is a glycolytic enzyme (GAPDH).

sHSP predominate in the HSR of plants (Wang et al., 2004) and are involved in the stabilisation and prevention of protein aggregation under stress conditions. Differences in the quantity, quality and the accumulation rate of HSPs are correlated with differences in the level of acquired thermotolerance (Downs et al., 1998). Thermotolerant and thermosensitive genotypes exhibit qualitative and quantitative differences in HSP expression (Fender and Oconnell, 1990; Ristic et al., 1991; Park et al., 1996; Skylas et al., 2002; Wang and Luthe, 2003; Ahn et al., 2004) and sHSP isoforms specific to heat tolerant cultivars or lines have been described for several species including maize (Ristic et al., 1991), wheat (Weng and Nguyen, 1992; Skylas et al., 2002), bentgrass (Park et al., 1996; Wang and Luthe, 2003) and tomato (Fender and Oconnell, 1990). The existence of sHSPs specifically or stronger regulated during the recovery phase in seedlings from the low elevation provenance might therefore point to a better adaptation of those plants to extreme temperatures. Similar patterns of thermotolerance variation with altitude are known in other species (Smillie et al., 1983).

The two proteins specifically up-regulated during the recovery from heat stress of the high elevation seedlings hint on the other hand towards an activation of oxidative stress protection mechanisms.
MDHAR (spot 78) is a key enzyme of the pathway responsible for the recycling of oxidised ascorbate, a major antioxidant that accumulates in plants in response to oxidative stress (Noctor and Foyer, 1998) including heat stress induced oxidative stress (Munne-Bosch et al., 2004). MDHAR expression and/or activity respond in a species-specific manner to physiological (senescence) (Jimenez et al., 1998) or environmental stresses like mechanical treatment (Ben Rejeb et al., 2004), osmotic stress (Lunde et al., 2006) or UV-B exposure (Kubo et al., 1999). MDHAR transcripts accumulate in *Brassica campestris* in response to oxidative stress generated by hydrogen peroxide, salicylic acid, paraquat and ozone (Yoon et

al., 2004). Mildly increased temperatures do not appear to affect MDHAR activity in *Arabidopsis thaliana* leaves (Kubo et al., 1999). Nevertheless, temperatures as high as 45°C applied for 1h to *Sinapis alba* seedlings result in a decreased MDHAR activity during the first 2h after the heat shock followed by an increase it above control levels (Dat et al., 1998) between 3 and 6h after the heat treatment, a timing that parallels our observations on the roots of high elevation seedlings.

Cytosolic GAPDH (spot 76) is a glycolytic enzyme with cell-type specific expression (Yang et al., 1993). Its early up-regulation during the recovery phase indicates an increase in the glycolysis rate, possibly in order to supply the plant with the ATP and the NADH necessary for the recovery from the stress period. Expression of glycolytic enzymes often increases during stress periods (Velasco et al., 1994; Plaxton, 1996), including heat shock (Russell and Sachs, 1989). Exposure of plants to heat or anaerobic stress resulted in an increased cytoplasmic GAPDH mRNA level in *Atriplex nummularia* (Niu et al., 1994) and *Arabidopsis* (Yang et al., 1993). Three different genes have been identified for cytosolic GAPDH in maize (Russell and Sachs, 1989), one of which is up-regulated more than 5 folds in roots and shoots during anaerobic treatment and heat stress. Except its function as a glycolytic enzyme, there is accumulating evidence that (at least in mammals) GAPDH is also involved in DNA replication and repair, translational control and apoptosis (Sirover, 1997). These secondary roles might better explain GAPDH regulation under stress conditions.

A group of proteins exhibiting root specific induction or up-regulation proved to have deviating behaviour between the two provenances as well. Out of this group of neighbouring proteins we identified four as γ-TMT precursors. γ-TMT catalyses the final step of vitamin E synthesis pathway, producing α-tocopherol from γ-tocopherol. Tocopherols are lipid soluble antioxidants and membrane stabilisers synthesized in plastids, of which α-tocopherol is the most abundant isoform. It is considered the most important antioxidant involved in fatty acid protection against oxidative stress (Fryer, 1992). Its level increases in response to developmentally dependent (Franzen et al., 1991) or environmental stress induced oxidative stress conditions (Bergmuller et al., 2003). Heat stress induces a 2 to 5-fold accumulation of α-tocopherol (Bergmuller et al., 2003; Munne-Bosch et al., 2004). In *Picea abies* needles, α-tocopherol accumulates with age (Franzen et al., 1991), probably an adaptation to an increased oxidative stress in aged tissues. Low elevation stands of *Picea abies* have a higher content of α-tocopherol compared to higher elevation stands, possibly indicating a higher antioxidative capacity (Franzen et al., 1991). Up-regulation of γ-TMT precursors during the heat stress in our experiments confirms that α-tocopherol is one of the antioxidants involved in the protection of *Picea abies* from heat induced oxidative stress. Meanwhile, the fact that the expression of γ-TMT precursors rapidly declined during the recovery from heat stress of low elevation plants but was maintained at a steady or even increasing level in the roots of high elevation plants, points to a comparatively longer state of oxidative stress experienced by the latter.

4.3. Differences in protein expression patterns between tissues

The most obvious dissimilarities in the expression patterns of needles and roots regarded the temporal dynamics and/or level of expression of proteins. More than half of the spots expressed in both tissues exhibited such differences, and there are several possible experimental and physiological factors that could explain them.

Firstly, we registered an apparent delay in the accumulation of several proteins in roots. This might reflect a delay in their regulation or low levels of expression at the beginning of the recovery period below the sensitivity of the method. Most of these proteins also exhibited a higher expression in needles. Plants react to the temperature of their tissues (Kimpel and Key, 1985) and since the roots were immersed in the Vermiculite layer saturated with nutritive solution, it might have taken longer for the roots to reach the threshold temperature that triggers a HSR in comparison with the needles. However, the delay we

observed was in the range of several hours, while the heat shock treatment lasted only one hour, so the experimental setup cannot fully explain the differences in the timing of reaction. Moreover, several other proteins exhibited higher levels of expression in roots immediately after the heat shock period, suggesting that there was small if any difference in the temperature profile experienced at root and needles level. Similar tissue-specificity of protein expression following stress was observed in pea (Chen et al., 1990), sunflower (Almoguera et al., 1993) and barley (Marmiroli et al., 1989), pointing to a tissue-specific control of the heat-shock response in the two organs (Chen et al., 1990).

Several proteins were constitutively expressed in only one of the tissues but highly expressed during the recovery from stress in both tissues. It is possible that these proteins are constitutively expressed in both organs, but their level falls below the sensitivity limit of 2DE in one of the tissues. However, similar organ-specific constitutive expression of sHSPs subsequently up-regulated in reaction to stress has been described in *Arabidopsis thaliana* (Bartling et al., 1992), *Papaver somniferum* (Facchini and Deluca, 1994), *Castanea sativa* (Lopez-Matas et al., 2004) and *Craterostigma plantagineum* (Alamillo et al., 1995). Moreover, plants can express in their natural environment proteins which are strictly heat inducible in controlled environment experiments (Knight and Ackerly, 2003). Four of the proteins exhibiting such pattern of expression in our experiments were identified as HSP22 (member of the HSP 20 family), two GRP1A and a probable MTD. As previously discussed, it is not unusual that HSPs exhibit tissue-specific regulation. The tissue-specific heat regulation of the other two proteins is however not as easy to explain.

Two isoforms of GRP1A were identified; one (spot 1) was constitutively expressed only in needles and one (spot 8) had no detectable constitutive expression. Interestingly, spot 1 was only regulated following the heat stress in roots (induced after 30h of recovery) while spot 8 was induced in both tissues and accumulated continuously during the 30h of recovery. GRPs are a group of diverse proteins developmentally and stress modulated, involved in RNA metabolism and gene regulation under various stress conditions (Sachetto-Martins et al., 2000; Gendra et al., 2004). Different members of this family exhibit tissue and age-specific expression (Sachetto-Martins et al., 2000) and are specifically regulated by cold stress (Kim and Kang, 2006), wounding (Sturm, 1992) and ABA (Nicolas et al., 1997), but also by the circadian rhythm (Heintzen et al., 1994). Heat, among other stresses, was shown to strongly increase the expression of GRP1 as well as GRP2 in maize (Didierjean et al., 1996) but it is not clear whether they have a role in the protection from heat induced damage or any other function relevant to the recovery from heat stress.

MTD is the first enzyme in the mannitol catabolism and regulates the concentration of mannitol, a phloem-translocated photoassimilate of many plants, with osmoprotectant and antioxidant functions (Pharr et al., 1999). Young actively growing tissues with high demand for carbon usually exhibit the highest MTD activity (Stoop and Pharr, 1992; Zamski et al., 2001). MTD was constitutively expressed in the needles of the spruce seedlings, and up-regulated (induced in roots, respectively) after 30h of recovery from heat stress.

Salt and osmotic stress as well as ABA and MeJa treatments repress MTD expression, resulting in an increase of mannitol concentration (Williamson et al., 1995; Zamski et al., 2001). In contrast, MTD activity and transcript level are increased by salicylic acid (Williamson et al., 1995). Interestingly, a study on the *Mtd* promoter regulation (Zamski et al., 2001) revealed a time-dependent up-regulation in response to SA (detected after 28h) that parallels our observations.

Jennings et al. (Jennings et al., 1998) suggested a possible involvement of MTD in active oxygen-mediated plant defence against pathogens. While this is clearly not its role during the recovery from heat stress, this is not the only reaction shared by heat stress and SA treatment. Both are known to induce thermotolerance and result in similar changes of antioxidants levels (Dat et al., 1998).

In addition to the proteins exhibiting tissue specific kinetics, the group of γ-TMT precursor isoforms already described were specifically induced or up-regulated in the roots. Previous studies comparing antioxidant enzymes activities following heat acclimation in *Sinapis alba* seedlings revealed that different parts of the plants had different levels of protection, with the leaves being better protected than stems and cotyledons (Dat et al., 1998). The fact that the enzyme was only heat regulated in roots, and there were no matching spots on the needles 2DE patterns, might indicate that other isoforms are active in needles, most probably constitutively expressed or that α-tocopherol was already stored in needles at levels high enough for the protection of needles from the level of oxidative stress induced under our experimental conditions. It also points to a tissue-specific regulation of this enzyme and to differences in the oxidative stress experienced by plants at root and needle level. The expression of all spots identified as γ-TMT decreased after low elevation plants were shifted to normal growth temperature, indicating that the state of oxidative stress developed in roots during the high temperature period but decreased during the recovery period. A comparatively longer maintenance of γ-TMT precursors expression in roots from high elevation plants points to an extension of the oxidative stress to at least 10h of the recovery phase in the plants from this provenance.

4.4. Relevance for the environmental adaptation of *Picea abies* provenances

This is to our knowledge the first comparison at proteome level of the reaction to and recovery from heat stress of tree provenances. *Picea abies* seedlings from both provenances exhibited changes in their protein expression patterns in response to heat stress. Several heat responsive proteins followed organ-specific kinetics. A state of oxidative stress was generated during the high temperature treatment in the roots of plants from both provenances, as suggested by the up-regulation of several isoforms of γ-TMT precursors. This state prolonged into the recovery phase in the case of high elevation seedlings. Concomitantly with the activation of antioxidative mechanisms involving α-tocopherol, the roots of the high elevation plants also up-regulated ascorbate mediated protective mechanisms. An isoform of the glycolytic enzyme GAPDH was also induced in the needles and roots of high elevation plants, either a sign of higher energy requirement, or of other secondary stress related roles of this enzyme.

Mechanisms of antioxidant protection are known to be interdependent and inter-regulated. The regulation of α-tocopherol level in response to stress can be influenced by the activation/inactivation of other complementary mechanisms (Munne-Bosch, 2005). Ascorbate for example has a role in α-tocopherol recycling (Munne-Bosch, 2005), and *Quercus ilex* was found to accumulate both α-tocopherol and ascorbate (Munne-Bosch et al., 2004) in response to heat stress.

The wider array and the stronger expression of some sHSPs synthesized in response to heat stress by the low elevation seedlings, seems on the other hand to offer a better protection against oxidative stress and to ensure a faster recovery. This observation correlates with known patterns of thermotolerance variation along altitudinal gradients (Smillie et al., 1983).

Investigation of protein expression patterns in response to and during the recovery from heat stress of *Picea abies* provenances from contrasting elevations revealed adaptations to local environmental conditions (including temperature and precipitation regimes), resulting in differences in their capacity to avoid and protect from heat induced oxidative stress that could affect their capacity to withstand and recover from elevated temperatures.

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Figure 1

Patterns of temporal dynamics of heat up-regulated proteins during the recovery from heat shock. Mean normalised spot volumes were plotted relative to the corresponding controls and to the constitutive level of expression of each protein.

a. early heat responsive proteins; b. late accumulating proteins.

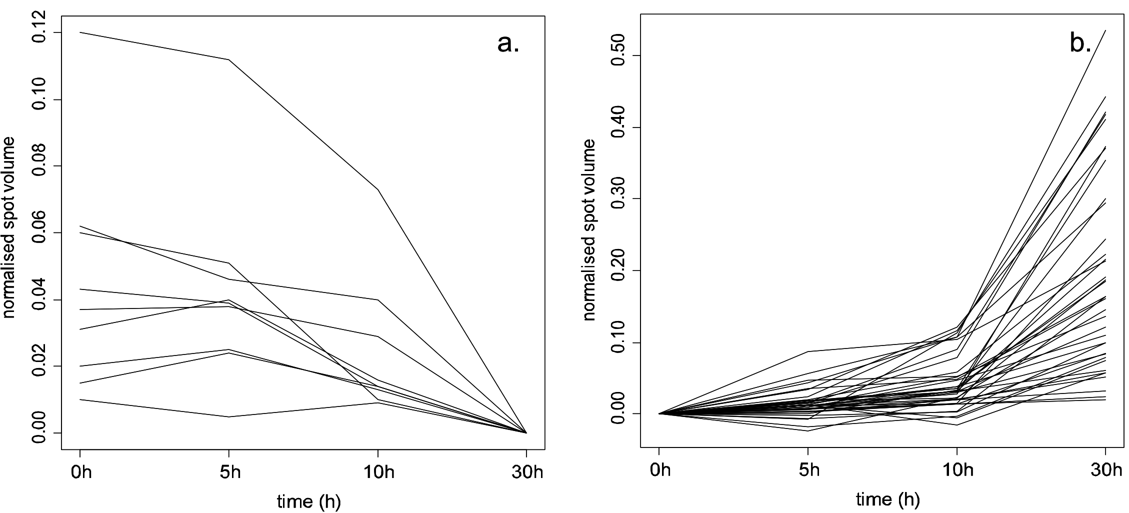

Figure 2

Protein spots exhibiting highly significant differences of expression level or kinetics between low and high elevation *Picea abies* provenances. Expression profiles in needles and roots are compared between provenances (heat shock over control ratios of mean spot volumes) in the left panel and exemplified by gel pictures in the right panel for one of the tissues.

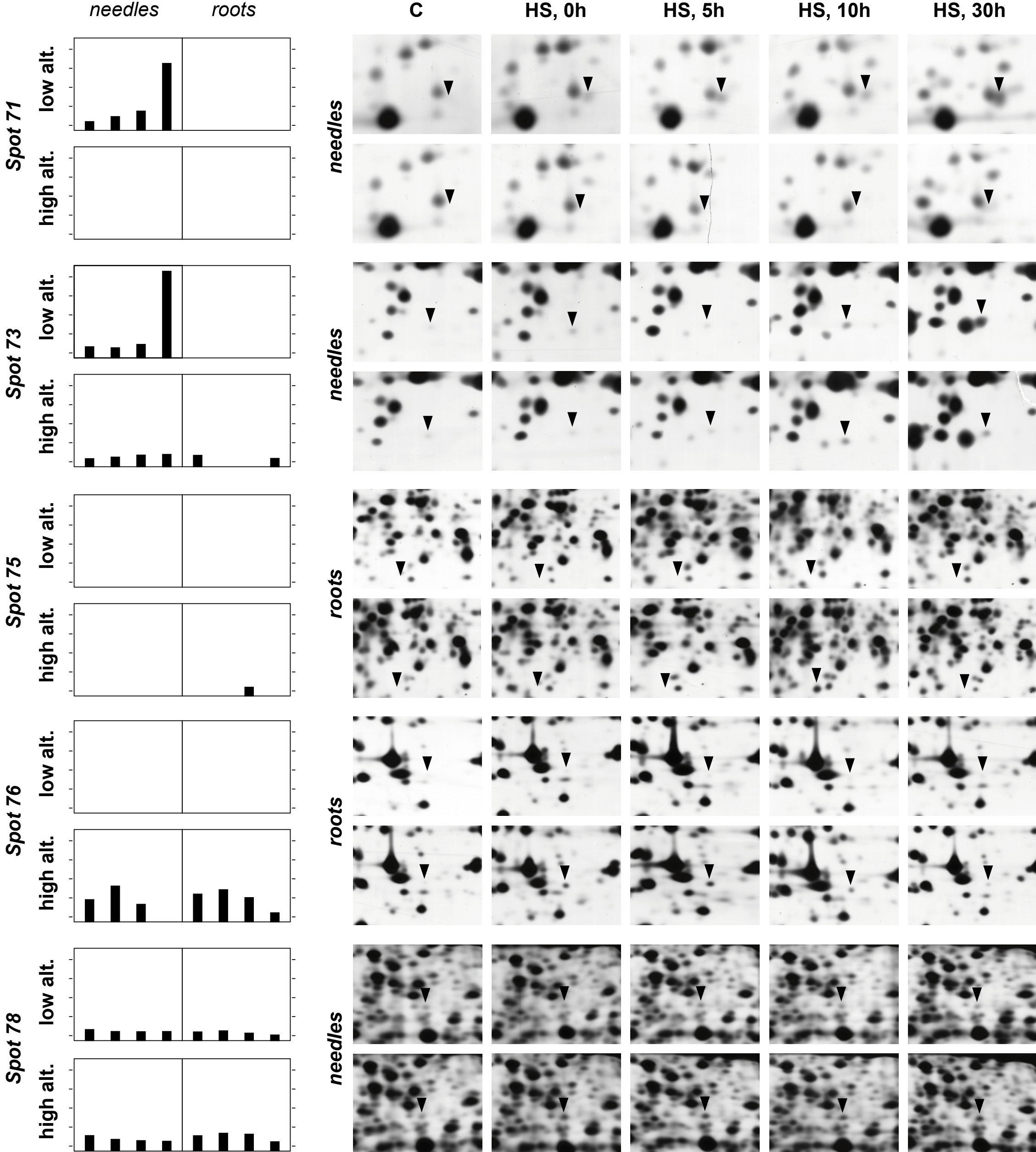

Table 1. Heat up-regulated spots that showed no differences in expression level and kinetics between provenances (exemplified for low altitude provenance).

No.	pH	MW (kDa)	Needles					Roots					Differences between organs
			Constitutive expression	0h	5h	10h	30h	Constitutive expression	0h	5h	10h	30h	
1	**8.7**	**17.3**	**+**	**0**	**0**	**0**	**0**					**i**	**L, H**
2	5.4	18			i	+	++	(+)	0	0	++	+++	H
3	5.7	18		i	+	+	+		i	0	+	++	H
4	6.4	18										i	
5	5.4	18.5					i					i	
6	5.6	18.5			i	+	++					i	
7	5.7	18.5		i	0	+	+				i	++	L, H
8	**8.2**	**18.5**		**i**	**0**	**+**	**++**			**i**	**+**	**++**	
9	5.6	19										i	
10	7.6	19				i						i	
11	7.7	19				i	+					i	
12	5.6	19.5	+	+	+	0	0	+	++	+	0	0	
13	8.7	19.5		i	0	0	+					i	
14	5.3	20						+	0	0	0	+	
15	5.4	20				i	+						
16	5.6	20	+	0	0	0	0	+	+	0	0	0	L
17	6.3	20	+	0	0	0	0	+	0	0	0	++	L
18	6.3	20		i	0	+	++					i	
19	6.5	20	+	0	+	+	+	+	0	+	+	++	
20	7.7	20		i	0	+	+			i	++	++	L
21	5.9	20.5					i					i	L
22	6	20.5	+	+	+	+	++	(+)	+	++	+++	+++	H
23	6.4	20.5	+	0	+	+	+	(+)	0	0	0	+++	
24	**6.4**	**21**										**i**	
25	6.5	21		i	0	0	+			i	+	++	L
26	**5.3**	**21.5**	**(+)**	**+**	**+**	**0**	**0**	**(+)**	**++**	**+**	**0**	**0**	
27	6.3	21.5	+	++	++	++	++	(+)	0	+	++	+++	
28	6.4	21.5			i	0	+			i	++	+++	H
29	6.5	21.5	+	0	0	0	+			i	++	+++	H
30	7.9	22.3	(+)	+	+	0	0						
31	4.9	22.5		i	+	+	++					i	H
32	5.1	22.5					i						
33	5.3	23					i					i	
34	6.5	24					i						
35	5.2	25			i	+	++					i	L, H
36	5.4	25				i	++					i	L, H
37	5.4	25		i	+	++	+++				i	++	H
38	5.5	25			i	+	++				i	+++	H
39	**5.7**	**25**	**(+)**	**0**	**0**	**0**	**+**					**i**	**L**
40	5.8	25	+	0	+	0	0	+	0	+	+	0	
41	6.1	25	+	0	+	+	++					i	H
42	5.4	25.5		i	0	++	+++				i	+++	
43	6.5	25.5					i						
44	5.6	27										i	
45	5.2	28			i	+	++				i	+++	
46	5.2	28						+	0	0	0	+	
47	5.3	28.5	+	0	0	0	+	+	0	0	0	0	L
48	6.6	29.5	+	0	0	0	+						
49	6.7	34	(+)	+	+	+	+				i	0	
50	**4.7**	**35.5**							**i, ++**	**0**	**0**		
51	4.7	36.5							i				
52	4.8	36.5							i				
53	4.8	36.5							i				
54	**4.7**	**37**							**i, +**	**0**	**0**		
55	5.4	37	+	+	+	0	0	+	+	+	+	0	
56	**4.7**	**37.5**						**(+)**	**++**	**++**	**+**	**0**	
57	4.8	37.5							i				
58	**4.8**	**38**							**i, +**	**0**	**0**		
59	6	39	+	+	+	+	0	+	+	+	0	0	
60	5.2	39.5				i	++				i	+	
61	5.7	40	+	0	0	0	+	+	0	0	0	0	
62	7.1	40	(+)	+	+	+	0						
63	6.8	40.5	+	0	0	0	0	+	0	0	0	0	
64	5.4	41										i	
65	6.2	43				i	0						
66	6.8	43.5								i			
67	**6**	**44**	**+**	**0**	**0**	**0**	**+**					**i**	**L, H**
68	6.4	44	+	+	0	0	0	+	+	+	+	0	
69	8.1	49	(+)	+	++	+	0						
70	6.9	50	+	+	+	+	0						

pH and MW presented are average values estimated from 2D gels. The constitutive expression of the proteins in needles and roots is marked with "+"; "(+)" denotes very low constitutive expression, in only part of the samples.

For constitutively expressed proteins the increase of the expression level is indicated relative to the control samples harvested at the same moment, as follows: "0": less than 2 times; "+": 2 to 5 times; "++": 5 to 20 times; "+++": more that 20 times. For proteins induced following heat stress, "i" indicates first moment of induction, "0", "+", "++" and "+++" indicate the increase in expression as for the constitutively expressed proteins, but relative to the lowest still detectable level of expression of the given protein throughout the time series. The last column indicates proteins that showed very significant differences in the expression level or kinetics between the needles and roots; "L" and "H" specify the provenances for which these differences were found, denoting the low elevation and high elevation provenance respectively.

Bolded lines indicate proteins that have been identified by tandem mass spectrometry.

Table 2. Heat up-regulated spots that showed differences in expression level and kinetics between provenances.

Provenance			Low elevation										High elevation										Provenance specificity of expression
			Needles					Roots					Needles					Roots					
No.	pH	MW (kDa)	Constitutive expression	0h	5h	10h	30h	Constitutive expression	0h	5h	10h	30h	Constitutive expression	0h	5h	10h	30h	Constitutive expression	0h	5h	10h	30h	
71	**6.5**	**20**		**i**	**+**	**+**	**++**					**i**											**L†**
74	6.4	34		i	0	0																	L‡
73	**5.6**	**25**	**+**	**0**	**0**	**0**	**+++**					**i**	**+**	**0**	**0**	**+**	**+**	**(+)**	**0**	**0**	**0**	**0**	**-**
72	7.6	20															i					i	H†‡
75	6.4	38																			i		H
76	**6.9**	**41.5**						**(+)**					**(+)**	**+**	**++**	**+**		**(+)**	**++**	**++**	**++**	**0**	**H**
77	8.2	41.5															i						H‡
78	**6.1**	**46**	**+**	**0**	**0**	**0**	**0**	**+**	**0**	**0**	**0**	**0**	**+**	**+**	**+***	**+**	**+**	**+**	**+**	**+**	**+**	**0**	**-**

Symbols legend as for table 1. † visible in some of the samples from the other provenance at high contrast; ‡ eliminated from further analysis due to low level of expression.; * just below 2 fold regulation.

Table 3. Proteins identified by tandem mass spectrometry.

Spot	Kinetics	Origin of identified spot	pH*	MW* (kDa)	MW† (kDa)	NCBI Accession No.	Species	Best homologue	Species	Localisation	No. of peptides	Xcorr‡	Coverage (%)
Proteins with different expression between tissues													
1	b	Needles	8.7	17.3	16	70337918	*Picea sitchensis*	Glycine-rich RNA-binding protein GRP1A	*Sinapis alba*	nucleus	5	50.23	31
8	b	Roots	8.2	18.5	16	89954687	*Picea abies*	Glycine-rich RNA-binding protein GRP1A	*Sinapis alba*	nucleus	2	20.24	25
24	b	Roots	6.4	21	18.2	49142899	*Picea glauca*	Class I sHSP (HSP 20 family)	*Pseudotsuga menziesii*	cytosolic	1	10.14	7
26	a	Roots	5.3	21.5	17.3	70311923	*Picea sitchensis*	Eukaryotic translation initiation factor 5A-4	*Solanum tuberosum*		4	40.19	28
39	b	Needles	5.7	25	26.8	70232652	*Picea sitchensis*	Small heat shock protein, precursor (HSP 20 family)	*Petunia hybrida*	plastid	3	30.21	18
50	a	Roots	4.7	35.5	38.1	83386189	*Picea glauca*	γ-Tocopherol methyltransferase, precursor	*Arabisopsis thaliana*	plastid	1	10.14	6
54	a	Roots	4.7	37	38.1	83389119	*Picea glauca*	γ-Tocopherol methyltransferase, precursor	*Arabisopsis thaliana*	plastid	2	20.16	11
56	a	Roots	4.7	37.5	38.1	69435675	*Picea sitchensis*	γ-Tocopherol methyltransferase, precursor	*Arabisopsis thaliana*	plastid	4	40.21	18
58	a	Roots	4.8	38	38.1	69435675	*Picea sitchensis*	γ-Tocopherol methyltransferase, precursor	*Arabisopsis thaliana*	plastid	2	20.2	13
67	b	Needles	6	44	39	69454601	*Picea sitchensis*	Probable mannitol dehydrogenase	*Medicago sativa*		2	20.18	10
					39.1	70630207	*Picea glauca*	Probable mannitol dehydrogenase	*Fragaria ananassa*		2	20.19	10
Proteins specifically regulated in the low elevation provenance													
71	b	Needles	6.5	20	18	49016297	*P. engelmannii x P. glauca*	Class I sHSP (HSP 20 family)	*Daucus carota*	cytosolic	2	20.23	17
73	b	Needles	5.6	25	25.3	70621592	*Picea sitchensis*	Small heat shock protein, precursor (HSP 20 family)	*Arabisopsis thaliana*	plastid	2	20.19	16
Proteins specifically regulated in the high elevation provenance													
76	c	Needles	6.9	41.5	36.5	90029148	*Picea abies*	Glyceraldehyde-3-phosphate dehydrogenase	*Pinus sylvestris*	cytosolic	3	30.23	28
76	c	Roots	6.9	41.5	36.5	83402450	*Picea glauca*	Glyceraldehyde-3-phosphate dehydrogenase	*Pinus sylvestris*	cytosolic	4	40.21	23
78	a	Roots	6.1	46	47	40764318	*Picea glauca*	Monodehydroascorbate reductase	*Arabisopsis thaliana*	cytosolic	1	10.23	11

The types of kinetics correspond to those described in Fig. 1: a – up-regulation during the high temperature treatment or at the beginning of the recovery phase, followed by a decline; b – accumulation during the recovery from heat stress; c – increase of expression during the recovery followed by a decline (see results).

* experimental values (averages of three 2DE replicates gels); † theoretical values; ‡ identifications with Xcorr values higher than 4.5 are considered to be certain.

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Supplementary material

Heat stress response in Norway spruce (*Picea abies*) provenances from contrasting elevations

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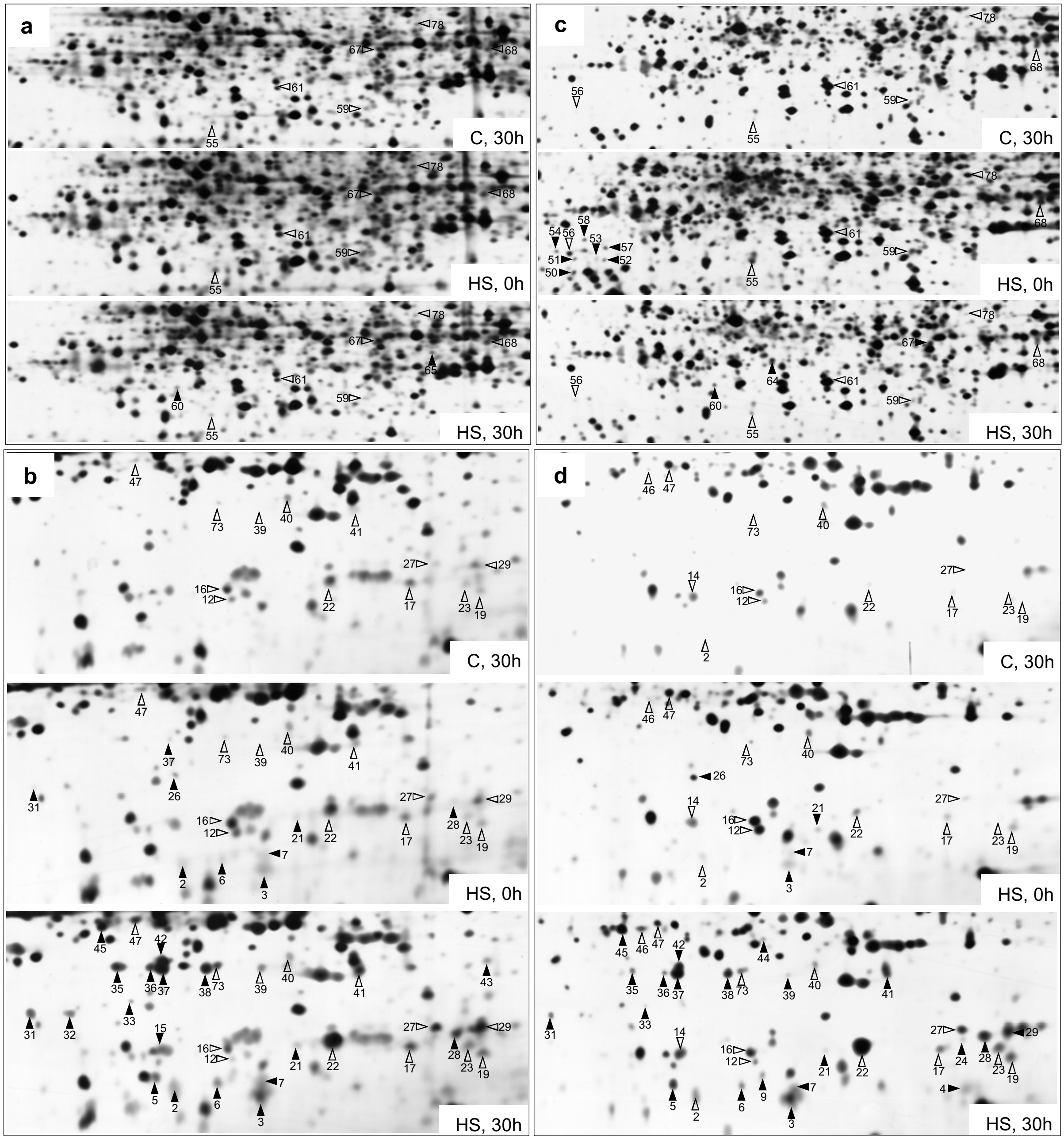
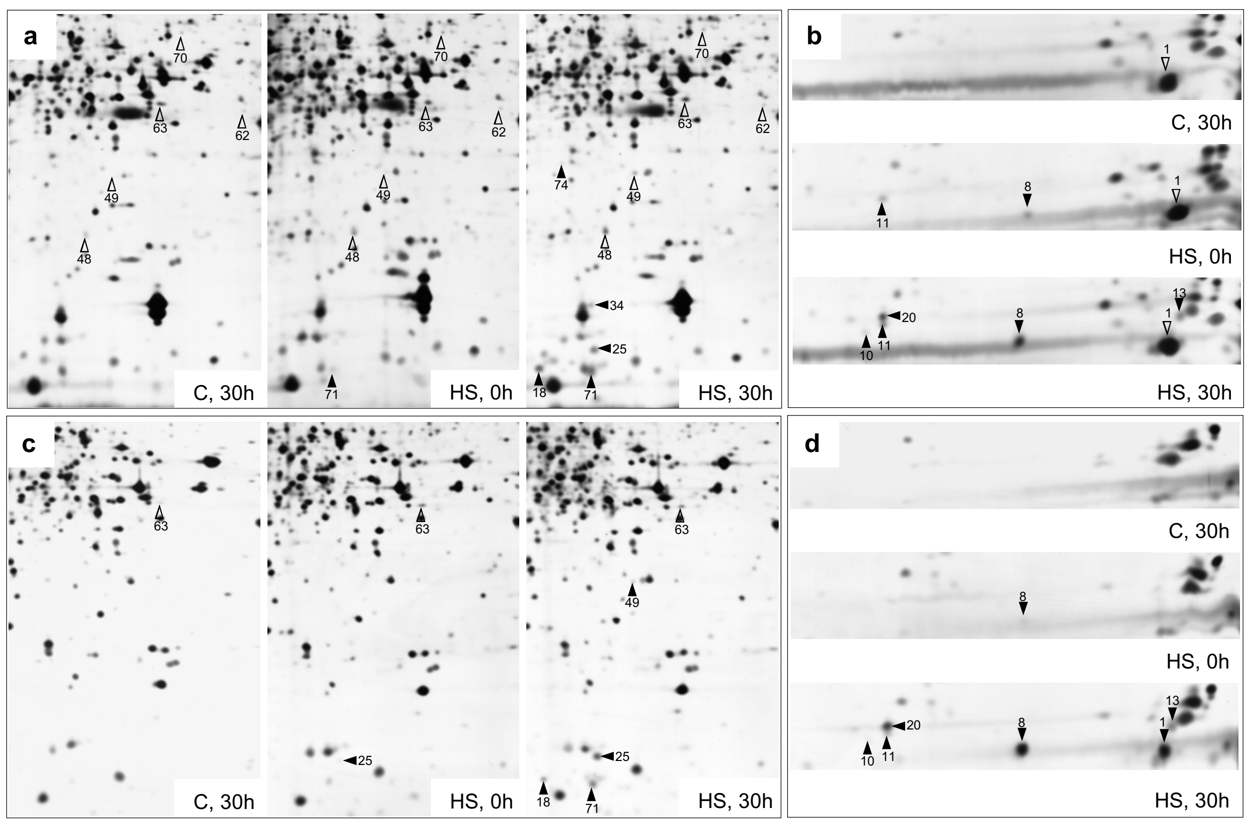
Heat up-regulated (empty arrow heads) and induced (solid arrow heads) proteins accumulated in needles (a, b) and roots (c, d) at 30h after heat shock, in the pH range 4.9-6.5 and MW ranges of 15-30kDa (b, d) and 35-47kDa (a, c) respectively, exemplified for the high elevation provenance. Expression level of marked proteins did not change in the control samples throughout the recovery period, therefore only one control gel is presented.



Heat up-regulated (empty arrow heads) and induced (solid arrow heads) proteins accumulated in needles (a, b) and roots (c, d) at 30h after heat shock, in the pH range 6.3-8.7 and MW ranges of 17-20kDa (b, d) and 20-50kDa (a, c) respectively, exemplified for the low elevation provenance. Expression level of marked spots did not change in the control samples throughout the recovery period, therefore only one control gel is presented.



List of peptides identified by means of tandem mass spectrometry.

Spot	NCBI Accession No.	MH+	Peptide sequence	Xcorr	DeltaCn
1	70337918	2106.86499	-.YGGGGGGGYGGGGYGGGGGSEGAWR.R	3.81	0.51
		1623.71077	R.GFGFVTFSDEQSM*R.D	3.93	0.39
		1467.66856	R.C#FVGGLAWATDDR.S	4.65	0.44
		1607.71582	R.GFGFVTFSDEQSMR.D	4.18	0.40
		1767.88000	R.SLQDAFSPFGEILESK.I	4.61	0.38
8	89954687	1623.71077	R.GFGFVTFSDEQSM*R.D	3.24	0.30
		1767.88000	R.SLQDAFSPFGEILESK.I	4.75	0.47
24	49142899	1446.72229	K.EASAIANTQIDWK.E	2.86	0.17
26	70311923	1088.58472	-.TFPQQAGAIR.K	2.38	0.35
		1369.73218	R.LPNDDQLVSQIK.D	3.47	0.40
		2207.11253	K.KLEDIVPSSHNC#DVPEVIR.T	3.78	0.40
		1437.71953	K.C#HFVAIDIFTSK.K	3.89	0.51
39	70232652	1157.67761	K.VSVEDGVLVIK.G	3.83	0.33
		1515.71479	R.LFDDAFM*LPTSSR.G	4.05	0.38
		1600.78528	R.TPWDIIENENELK.M	4.17	0.40
50	83386189	1261.66345	R.ATALTAAEGLSEK.V	2.88	0.40
54	83389119	1261.66345	R.ATALTAAEGLSEK.V	3.29	0.40
		1264.64531	R.M*AETLSLENIK.T	2.66	0.31
56	69435675	1264.64531	R.M*AETLSLENIK.T	3.22	0.27
		1248.65039	R.MAETLSLENIK.T	2.98	0.14
		1782.83026	R.IC#ALPAWC#STADYVR.M	4.17	0.38
		1463.73511	K.GVNPLSIWMDGFK.Q	2.53	0.40
58	69435675	1261.61329	K.LSSVFFESAM*K.A	2.85	0.43
		1782.83026	R.IC#ALPAWC#STADYVR.M	4.05	0.41
67	69454601	1007.55200	R.FVGGSSIGGVK.E	2.69	0.21
		1328.72083	R.EVLGADHFIISK.D	3.54	0.42
67	70630207	1292.62695	R.FEVEVEGWAAR.D	3.74	0.41
		1434.72229	R.DDSGILSPINFTR.R	2.25	0.17
71	49016297	1432.70667	K.EASAVANTQIDWK.E	4.15	0.45
		1461.76174	K.ASM*ENGVLTVTVPK.Q	4.59	0.38
73	70621592	1157.67761	K.VSVEDGVLVIK.G	3.49	0.33
		1600.78528	R.TPWDIIENENELK.M	3.78	0.35
76	90029148	1470.81628	R.VPTADVSVVDLTVR.L	4.09	0.52
		1775.80237	K.LVSWYDNEWGYSTR.V	4.52	0.58
		1882.95447	R.SSIFDAQAGIALSDNFVK.L	3.41	0.41
76	83402450	1749.82647	K.DAPM*FVVGVNEHQYK.S	3.37	0.56
		1390.73254	R.GAGFNIIPSSTGAAK.A	2.45	0.15
		1470.81628	R.VPTADVSVVDLTVR.L	4.15	0.51
		1775.80237	K.LVSWYDNEWGYSTR.V	4.14	0.53
78	40764318	1423.76917	K.YVIVGGGVAAGYAAR.E	4.65	0.54

Protein polymorphism between two *Picea abies* populations revealed by two-dimensional gel electrophoresis and tandem mass spectrometry

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Running title: Proteomics of local adaptation: a case study in *Picea abies*

Abstract

In species with high gene flow and consequent low inter-population differentiation over wide geographic ranges, deviating gene expression in response to ecological gradients often reveals adaptive significance. We investigated potential differences in gene expression between *Picea abies* ecotypes adapted to contrasting altitude conditions. Protein expression patterns were compared between needles and roots of two months old *Picea abies* seedlings, by means of two dimensional electrophoresis. Proteins exhibiting differential expression between the two ecotypes were analysed by tandem mass spectrometry. A total of 19 proteins exhibited qualitative or quantitative polymorphism between the two populations. These proteins exhibited organ specific expression, and the level of inter-population protein polymorphism was organ dependent. Among differentially expressed proteins we identified proteins involved in photosynthesis, photorespiration, root tracheary element differentiation and trans-mitochondrial membrane transport. Our results show that *Picea abies* seedlings from locally adapted ecotypes exhibit consistent differences in protein expression. The expression polymorphism of some of these proteins has potential adaptive significance.

Key words: ecotype, *Picea abies*, protein amount polymorphism, proteomics, two-dimensional gel electrophoresis

INTRODUCTION

Trees response to environmental selective pressure in terms of nucleotide substitution rates and speciation rates is relatively slow compared to other species, due to the high age of reproductive maturity and the long generation interval (Jump and Penuelas 2005; Petit and Hampe 2006). Compensatory mechanisms have therefore developed, that allow tree species to rapidly adapt to changing environmental conditions (Saxe et al. 2001). As most tree species, conifers maintain unusual high intra-population levels of genetic diversity, due to specific life-history characteristics (long-lived woody species with out-crossing breeding systems spread over large geographic ranges) (Hamrick 2004). They have also acquired high levels of phenotypic plasticity adding to their adaptive potential and increasing their chances of surviving extreme climatic events. In spite of the low genetic differentiation between populations (reviewed by Hamrick 2004), conifers exhibit rather strong inter-population variations of phenotypic characters including morphologic and physiologic features (Oleksyn et al. 1998) but also of phenotypic traits relevant for climatic adaptation such as frost hardiness (Daehlen and Johnsen 1995; Skroppa et al. 1994). Parental effects act synergic with phenotypic plasticity, contributing to the maintenance of a high genetic variability in population, while exhibiting a phenotype "tuned" to the local environmental conditions (Skroppa and Johnsen 2000). Thus, phenotypic differences between populations are not only a result of genotype selection but also of the environmental influence upon gene expression through imprinting (Johnsen and Skroppa 1996) and of phenotypic plasticity.

Besides assessing between and within-species genetic variation based on molecular markers, targeting the protein level should bring valuable information on mechanisms behind adaptation and evolution of populations. Although genetic markers are extremely useful for establishing genetic relationships and history of populations (e.g. Achere et al. 2005), they only account for the presence/absence of the given marker at individual level, ignoring quantitative traits. A more complete picture of within- and between-population variability can be acquired at gene expression level i.e. transcriptome and proteome level. This type of information is highly relevant for the processes of adaptation and evolution of natural populations. Qualitative polymorphism of a protein (e.g. presence/absence variation) most often mirrors variation of the structural genes, while quantitative variation (i.e. polymorphism of protein amounts) is the result of a complex network of regulatory mechanisms, subject themselves to genetic variation and interaction with the environment (Rockman and Kruglyak 2006; Thiellement et al. 2002). Patterns of protein expression integrate thus the genetic background of the individual (structural and regulatory variation), with the environmental influence.

Two-dimensional gel electrophoresis (2DE) is an efficient tool for such investigations allowing the separation, simultaneous display and quantification of a large number of proteins. This method has a large yet underexploited potential to address scientific hypothesis specific to population ecology, phylogenetic and evolutionary studies (Biron et al. 2006; Navas and Albar 2004; Karr 2007). Proteomic-like approaches have so far been successfully applied for investigating variation or establishing genetic relationships both at species or above-species level (Marquès et al. 2001; Lum et al. 2002; Barreneche et al. 1996) and below-species level (Chevalier et al. 2004; Rocco et al. 2006; Jacobsen et al. 2001; Bahrman et al. 1994; Jorge et al. 2006).
For species characterised by strong genetic flow over wide geographical ranges like trees, genetic variation with potential adaptive significance often develops in response to ecological gradients (Lexer et al. 2007). Genes whose expressions deviate under the pressure of selection in spite of the strong gene flow might underlie characters that confer adaptive advantage under the given environmental conditions (Lexer et al. 2007). We therefore used a proteomic approach (2DE followed by tandem mass spectrometric identification of proteins) to investigate the level of differentiation between two *Picea abies* ecotypes adapted to different environmental conditions. As most conifers, *Picea abies* exhibits a low level of population genetic differentiation across its geographical range (Achere et al. 2005). However,

differences in allelic distribution have been described between populations for several isozymes loci (e.g. Mitton and Duran 2004; Puglisi et al. 1999). In the present study our objective was to verify if populations adapted to different environmental conditions also exhibit more consistent differences in protein expression, in addition to the rather subtle differences in allelic distribution previously described. For this, we compared 2DE patterns obtained for of needles and root samples from 2 months old seedlings from a typical low altitude *Picea abies* ecotype and an autochthonous population adapted to high altitude conditions. Proteins displaying consistent variation between the two ecotypes were identified by means of tandem mass spectrometry.

MATERIAL AND METHODS

Plant material

Two different *Picea abies* populations were selected: an autochthonous sub-alpine population (84030, close to Füssen, at 1300-1600m a.s.l.) and a sub-montane ecotype recommended as particularly well adapted low elevation climatic conditions (84008, near Westerhof, at 300m a.s.l.; EU category "Tested reproductive material") (Recommendation of Niedersächsische Forstliche Versuchsanstalt*** 2004). The two locations have different temperature and precipitation conditions (average annual temperatures (1961-1990): Füssen 4.5°C, Westerhof 8.5°C; average annual precipitations: Füssen 1750mm, Westerhof 800mm; climatic data available at www.klimadiagramme.de)(Enders 1996).

Seed samples collected from *Picea abies* stands from these two ecotypes were supplied by the Staatliche Samenklenge Laufen, Germany. After vernalisation, seeds were soaked over night in sterile demineralised water and surface sterilised for 30 minutes with 30% hydrogen peroxide. Floating seeds were discarded and the remaining seeds were sown in sterile Phytatray boxes (Sigma-Aldrich) on Vermiculite soaked with sterile nutritive solution (Ingestad 1979). Seeds germinated and seedlings grew in growth chambers at 16°C, 100% humidity and 16h photoperiod (30 $\mu molm^{-2}s^{-1}$; Osram True light T8, 5500 Kelvin). After two months, needle, stem and root length were measured to nearest mm for 40 seedlings per ecotype.

For 2DE, needles and roots were harvested, frozen in liquid nitrogen within 30 seconds to avoid proteolysis and stored at -76°C until protein extraction. Equal amounts of biological material from 30 to 40 seedlings were pooled per aliquot. The pooling procedure is known to be useful for the comparison of natural populations by means of DNA markers (e.g. RAPD patterns for *Picea abies* populations, (Scheepers et al. 1997)) but has also been successfully used in proteomic studies (Rocco et al. 2006; Thiellement et al. 1989; David et al. 1997; Weinkauf et al. 2006).

The choice of young seedlings as biological material was based on the need of minimising environmental and individual life history related bias of gene expression patterns, since it is recognised that gene expression depends not only on the genotype but also on the particular environmental conditions of germination (Johnsen et al. 2005) and ontogeny (Gion et al. 2005).

Protein extraction

Proteins were precipitated with TCA/acetone and extracted in buffer containing 5M urea, 2M thiourea, 100mM DTT, 2% CHAPS, 2% SB3-10 and 0.5% Pharmalyte 3-10 for the needle samples and 7M urea, 2M thiourea, 2mM TCEP, 50mM DTT, 2% DM and 0.5% Pharmalyte 3-10 for the root samples. Protein concentration was measured using the RC-DC Protein Assay (Bio-Rad). A detailed description of the protocol is presented elsewhere (Vâlcu and Schlink 2006b; Vâlcu and Schlink 2006a).

Two-dimensional gel electrophoresis

For optimal resolution, acidic and basic proteins were focused on separate gradients, following previously optimised protocols (Vâlcu and Schlink 2006b; Vâlcu and Schlink 2006a). Samples were cup-loaded near the cathode for the 4-7 IPGs and near the anode for the 6-11 IPGs, on 24cm long IPG strips (GE Healthcare) previously rehydrated for at least 12h. The rehydration buffer contained 100mM HED, except for the focusing of root proteins, where it contained the same reducing agents as the extraction buffer. The

rehydration buffer was supplemented with 10% isopropanol for the focusing of basic gradients. Focusing was performed in an Ettan IPGPhor Cup Loading Manifold at 20°C and 50mA/IPG gel strip for a total of 55kVh in case of acid IPGs and 60kVh in the case of basic ones. After a two step equilibration of the strips with buffer containing 2%DTT and 4% iodoacetamide, respectively, the second dimension was performed on 12.5% polyacrylamide 1mm thick gels in an Ettan Dalt 6 electrophoresis chamber (GE Healthcare). Gels were silver stained according to Heukeshoven and Dernick (Heukeshoven and Dernick 1988), with the acetic acid in the stop solution substituted for 1% glycine.

Image and data analysis

Silver stained gels were scanned under blue light, at 300 dpi, with ImageScanner (GE Healthcare). ImageMaster 2D Platinum (GE Healthcare) was used for spots identification, quantitation and matching. A WACOM digital plate was used for a more accurate manual editing of spots. Mismatches were manually corrected. Relative spot volumes (normalised per gel) were compared for each organ/ecotype/IPG between five biological replicates by means of paired t-tests. Statistical analysis were performed with R2.2.1 (R-Development-Core-Team 2006). In order to avoid type I errors, only spots showing very significant ($p < 0.01$) differences between the two ecotypes were selected. Among those, spots whose relative volumes differed by a factor of at least 2 were kept for further characterisation. The spots thus selected were validated by comparison with a set of further 15 samples.

Protein identification by mass spectrometry

Preparative electrophoresis was performed similarly to the analytic one, except for the higher protein amount (between 500 and 800µg per gel) loaded by paper bridge loading and the prolonged focusing (65kVhr for acidic gradient and 72kVhr for basic gradient). Colloidal Coomassie G-250 staining was performed according to Anderson (Anderson et al. 1991). Weaker spots were cut from gels stained with MS-compatible silver staining (with glutaraldehyde and formaldehyde excluded from the sensitising and silver solution, respectively). Spots were cut from gels using a scalpel, destained with 40% ethanol / 50mM ammonium bicarbonate (Coomassie stained spots) or in a 30mM potassium hexacyanoferrate / 100mM sodium thiosulphate 1:1 mixture (silver stained spots), then dehydrated with acetonitril and dried for 30 min under vacuum. After reduction and alkylation (Shevchenko et al. 2006) spots were washed and dehydrated with acetonitrile, dried under vacuum and stored at -20°C until analysis. In-gel digestion with trypsine was performed over night at 37°C. The resulting digests were analysed with a nanospray LCQ ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) and peptide identification was performed with SEQUEST against all *Picea* ESTs available at dbEST in January 2007. Validation filters were set for Xcorr values higher than 1.90 for +1 peptides, 2.20 for +2 peptides and 3.75 for +3 peptides, and Delta CN higher than 0.1.

SEQtools software package by Søren W. Rasmussen (www.seqtools.dk) was used for DNA sequence translation and protein sequence alignments. If necessary CLUSTAL W alignments (Thompson et al. 1994) were optimized using T-Coffee (Notredame et al. 2000). Protein pattern and motif searches were performed using InterProScan (http://www.ebi.ac.uk/InterProScan/) (Zdobnov and Apweiler 2001), Motif Scan of the Swiss Institute for Experimental Cancer Research (ISREC) provided by the Swiss Institute of Bioinformatics website (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and Pfam Protein Search (http://pfam.janelia.org/hmmsearch.shtml) (Sonnhammer et al. 1998).

RESULTS

Morphometric comparison

Two months old seedlings from Westerhof and Füssen populations grown under identical conditions were compared with respect to their morphometric characteristics. A linear model with organ length as dependent variable and the organ and the ecotype as fixed factors showed highly significant differences between the seedlings from the two ecotypes ($F_{3,371} = 378.9$, $p < 0.001$). The length of above-ground

organs was higher for seedlings from the lower elevation population and the differences were highly significant for both needles ($t_{1,123} = -3.2899$, $p = 0.001$) and stems ($t_{1,123} = -13.1852$, $p < 0.001$) (Fig. 1). Roots of the high elevation population were longer in comparison with the low elevation population, though the difference was not significant ($t_{1,123} = 0.107$, $p = 0.915$). A Wilcoxon rank sum test with continuity correction was used to compare the root to stem length ratio. The seedlings from the high elevation population were characterised by higher values of the ratio as compared with seedlings from the lower elevation population ($W = 2673$, $p < 0.001$) (Fig. 1).

Needle and root 2DE patterns

Highly reproducible 2D patterns were obtained for needles and roots of *Picea abies* seedlings from Westerhof and Füssen ecotypes. More than 1150 spots were reproducibly detected on acidic gradients and more than 650 on the basic gradients for the root samples. Needle 2DE patterns were richer in spots with approximately 40% in the acidic gradient and 20% in the basic gradient.

Following the criteria defined in the material and methods section, a total of 19 protein spots were identified, with estimated pH values ranging between 5 and 7.3 and MW between 17.5 and 104.2, exhibiting differential constitutive expression between Westerhof and Füssen ecotypes. Ten proteins had a higher level of expression in seedlings from Füssen plants (four in needles and six in roots, see Fig. 2, Fig. 3 and Table 1) and seven proteins were over-expressed in Westerhof (all in root samples, see Fig. 3 and Table 1). We also identified two proteins (spots 9 and 18) that appeared to be uniquely expressed in seedlings from Füssen, or else their level of expression in Westerhof plants was below the level of detection.

Mass spectrometric identification of proteins

Out of the 19 spots exhibiting differential expression between the two ecotypes, seven different protein functions were identified by tandem mass spectrometry corresponding to eight spots (Table 1). Peptides were mapped on the sequences of the *Picea* clones used for protein identification (supplementary material) and alignments of these clones with the best GenBank matches were build to verify function assignments (e-values in Table 1). Four of the identified proteins had higher expression levels in the seedlings from Füssen: glycine cleavage system T protein (spot #3) and protochlorophyllide reductase (spot #4) in the needles, glyoxysomal malate synthase (spot #16) and a homolog of the r40c1 protein (spot #9) in the roots. The remaining four spots were expressed at higher levels in the roots of seedlings from Westerhof and were identified as a tracheary element differentiation protein (TED2, spot #7), a voltage-dependent anion-selective channel (Porin, spot #14) and two isoforms of a NAD(P)-binding non-metallo dehydrogenase (spots #12 and #19) sharing five peptides.

DISCUSSION

2DE is known as a powerful technology for the investigation of qualitative and quantitative gene expression variation between different genetic units. Comparison of the 2DE patterns between populations on an individual basis can nevertheless be a laborious process especially when large numbers of individuals are to be investigated. The use of pooled samples allows for larger sample sizes and results in a more representative 2DE pattern for the compared populations (David et al. 1997) when the interest lies in identifying major differences in protein expression at the population level and not in comparing levels of polymorphism between individuals.

In our study, protein expression patterns were compared in a differential display experiment between pooled samples of needles and roots of *Picea abies* seedlings from two ecotypes adapted to contrasting elevations. This approach revealed both qualitative and quantitative protein polymorphism between the two ecotypes. The level of inter-population protein polymorphism was organ dependent. The significance of these differences is discussed below.

Morphometric comparison

The differences in the morphometric characteristics of seedlings from the two ecotypes are consistent with known altitudinal and latitudinal trends. Plant height declines with the altitude or latitude (Oleksyn et al. 1998; Danusevicius and Gabrilavicius 2001), while plants from the higher altitude allocate comparatively more biomass to roots than plants from lower elevations (Oleksyn et al. 1998). These differences could result from genetic differentiation between the two populations, environmental effects on plant growth (Lindgren and Wei 1994) or both. The longer stems and primary needles of Westerhof plants as well as the higher values of the root to stem ratios for the Füssen seedlings confirm that this phenotypic differentiation appears in *Picea abies* as early as 2 months of age.

Qualitative and quantitative inter-population protein polymorphism

Most of the between-ecotype variation found in the present study was quantitative (17 out of 19 spots). Only two cases of qualitative variation were identified between the two ecotypes, both proteins being specifically expressed in the roots of seedlings from the high elevation ecotype (Füssen).

Qualitative protein polymorphism among individuals (i.e. presence / absence or shift in the position of a protein spot) is often attributed to allelic variation but can also reflect quantitative variation close to the level of detection or post-translational modification of proteins. Quantitative variation (i.e. variation in spot intensity) on the other hand, may result from the concerted action of several factors including the stability of the protein or its level of expression, which can be under the control of multiple loci (Damerval 1994). Levels of qualitative and quantitative variation estimated from 2DE patterns of single individuals vary between species. *Zea mays* lines for example have comparable levels of qualitative and quantitative protein polymorphism (de Vienne et al. 1988; Leonardi et al. 1988). The polymorphism of protein amount between *Picea abies* ecotypes on the other hand seems to be larger than the variation in gene structure measured as presence/absence or shift in the spot position (Sieffert 1988).

By pooling several individuals per sample, the information concerning allele frequency and inter-individual variations in the protein expression level is discarded and differences relevant at between-population level become apparent. Quantitative polymorphism between pooled samples can therefore reflect differences in the proportion of individuals that express a given protein, differences in the actual level of protein expression at individual level (rates of synthesis and degradation, stability) or both. The number of proteins that discriminate the populations will be correspondingly reduced as compared to polymorphism assessed at individual level (Sieffert 1988).

Such quantitative variation of protein expression at population level is essential when comparing plants under different environmental pressure. Regulation of gene expression is a multilevel complex process exhibiting a wide range of genetic variability, probably reflecting the large basis it offers for mutations (de Vienne et al. 1988) and is likely to be more involved in adaptive or morphological evolution than structural variations of proteins (MacIntyre 1982). This is supported by the fact that, at least in some species (i.e. *Zea mays*), morphological distances are correlated with polymorphism of protein amounts rather than with qualitative distances between lines (Damerval et al. 1987).

Variability of organ-specific and organ-non-specific peptides

All spots found to consistently and reproducibly distinguish the two ecotypes were organ-specific. Most of the proteins differentially expressed between the two ecotypes (79%) were root specific proteins as compared to only 21% needle-specific proteins. Previous studies on plant proteome demonstrated that the variability of protein expression level is under genetic control (reviewed by Thiellement et al. 1999) and depends on the organ or type of tissue. *Pinus pinaster* needle, pollen and bud-specific proteins for example were found to exhibit levels of polymorphism of 44%, 58.1% and 70.4% respectively (Bahrman and Petit 1995). Similar differences in the level of variability were described between *Zea mays* second leaf blade, mesocotyl and sheath (7.5%, 12.6% and 13.2% respectively) (Leonardi et al. 1988). Moreover, the latter study also showed that the dominant inheritance of most polypeptides is organ-specific.

These findings are not surprising, since it has been repeatedly shown that organ-specific proteins also exhibit a larger genetic variation than organ-non-specific ones (de Vienne et al. 1988; Bahrman and Petit 1995; Klose 1982; Leonardi et al. 1988). It has been hypothesised (Klose 1982) that „housekeeping“ proteins expressed in all organs are more critical for the individual's metabolism and therefore under higher selective pressure than organ-specific proteins. This would explain why they are less variable than the latter. Alternatively, de Vienne *et al.* (1988) proposed that the higher variability of proteins displaying organ-specific level of expression might reflect the higher number and complexity of regulatory mechanisms needed for the control their expression, each of them subject to genetic variability.

Function of proteins differentially expressed between *Picea abies* ecotypes

It is, at least for some of the identified proteins, difficult to speculate whether their differential expression has any adaptive significance for *Picea abies* ecotypes located at different elevations. A closer inspection of their functions will nevertheless offer some insight into the putative significance of their expression patterns in the two populations investigated. Detection of molecular signature of natural selection at the nucleotide level could constitute a further step toward the validation of the adaptive significance of the detected proteins (Wright and Gaut 2004; Ehrenreich and Purugganan 2006).

Isoforms of protochlorophyllide reductase and glycine cleavage system T protein exhibited higher expression in the needles of the Füssen ecotype. Protochlorophyllide reductase (spot #4, POR) catalyses a late light-dependent step in the synthesis of chlorophyll (Heyes and Hunter 2005). In gymnosperms are known two *por* gene subfamilies (A and B) involved in different developmental stages of the green tissue and with different (light dependent/independent) expression regulation (Skinner and Timko 1998). In *Pinus taeda*, mature needles only express PORB but both isoforms are detectable in cotyledons and primary needles (Skinner and Timko 1999). First crown needles in *Picea abies* might also express both POR genes, but we could not distinguish whether the protein differentially expressed in the needles of seedlings from the two spruce ecotypes is a PORA or a PORB ortholog.

Glycine cleavage system T protein (spot #3) is one of the four components of the mitochondrial glycine decarboxylase (GDC) multienzyme system (Douce et al. 2001) which catalyses the oxidative decarboxylation and deamination of glycine produced in the peroxisomes during photorespiration. All the components (P-, H-, T- and L-proteins) are highly abundant in mitochondria from green tissues. T-protein is light induced (Vauclare et al. 1998) and developmentally regulated (Vauclare et al. 1996; Thompson et al. 1998) and its expression was shown to be under posttranscriptional control (Vauclare et al. 1996). A higher expression of the T-protein in Füssen seedlings needles might indicate higher photorespiration rates. Similarly elevated photorespiration rates have been described both in herbaceous (Streb et al. 2005; Streb et al. 1998; Kumar et al. 2006) and woody plants (Oleksyn et al. 1998) as adaptation to high altitude conditions.

Two of the proteins showing specific or higher expression in Füssen seedlings roots were identified as a homolog of the r40c1 protein carrying a lectin domain (spot #9) and glyoxysomal malate synthase (spot #16) respectively. Glyoxysomes are found in cells of storage organs like endosperm (Cooper and Beevers 1969) and during post-germinative growth of oil-seed plants. Malate synthase is part of the glyoxylate cycle, which converts storage lipids into sucrose (Beevers 1961). The sucrose is then transferred to shoot and root apical meristems, and provides the carbon source necessary for growth before the plants start photosynthesis (Hayashi 2000). In roots, malate synthase expression was shown to be activated in response to carbohydrate deprivation (Ismail et al. 1997) and may confer adaptive advantage to plants grown in low carbohydrate conditions.

From the proteins over-expressed in the roots from Westerhof seedlings, we identified four spots as a tracheary element differentiation protein (TED2), a voltage-dependent anion-selective channel (VDAC)

and two isoforms of a NAD(P)-binding non-metallo dehydrogenase belonging to the short-chain dehydrogenase/reductase family (SDRs).
TED2 (spot #7) is a quinone reductase involved in differentiation of tracheary elements and considered a marker for the development of the root vascular system (Demura and Fukuda 1994). The protein is under a strict temporal and spatial regulation (Demura and Fukuda 1994) and is expressed in early stages of differentiation of procambial cells into immature xylem or phloem cells. Given its narrow time and space frame of expression, the differential expression of TED2 in roots from the two ecotypes might indicate asynchrony in their root systems development, a fact which is in accordance with the morphometric measurements on the root length.
VDAC (spot #14) is a 30kDa protein that forms homodimers in the mitochondrial outer membrane (Thomas et al. 1991). It is believed to provide the major pathway for metabolite flux through the outer membrane (Colombini 1997; Manella and Colombini 1984; Manella et al. 1992) and reduction of its permeability can regulate mitochondrial respiration (Liu and Colombini 1992).
Two spots (#12 and #19) were identified as isoforms of a member of the short-chain dehydrogenase/reductase family (SDRs). More that 3000 members of these superfamily of enzymes with NAD(P)(H)-dependent (Rossmann-fold domains*)* oxidation/reduction activities have been described. They have a broad substrate spectrum, ranging from alcohols, sugars, steroids, aromatic compounds to xenobiotics (Kallberg et al. 2002). For the two proteins shown here to have higher expression in low elevation plants, the matches to the Panther PTHR19410 extension indicate that the proteins are not alcohol dehydrogenases, but another type of dehydrogenase or reductase.

Significance of between-population protein polymorphism in *Picea abies*

Picea abies covers a large natural range across Europe. The large scale geographical patterns of genetic variation over its natural range confirm the three major genetic domains resulting from post-glacial re-colonisation (Achere et al. 2005; Collignon et al. 2002). Similarly to other woody plant species, *Picea abies* exhibits high levels of within-population variation in contrast with a low between-population differentiation (Achere et al. 2005). Both ecotypes used in the present study belong to the Alpine domain (Achere et al. 2005) and can therefore be assumed to show low genetic differentiation.
However, even in genetically similar coniferous populations, genetic structure is known to be influenced by environmental conditions such as water or nutrient availability (Stutz and Mitton 1988; Cobb et al. 1994; Mitton and Duran 2004). Clinal variations of isozymes allele frequency along latitudinal (Bergmann and Gregorius 1993) or altitudinal gradients have been described in several coniferous species (Puglisi et al. 1999; Mitton et al. 1980; Grant and Mitton 1977) including *Picea abies* (Lundkvist 1979; Bergmann 1978).
Protein expression polymorphism between ecotypes locally adapted to different environmental conditions can be the result of genetic differentiation (at structural and/or regulatory loci) between the two populations, but can also reflect environmental influence upon gene expression through imprinting. Irrespective of the underlying mechanisms, these characters are potentially under selective pressure and might constitute the material for subsequent population divergence.

Overall, our results show that two-dimensional gel electrophoresis performed on pooled samples can reveal qualitative and quantitative between-population protein polymorphism with putative adaptive significance. *Picea abies* ecotypes adapted to contrasting elevations exhibit differential expression of proteins potentially conferring adaptive advantage under selective pressure.

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Table 1.

Spots showing different expression in Westerhof and Füssen ecotypes. pH and MW were estimated from 2DE gels and averaged across three gels.

Spot	Origin of identified spot	Stronger expression	Provenance specific	No. of peptides	Xcorr	Coverage (%)	NCBI Accession No.	Species	Best homologue	Species	Localisation	pH*	MW* (kDa)	pH**	MW** (kDa)
1	needles	Füssen	-									5.33	17.5		
2	needles	Füssen	-									6.01	38		
3	needles	Füssen	-	6	60.26	30	70281181	*Picea sitchensis*	Aminomethyltransferase, mitochondrial precursor (EC 2.1.2.10) (Glycine cleavage system T protein) (e-value 2e-98)	*Flaveria pringlei*	mitochondria	6.7	44.5	8.53	44
				4	40.26	22	40766142	*Picea glauca*	Aminomethyltransferase, mitochondrial precursor (EC 2.1.2.10) (Glycine cleavage system T protein) (e-value 4e-54)	*Oryza sativa*				8.91	44.4
4	needles	Füssen	-	4	40.30	16	70220849	*Picea sitchensis*	Protochlorophyllide reductase, chloroplast precursor (EC 1.3.1.33) (PCR) (NADPH-protochlorophyllide oxidoreductase) (POR) (e-value 2e-146)	*Pinus mugo*	chloroplast	7.01	38	8.96	43.5
5	roots	Westerhof	-									4.99	47		
6	roots	Westerhof	-									5.97	30		
7	roots	Westerhof	-	2	20.23	14	70329853	*Picea sitchensis*	TED2 (e-value 1e-74)	*Vigna unguiculata*		6.55	39	6.46	34.6
8	roots	Westerhof	-									6.58	37		
9	roots	Füssen	+	2	20.23	9	70229422	*Picea sitchensis*	Putative r40c1 protein (e-value 2e-45)	*Oryza sativa*		6.33	43.5	6.25	41.7
10	roots	Füssen	-									5.31	90		
11	roots	Füssen	-									5.54	82		
12	roots	Westerhof	-	7	70.30	36	49025207	*P. engelmanii x P. glauca*	oxidoreductase (short-chain alchool dehydrogenase) (e-value 4e-59)	*Arabidopsis thaliana*		6.6	36	6.26	32.2
				6	60.28	26	68773949	*P. engelmanii x P. glauca*	oxidoreductase (short-chain alchool dehydrogenase) member, Tasselseed 2 (e-value 4e-68)	*Buchloe dactyloides*				6.17	33.2
13	roots	Füssen	-									6.63	33		
14	roots	Westerhof	-	4	40.18	17	70635044	*Picea glauca*	36 kDa outer mitochondrial membrane protein porin (Voltage-dependent anion-selective channel protein) (e-value 4e-59)	*Prunus armeniata*	mitochondria	6.63	33	7.06	29.7
				3	30.23	12	70318841	*Picea sitchensis*	36 kDa outer mitochondrial membrane protein porin (Voltage-dependent anion-selective channel protein) (e-value 6e-69)	*Solanum tuberosum*				7.78	29.4
15	roots	Füssen	-									7.3	42		
16	roots	Füssen	-	3	30.21	11	70222904	*Picea sitchensis*	Malate synthase, glyoxysomal (EC 2.3.3.9) (e-value 4e-84)	*Brassica napus*	glyoxysome	6.29	61	8.45	65
				2	20.24	12	49024400	*P. engelmanii x P. glauca*	Malate synthase, glyoxysomal (EC 2.3.3.9) (e-value 7e-86)	*Ricinus communis*				8.53	64.3
17	roots	Füssen	-									6.61	56		
18	roots	Füssen	+									6.97	104		
19	roots	Westerhof	-	6	60.31	28	68773949	*P. engelmannii x P. glauca*	oxidoreductase (short-chain alchool dehydrogenase) (e-value 5e-67)	*Tripsacum dactyloides*		6.96	35	6.90	34.4

Figure 1.

Two months old *Picea abies* seedlings from Westerhof and Füssen ecotypes grown under identical conditions exhibit statistically significant differences in the length of needles, roots and stems.

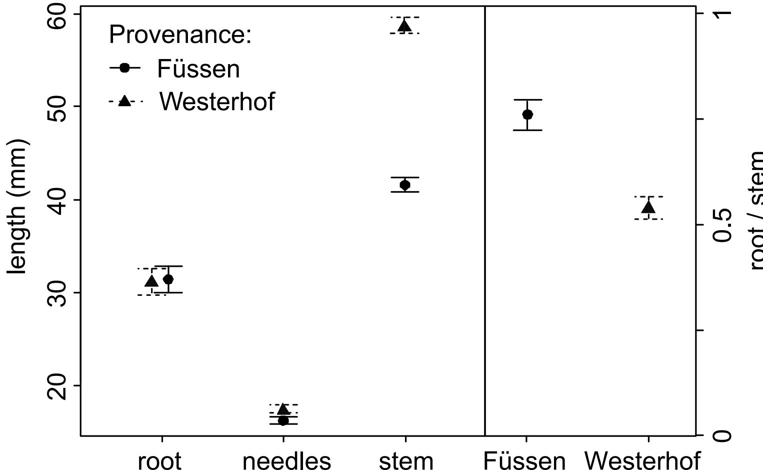


Figure 2.

Qualitative and quantitative differences in protein expression between the needles of two months old *Picea abies* seedlings from Westerhof and Füssen ecotypes.

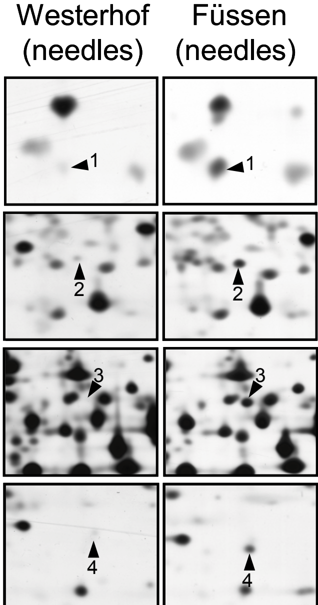
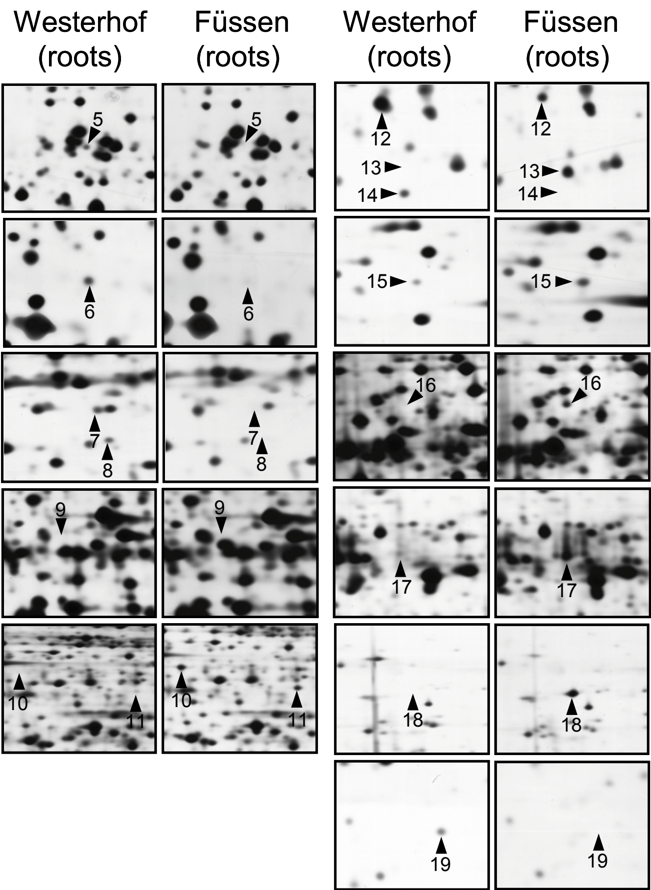


Figure 3.

Qualitative and quantitative differences in protein expression between the roots of two months old *Picea abies* seedlings from Westerhof and Füssen ecotypes.



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Supplementary data

Protein polymorphism between two *Picea abies* populations revealed by two-dimensional gel electrophoresis and tandem mass spectrometry

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The peptides on the basis of which the spots were identified by mass spectrometric analysis were numbered according to their mass in ascending order for each spot and mapped on the matching *Picea* clones.
GeneDoc (Nicholas & Deerfield) was used for sequence alignment shading.
For each protein spot peptides are numbered according to their mass in ascending order.

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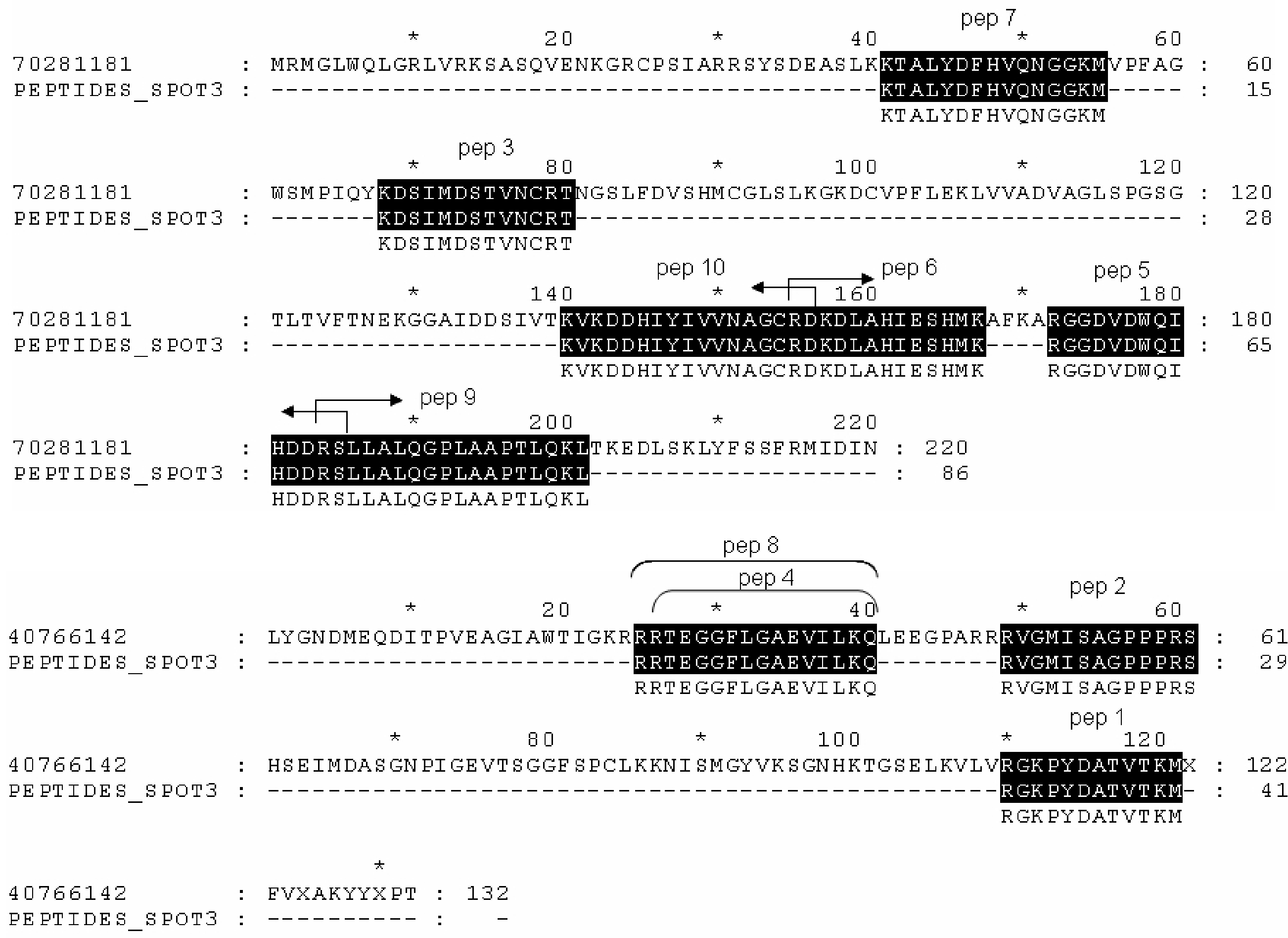

Fig. S1. Peptides of spot 3 mapped on *Picea sitchensis* clone GI 70281181 and *Picea glauca* clone GI 40766142. Clone GI 70281181 is lacking the C-terminus while clone GI 40766142 is the lacking the N-terminal sequence.

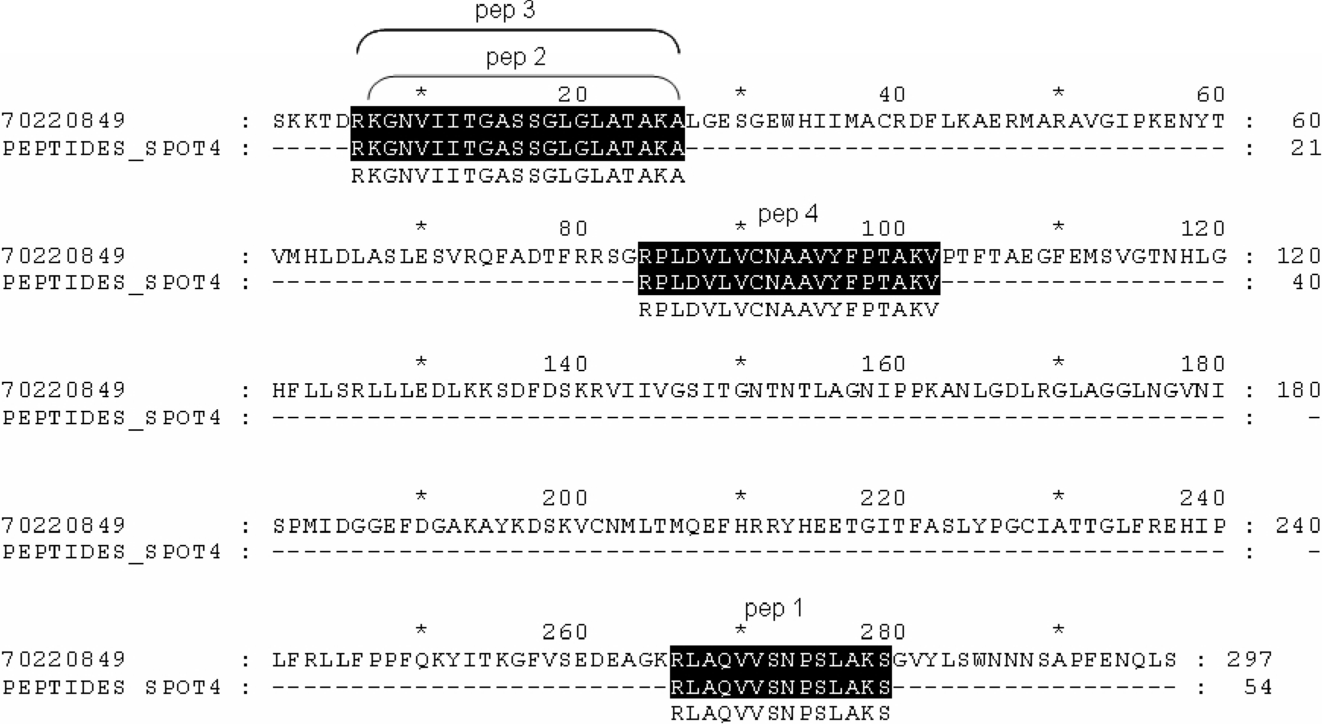

Fig. S2. Peptides of spot 4 mapped on *Picea sitchensis* clone GI 70220849

```
                                   pep 2
                         *        20        *        40        *        60
70329853        : MVKAITVHELGGPEVLKWEDVEVGEPGEGEILVKHKAIGVNFIDVYYRKGVYKAEKLPFI :  60
PEPTIDES_SPOT7  : ----------------KWEDVEVGEPGEGEILVKH------------------------- :  19
                                  KWEDVEVGEPGEGEILVKH
                       pep 1
                         *        80        *       100        *       120
70329853        : PGREAVGVVTAVGQGLTGRKVGDLVAYAGNPMGSYAEEQILPANQVVPFPSTLDPILGAA : 120
PEPTIDES_SPOT7  : --REAVGVVTAVGQGLTGRK---------------------------------------- :  37
                    REAVGVVTAVGQGLTGRK

                         *       140        *       160        *       180
70329853        : VMLKGMTVQYLLRSCFKVEPGHTILVHAAAGGVGSLLCQWGEALGATVIGSVSNEEKAAQ : 180
PEPTIDES_SPOT7  : ------------------------------------------------------------ :   -

70329853        : AAEDGCH : 187
PEPTIDES_SPOT7  : ------- :   -
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Fig. S3. Peptides of spot 7 mapped on *Picea sitchensis* clone GI 70329853

```
                                                      pep 2
                         *        20        *        40        *        60
70229422        : MHNPFGHHQPEPQQPYQAPHGGQYPPPIQGETVKIYCEANPDFLLASRNESVVMVPANES :  60
PEPTIDES_SPOT9  : ---------------------------------KIYCEANPDFLLASRN----------- :  16
                                                 KIYCEANPDFLLASRN

                         *        80        *       100        *       120
70229422        : DPSQQWIMDTSWSVKAKDDAGFPAFALVNKATGQALRHGRSEKDKVTLGPYHPDDLNEAV : 120
PEPTIDES_SPOT9  : ------------------------------------------------------------ :   -

                         *       140        *       160        *       180
70229422        : LFTQSADVGKGYQCIRPVNNTHLNLDASAGDDKHHVALSEGTEIVLCKWNKKESQKWKIS : 180
PEPTIDES_SPOT9  : ------------------------------------------------------------ :   -

                                                                pep 1
                         *       200        *       220        *       240
70229422        : PILGSSHGAYPPRGEPSSFFPQANVEPQGSTVRIHCEANPEFFLAARGDVAVLAPENPRD : 240
PEPTIDES_SPOT9  : ---------------------------------------------RGDVAVLAPENPRD :  30
                                                             RGDVAVLAPENPRD

70229422        : PHQQWIKV : 248
PEPTIDES_SPOT9  : -------- :   -
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Fig. S4. Peptides of spot 9 mapped on *Picea sitchensis* clone GI 70229422

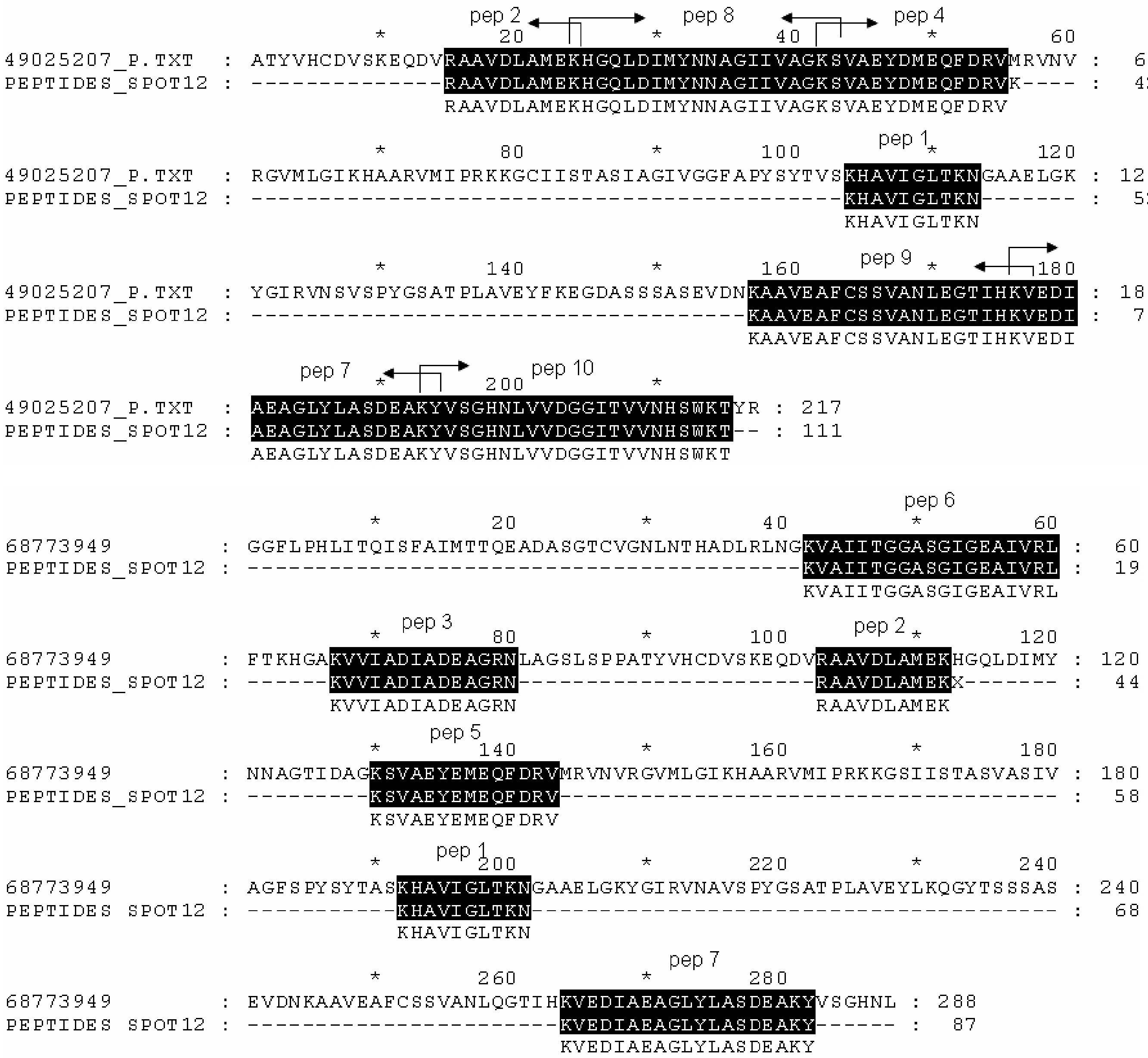

Fig. S5. Peptides of spot 12 mapped on *Picea engelmanii x P. glauca* clones GI 49025207 and GI 68773919

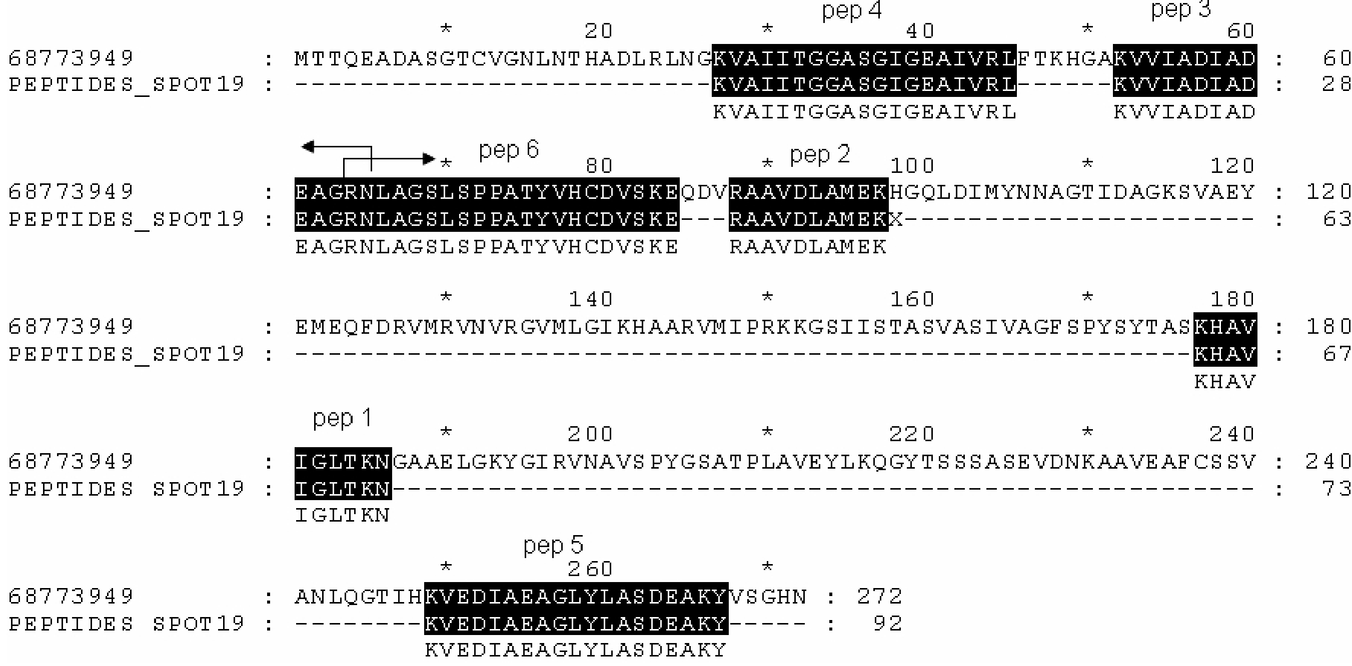

Fig. S6. Peptides of spot 19 mapped on *Picea engelmanii x P. glauca* clone GI 68773949

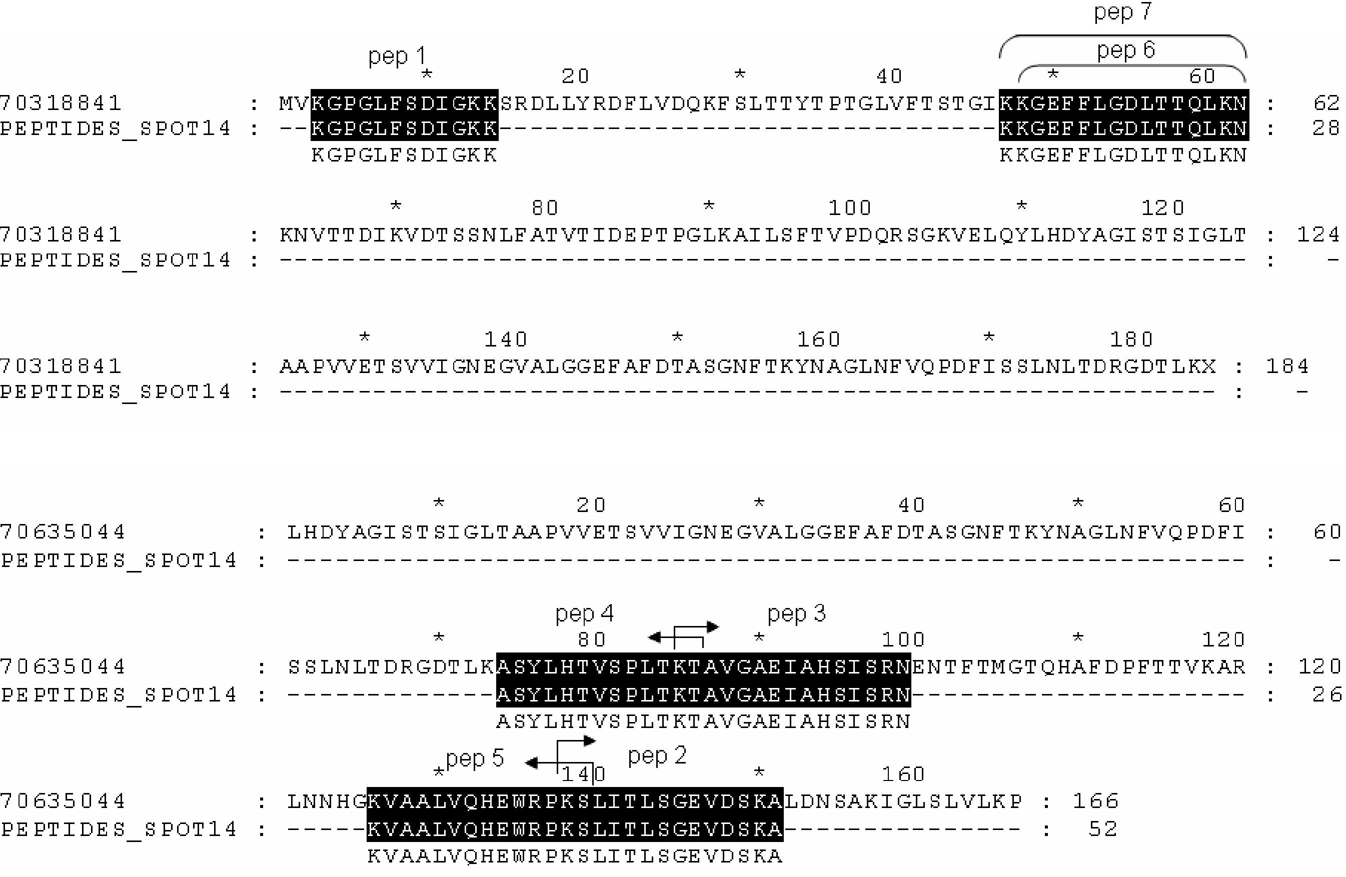

Fig. S7. Peptides of spot 14 mapped on *Picea sitchensis* clone GI 70318841 and *Picea glauca* clone GI 70635044

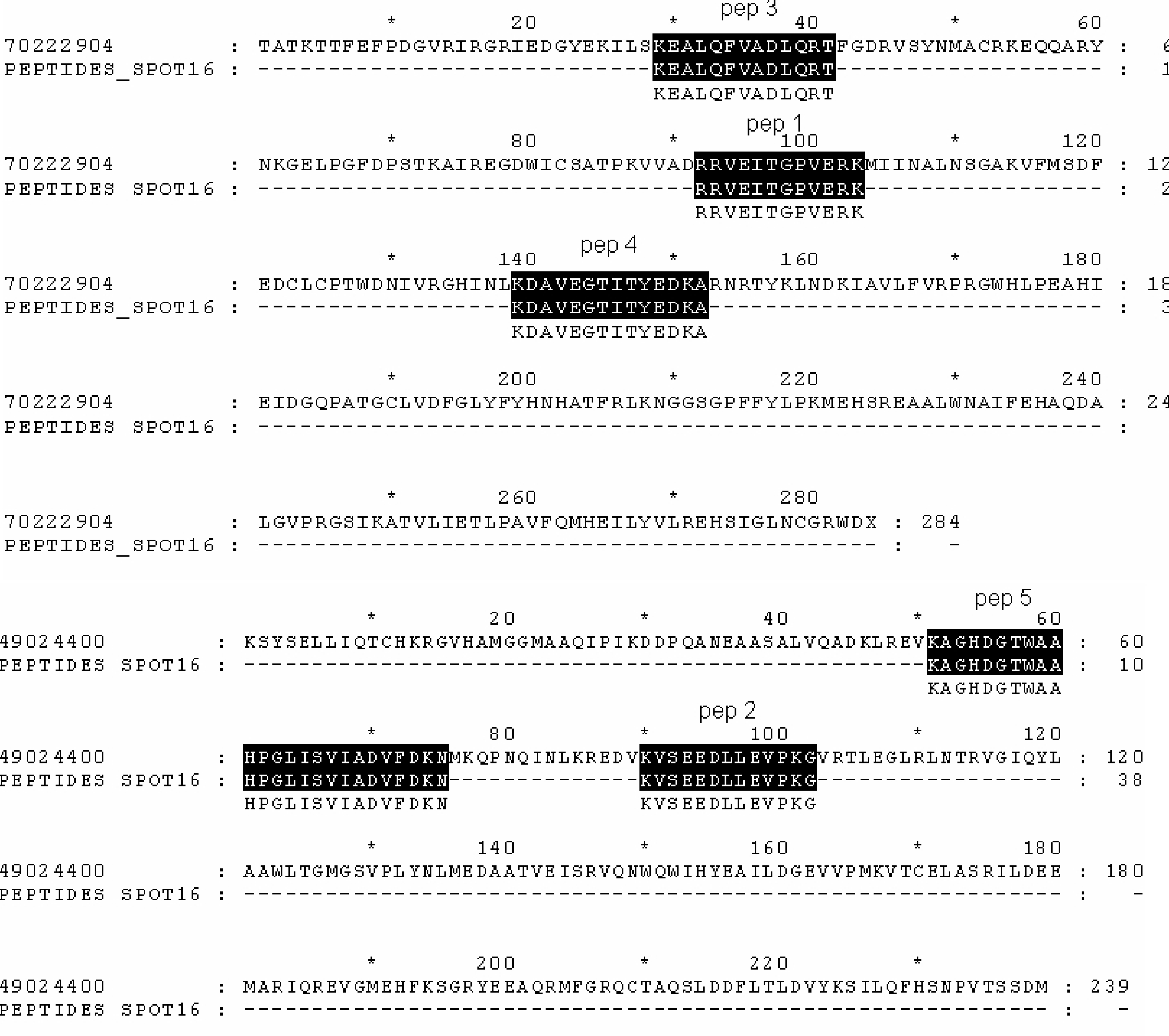

Fig. S8. Peptides of spot 16 mapped on *Picea sitchensis* clone GI 70222904 and *Picea engelmanii x P. glauca* clone GI 49024400. Clone GI 70222904 is lacking the C-terminus while clone GI 49024400 is lacking the N-terminal sequence.

Local and systemic changes in European beech (*Fagus sylvatica*) proteome following infection with *Phytophthora citricola* and wounding

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Running title: Changes in *Fagus sylvatica* proteome following pathogen attack and wounding

Abstract

Defence responses of *Fagus sylvatica* seedlings elicited by infection with the root pathogen *Phytophthora citricola* and wounding were compared at local and systemic levels in differential display experiments using two-dimensional gel electrophoresis of proteins. In order to enable the direct comparison of plant reaction following the two types of stress infection and wounding experiments were carried out in parallel under similar conditions. Several overlaps were observed between the protein expression patterns in response to pathogen attack and wounding, consisting of co-regulated as well as differentially expressed proteins. The results are in accord with known inter-regulation of signalling and defence pathways elicited by pathogens and herbivores and suggest antagonistic connections between them.

Key words: proteomics, stress, pathogen, wounding

1 Introduction

Investigations of plant response to different biotic and abiotic stressors in the last decades have revealed the existence of plant defence mechanisms of high complexity. Plant responses to different types of stress are not seen as linear series of independent events any longer but rather as overlapping inter-regulated networks strongly influenced by plant growth and development [1] as well as by environmental conditions [2]. The existence of crosstalk between signalling pathways triggered by pathogen attack and leading to salicylic acid (SA)-dependent systemic acquired resistance (SAR) or by herbivores/wounding and leading to jasmonic acid (JA)/ethylene-dependent induced systemic resistance (ISR) has been widely documented [3-9]. The best way to understand such complex defence systems is the parallel investigation, in comparable conditions, of plants' reactions to different stressors.

In case of incompatible interactions, plant responses to pathogens often involve the rapid and localised cell death at the site of pathogen penetration, known as hypersensitive response (HR). HR prevents the spread of the pathogen and, with few exceptions [10,11] is coupled with SA accumulation [12,13]. Subsequently a state of enhanced resistance (SAR) is established at systemic level, also believed to involve SA signalling [14,15]. Although patterns of response to pathogen attack common to many species have been described, pathogen recognition as well as the defence mechanisms activated in the plant following this recognition [12,16] are often pathosystem-specific. Compatible plant-pathogen interactions are established when the plants fail to recognize the pathogen and result in plant susceptibility to the given pathogen. Local pathogen-induced necrosis can in this case be the inducer of SAR [3]. Also in this type of interaction, plant's defence reactions against the pathogen are largely host and pathogen specific.

Plant response to herbivores/wounding is as complex as the response to pathogens. Depending on the attack/wounding site, different parts of the plant respond differently [17], and the response patterns are often species-specific [18]. In response to herbivores/wounding, plants can increase their direct resistance against herbivores [19-21] or attract parasitoids or predators of the herbivores [22-24]. The same effects can be induced in unharmed neighbouring plants [25] following perception of volatile organic compounds (VOC) [26,27] including methyl jasmonate [28,29] emitted by individuals under attack.

The aim of this study was to compare the plant-pathogen and plant-herbivore interactions and to identify and characterise common and specific patterns of gene expression. As model systems we chose wounding as elicitor of herbivore defence response and the pathosystem *Fagus sylvatica* - *Phytophthora citricola*.

Phytophthora is a oomycetes genus of which more than 60 species have been described to date [30]. Most of them are destructive pathogens covering a wide range of host specificity: from species specific, like *P. quercina* – for *Quercus* genus [31] or alder *Phytophthora* – for *Alnus* genus [32] to pathogens which can attack hundreds of hosts, like *P. cinnamomi*, *P.parasitica* / *nicotianae* or *P. cactorum* [30]. Soilborn species of this genus have been suggested to be involved in the *Fagus sylvatica* and *Quercus* sp. decline syndromes [31,33-36] and in the Ink Disease of *Castanea sativa* [37].

P. citricola is one of the most frequent *Phytophthora* species isolated from forest sites across Europe [31,33-35,37-40] and around the world [41-46] and has a broad host range, from agricultural to tree species. Among woody plants susceptible to *P. citricola* infection we can count species from more than 20 genera [a]. In woody plants like *Eucalyptus calophylla* [61], *Theobroma cacao* [62] or *Prunus dulcis* [63], the defence reaction

[a] e.g. *Abies fraseris* [47], *Abies procera* [48], *Actinidia deliciosa* [49], *Alnus glutionosa* [50], *Banksia hookeriana, B. ashbyi, B. coccinea, B. menziesii, B. prionotes* [51], *Castanea sativa* [32,37], *Chamaecyparis lawsoniana* [32], *Eucaliptus marginata* [41], *Fagus sylvatica* [32], *Hevea brasiliensis* [46], *Juglans hindsii* [43], *Juglans nigra* [52], *Laurus nobilis* [39], *Malus domestica* [53], *Mangifera indica* [40], *Persea indica* [54], *P. americana* [55], *Picea abies* [56], *Pieris japonica* [45], *Pinus radiata* [57], *Pyrus communis* [53], *Prunus cerasus* [58], *Prunus dulcis* [59], *Quercus cerris*, *Q. petraea* [31,34], *Q. ilex*, *Q. frainetto*, *Q. pubescens*, *Q. robur* [31,33], *Rhododendron sp.* [60]

of resistant plants against infection with *Phytophthora* species includes increased levels of phenylalanine ammonia lyase (PAL) activity in roots and cell wall reinforcement by deposition of lignin at the infection site. However, the defence reaction of infected woody plants during compatible interaction with *P. citricola* has been little studied [64].

F. sylvatica is the most abundant and dominant broad leafed tree species in Central and Eastern Europe, and exhibits high susceptibility to *P. cambivora*, *P. cinnamoni*, *P. citricola* [32], *P. kernoviae*, *P. gonapodyides*, *P. ramorum* [65] and *P. cactorum* [66]. Among these species, *P. citricola* is particularly aggressive [66] and the morphological and physiological consequences of *F. sylvatica* root infection with this pathogen have been addressed in several studies [64,67,68]. The intimate mechanisms of the plant-pathogen interaction are however, not known. More detailed investigations of this pathosystem are necessary in order to better understand the pathogenesis and the defence mechanisms involved.

Proteomic techniques emerged in the last years as very powerful tools for the study of plant's reaction to different stress factors, including plant-pathogen interactions [69-75], plant-herbivores interactions [76] and wounding [76,77]. Although rather laborious as compared for example with transcriptome analysis using microarrays, investigations at proteome level have the advantage to supply information on the actual protein expression level, isoforms and post-translational modifications [78]. We therefore undertook a proteomic approach in order to compare local and systemic changes in protein expression following infection of *F. sylvatica* seedlings with *P. citricola* and following leaf and root wounding. A set of wound experiments was designed to allow the identification of different wound response patterns and the estimation of the degree to which they overlap with plant's response to root infection.

2 Material and methods

2.1 Chemicals and equipment

2-DE electrophoresis and image digitisation were performed with equipment purchased from GE Healthcare (Freiburg, Germany): Ettan IPGPhor, Ettan Dalt6, ImageScanner. IPG strips, acrylamide and bisacrylamide, Tris, glycine, glycerol, SDS, urea, DTT, CHAPS, Pharmalyte 3-10 and DeStreak reagent were also from GE Healthcare. IAA was from Merck (Darmstadt, Germany). TCA was from Roth (Karlsruhe, Germany). TCEP, thiourea, isopropanol, dodecyl maltoside and SB 3-10 were from Sigma-Aldrich (Taufkirchen, Germany). Protein RC-DC Protein Assay was from Bio-Rad (München, Germany). All chemicals used were of analytical grade. MilliQ water was used for all solutions.

2.2 Plant material

F. sylvatica seeds (R0016812 FOD Gessertshausen, elevation: 540-600m a.s.l.) were washed two times with sterile distilled water and floating seeds were discarded. For germination seeds were placed on paper towels moistened with sterile distilled water and kept in dark, at 10°C. When roots reached approximately 1cm, the seeds were gradually transferred to normal light and room temperature conditions, in individual tubes containing sterile tap water (liquid system) or in individual pots (soil system).

2.3 Experiment design

In order to directly compare plant reaction following pathogen attack and wounding, infection and wounding experiments of *F. sylvatica* saplings were carried out in parallel under similar conditions as described below.

2.3.1 Infection experiments

Three months old *F. sylvatica* saplings were infected with the root pathogen *P. citricola* in soil and in liquid system.

For experiments in soil system, a 1:1 peat:vermiculite mixture was used to ensure appropriate pH conditions (4.4 - 5) for the pathogen development [31]. *P. citricola* was cultured on V8-juice agar plates and inoculation of plants was performed according to [79]. To stimulate zoospores production and release, the pots were flooded 1cm above soil level for 6 days. When first symptoms of leaf wilting appeared in some plants, leafs were sampled from the remaining healthy looking plants. *P. citricola* was reisolated from infected roots on selective agar medium [79] and verified in a PCR reaction using CITR1/CITR2 primers [80].
For the experiments in liquid system, seedlings with two fully developed leaves were transferred for accommodation into 2 litre containers (15 seedlings per container), in which the infection was performed. For inoculation, 7mm diameter discs were cut from the edge of a 5 days old *P. citricola* culture that was rinsed with sterile water for the past 24h (in order to remove nutrients and stimulate zoospore formation). Containers were inoculated with 1 disc per plant; control plants received discs from non-inoculated plates treated as the *P. citricola* culture. Sampling was performed at several stages of infection: early (3h to 24h), middle (2 days) and late, at the appearance of first symptoms of root tip browning (4 to 6 days). Root infection was verified as above. The infection experiment in liquid system was repeated in the subsequent year for validation.
For 2DE, proteins were extracted from P. citricola grown in liquid medium [81].

2.3.2 Wounding experiments

Three months or one year old *F. sylvatica* saplings were subjected to wounding experiments under different experimental setups in order to distinguish different wound response patterns depending on the wounded organ. Leaf wounding was performed with a cork borer of 15mm diameter; the leaf discs thus obtained were used as control samples. Roots were wounded using scissors. Samples were harvested at 3h after wounding.
Local and systemic (leaves) plant response to leaf wounding was investigated in three month old plants grown in soil system. The systemic response was also compared for leaves above and below the wounded leaf using one year old plants grown in soil. Seedlings response to volatile signals was investigated in plants that “witnessed” the wounding of neighbouring plants. The systemic (at leaf level) and local (at root level) response to root wounding was characterised for plants grown in liquid system, which allowed a “stress free” access to the root samples.

2.4 Protein sample preparation

Leaf samples from all experiments were harvested with a cork borer with 15mm diameter while roots where blotted dry gently and cut with a scalpel. All plant material was frozen in liquid nitrogen within 30 seconds. Each sample consisted of equal amount of plant material pooled from several individuals (15 individuals for the experiments in liquid system and 35 for experiments in soil system). All samples were harvested in duplicate; the duplicates were processed independently as technical replicates.

Sample preparation for 2-DE and the composition of the extraction buffer were previously optimised for all samples [82,83]. After grinding in liquid nitrogen, over night precipitation with 10%TCA, 1%PVPP, 0.07% ME in acetone and two times washing with 0.07% ME in acetone, samples were extracted in sample buffer (1.5ml/0.1g tissue powder for the leaf samples and 1ml/0.1g tissue powder for the root samples) and sonicated for 30 min in a water-bath sonicator. Leaf proteins were extracted in buffer containing 7M urea, 2M thiourea, 100mM DTT, 2% OG and 0.5% Pharmalyte 3-10. For the extraction of root proteins, the sample buffer contained 7M urea, 2M thiourea, 2mM TCEP, 50mM DTT, 2% DM and 0.5% Pharmalyte 3-10. After centrifugation the supernatant was collected and stored at -76°C until IEF and the pellet was further processed for the extraction of proteins with low solubility. After two times washing with 40mM pH8.8 Tris-HCl buffer for the removal of urea, the pellet was extracted in hot (95°C) SDS buffer (0.4M pH8.8 Tris-HCl, 2% SDS, 3% EDTA, 1% DTT), sonicated for 30 min in a water bath sonicator and boiled for 5 min. Proteins were alkylated with 4% IAA (20mg solid IAA were added to

500µl extract and incubated in dark, at room temperature for 30 min) and separated by SDS-PAGE. Protein concentration was measured using the RC-DC Protein Assay (Bio-Rad).

2.5 Two-dimensional gel electrophoresis

Samples were applied by cup-loading on IPG strips previously rehydrated for at least 12h. Cups were applied near the cathode for the 4-7 IPGs (24cm) and near the anode for the 6-11 IPGs (18cm). Leaf proteins were focused in buffer containing 7M urea, 2M thiourea, 100mM HED, 2% OG, 0.5% Pharmalyte 3-10 and 0.002% BPB. In the case of root proteins, 2% CHAPS was used during focusing instead of 2%DM. Acidic root proteins were focused in buffer containing two reducing agents, as in the extraction buffer (2mM TCEP and 50mM DTT) but basic IPG strips were rehydrated with buffer containing 100mM HED, as for leaf proteins. 10% isopropanol was added to the rehydration buffer for the basic samples. Focusing was performed at 20°C and 50mA/IPG gel strip in an Ettan IPGPhor Cup Loading Manifold for a total of 55kVhr or 60kVhr for the acidic and basic gradients, respectively. After focusing, IPG strips were stored at -76°C until the second dimension.

A two step equilibration was performed before the second dimension, 2%DTT being added to the equilibration buffer (50mM Tris HCl pH 8.8, 6M urea, 2% SDS, 30% (w/v) glycerol, 0.002% BPB) in the first step, and 4% iodoacetamide supplementing the same buffer in the second step. The second dimension was performed using Laemmli's buffer system, in 10%, 1mm thick polyacrylamide gels. Silver staining was performed according to Heukeshoven and Dernick [84], with the exception that the acetic acid in the stop solution was replaced with 1% glycine.

2.6 One-dimensional electrophoresis

Proteins with low solubility were separated by SDS-PAGE in 15% 1mm thick gels, using Laemmli's buffer system. Six µg of protein were loaded per lane and gels were stained with silver following the same protocol as for the 2DE gels.

2.7 Image and data analysis

Gels were scanned under blue light at 300dpi, with ImageScanner (GE Healthcare). Spot identification and quantitation were performed with Progenesis SameSpots (Nonlinear Dynamics). In order to compensate for small between-gels variation due to differences in protein load and/or staining, spot volumes normalised per gel were exported and z-scores computed per gel were used for all statistical analysis. One dimensional SDS-PAGE gels were analysed with ImageQuant 5.2 (Molecular Dynamics). All statistical analyses were performed with R2.4.0 [85] using the following packages: *clValid* [86], *vcd* [87], *effects* [88] and *nlme* [89].

2.8 Sample preparation for mass spectrometric analysis

For preparative 2-DE, samples were loaded by paper bridge loading and focusing was prolonged to 65kVhr for acidic gradient and 72kVhr for basic gradient. Gels were stained with Colloidal Coomassie G-250 staining according to Anderson [90] or with MS-compatible silver staining. Protein spots cut from the Coomassie stained gels were destained with 40% ethanol / 50mM ammonium bicarbonate; spot cut from silver stained gels were destained in a 30mM potassium hexacyanoferrate / 100mM sodium thiosulphate 1:1 mixture. After dehydration with acetonitril, drying for 30 min under vacuum, reduction and alkylation [91] spots were washed and dehydrated with acetonitrile, dried again under vacuum and stored at -20°C until analysis.

3 Results

3.1 Infection of *Fagus sylvatica* seedlings with the root pathogen *Phytophthora citricola*

Infection symptoms following the inoculation with the root pathogen *P. citricola* were recorded at leaf level for the experiments in soil system and at root level for the experiments in liquid system. Root tip browning was observed after 4-6 days from inoculation, while leaf wilting of plants infected in soil

system was recorded 7 days after inoculation (one day after the end of the flooding period). In the case of plants infected in soil system, only one leaf was sampled from apparently healthy plants when first wilting symptoms were recorded in the infected lot, allowing us to continue the record of above ground symptoms (Figure 1). 90% of the inoculated plants showed wilting symptoms within the first two weeks from inoculation and all except one had clear symptoms before 46 days (40 days from the end of the flooding period). All plants died within 2-3 days from the appearance of first symptoms. Only two control plants showing symptoms different from the infected plants died within this period (at 21 and 46 days from inoculation, respectively).

3.2 Protein regulation following pathogen attack and wounding

Proteins with low solubility separated by SDS-PAGE did not exhibit any consistent differences in expression levels between the control and treated samples neither in the infection nor in the wounding experiments (not shown).

Protein spots separated by 2-DE and regulated following stress were identified based on two criteria. Firstly, differences in protein expression were assessed between control and treatment samples using t-tests (paired t-tests in case of samples originating from the same plants, as in the wound experiment) or linear mixed effect models [92] (for nested designs as in the infection experiment in liquid system) with the z scores of spot normalised volume as dependent variable, and the runs nested in samples as random factors. Secondly, the protein spots that exhibited statistically significant differences between the control and treated plants were subjected to further statistical investigation in order to establish if the observed fold variation could originate solely from biological and/or experimental variation.

The level of spot volume variation that can occur by chance alone between biological replicates was expressed as the 95% quantile of the fold regulation distribution estimated for each spot based on pairs of two replicates randomly chosen form a pool of six independent biological replicates. This quantile was used together with each spot's parameters to calculate spot-specific levels of intrinsic variation (see Supplementary material 1), used as thresholds above which differences in spot volumes between control and treated samples can be considered to represent protein expression regulation in response to stress.

Protein spots exhibiting both statistically significant differences and a fold variation that exceeded the spot-specific threshold calculated as described above were considered regulated in response to the treatment applied (pictures in Supplementary Material 2).

Additional controls were designed in order to distinguish protein spots which belonged to the *P. citricola* proteome among the spots that appeared up-regulated or induced on the 2-DE gels of the infected root samples. Investigation of 2DE protein expression patterns characteristic to different developmental stages of *P. palmivora* revealed that only approximately 1% of proteins are specific to a particular stage [93]. We therefore considered that the 2-DE pattern established for *P. citricola* grown in liquid culture would constitute an appropriate control for identifying the majority of proteins expressed by the pathogen. For this, samples of *P. citricola* grown in liquid culture were processed and separated on 2-DE gels in parallel with the *F. sylvatica* samples. To make the correct assignment of spots to the *P. citricola* or *F. sylvatica* proteome possible, a 1:1 mixture of their protein extracts was separated in parallel and matched to the individual gel samples.

Out of the protein spots that appeared as up-regulated or induced in the root samples particularly during the late stages of infection, 86 spots (50 on the acidic pH gradient and 36 on the basic pH gradient) clearly matched spots of the *P. citricola* proteome, all of them prominent spots both on the *P. citricola* gels and on the *F. sylvatica/P. citricola* 2-DE gels and were therefore eliminated from further analyses.

3.3 Patterns of protein expression following pathogen attack and wounding

A total of 188 protein spots (142 leaf proteins and 46 root proteins) exhibited significant changes in their level of expression following root infection with *P. citricola* or wounding (Table 1). Among them, 134

were specifically regulated following pathogen attack, 36 specifically responded to wounding and 18 responded to both stressors (Figure 3, Supplementary material 2).

In order to identify and compare general and specific patterns of protein expression in response to root infection and leaf or root wounding, proteins were clustered on the basis of their fold regulation and expression patterns in all experimental variants (Figure 2). The clustering method was selected using clValid package [86]. Proteins were clustered by hierarchical clustering based on a similarity matrix calculated using Pearson correlation coefficient. Clusters and heat maps (Figure 2) were built with vcd package [87] using the fold regulation values for each protein and experimental variant respectively. The fold regulation values were positive for the up-regulated proteins and negative for the down-regulated proteins and were standardised per protein on a scale [-1, 1]. Values corresponding to experimental variants which did not satisfy both the threshold and the significance criteria were replaced with "0". In the clustering were included all proteins found to be regulated (both criteria satisfied) following any of the applied treatments as well as four proteins that satisfied the threshold criterion and exhibited marginally significant differences in their expression in several experimental variants. The cluster also included four protein spots with marginally significant regulation, suspected to be isoforms of neighbouring spots already selected as being regulated following one of the applied treatments. The clusters revealed several patterns of protein expression regulation in response to infection and wounding, as illustrated in Figure 3.

3.4 Defence response against *Phytophthora citricola*

Plants grown in liquid system responded to infection with the local regulation (roots) of 34 protein spots (22 up-regulated and 12 down-regulated) and the systemic regulation (leaves) of 48 protein spots (31 up-regulated and 17 down-regulated). Protein expression patterns were temporally specific. Different sets of proteins were systemically regulated during early and late stages of infection (12 and 36 proteins, respectively). The local response during early and late stages of infection (consisting of 8 and 30 proteins, respectively) shared only four regulated proteins (three up-regulated and one down-regulated) (Figure 2).
A higher number of proteins were systemically regulated following infection in the leaves of plants infected in soil system: 102 protein spots (of which 65 up-regulated and 37 down-regulated). Of these proteins, a total of 30 (23 up-regulated and 7 down-regulated) responded both in soil system and in liquid system experiments (Figure 2). Overall, similar proportions of the local (leaves) and systemic (root) response to infection consisted in up-regulation (65% and 67% respectively) or down-regulation (35% and 33% respectively) of protein expression (Figures 2 and 3; for pictures, see Supplementary material 2).

3.5 Defence response against wounding

31 protein spots significantly changed their level of expression following root wounding (16 local – roots and 15 systemic – leaves) and 26 following leaf wounding (2 local, 14 systemic and 10 both local and systemic – all leaves). Systemic response to the wounding of roots and of leaves shared only three protein spots, all of them down-regulated. Individuals "witnessing" wounding of the neighbouring seedlings (denoted "listening tree" in Figures 2 and 3) also exhibited changes in their protein expression patterns, a large part of these changes overlapping with the local or systemic response to infection or wounding (22 protein spots of which only three specific) (Figures 2 and 3).
We found only limited evidence for a vertical asymmetry in the systemic response: two protein spots, slightly stronger up-regulated in leaves below the wounded leaf as compared to leaves above the level of the wounded leaf (for pictures, see Supplementary material 2).

3.6 Overlaps in the *Fagus sylvatica* response to infection and wounding

Local infection-induced and wound-induced protein expression patterns partly overlapped: five spots, of which three exhibited opposite directions of regulation in response to the two different stressors. Similarly

shared responses were observed at systemic level between plant reactions to infection and to root (seven proteins) and leaf wounding (six proteins) (Figures 2 and 3). However, most of the stress-responsive proteins (90.4%) were stress specific.
Roots and leaves expressed different sets of stress regulated proteins following both infection and wounding, in full accord with previous investigations at transcriptome level, which showed that plant response to different types of stress is largely organ-specific [2].

4 Discussion

4.1 Symptoms of *Fagus sylvatica* infection with *Phytophthora citricola*

Among *Phytophthora* species, *F. sylvatica* is particularly sensitive to *P. citricola*, which severely damages plant's root system causing local necrosis [67,68]. The pathogen exhibits a higher capacity to colonise *F. sylvatica* phloem tissues laterally as compared for example to species usually recognised as aggressive like *P. cambivora* and *P. cinnamoni* [32]. *P. citricola* causes 100% root rot in young seedlings as compared to 80% root damage produced by *P. cambivora* and less than 25% caused by *P. syringae* and *P. undulata* [64]. The same study found the decrease of water use efficiency to be more severe in saplings infected with *P. citricola* than in saplings infected with any of the other three pathogens [64].

P. citricola isolates have been morphologically and/or genetically divided into several groups, suggesting that they form a species or subspecies complex [34,35,41,94]. The virulence largely differs among types and isolates [34] and plant susceptibility to infection varies between species and ecotypes [95] as well as seasonally [32]. Several tree species have been found to exhibit increased susceptibility to *P. citricola* during the period of maximum growth, e.g. *Juglans hindsii* [43], *Persea americana* [44]. The incidence of *Phytophthora* mediated diseases is significantly increased when infection is associated with other environmental stressors [33,37] like flooding, particularly when this occurs in the period of active plant growth [32,43,44]. The amplification of the hydrological cycle [96] predicted to result from climatic changes are thus likely to amplify the risks associated to such diseases in both natural and managed ecosystems (reviewed in [97]).

Morphologically, the infection of *F. sylvatica* roots with *P. citricola* causes a significant reduction of the belowground biomass i.e. a reduction with almost 50% of the fine root length (<0.5mm diameter) [67,68]. Above ground symptoms are generally unspecific, consisting mainly in chlorosis and wilting as for example in *Juglans* seedlings [68] or *Fagus* [64]. At physiological level, the infection results in weakened rhizosphere activity and nutrient uptake [67,68] accompanied by a severe reduction of photosynthesis and transpiration [64] and a reallocation of nutrients out of the damaged roots systems [67]. Plants were also shown to suffer from severe drought [64] and oxidative stress [68] symptoms.

In our experiments, *F. sylvatica* seedlings infected with *P. citricola* exhibited root and leaf symptoms similar to those observed in the studies described above. In the liquid infection system root tip necrosis was observed in the present study after 4-6 days from inoculation with *P. citricola*. Leaf wilting symptoms were first recorded after 7 days post-inoculation, and continued to appear until 45 days in the soil infection system. After 48 days from the on-set on the experiment, all but one plant have died. This high rate of mortality (99.5%) of infected seedlings is in accord with previous reports on the high sensitivity of young *F. sylvatica* seedlings to in infection with *P. citricola* as compared to 1 year old saplings [64].

4.2 Protein regulation following pathogen attack and wounding

Two criteria were used for the identification of differentially expressed proteins: proteins had to exhibit statistically significant differences in their level of expression between control and treated samples, and their fold regulation had to exceed a spot-specific threshold of variation that could appear by chance alone

in the study population. This method is a rather conservative approach for identification of differential protein expression. We preferred such an approach for our experiments because it is restrictive in what concerns the number of false positives resulting from experiments performed on low number of replicates.

The efficiency of few clustering methods in the identification of patterns of protein expression across different treatments was tested (not shown). The best results were obtained when the fold regulation values were standardised per protein as described in the results section (3.3). This approach represents a slight modification of the method suggested by Lippert *et al.* [76] and can reveal patterns of protein expression independent of the absolute protein abundance and fold regulation.

4.3 *Phytophthora*-induced responses

Ample changes in *F. sylvatica* protein expression were induced following root infection with *P. citricola* at both local and systemic levels. Although relatively more proteins were regulated as part of the systemic response to root infection, it is possible that the amplitude of the local response was underestimated due to technical limitations. The simultaneous separation of *F. sylvatica* and *P. citricola* proteomes on the same gel in the case of infected roots resulted in a high level of spot overlap. Although additional controls were designed to circumvent this issue (see section 3.2) it is still likely that many regulated protein spots could not be detected. Narrow range IPGs could improve the detectability of such spots [98], if enough biological material is available and/or if sample prefractionation methods are used prior to 2-DE [99].

Comparatively more systemic stress-responsive proteins were identified in the leaves of plants infected in soil system. This was most likely due to the higher number of biological replicates which increased the confidence of the differential expression identification. Systemic response to infection of plants from the liquid system and soil system experiments shared 30 proteins, representing 63% and 29% of the protein regulated in the two types of experiments, respectively. The differences in stress-induced expression patterns between different experiments are not unexpected, since it is known that plant's response to stress is largely determined by the physiological status and environmental/experimental conditions [2].

As much as 16% of the proteins regulated following *Phytophthora* infection were also regulated following plant wounding (Table 1). This suggests the capacity of *Phytophthora* infection to elicit not only classical pathogen-induced plant defences but also herbivore/wound-induced reactions. Indeed, *Phytophthora* species were confirmed to induce both SA-dependent and independent responses in several host species. Soybean for example up-regulates in response to elicitation with the β-1,3/1,6 glucan elicitor from *P. sojae* cell wall (WGE) not only the expression of PR-1a protein but also of several JA/ET dependent PR proteins which are induced by wounding (PR-2, PR-4 and PR-10) [100]. During an incompatible interaction with tobacco, PB90 - a 90kDa protein elicitor of *P. boehmeriae* - induces HR and SAR to fungal, bacterial and viral pathogens [101]. Both reactions were shown to involve formation of H_2O_2. Although the elicitor induces local and systemic accumulation of SA, only SAR is mediated by SA [102]. PB90 induces both tobacco PR-1a (SA-dependent) and PR-1b (JA-activated), suggesting the activation of either SA-dependent and SA-independent signal transduction pathways [102].
Tobacco plants infected with *P. parasitica nicotianae* [103] and *Solanum* cultivars infected with *P. infestans* [104] up-regulate the expression of lipoxygenase (LOX) during compatible as well as incompatible interactions. LOX is presumably involved in JA-dependent defence against pathogens. The earlier [103] and stronger [104] activation of LOX during incompatible as compared to compatible interactions supports its contribution to plant resistance.
Another class of elicitors secreted by the majority of *Phytophthora* species are the elicitins, 10kDa sterol carrier proteins [105-107] known to induce HR and SAR [108]. Elicitins are translocated through the plant [109,110] and induce local and systemic synthesis of both acidic (SA-dependent) and basic (ethylene-dependent) PR

proteins [110]. SAR induction by elicitins is SA-dependent [110] and might not require a plant messenger but could be mediated by the translocated elicitin itself [109].
A glycoprotein elicitin from *P. megasperma*, inducer of HR and SAR in tobacco was shown to increase local but not systemic O-methyltransferase (enzyme on the phenylpropanoid pathway) activity [111]. The same elicitin causes local (PR1, PR2, PR3 and PR5) and systemic (PR1, PR2 and PR5) accumulation of acidic (SA-dependent) PR proteins as well as local accumulation of basic (ethylene dependent) isoforms (PR1, PR2, PR3 and PR5), suggesting that multiple defence pathways are activated also in this pathosystem. An elicitin isolated from *P. citricola* (citricolin) was shown to cause necrosis and to decrease gas exchange in tobacco leaves [112]. Results from the same study obtained on tobacco cell suspension culture suggest that citricolin might also be responsible for inducing oxidative burst [112]. However, none of the citricolin's effects on tobacco have been observed on *F. sylvatica*.

Significant quantitative and qualitative differences have been observed between the responses to *Phytophthora* of genotypes characterised by different levels of tolerance. For example, resistant varieties of *Capsicum annuum* respond to *P. capsici* elicitors with higher accumulation of lignin and activation of peroxidases, as compared to the sensitive varieties [113]. A PR protein with peroxidase activity is also specifically activated in the resistant variety.
Gene expression patterns in response to infection with *P. infestans* were shown to differ between *Solanum* cultivars with different levels of resistance [114,115]. These cultivars exhibit differences in the constitutive expression of proteins with a putative role in the resistance e.g. PR-1a (SA-dependent), PR-1b (JA-activated) and a lipid desaturase (modulator of JA signalling) are expressed at higher level by the resistant cultivars [114]. Additionally, the susceptible cultivars induce a specific set of wound responsive genes in response to infection [114], possibly as result of the pathogen's growth inside the plant's tissue.
Early stages of susceptible interactions (e.g. soybean - *P. sojae*) do not consist in HR but in a biotrophic phase in which the pathogen colonises host cells and plant defences like phenylpropanoid methabolism and SA-dependent PR1a are activated [116]. This stage is followed by a necrotrophic phase associated with the apparition of necrotic lesions which can subsequently induce SAR [3]. Gene expression patterns change during different stages of the compatible interaction reflecting this shift of the pathogen from the biotrophic to the necrotrophic stage [116]. This could also explain the high temporal specificity we observed in the protein regulation patterns induced by *P. citricola* at both local and systemic levels (Fig. 2).

4.4 Wound-induced responses

Wounding of roots and leaves induced local and systemic changes in *F. sylvatica* protein expression patterns that partly overlapped (Fig. 3). Partial site specificity of plants' response to wounding has been already described for other species[17]. The overlap between the local and systemic response to wounding is also not surprising, since secondary metabolites like phytoalexins as well as proteins like proteinase inhibitors, α-amilase inhibitors, polyphenol oxidase, lectins and basic PR proteins are known to accumulate after wounding locally and systemically [117]. The involvement of volatile signalling in plant resistance to pathogens is however less known. Investigations at transcriptome level suggest that although no accumulation of defensive chemicals or proteins occurs, *Nicotiana attenuata* transplanted close to clipped *Artemisia tridentata tridentata* is primed for defence response against herbivores and accumulates faster trypsin proteinase inhibitors after herbivore attack [118]. Different studies on the same species also found elevated levels of polyphenol oxidase activity and confirmed the increased resistance to herbivores of *Nicotiana* plants that witnessed clipping or herbivore attack on *Artemisia* [119]. Experiments on *Alnus glutinosa* demonstrated that perception of volatile signals results in increased phenolic content and increased catalase and proteinase inhibitors activity [29]. In *F. sylvatica*, all proteins shared between the pathogen induced reaction and the "listening tree" reaction (7 proteins of which 3 also shared with the reaction of wounded plants) exhibited opposite direction of regulation, suggesting a possible antagonistic connection between the two reactions. However, since these effects were observed on different

individuals, further investigations on multiple stressed plants are needed to confirm a possible trade off between the two types of stress response.

Changes in protein expression shared between *F. sylvatica* response to wounding and pathogen attack amounted to almost 60% of the wound-induced response. This extensive overlap could probably explain observations that wounding can induce systemic resistance to pathogens [120] while pathogens can activate wound responses [121] and confer partial resistance to herbivores [122]. This type of resistance is however pathogen and host specific, since wounding does not enhance the resistance of *Theobroma cacao* to infection with *P. megakarya* [123] and wound-activated responses can be inhibited in tobacco by pathogens [124]. Even within the same plant-pathogen-herbivore interaction, cross reactions can be asymmetric [122]. These data indicate that the overlap of the responses to different stressors does not necessarily imply synergic effects [125] but can result in antagonistic effects [126]. This is supported by the fact that SA- and JA-dependent defence pathways are inter-regulated at different levels (e.g. NPR1 [7] or fatty acids [127]) while SA and JA are mutual antagonists [117] and might explain why 13 of the 22 *F- sylvatica* proteins changing expression in response to both infection and wounding were regulated in opposite direction (Table 1, Fig. 3).

5 Conclusions

Plants' needs to simultaneously cope with multiple environmental stressors have evolved in complex and effective defence mechanisms. Signalling and defence pathways against different types of aggressors like pathogens and herbivores are inter-regulated at different levels forming intricate networks designed to ensure a maximal chance of survival with a minimal investment of energy. This high level of inter-regulation was observed at proteome level in *F. sylvatica* as a relatively high degree of overlap in protein expression regulation patterns following infection with the root pathogen *P. citricola* and wounding. Plant responses to the two stressors shared co-regulated proteins as well as proteins differentially regulated at both local and systemic level, suggesting possibly antagonistic effects between the pathogen and herbivore-induced defences.

Table 1 Overview of protein expression regulation following infection with *Phytophthora citricola* and/or wounding.

Expression regulation		Specificity:	Infection	Wound	Unspecific	Total
	Leaves	up-regulated	64	7	4	**142**
		down-regulated	35	19	3	
		opposite*			10	
		total	*99*	*26*	*17*	
	Roots	up-regulated	20	5	1	**46**
		down-regulated	10	6	1	
		opposite*			3	
		total	*30*	*11*	*5*	

*Opposite regulation directions in pathogen response compared to wounding.

Figure 1

Record of above ground symptoms following infection in soil system of *Fagus sylvatica* seedlings with the root pathogen *Phytophthora citricola*.

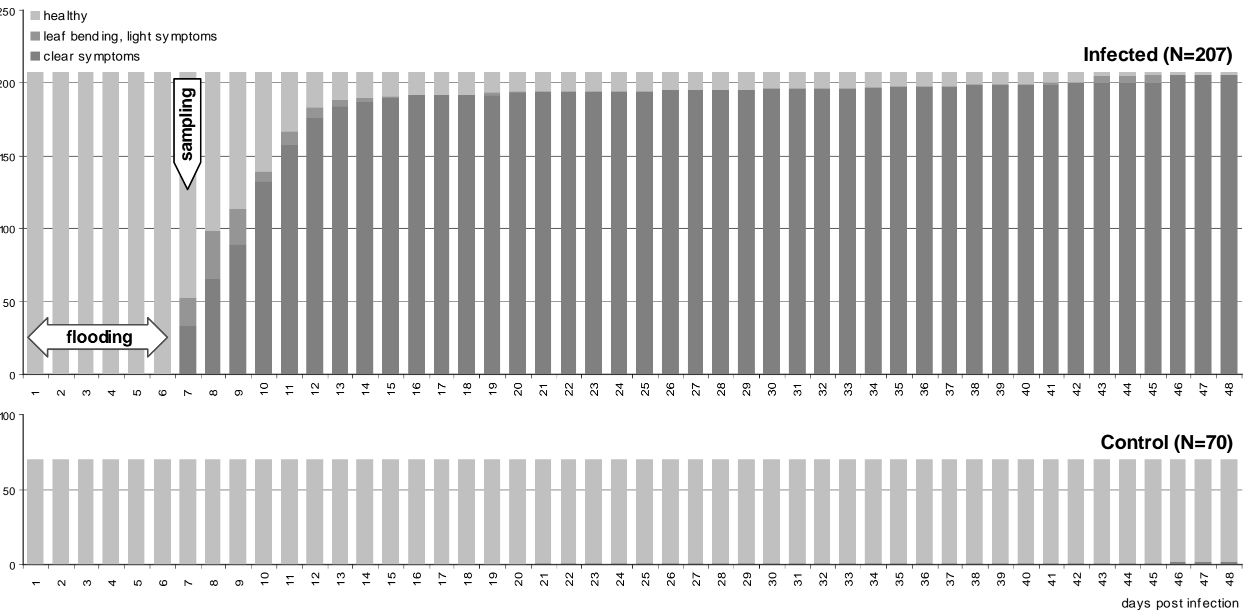


Figure 2

Cluster analysis of *Fagus sylvatica* proteins regulated following root infection with *Phytophthora citricola* and/or wounding.

A. Leaves: 1 - Late systemic response to infection in soil system; 2 - Early systemic response to infection in liquid system; 3 - Late systemic response to infection in liquid system; 4 - Local response to leaf wounding; 5 - Systemic response to leaf wounding; 6 - „Listening trees“; 7 - Systemic response to root wounding

B. Roots: 1 - Early local response to infection; 2 - Late local response to infection; 3 - Local response to root wounding

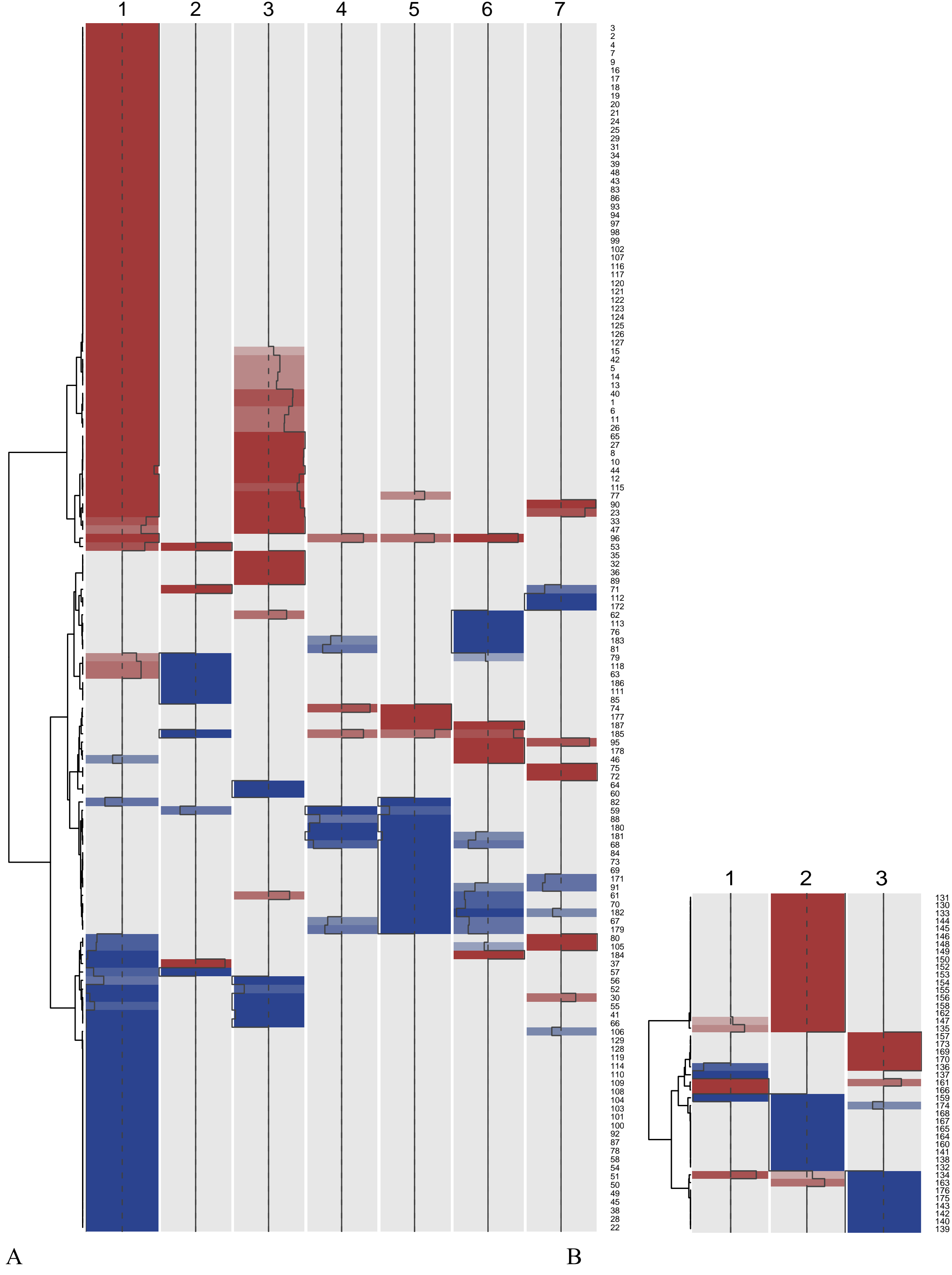
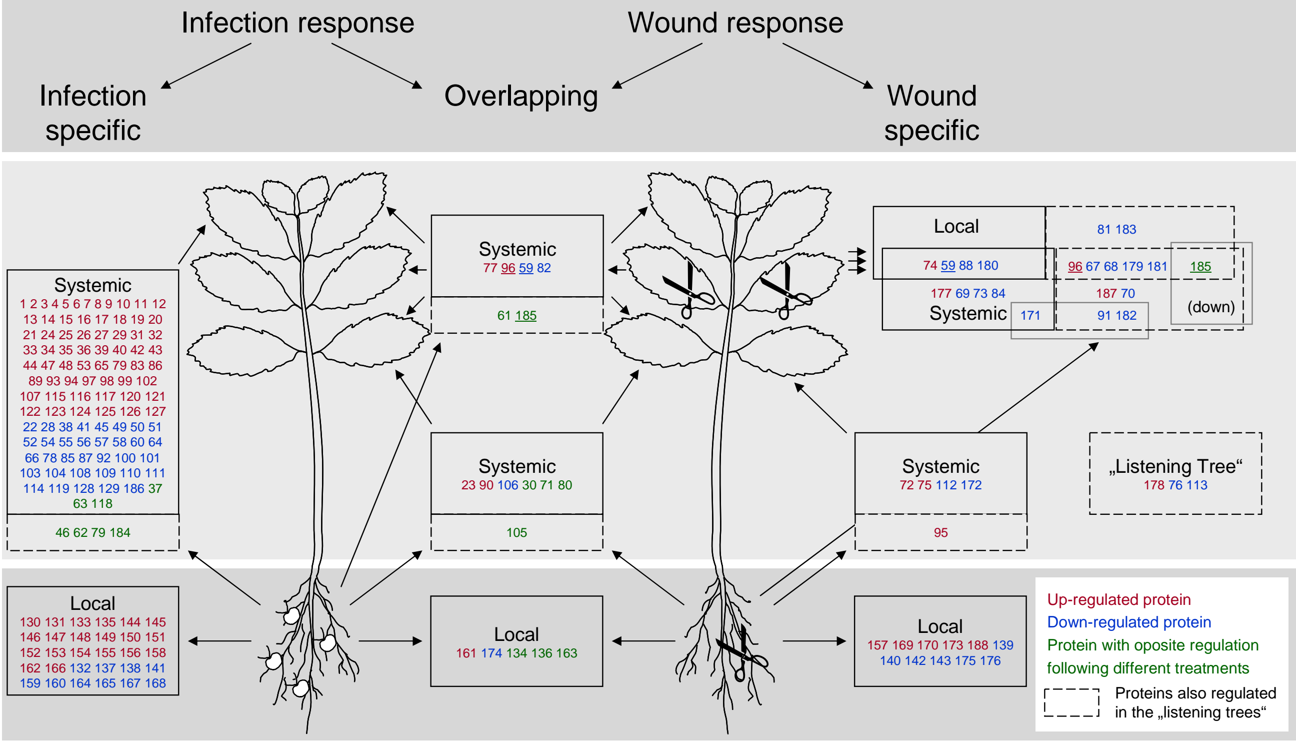


Figure 3

Overview of protein spots exhibiting expression regulation following infection with *Phytophthora citricola* and/or wounding. Spot numbers are the same as in Figure 2 and in Supplementary material 2.



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Supplementary material 1

Predictors of spot volume variation

The level of normalised spot volume variation which occurs solely as a result of biological and experimental variation was established based on a set of 6 independent biological replicates. We used 1396 spots separated on acidic (4-7) pH gradients and 845 spots from basic (6-11) pH gradients. For each spot, two groups of duplicates were randomly extracted from the set of 6 replicate normalised spot volumes. The fold up-regulation between the two groups was calculated as the ratio between their means (the higher over the lower mean). The random extraction was repeated 1000 times per spot and the 95% quantile of the fold regulation distribution was retained for each spot. For each spot, the 95% quantile of the fold regulation (hereafter the 95% quantile) represents the level of fold difference that can occur by chance alone, due to biological and experimental variation in the given experimental conditions.
Further we used this quantile as a measure of intrinsic spot variability. Linear models with the 95% quantile as dependent variable and mean normalised volume, log transformed spot area, circularity and position on the 2DE gel (second order polynomials of x, y coordinates to account for non-linear dependencies) as predictors were fitted separately for the acidic and the basic gradients. Note that, in Progenesis SameSpots, spot parameters (area, x and y coordinates) are invariable per spot, when all gels have been analysed within the same experiment. The distribution of the 95% quantile across all spots was extreme left skewed and therefore the quantile was Box-Cox (Box and Cox 1964) transformed for normalisation ($lambda_{acidic}$ = -1.77, $lambda_{basic}$ = -1.25). The simplified models differed slightly for the spots on the acidic and on the basic gradients.

Minimal model for acidic gradient
95% quantile = 0.4932 - 0.0000156*mean normalised spot volume - 0.04009*log area + 0.1525*circularity -0.2923*x + 0.7894*x^2 + 0.8721*y + 0.9596*y^2

Minimal model for basic gradient
95% quantile = 0.4408 -0.00002166*mean normalised spot volume -0.0351*log area + 0.1453*circularity + 0.00006237*x + 0.06521*y + 0.7358*y^2

Effect displays of the high-order terms in the linear models (Fig. S1 and S2) were built using the R add-on package *effects* (Fox 2006).
These models were further used to calculate the level of expected spot variance based on each spot's parameters and to set spot-specific thresholds above which differences in spot volumes between control and treated samples can be considered to represent protein expression regulation in response to stress.

Figure S1. Effect displays plots of the minimal model for acidic gradient.

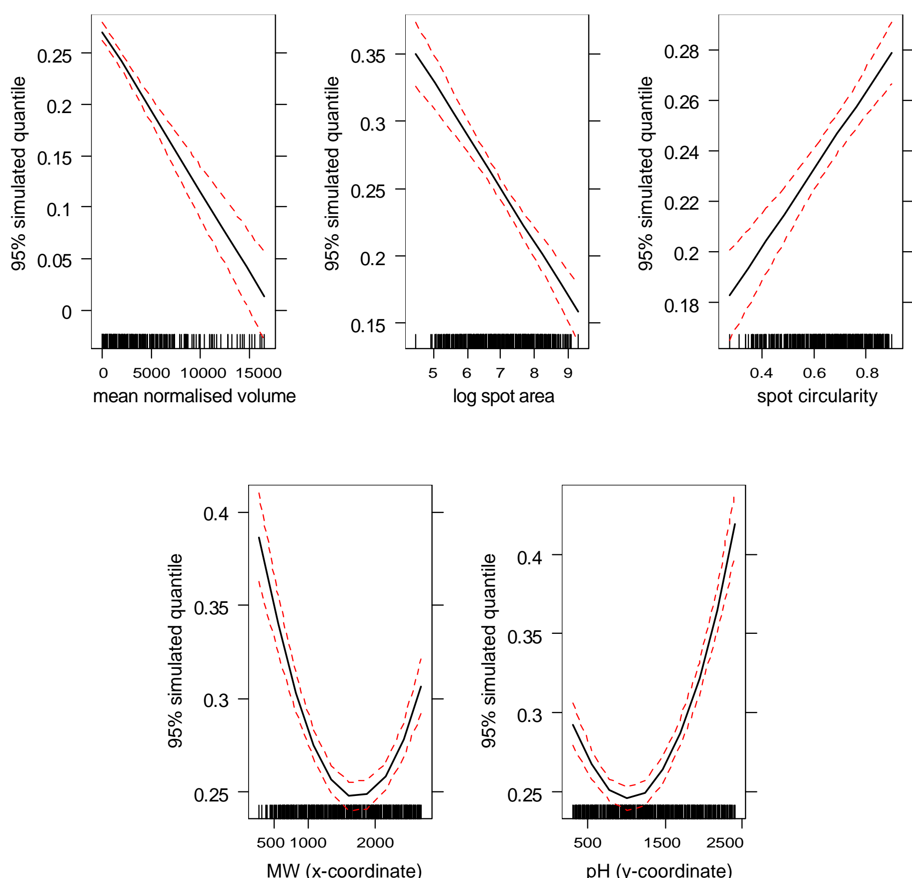
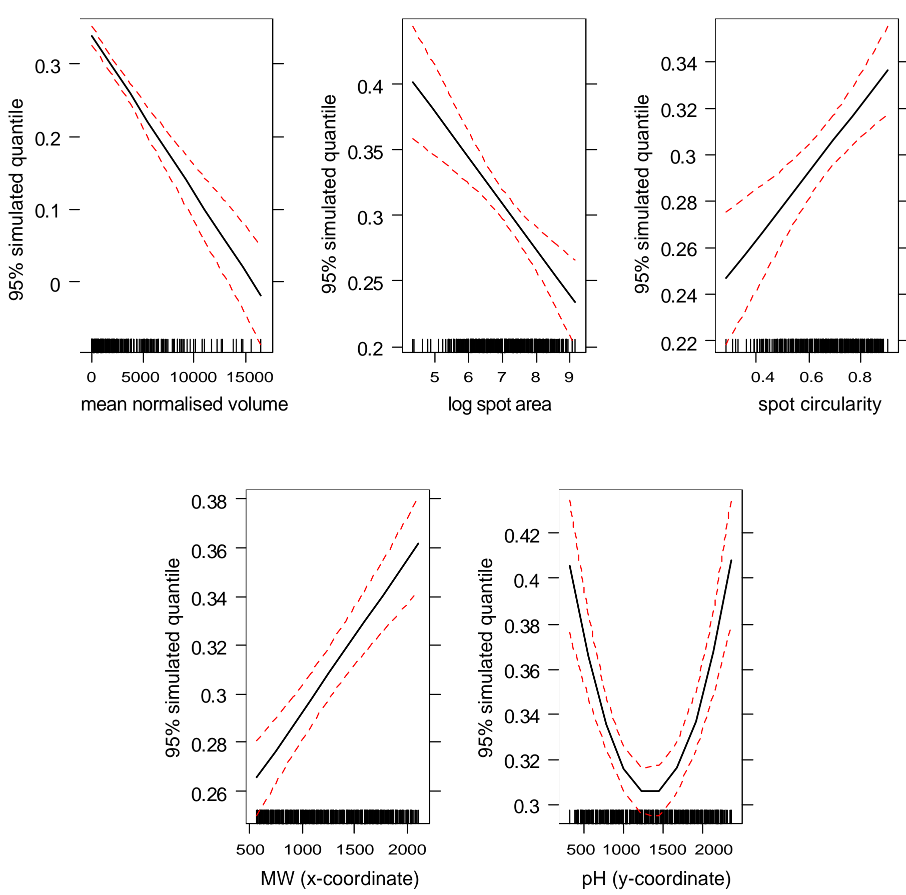


Figure S2. Effect displays plots of the minimal model for basic gradient.



Supplementary material 2

Systemic response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **root infection** with *Phytophthora citricola*: proteins separated on **acidic** IPGs (4-7)

Control seedlings	Infected seedlings	Control seedlings	Infected seedlings	Control seedlings	Infected seedlings

Systemic response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **root infection** with *Phytophthora citricola*: proteins separated on **basic** IPGs (6-11)

Control seedlings	Infected seedlings	Control seedlings	Infected seedlings	Control seedlings	Infected seedlings

Systemic response (leaves) of *Fagus sylvatica* seedlings grown in liquid system to **root infection** with *Phytophthora citricola*: proteins separated on **acidic** IPGs (4-7)

Early response

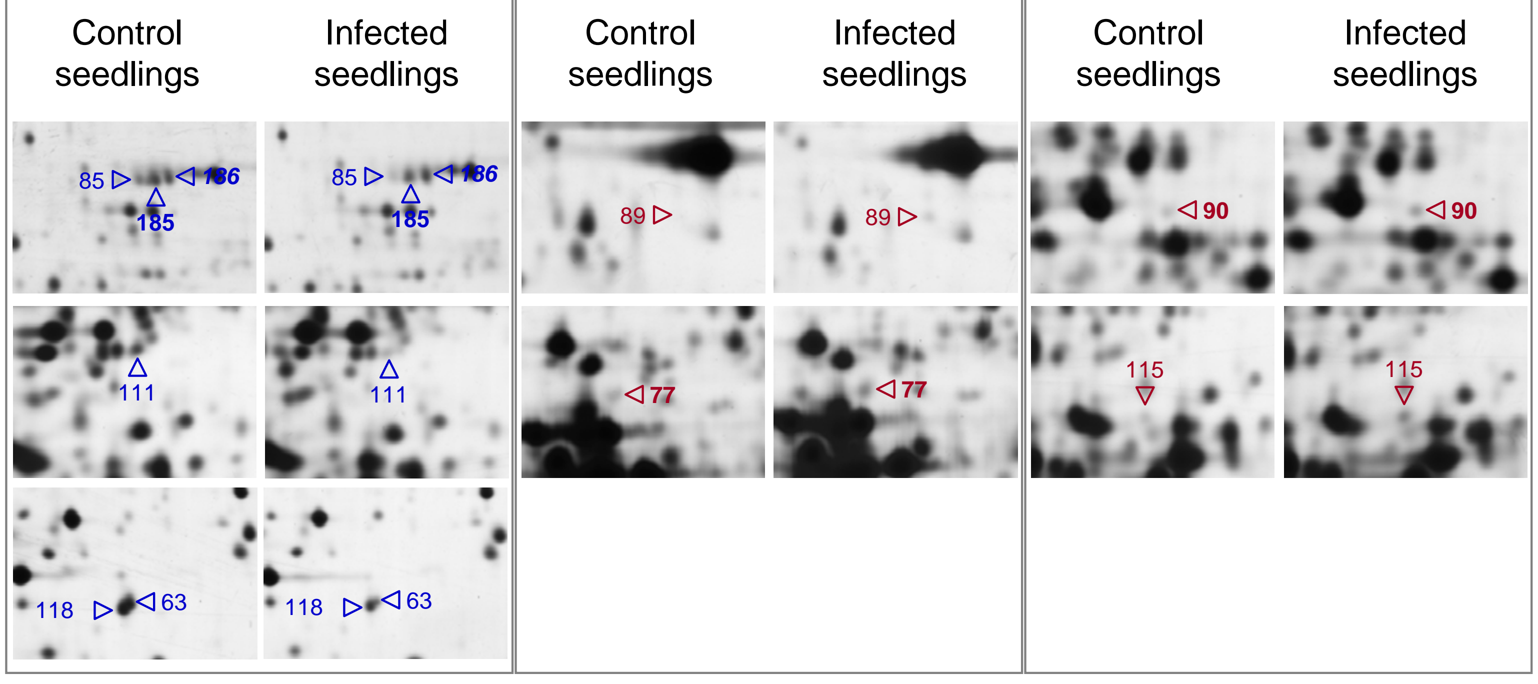
Control seedlings	Infected seedlings
37	37
53	53
57	57
71	71
58	58
59	59

Late response

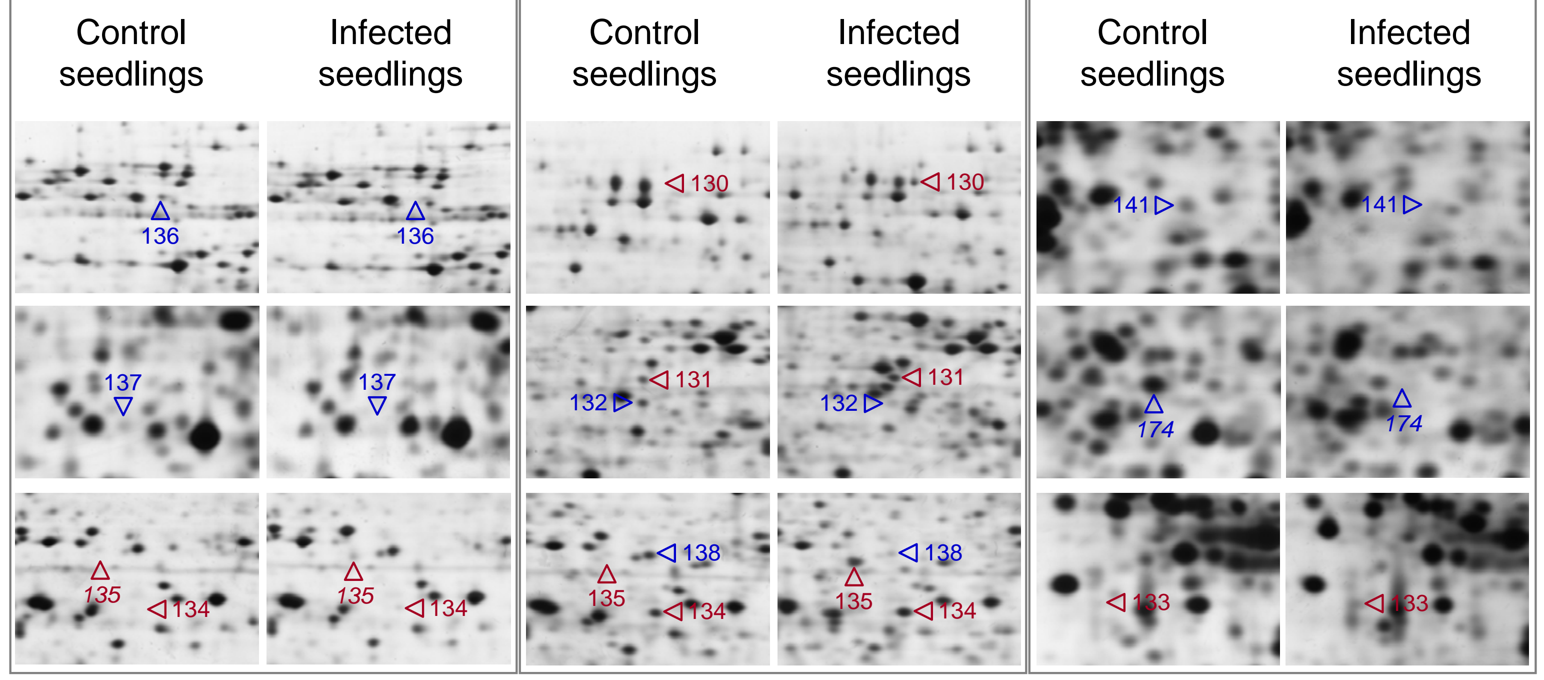
Control seedlings	Infected seedlings
27, 5, 35, 40, 1	27, 5, 35, 40, 1
13, 36, 6, 23	13, 36, 6, 23
8	8
52, 10	52, 10
12, 11	12, 11
14	14
15	15
26	26
30	30
32	32

Control seedlings	Infected seedlings
41, 55	41, 55
42, 47	42, 47
44, 33	44, 33
56	56
60, 64	60, 64
61	61
62	62
65	65
66	66

Systemic response (leaves) of *Fagus sylvatica* seedlings grown in liquid system to **root infection** with *Phytophthora citricola*: proteins separated on **basic** IPGs (6-11)



Local response (roots) of *Fagus sylvatica* seedlings grown in liquid system to **root infection** with *Phytophthora citricola*: proteins separated on **acidic** IPGs (4-7)



Local response (roots) of *Fagus sylvatica* seedlings grown in liquid system to **root infection** with *Phytophthora citricola*: proteins separated on **basic** IPGs (6-11)

Early response

Control seedlings | Infected seedlings

166 159
161

Late response

Control seedlings | Infected seedlings

159 144
145
146 160
165 147 153
148 156
155 149
150 154
151

Control seedlings | Infected seedlings

152
158
162
163
164
168
167

Systemic response (leaves) of *Fagus sylvatica* seedlings grown in liquid system to **root wounding**: proteins separated on **acidic** IPGs (4-7)

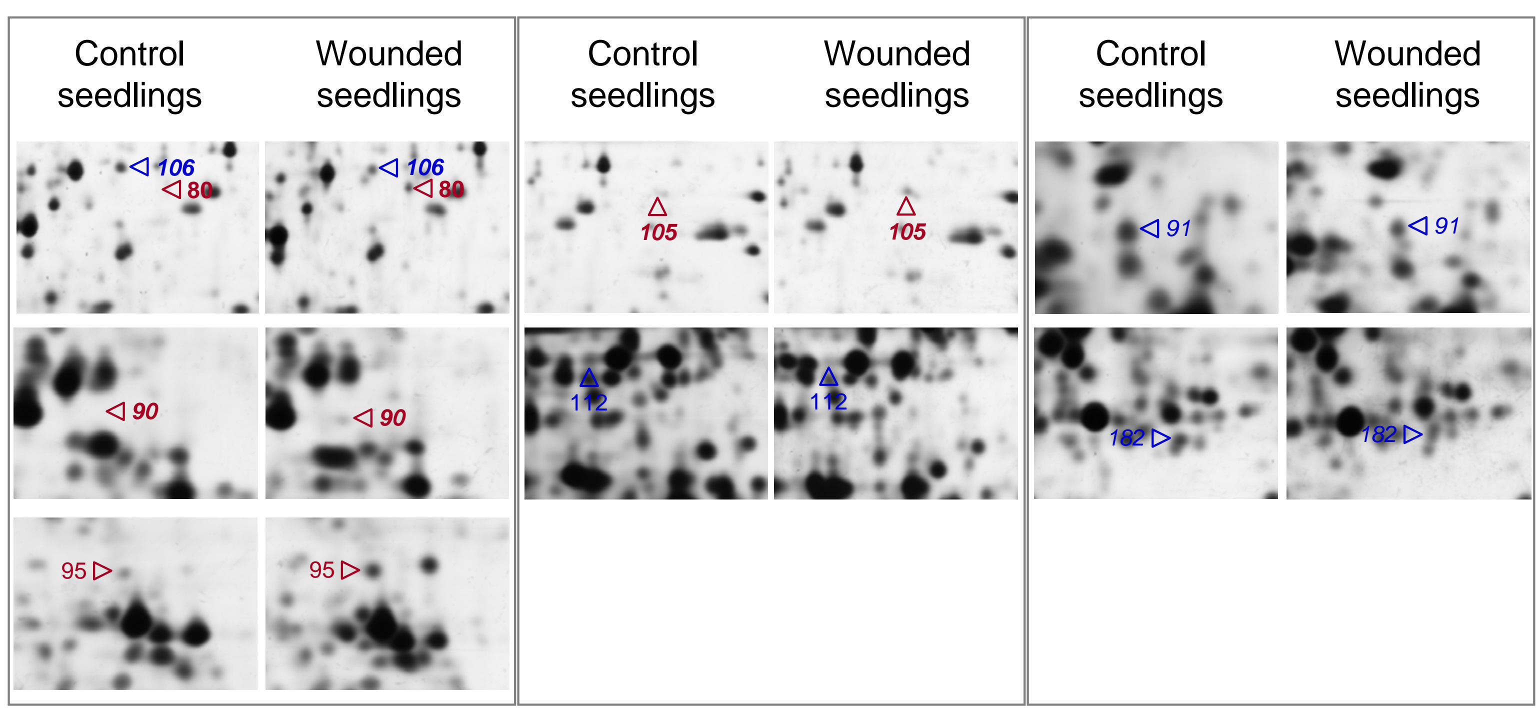
Control seedlings | Wounded seedlings | Control seedlings | Wounded seedlings | Control seedlings | Wounded seedlings

23 23 75 75 71 71

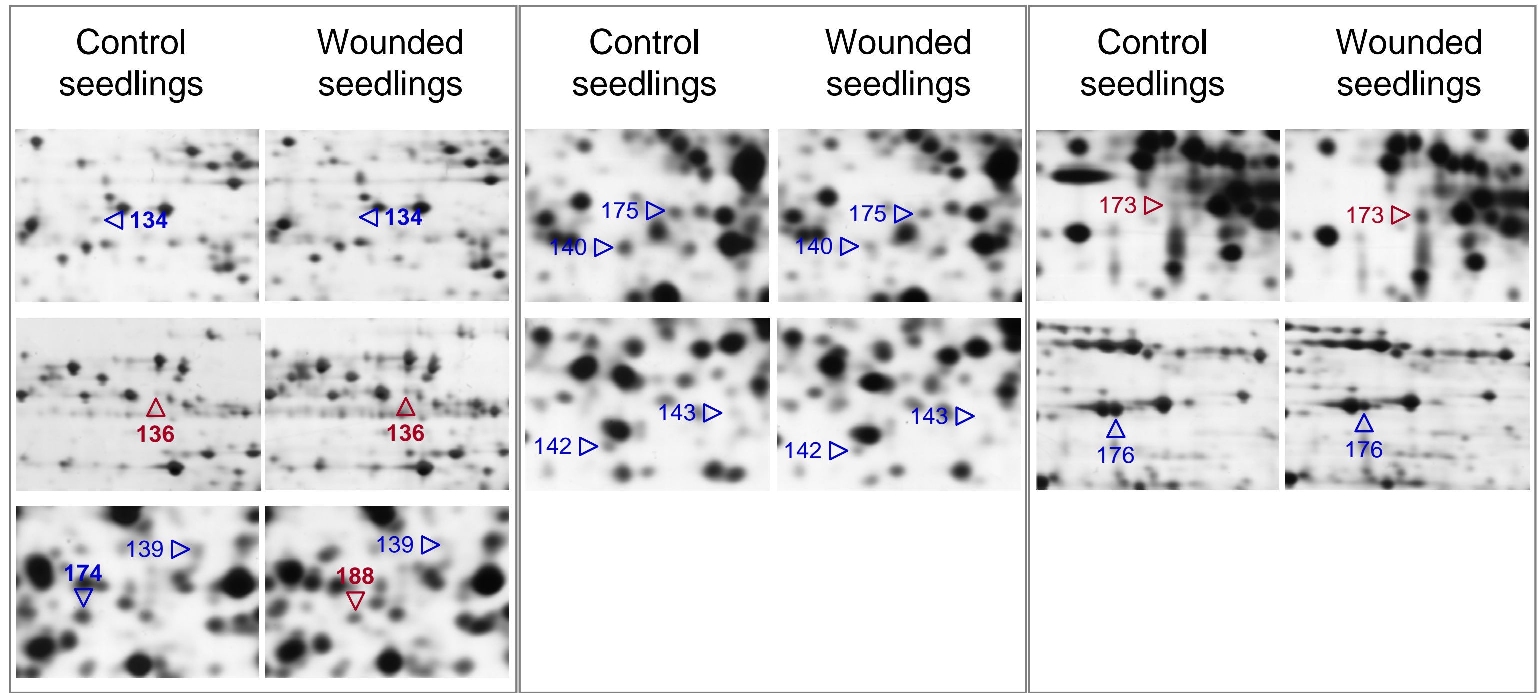
30 30 171 171 172 172

72 72

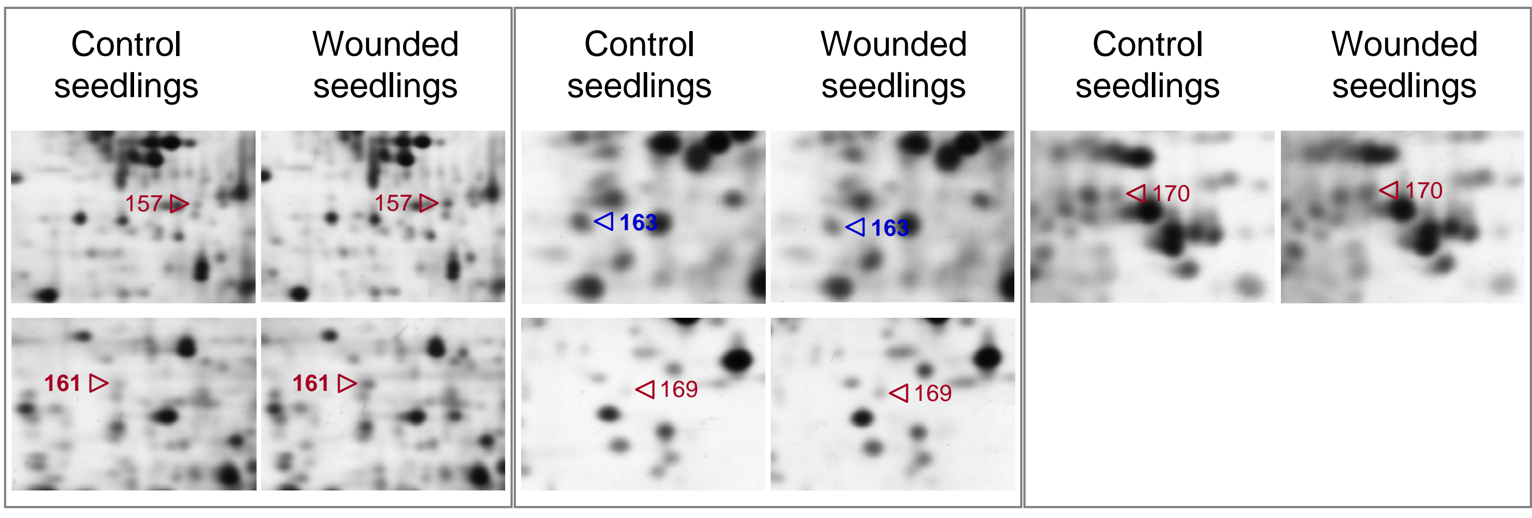
Systemic response (leaves) of *Fagus sylvatica* seedlings grown in liquid system to **root wounding**: proteins separated on **basic** IPGs (6-11)



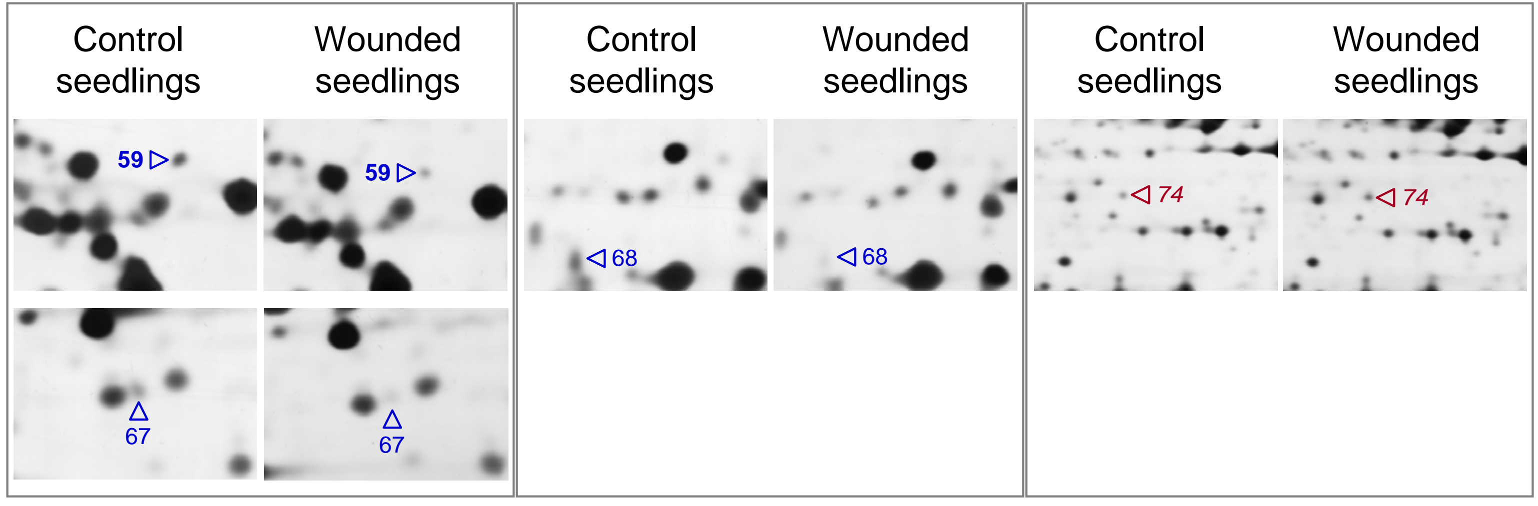
Local response (roots) of *Fagus sylvatica* seedlings grown in liquid system to **root wounding**: proteins separated on **acidic** IPGs (4-7)



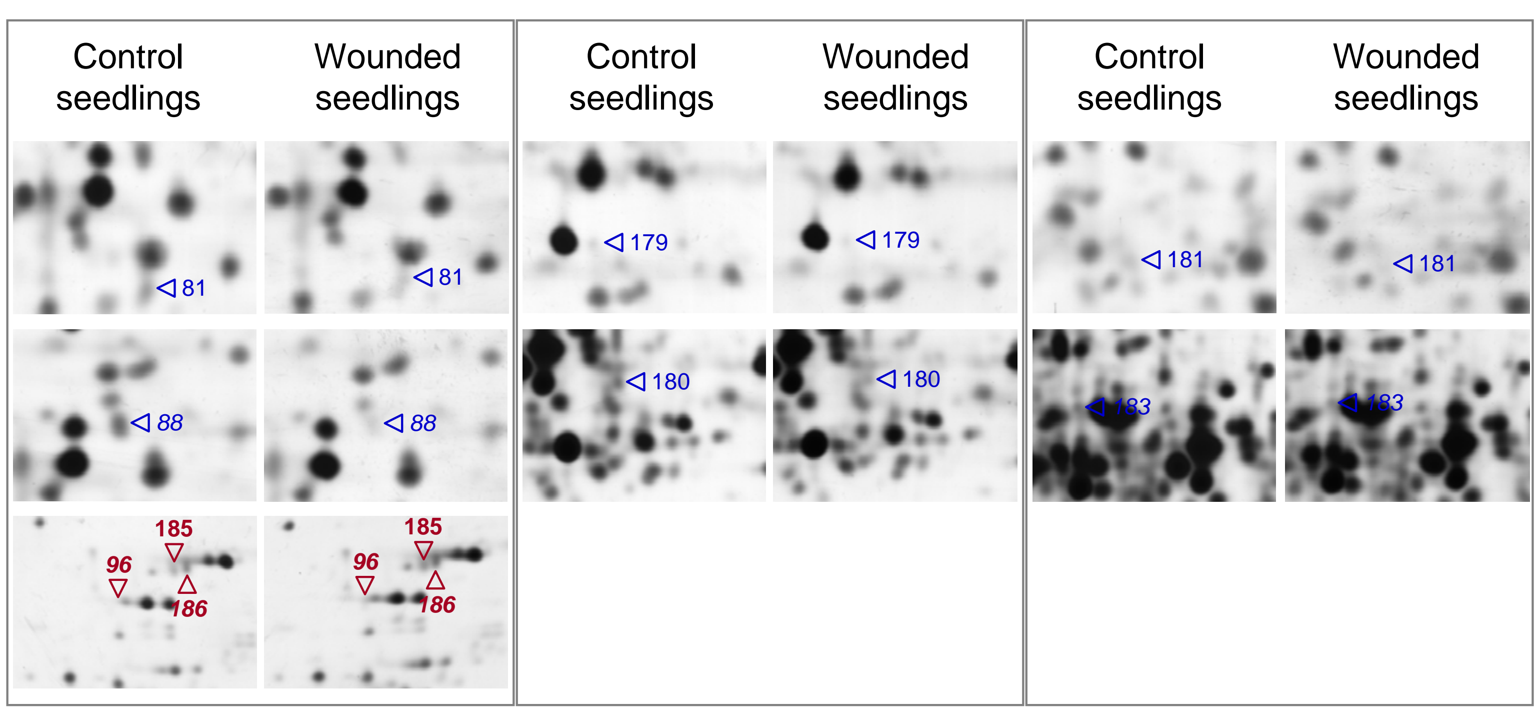
Local response (roots) of *Fagus sylvatica* seedlings grown in liquid system to **root wounding**: proteins separated on **basic** IPGs (6-11)



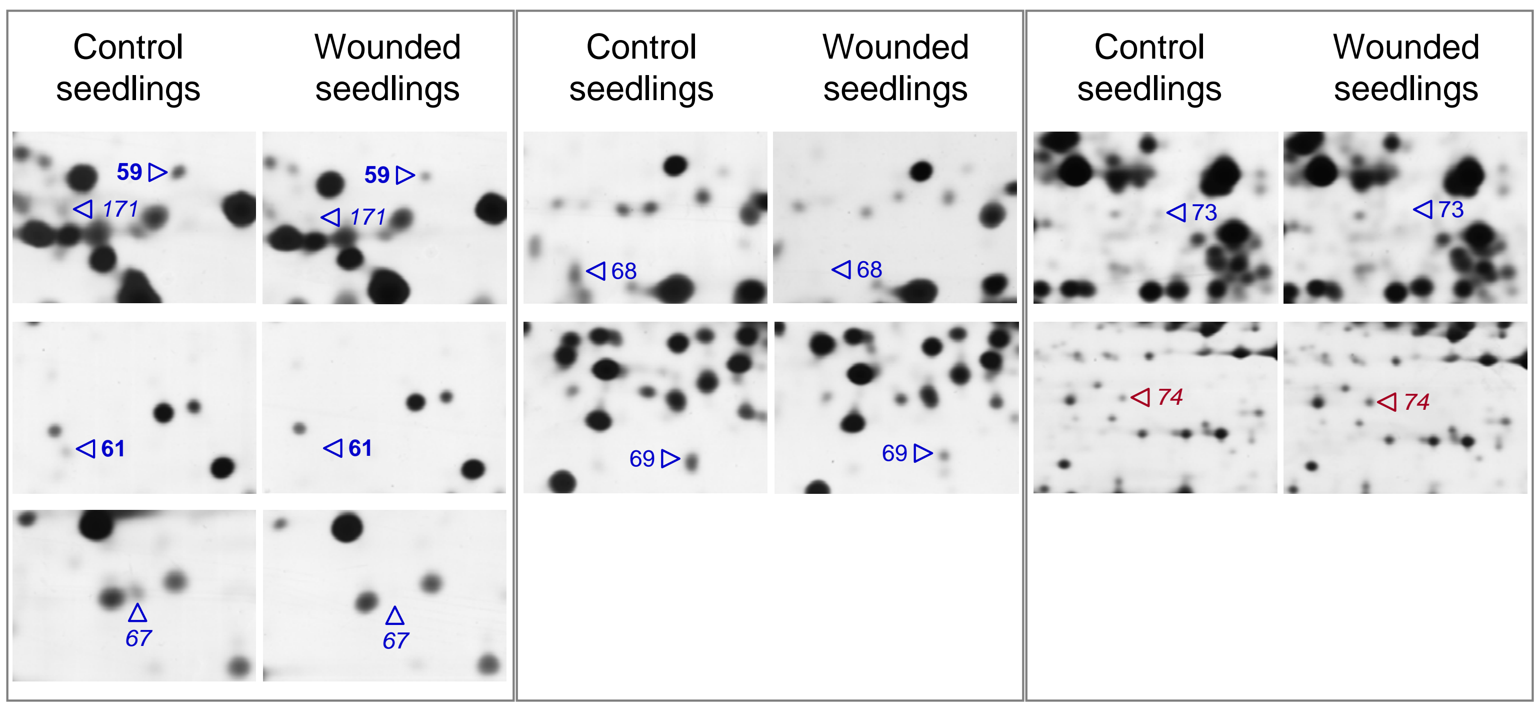
Local response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **leaf wounding**: proteins separated on **acidic** IPGs (4-7)



Local response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **leaf wounding**: proteins separated on **basic** IPGs (6-11)



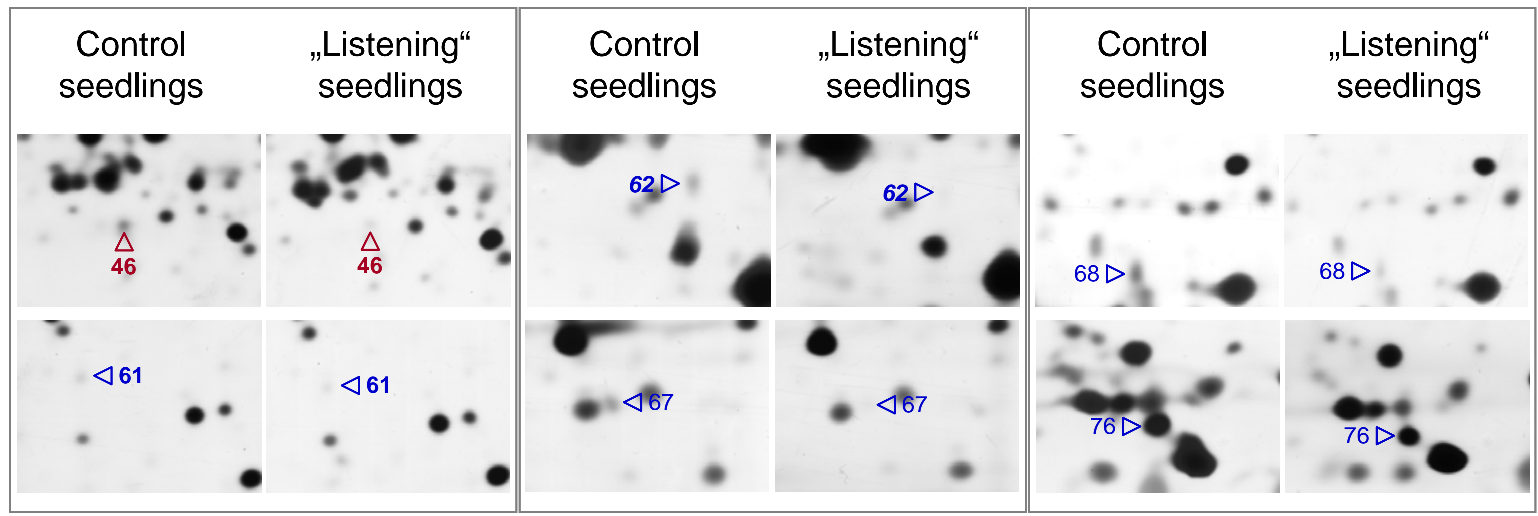
Systemic response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **leaf wounding**: proteins separated on **acidic** IPGs (4-7)



Systemic response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **leaf wounding**: proteins separated on **basic** IPGs (6-11)

Control seedlings
Wounded seedlings
Control seedlings
Wounded seedlings
Control seedlings
Wounded seedlings
180
187
182
70
77
82
91
84
181
88
185
96
186
177
179

Response (leaves) of *Fagus sylvatica* seedlings grown in soil system to the **wounding of neighboring seedlings**: proteins separated on **acidic** IPGs (4-7)



Response (leaves) of *Fagus sylvatica* seedlings grown in soil system to the **wounding of neighboring seedlings**: proteins separated on **basic** IPGs (6-11)

Control seedlings
„Listening" seedlings
Control seedlings
„Listening" seedlings
Control seedlings
„Listening" seedlings
81
81
105
105
183
183
91
91
113
113
187
184
182
70
187
184
182
70
95
178
95
178
179
179
185
96
186
185
96
186
181
181

Response (leaves) of *Fagus sylvatica* seedlings grown in soil system to **leaf wounding** : proteins separated on **basic** IPGs (6-11)

Evidence for asymmetric transmission of wounding signal.
1: leaves above the wounded leaf
2: leaves below the wounded leaf