

Responses of the pea (*Pisum sativum* L.) leaf metabolome to drought stress assessed by nuclear magnetic resonance spectroscopy

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Abstract While many compounds have been reported to change in laboratory based drought-stress experiments, little is known about how such compounds change, and are significant, under field conditions. The *Pisum sativum* L. (pea) leaf metabolome has been profiled, using 1D and 2D NMR spectroscopy, to monitor the changes induced by drought-stress, under both glasshouse and simulated field conditions. Significant changes in resonances were attributed to a range of compounds, identified as both primary and secondary metabolites, highlighting metabolic pathways that are stress-responsive. Importantly, these effects were largely consistent among different experiments with highly diverse conditions. The metabolites that were present at significantly higher concentrations in drought-stressed plants under all growth conditions included proline, valine, threonine, homoserine, myoinositol, γ -aminobutyrate (GABA) and trigonelline (nicotinic acid betaine). Metabolites that were altered in relative amounts in different experiments, but not specifically associated with drought-stress, were also identified. These included

glutamate, asparagine and malate, with the last being present at up to 5-fold higher concentrations in plants grown in field experiments. Such changes may be expected to impact both on plant performance and crop end-use.

Keywords Pea leaf metabolome · Drought-stress · NMR spectroscopy

1 Introduction

A major limitation to yield and quality in many crop species is water availability throughout or at critical times in the growing season (Parry et al. 2005; Morison et al. 2008). In the face of a mild soil water deficit, plants can maintain photosynthesis and turgor for a short period (Boyer 1970; Morison et al. 2008). However, with extended periods of negative soil water potential, plants stop growing, show decreased photosynthesis and initiate a series of measures that ensure their survival and/or re-direct resources to flowering and seed production (Boyer 1970; Lawlor and Cornic 2002; McKay et al. 2003; Forster et al. 2004; Morison et al. 2008). Across the spectrum of mild to severe drought conditions, plants employ a range of specific responses to minimise water loss or increase the rate of water uptake (Morison et al. 2008). These include regulation of stomatal conductance (Davies et al. 2002; Buckley 2005), maintenance of cell turgor and osmotic adjustment (Zhang et al. 1999), and protection of cellular macromolecules, membranes and enzymes from oxidative damage (Shen et al. 1997; Kranner et al. 2002; Srivalli et al. 2003; Quan et al. 2004; Munné-Bosch and Lalueza 2007; Hura et al. 2007). Maintenance of tissue water content by altering developmental processes is also important and includes altered canopy morphology (Davies

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et al. 2002; Morison et al. 2008), suppression of shoot growth (Boyer 1970; Sharp 2002; Davies et al. 2002; Morison et al. 2008), altered leaf senescence (Lefi et al. 2004; Munné-Bosch and Allegre 2004; Harris et al. 2007) and increased root proliferation (Sharp 2002; Fitz-Gerald et al. 2006).

The responses and acclimation of plants to soil water deficit may be governed by the action of small molecules or metabolites, including protective compounds and hormones. Changes in metabolites in response to drought may reflect changes in photosynthesis (Boyer 1970; Lawlor and Cornic 2002; Maroco et al. 2002; Villadsen et al. 2005) or the activation of futile cycles to prevent over-reduction of photosynthetic electron transport chain components, including those contributing to the photorespiratory cycle and the malate valve (Asada 1999; Cornic and Fresneau 2002; Scheibe 2004). Chemical signalling from roots to shoots via the xylem stream to influence growth, timing of reproduction and stomatal function is an important part of plants' early responses to soil drying (Davies et al. 2002; Sharp 2002; Morison et al. 2008). This chemical signalling is achieved principally by several plant hormones (Correia et al. 1997; Davies et al. 2002; Sharp 2002; Munné-Bosch et al. 2002; Trouverie et al. 2003; Munné-Bosch and Allegre 2004; Young et al. 2004; Liu et al. 2006), with the stimulation of secondary root growth in response to soil drying mediated at least in part by brassinosteroid hormones (Müssig et al. 2003).

Several compounds synthesised by plants have been identified as significant in responses to dehydration stress. Variation in plant tolerance to drought is correlated with compounds as diverse as ABA, sugars and ferulic acid (Sanguinetti et al. 1999; Pelleschi et al. 2006; Hura et al. 2007). Maintenance of a positive leaf osmotic potential is achieved by accumulation of so-called compatible solutes such as mannitol, raffinose oligosaccharides (RFOs), trehalose, proline and glycinebetaine (Shen et al. 1997; Zhang et al. 1999; Almeida et al. 2005; Karim et al. 2007; Verslues et al. 2007). These compounds may also protect cellular components such as membranes and enzymes (Shen et al. 1997). The oxidative stress associated with drought can be minimised by an increase in antioxidants such as glutathione, ascorbate and phenolic compounds (Srivalli et al. 2003; Quan et al. 2004; Hura et al. 2007).

Despite the benefits to plants that have been demonstrated for many compounds in the laboratory, to our knowledge, none has been the basis of a commercially available crop genotype with enhanced performance in the field. The many varied observations, based on isolated studies of individual compounds, suggest that a metabolite profiling approach is highly applicable to the characterisation of the range and diversity of synergistic metabolic

responses exhibited by plants resulting from drought-stress under glasshouse and field conditions. Consistency of response in both growth environments would demonstrate a role for specific, possibly novel, drought-induced metabolites that would warrant further investigation. Therefore, the aim of this study was to define a metabolic plant phenotype for the drought-stressed state, information that then could be utilised further to identify genotypes that are differentially responsive and therefore perhaps more tolerant to changes in water availability. In this paper, we report a detailed exploration of variation in the pea leaf metabolome in response to controlled drought-stress under glasshouse and simulated field conditions. The work has focussed on an analysis of leaf responses and is restricted to the vegetative phase of plant growth, prior to the major resource re-allocation that occurs during pod and seed development (Schiltz et al. 2005; Larmure et al. 2005).

The metabolite profiling approach described here employed nuclear magnetic resonance (NMR) spectroscopy as a tool for the unbiased assessment of changes in response to drought-stress. We have previously employed this technique to assess the effect of genetic modification in peas (Charlton et al. 2004). The use of NMR spectroscopy for studying plant metabolism in a holistic manner predates the term "metabolomics" (Fiehn et al. 2000) by some years (Moore et al. 1983; Belton and Ratcliffe 1985; Ratcliffe 1987; Fan 1996). It is now widely acknowledged that metabolic profiling provides a useful and rapid method for assessing the changes that occur in the metabolome as a consequence of the interaction of plant genotype and environment (Messerli et al. 2007). In particular, the use of NMR spectroscopy has been successful in the analysis of field samples of wheat (Baker et al. 2006) and therefore is the method of choice to allow the efficient analysis of a large number of samples that is necessary to deal with the expected intrinsic variability of such material.

2 Materials and methods

2.1 Chemicals

All materials were of analytical grade ($\geq 98\%$ purity) and obtained from reputable chemical suppliers. Specifically, 3-trimethylsilyl[2,2,3,3-D₄] propionic acid (TSP) was obtained from Avocado (Morecambe, UK), deuterium oxide (²H₂O) from Goss Scientific (Great Baddow, UK), sodium azide (NaN₃) from Sigma-Aldrich (Poole, UK), potassium dihydrogen phosphate (KH₂PO₄) and dipotassium hydrogen phosphate (K₂HPO₄) were from BDH (Poole, UK). All metabolite standards were from Sigma-Aldrich.

2.2 Plant materials

A set of transgenic pea lines, expressing a drought-responsive transgene, was included in the experiments to facilitate the monitoring of drought responses. Transgenic lines were derived by *Agrobacterium*-mediated transformation of *Pisum sativum* (cv. Puget) using a construct composed of a drought-responsive promoter from a trypsin inhibitor (*TII*) gene to control the expression of β -glucuronidase (GUS), together with a selectable marker gene, *bar* (Welham and Domoney 2000). Four independent transgenic lines and three null segregant lines (see below) were grown under both drought-stressed and well-watered conditions in the different experiments (PT1 and PT2 were experiments in a polytunnel in two different years; GH1 and GH2 were experiments in a glasshouse in two different years). The null segregant lines lacked the transgene at either T₂ or T₃ generations. The induction of the *TII* gene promoter in roots and, to a lesser extent in leaves, under conditions of drought-stress has been described (Welham and Domoney 2000). Four transgenic lines, 6C:4, 10B:10, 12A:4 and 14A:11, and three null segregant lines, 6C:1, 12A:3 and 14A:4, were used in every experiment (6 replicates of every line, except 12A:3, where 12 were used, per experimental block, see below). Since a null segregant line had not been identified among the segregants of the original 10B:10 hemizygous line, additional 12A:3 seeds were substituted as null segregant controls for this line. Four plants of the equivalent of eight lines were grown in every one of four blocks per treatment (128 plants per treatment). For the PT experiments, non-transgenic plants (cv. Puget) were used as guard plants in the outer rows of the raised beds to minimise edge effects.

2.3 Plant growth conditions and leaf harvesting

The growth medium for plants throughout was John Innes No. 1 compost (Arthur Bowers) with additional grit for drainage (50:1, by volume). Seeds were germinated in jiffy pots and grown for 16 days in a greenhouse before transplantation either to 10 cm² pots, which were maintained in a greenhouse, or to raised beds in a polytunnel at a planting density of 132 plants per m². Plants were transplanted according to a four-block randomisation scheme for each of two treatments (well-watered or drought-stress). All plants were watered normally twice daily for 10 days, after which time a drought-stress regime was applied to half of the plants, i.e. to four blocks within one of the two beds in the polytunnel experiments or to four randomised blocks of pots.

The experiments reported here were all conducted between late spring and during summer months (April–July) using growth facilities on the John Innes site (grid

reference: 181 074). Daily mean temperatures and minimum temperatures for the experiments were as follows (minimum in brackets): 20.4 ± 2.9 (12) for GH1; 24.9 ± 5.5 (6) for PT1; 19.4 ± 5.2 (1) for PT2 and 21.0 ± 2.6 (5) for GH2. Supplementary heat or light were not provided in any experiment. Plants did not require sprays against any diseases. Plant and soil measurements were taken routinely between 14:00 and 15:00 (BST) and, following this, water was applied to plants, where appropriate and as outlined below. Control plants were watered twice daily, with an earlier watering at 08:00 (BST).

In order to establish conditions for controlled plant abiotic stress responses, and allow for adequate flowering later on and seed set, a pilot drought-stress project was conducted first in a greenhouse. In this pilot experiment, plants were grown in pots (10 cm², as above) and then subjected to one of the following four treatments: normal watering (twice daily), or water was withheld for 9, 12 or 14 days. Following the period without watering, the three groups of plants were treated in one of two ways: water was supplied at a rate of either 20 ml or 40 ml plant⁻¹ day⁻¹. From the six water stress treatments, delivery of 20 ml plant⁻¹ day⁻¹ to plants that had received no water for 12 days maintained the soil water potential at approximately 50% of that of the well-watered plants, led to retarded plant growth relative to control plants but allowed for set seed (data not shown).

These conditions (12 days without water, 20 ml plant⁻¹ day⁻¹ recovery) were adopted subsequently for drought-stress treatment under glasshouse/greenhouse (GH) conditions. For the polytunnel (PT) drought experiments, where plants were in raised beds, the total water required to give equivalence to the GH experiments above was calculated for recovery conditions, and applied evenly across groups of plants. In PT1, recovery conditions were applied after 10 days without water (due to the higher ambient temperatures) whereas, in PT2, the standard 12 day period without water was used. Soil moisture content was monitored throughout all experiments, using soil moisture probes suitable for pots or beds. For the remaining period of the drought-stress treatments, soil water potential was maintained at approximately 50% of that of the well-watered plants; slight modulation of the amounts of delivered water was sufficient to ensure that this value was not exceeded. [For example, the average soil water potential at 10 cm throughout the drought-stress recovery period in PT1 (Supplementary Fig. 2S) was 48.8%]. Measurements were taken daily at 10 and 20 cm within three permanent tubes inserted per block within every random block of plants in PT1 and PT2 experiments. For pots, 10 cm soil moisture readings were monitored. Stomatal conductance was monitored daily for ten plants at random within every block, using a hand-held porometer.

The onset of flowering occurred for all plants within 2–3 days of the start of the drought-stress recovery period (at approximately 40 days from sowing). Onset of flowering was determined as the time when the first flower had opened fully, and leaves were harvested from every individual plant within 3 h of this stage being attained. Since flowering was not synchronous within or between lines, plant harvest occurred over a period of 2–3 days. Leaves were harvested from all the plants within four randomised blocks, i.e. two blocks each of well-watered and drought-stressed plants. Leaves were frozen immediately in liquid nitrogen. All leaves from an individual plant were pooled and stored at -80°C before being lyophilised (see below). The remaining blocks of plants were maintained under the same conditions (well-watered or stress) until seeds had set and matured, when a set of harvest traits was measured (Supplementary Table 1 for PT1 and PT2 experiments).

A subset of the plants that had been used for leaf harvest was removed carefully from the soil. The roots were removed, washed briefly to remove adhering soil and either frozen in liquid nitrogen or used directly for histochemical staining of GUS activity. Leaves were harvested for RNA preparation from one or two plants of every line grown under all conditions to monitor the induction of dehydration marker genes.

2.4 Measurement of GUS activity

GUS activity in pea root extracts was measured quantitatively, using either a Tropix light assay kit (Welham and Domoney 2000) or assays based on hydrolysis of 4-methylumbelliferyl glucuronide (4-MUG) to yield the fluorescent 4-methylumbelliferone. Additionally, a selection of tips and elongating zones from roots of individual plants were placed overnight in GUS staining solution (5-bromo-4-chloro-3-indolyl glucuronide in DMSO) at room temperature in the dark. The extent of staining was assessed visually and recorded photographically.

2.5 RNA extraction

RNA was prepared from pea leaves, using the TRI RNA isolation reagent (Sigma), according to the manufacturers' instructions, using 80 mg powdered frozen leaf per extract. Northern blot analyses were performed, as previously described (Welham and Domoney 2000), using a pea dehydrin cDNA that was a gift from Dr P. Chandler, CSIRO.

2.6 Metabolite extraction protocol

The frozen leaves were stored at -80°C for a maximum period of 8 weeks before being lyophilised overnight and ground to a fine powder using a coffee grinder. The

resulting powder was sieved carefully to remove any large residual particles and thoroughly mixed. The samples were then stored in a desiccator for between 1 day and three months prior to extraction. The leaf sample (150 ± 1 mg) was placed in a labelled 6 ml crimp top vial. $^2\text{H}_2\text{O}$ (3 ml) containing 1 mM TSP, was added to each vial. A deuterated solvent was used to reduce the water signal and to provide a magnetic field lock for subsequent NMR measurements. TSP provided a chemical shift and intensity reference. The samples were extracted using a shaking platform for 90 min at room temperature. Sample extracts were centrifuged at 2328g for 15 min, yielding a liquid phase containing the water-soluble metabolites and a pellet of the remaining leaf material. The supernatant liquid was decanted into the barrel of a plastic polypropylene syringe, and the liquid was filtered through a 0.45 μm followed by a 0.2 μm PTFE syringe filter into a labelled 1.5 ml Eppendorf tube. Sodium azide solution (60 μl , 10 mM in $^2\text{H}_2\text{O}$) was added to 540 μl of the filtered extract and the resulting solution was transferred to a labelled 5 mm diameter NMR tube.

2.7 NMR spectroscopy

Experimental acquisition and processing parameters are provided in accordance with the recommendations in Sumner et al. (2007). Where parameters have been omitted they are superfluous to the accurate reproduction of the experimental results. Similarly, those parameters that are not included in the minimum reporting standards but are key to accurate experimental reproduction have been included here.

High-resolution solution state NMR was used throughout and sample temperature was maintained at 300 K using compressed air and a VT unit. The deuterium signal from $^2\text{H}_2\text{O}$ in the extraction solvent was used to lock the magnetic field and samples were not rotated.

2.8 NMR data acquisition and processing for profiling

One-dimensional (1D) ^1H NMR spectra were acquired for every pea leaf extract. Spectra were acquired on a 11.7 T Bruker ARX-500, analogue, NMR spectrometer using a 5 mm broad-band direct detection probe tuned to detect ^1H resonances (500.13 MHz). The probe was manually tuned and matched using a representative pea leaf extract prior to every analysis batch.

Magnetic field homogeneity was optimised using the first sample in every batch and 17 shims were used as a reference set for automation.

One thousand and twenty-four free induction decays (FIDs) were recorded as 32,768 complex data points using a 30° observation pulse calculated from a calibrated 90°

pulse length of 12.1 μs . A 3.5 s relaxation delay was found to be sufficient for the acquisition of quantitative data (Weljie et al. 2006) for all resonances. Typically, data from 12 samples were acquired per batch using an automation routine.

Data collected from all samples were processed using FELIX software (Accelrys, USA). A sine-bell shaped window function phase shifted by 90° was applied over the first 16,384 points prior to Fourier transformation, phase and baseline correction. All data were referenced to the TSP peak at 0 ppm. The area of the TSP peak was set to unity for all spectra acquired. Magnetic field homogeneity was assessed by measuring the line width at half-height of the internal standard. An upper acceptable line width at half-height for the TSP standard of 1.8 Hz was determined to be within two standard deviations of the mean width for the initial data set. Samples for which the TSP line width was greater than 1.8 Hz were re-extracted and new data acquired such that all data in the final analysis had a TSP line width of less than 1.8 Hz.

2.8.1 PT1, PT2 and GH2

Spectra were acquired using the Bruker pulse program zgpr, which uses on-resonance solvent presaturation to remove the residual water signal. The following acquisition parameters were used for data collection; spectral width: 14.0806 ppm, acquisition mode: sequential quadrature detection (QSEQ), unrecorded FIDs: 16, offset frequency: 4.776 ppm. These parameters gave a total experiment time of 1h 41m 11s.

2.8.2 GH1

Spectra were acquired using the Bruker pulse program zg30. The following acquisition parameters were used for data collection; spectral width: 10.3064 ppm, acquisition mode: simultaneous quadrature detection (QSIM), unrecorded FIDs: 2, offset frequency: 4.315 ppm. These parameters gave a total experiment time of 1h 54m 23s.

2.9 NMR data reproducibility and repeatability

The variance in the ^1H NMR profiles due to the extraction protocol (reproducibility) and due to the NMR measurement (repeatability) was assessed. Extracting aliquots of the same pea leaf sample on ten occasions and obtaining the 1D ^1H NMR spectrum from these samples determined the extraction variability. These data were then compressed using principal components analysis (PCA) and the variance in the scores assessed to determine the reproducibility of the profiles. NMR data were obtained from one sample on ten different occasions to test variation in instrumental

performance. Under optimal conditions such as those used here, the repeatability of the NMR measurement was reliable (data not shown). The extraction protocol was judged to be reproducible when the variation due to extraction gave a similar variance in the PCA to that introduced by repeated measurements of the same sample. This was determined by inspection of PCA score plots.

2.10 NMR data acquisition and processing for metabolite identification

Spectra were acquired on a 11.7 T Bruker Avance, digital, NMR spectrometer equipped with a 16 bit digitiser (maximum sampling rate of 2 MHz) capable of generating Z magnetic field gradients of up to 50 G cm^{-1} . A 5 mm cryoprobe with cooled ^{13}C and ^1H preamplifiers (Bruker TCI cryoprobe) was tuned to detect ^1H resonances at 500.13 MHz and ^{13}C resonances at 125.76 MHz. The probe was manually tuned and matched and the magnetic field homogeneity optimised using up to 34 shims. Manual data acquisition was used throughout.

For 1D ^1H NMR, spectra were acquired using the Bruker pulse program zgpr. The following acquisition parameters were used for data collection; 90° observation pulse length: 9.2 μs , spectral width: 15.01 ppm, recorded FIDs: 128, unrecorded FIDs: 2, data points in FID (complex): 32,768, relaxation delay: 10 s, acquisition mode: digital quadrature detection (DQD), offset frequency: 4.647 ppm,

These parameters gave a total experiment time of 26 min 32 s.

A sine-bell shaped window function phase shifted by 90° was applied over all points prior to Fourier transformation, phase and baseline correction.

Two-dimensional phase cycled ^{13}C - ^1H heteronuclear single quantum coherence (HSQC) (Bodenhausen and Ruben 1980) spectra were acquired using the Bruker pulse sequence hsqcphpr. This experiment performs on-resonance presaturation of the ^1H signal from residual water and an INEPT sequence for spectral editing. The following acquisition parameters were used; indirect nucleus (F1): ^{13}C , direct nucleus (F2): ^1H , decoupling method: GARP, decoupled nuclei: ^{13}C - ^1H , decoupling bandwidth: $\pm 15\text{ kHz}$, 90° ^1H pulse length: 9.2 μs , 90° ^{13}C pulse length: 16.5 μs , J_{CH} : 145 Hz, ^1H spectral width: 13.330 ppm, ^{13}C spectral width: 179.990 ppm, relaxation delay: 2 s, recorded FIDs per t_1 increment: 64, unrecorded FIDs: 16, number of t_1 increments: 395, data points per FID: 1536 (complex), ^1H offset frequency: 4.647 ppm, ^{13}C offset frequency: 90 ppm, acquisition mode: DQD, t_1 : 3 μs , t_1 increment: 22.09 μs , quadrature detection: States-TPPI. These parameters gave a total experiment time of 15h 24m 30s.

Two-dimensional phase cycled ^1H – ^1H total correlation spectroscopy (TOCSY) (Bax and Davis 1985) spectra were acquired using the Bruker pulse sequence *mlevphpr*. This experiment performs on-resonance presaturation of the ^1H signal from residual water. The following acquisition parameters were used; indirect nucleus (F1): ^1H , direct nucleus (F2): ^1H , 90° ^1H pulse length: 10.13 μs , ^1H spectral width (F1 and F2): 13.330 ppm, relaxation delay: 1.5 s, recorded FIDs per t_1 increment: 32, unrecorded FIDs: 16, number of t_1 increments: 512, data points per FID: 4096 (complex), ^1H offset frequency: 4.709 ppm, acquisition mode: DQD, t_1 : 3 μs , t_1 increment: 150 μs , spinlock duration: 100 ms, trim pulse length: 2 ms, spinlock field strength: 7.142 kHz, quadrature detection: States-TPPI. These parameters gave a total experiment time of 8h 55m 26s.

HSQC and TOCSY spectra were processed using a sine-bell shaped window function phase shifted by 90° over all points. These data were zero filled to give a real data matrix size of 4096×2048 points prior to Fourier transformation, phase and baseline correction.

Data acquisition and processing for peak picking and compound identification was performed using Topspin v 1.3 (Bruker, Germany).

2.11 Data analysis

2.11.1 Software

Statistical analysis, binning and visualisation of the results were performed using an in-house toolbox for Matlab® (The Mathworks), known as Metabolab™.

2.11.2 Categorical variables

For statistical analysis, the data derived from the 1D ^1H NMR measurements described above were classified as drought-stressed (D) or well-watered (W), transgenic (T) or null segregant (N), and from experiment (PT, GH). The samples obtained for plants grown under GH conditions were assessed independently of the PT samples. Analysis of the two environments provided an independent statistical analysis of the results obtained in either. The categorical (or Y) variables were therefore the watering regime, the individual experiment and the genotype (transgenic or null segregant) of the plants.

2.11.3 Adaptive binning

Prior to statistical analysis, the data were binned using the adaptive binning procedure of Davis et al. (2007). Briefly, this method identifies the range over which peak positions

vary within the 1D NMR dataset and applies these ranges to be the start and end points for data binning. Therefore subsequent statistical analysis was performed on the NMR resonance peaks. This has been shown to have major advantages over binning the data using fixed width bins and also over using an unbinned dataset. The advantages include: accounting for small changes in chemical shift, improved classification rates due to reduced intra-class variation, better interpretability of the data, improved alignment of the data with multidimensional NMR spectra and a reduced risk of misinterpreting statistical results due to misaligned data.

Following adaptive binning to wavelet level 3, 656 data bins were identified and these constituted the input variables in subsequent statistical analyses.

2.11.4 Student's *t*-tests

Two-tailed Student's *t*-tests were used to determine statistical differences between the input variables (the binned NMR data) when grouped according to the categorical variables. The *P* values from these tests were used to identify regions of the spectral data that were most likely to be derived from metabolites that were altered as a consequence of experiment, treatment or genotype.

2.11.5 Principal components analysis (PCA)

PCA was used to present an overview of the variance in the data matrix. The categorical variables were used to encode the data, such that obvious trends were highlighted readily. Ten PC scores were calculated.

2.11.6 Partial least squares linear discriminant analysis (PLS-LDA)

PLS-LDA was used to determine the presence of data trends in the NMR spectra that were correlated with the categorical variables. Prior to PLS calculation, the data were set to unit variance (the correlation method). Linear discriminant analysis was performed, using the Mahalanobis distance metric operating on the PLS scores. For every PLS calculation, ten scores were determined. PLS results were validated using the Venetian blind method and, in this case, a random 10% of the data set was omitted from the PLS calculation during each validation round until all of the data had been omitted once (ten rounds). The classification rate upon cross-validation was used to assess whether or not the PLS model was over-fitting and, therefore, to determine the number of PLS scores used. Over-fitting was determined when the addition of a PLS score to the calculation did not result in improved cross-

validation rates. Both PCA and PLS were conducted using the NIPALS algorithm.

3 Results

3.1 Plant growth conditions and responses

The pea (*Pisum sativum* L.) plants analysed in these experiments were grown either in a greenhouse (GH) or under simulated field conditions within a polytunnel (PT). A set of independent transgenic pea lines was included in all experiments to facilitate comparisons of plant responses to drought under different conditions. The transgene comprised a drought-responsive promoter from a trypsin inhibitor gene controlling the expression of the marker enzyme, β -glucuronidase (Welham and Domoney 2000). Phenotypic, biochemical and metabolic comparisons (see below) of near-equivalent transgenic and null segregant lines were carried out to ensure pleiotropic effects of drought-induced transgene expression were not being monitored.

The raised bed system (Supplementary Fig. 1S), used for the PT experiments, permitted a controlled simulation of field conditions, and a differential in soil water status to be maintained between the well-watered (W) and drought-stress (D) conditions (Supplementary Fig. 2S). Major phenotypic differences were obvious between the D and W plants in all experiments, including a lower plant stature, earlier flowering, and reduced seed number. Analysis of the impact of the drought-stress regime on a range of agronomic characters showed significant differences for all measured characters under the two growth conditions (W vs. D); data for two combined field experiments, PT1 and PT2, are given in Supplementary Table 1. Differences in numbers of pods, total seed weight and above ground biomass were apparent between the two transgenic lines 10B:10 and 14A:4, when grown under well-watered conditions. This reflected a lower plant height for the line 10B:10, that was not apparent under drought-stress conditions (Supplementary Table 1).

Stomatal conductance was decreased for the D compared with W plants throughout all experiments (PT2 shown in Supplementary Fig. 3S). A direct measure of the effect of the drought-stress on plants was the extent of induction (7- to 10-fold) of the marker gene, under the control of the drought-inducible *TII* gene promoter, in roots of the transgenic plants. Transgene induction was observed histochemically (not shown) and determined quantitatively; data from four experiments are shown in Fig. 1a. This reflected an induction of *TII* mRNA (not shown). Induction of dehydrin mRNA was evident in leaves of drought-stressed plants in all experiments (Fig. 1b).

3.2 Global analysis of NMR metabolite profiles of leaves from drought-stressed and well-watered plants

Analysis and comparison of metabolite profiles from different groups of plants utilised adaptive binning of the 1D ^1H NMR datasets (Davis et al. 2007). Up to 656 data regions were identified within leaf NMR spectra that related to NMR resonance peaks. Resonances that were derived from different molecules, but showed overlapping 1D NMR data, were resolved subsequently by 2D NMR.

The data analysis primarily reported is based on the PT plants. Data from the GH plants were used to validate the changes in the concentration of metabolites found when the watering regimes of the PT plants were compared. All metabolites that are reported to differ as a result of watering regime in the PT1 and PT2 experiments were also found to differ significantly ($P < 0.01$) in the GH1 and GH2 experiments. Since the NMR data acquisition parameters were improved substantially following the acquisition of the GH1 data, it is not useful here to provide a direct comparison of the data from GH1 with those from GH2.

Variation within datasets was assessed using exploratory data analysis by PCA. PCA was performed on the adaptively binned data and thus the input variables were 656 bins relating to the NMR spectral peak positions. The total variance captured by the calculation of 10 PC scores was 98.84%. PC1 and PC2 were used for data interpretation and these captured 40.38% and 29.69% of the variance in the data, respectively. Figure 2 shows a biplot of PC scores 1 and 2 calculated using the NMR data from samples from two experiments (PT1, PT2), either as a scatter plot with each point representing a different NMR data set (Fig. 2a), or showing the mean and the standard deviation for the PC scores (Fig. 2b). In the latter, the mean for every group is shown by the central symbol with the standard deviation represented by the ellipse that passes through ± 1 s.d. on both PC axis 1 and 2. (As PCA is an unsupervised technique, it does not use the categorical variables to separate the data groups and is simply a representation of the variance within the dataset, regardless of its source). The NMR data shown in Fig. 2 comprise four distinct groups, reflecting differential plant responses in two experiments and, within experiment, treatment: W(PT1), D(PT1), W(PT2) and D(PT2). Thus a divergence is evident between the responses of plants grown in the two experiments, regardless of the watering regime used or plant genotype. Equally, there was divergence of the responses of plants to water status, regardless of growth year or genotype (Fig. 2b). There was no separation of NMR data when transgenic (T) and null segregant (N) plants were compared, when up to ten PC score combinations (PC1/PC2 for

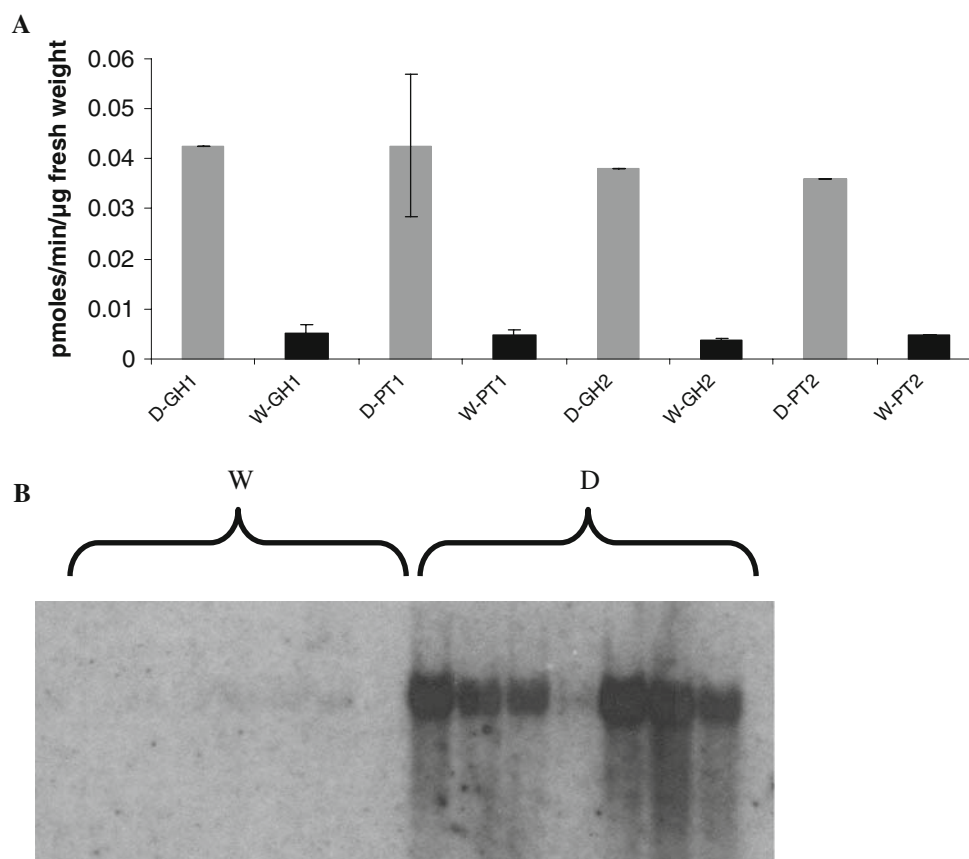


Fig. 1 (a) Induction of marker enzyme (β -glucuronidase) activity in roots of drought-stressed (D, grey bars) compared with well-watered (W, black bars) plants of the line 14A:11 in four independent experiments (GH, greenhouse, PT, polytunnel). (b) Induction of

dehydrin RNA in leaves of drought-stressed (D), compared with those of well-watered (W) plants; 3 μ g total RNA were analysed for six plants of each group

T and N shown in Fig. 2) were used. This was judged by viewing all combinations of scores plots. In summary, PCA identified variance in the NMR data that was ordered thus: growth year as the most significant source of variance, followed by water status.

The results of a PCA using plants grown under greenhouse conditions showed a high degree of consistency with the data from the PT experiments (data not shown). Again the data were clustered according to the watering regime and showed no significant patterns that could be ascribed to the presence or absence of the transgene.

3.3 Identification of NMR spectral regions that distinguish drought-stressed and well-watered plants

Figure 3a shows the average NMR spectrum obtained for samples from two field experiments (PT1, PT2). Two-tailed Student's *t*-tests were performed on the data within every bin, based on groupings defined by experiment, watering regime and plant genotype. The panel in Fig. 3b shows a representation of the *t*-test results (*P* values) as

shaded areas aligned with the NMR spectrum, according to the chemical shift at which the test was performed. This representation allows rapid assessment of the data to determine variance at specified chemical shifts, where the darker the shaded area the less significant the test results and vice versa. Data bins for which $P < 0.01$ are highlighted in Fig. 3b, and thus only the most significant results are displayed as white bars. The number of bins with $P < 0.01$ in any comparison illustrates the extent to which the metabolome is altered. The non-normal distribution of the data in some bins means that the absolute probabilities are not a precise statistical measure of alterations to the metabolome, but rather provide a comparative index for identifying changes in the datasets. A summary of the *t*-test results (Table 1) shows a large impact on the metabolome associated with two field experiments and watering regime, with up to 50% of the bins differing significantly (270 and 335, respectively). In contrast, few regions of the NMR spectra differed significantly between the two genotype classes (Table 1). These results provide an explanation for the separation of the W and D groups identified in the PCA (Fig. 2).

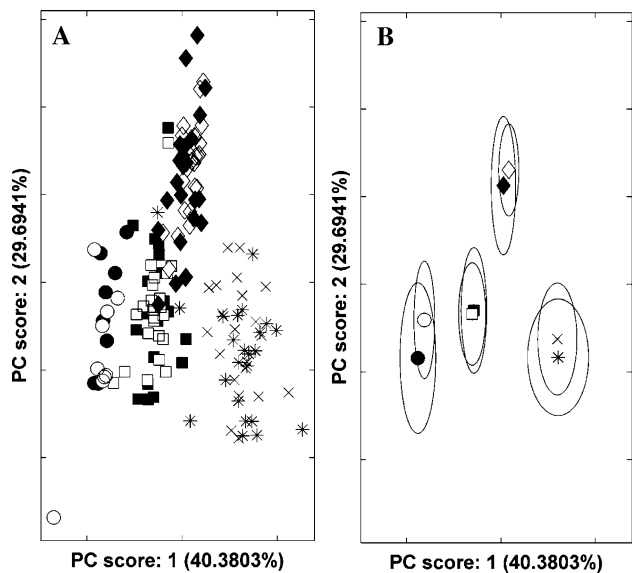


Fig. 2 Principal components analysis scores biplot of the ¹H NMR data collected for two field experiments (PT1, PT2). PC scores 1 and 2 are shown with the variance captured by each score given in parentheses. *, D(PT1 N); ×, D(PT1 T); ◇, W(PT1 N); ◆, W(PT1 T); ■, D(PT2 T); □, D(PT2 N); ○, W(PT2 N); ●, W(PT2 T). D = drought-stressed, W = well-watered. T and N are transgenic and null segregant plants, respectively. The mean and the standard deviation for the PC1/PC2 NMR data set scores (a) are shown (b)

Table 1 Summary of the *t*-test results obtained for data from two field experiments

| Comparison | <i>n</i> | Number of bins (<i>P</i> < 0.01) |
|-------------|----------|-----------------------------------|
| W vs. D | 157 | 335 |
| PT1 vs. PT2 | 157 | 270 |
| N vs. T | 157 | 25 |

W and D are the different water regimes, either well-watered (W) or drought regime (D). PT1 and PT2 represent two different experiments in a polytunnel. N and T differ according to absence (N) or presence (T) of the transgene (*n* = number of plants analysed)

3.4 Validation of the NMR metabolite profiles of leaves from transgenic indicator plants

Comparisons between the transgenic (T) and the null segregant (N) plants were carried out to validate the inclusion of the former as indicators of drought-stress responses. The NMR spectral profiles of the N plants were shown to be very similar to those of the T plants, with significant differences between these groups in approximately 4% of the tests performed, or 25 chemical shift ranges (Table 1). The type I error associated with these tests, at the 99% significance level, is 1%, implying that 3% of the data bins did

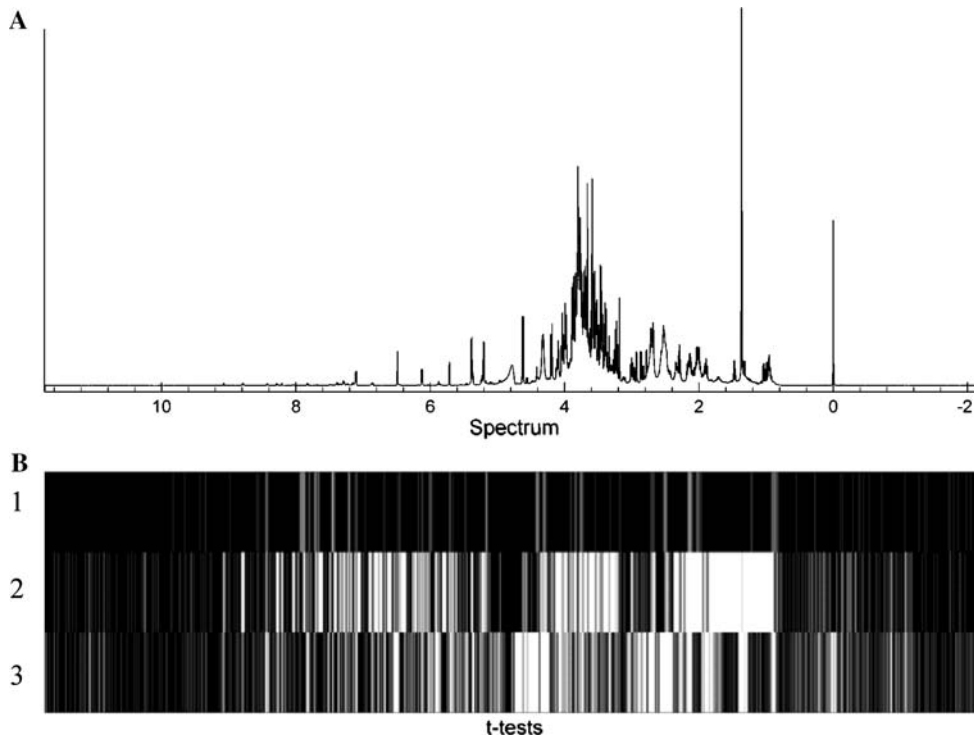


Fig. 3 (a) The average NMR spectrum of the PT1 and PT2 samples. The panel in (b) shows *P* values from the *t*-tests between data from the transgenic and null segregant plants (1), the well-watered and the drought-stressed plants (2) and plants from PT1 and PT2 (3). The *P*

values for each test are represented as shaded areas aligned with the NMR spectra according to the chemical shift at which the test was performed. The darker the shaded area the less significant the test results and vice versa

not differ by chance. The data from the 25 bins were compared for all sets of plants.

Data from 15 of the 25 resonance peaks also differed significantly in comparisons of water regimes or experiments and, therefore, could not be considered to be specific to transgene expression. The ten remaining bins corresponded to peaks of low intensity with a signal to noise ratio of less than 10:1. This limited their characterisation to an assessment of the ^1H chemical shift. Seven of these bins were in the region of the NMR spectrum populated by aromatic compounds (7.520–7.613 and 7.172–7.218 ppm), indicating that the two groups of data are distinguished by phenolic-type metabolites. The three remaining bins corresponded to peaks with very low signal to noise ratios ($\leq 3:1$) and/or were biased by an anomalous peak in very few samples (not shown).

Student's *t*-tests, performed separately on the two experiments, PT1 and PT2, revealed that none of the 25 bin positions differed significantly according to genotype (T and N) in both experiments. Similarly, *t*-test results obtained for NMR datasets obtained for plants grown under GH conditions showed that less than 1% (i.e. below the type 1 error rate) showed differences between T and N genotypes (data not shown).

The classification rates obtained from PLS-LDA calculations of the NMR datasets from the field experiments are shown in Table 2. In every case, ten PLS scores were calculated and the classification rate upon Venetian blind cross validation is presented for models that contained the maximum number of PLS scores and that were not over-fitted. The classification rates were calculated as the mean value from the ten cross-validation rounds. A classification rate of 50% would be expected purely by chance in these two group models. The rates indicate that the data contained resonances from metabolites that can differentiate between the watering regime (96.2%) and the experiment (98.7%). The classification rate for genotype (50.3%) was only marginally higher than would be expected by chance, in agreement with the conclusions from the *t*-tests. Further analysis of all the data using variable importance of project

(VIP) scores revealed no clear combination of variables that was significant in the classification of genotype (data not shown).

Therefore, the combined results of the *t*-tests and PLS-LDA indicate that the metabolome has not been perturbed significantly by the presence of the transgene, with the possible exception of seven unassigned aromatic resonances of low intensity. Thus inclusion of transgenic indicator plants did not lead to pleiotropic metabolic effects in any experiment reported here and it can be concluded that transgene expression did not alter the leaf metabolome significantly.

3.5 Identification of metabolite NMR resonances

responsive to environmental effects: Drought-stress responsive metabolites

The PLS-VIP scores derived from the comparisons between treatment and experiment were explored further in order to determine the metabolic basis for the separation of the groups. A detailed 2D NMR study of the effect of drought-stress on the pea leaf metabolome was undertaken. The ^1H and ^{13}C chemical shifts of resonances (from the ^{13}C - ^1H HSQC spectra), together with *J* coupling data and signal intensity (from the ^1H 1D spectra), and spin system information (from the ^1H - ^1H TOCSY spectra) enabled the characterisation of metabolites. Identification was confirmed by performing the same NMR experiments using standard metabolites under the same experimental conditions. Spiking experiments were also used to confirm any remaining ambiguous metabolite identities.

This approach was used to determine that the following metabolites were affected strongly by drought-stress: proline, leucine, isoleucine, valine, threonine, γ -aminobutyrate (GABA), homoserine, myoinositol and trigonelline (Table 3). All of these metabolites were present at elevated concentrations in the drought-stressed plants from the field experiments (PT1 and PT2). Inspection of the data derived from plants grown under greenhouse conditions showed that all of these metabolites, with the exception of leucine and isoleucine, were also present at elevated concentrations in the drought-stressed plants. The most substantial difference in concentration between W and D plants in all experiments was for myoinositol, where an approximately two-fold increase in mean concentration was apparent in the drought-stressed plants (data not shown).

PLS-LDA highlighted several resonance peaks that were reduced in concentration under drought-stress conditions, i.e. these were present at elevated concentrations in the W plants. As most of these were present at relatively low concentrations, limiting the application of 2D NMR techniques to the unfractionated samples, these peaks were not identified. The resonance peaks showing consistently

Table 2 Summary of the classification rates obtained by PLS-LDA for data from two field experiments

| Comparison | <i>n</i> | Scores (variance) | Classification | |
|-------------|----------|-------------------|----------------|------|
| | | | Train | Test |
| W vs. D | 157 | 2 (88.34%) | 98.7 | 96.2 |
| PT1 vs. PT2 | 157 | 2 (92.94%) | 99.4 | 98.7 |
| N vs. T | 157 | 2 (36.62%) | 77.7 | 50.3 |

W and D are the different water regimes, either well-watered (W) or drought regime (D). PT1 and PT2 represent two different experiments in a polytunnel. N and T differ according to absence (N) or presence (T) of the transgene (*n* = number of plants analysed)

Table 3 Single bond ^1H - ^{13}C chemical shift correlations used to identify metabolites in the pea leaf extracts that were significantly affected by drought-stress and/or by experiment. Chemical identification codes are given in every case (CID; <http://pubchem.ncbi.nlm.nih.gov/search/search.cgi>)

| Metabolite | Chemical shift correlations (ppm) |
|--|--|
| Proline (CID:614) | 4.133–63.87; 3.342–48.72; 3.423–48.68; 2.071–31.55; 2.356–31.61; 2.009–26.41 |
| Leucine (CID:857) | 1.712–42.34; 0.969–24.77; 0.957–23.68 |
| Isoleucine (CID:791) | 1.013–17.24; 0.938–13.55 |
| Valine (CID:1182) | 3.615–63.25; 2.289–31.84; 1.049–20.64; 0.999–19.37 |
| Threonine (CID:6288) | 4.259–68.56; 1.336–21.90 |
| γ -aminobutyrate ^a (CID:5460232) | 3.012–41.92; 2.311–36.91; 1.904–26.21 |
| Homoserine (CID:779) | 3.858–56.13; 3.783–61.40; 2.152–34.82; 2.033–34.78 |
| Myoinositol (CID:892) | 4.061–74.95; 3.624–75.24; 3.544–73.89; 3.270–77.14 |
| Trigonelline (CID:5570) | 9.132–148.40; 8.841–147.50; 8.821–148.57; 8.089–130.17; 4.437–50.92 |
| Malate (CID:525) | 4.364–72.72; 2.737–44.51; 2.536–44.41 |
| Glutamate (CID:104813) | 3.759–57.35; 2.374–35.97; 2.134–29.60; 2.066–29.55 |
| Asparagine (CID:236) | 4.009–53.90; 2.977–37.16; 2.943–37.12; 2.883–37.12; 2.846–37.07 |

^a change significantly between treatment and experiment

elevated concentrations in the well-watered plants in all experiments were present at chemical shifts of 6.938–6.943, 6.841–6.881, 6.438–6.477 and 5.817–5.851 ppm.

The ^{13}C and ^1H chemical shift assignments for the assigned metabolites that were elevated under drought-stress conditions are listed in Table 3. Comparison of the extent to which these compounds are increased in concentration showed a remarkable consistency in the different experiments. An example is shown in Fig. 4. A plot of the peak intensity corresponding to a region of the 1D ^1H NMR spectrum populated by a trigonelline resonance (8.072–8.089 ppm) is shown for field data in Fig. 4a, whereas data acquired for the trigonelline resonance at 4.437 ppm from plants grown under greenhouse conditions are presented in Fig. 4b. These data show firstly that trigonelline has been confirmed as a drought-responsive compound in both greenhouse and field experiments. Secondly, the relative concentration of this compound is remarkably similar under the very different growth conditions, with almost a doubling of the relative amount under drought-stress field conditions (Fig. 4b). The means (solid lines in Fig. 4a, b) show that the increase was larger under field conditions (Fig. 4a). A wider range of concentration of this compound was also observed in these plants (Fig. 4a).

3.6 Identification of metabolite NMR resonances responsive to environmental effects: Metabolites responsive to additional experimental conditions

The metabolites that were primarily responsible for the differentiation of data originating from the different experiments were also investigated, based on their VIP scores from the PLS-LDA calculations. The most significant metabolites within this group were malate, glutamate, asparagine and GABA, with the last being significant also

in differentiating the drought-stressed from the well-watered plants in all experiments (see above). The single bond ^{13}C and ^1H NMR chemical shift correlations for these compounds are also listed in Table 3.

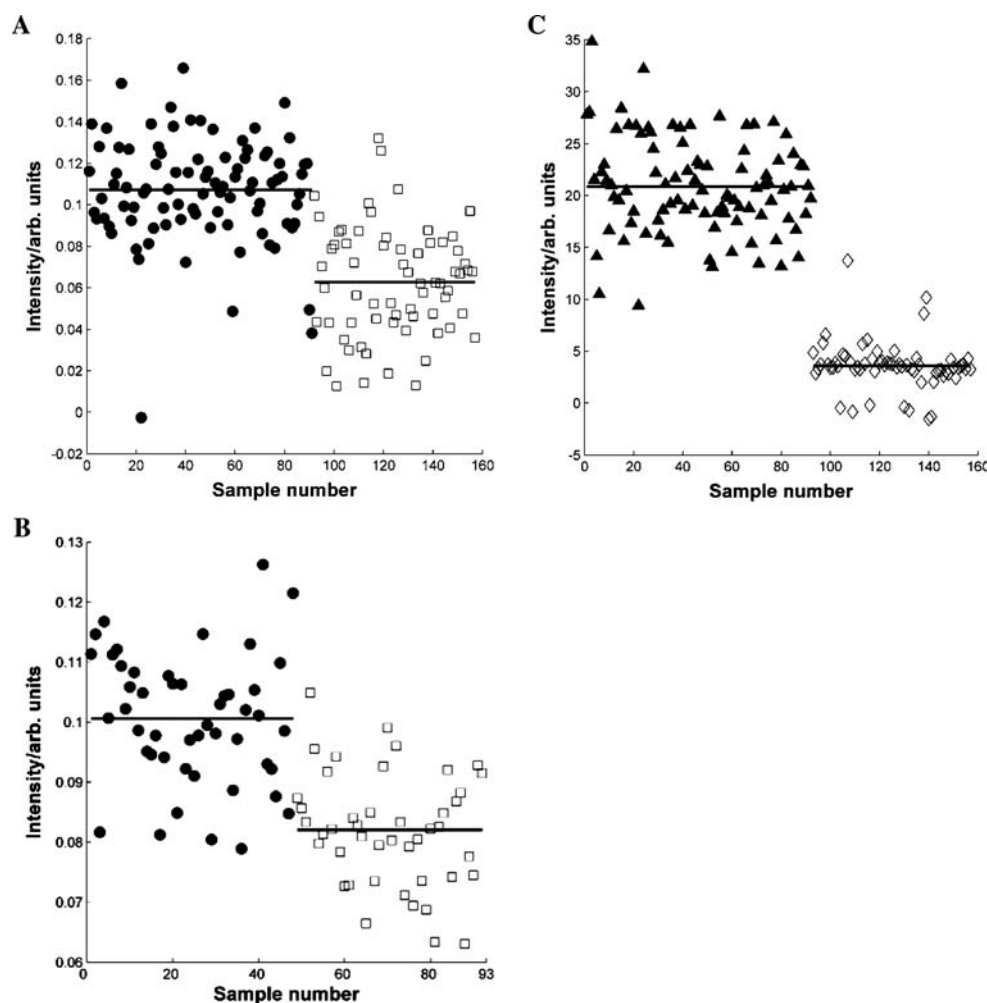
The concentration of malate, for example, was significantly higher in samples originating from the PT1, compared with the PT2, experiment. The resonances from malate dominated these PLS calculations, with all of the resonances from this compound present in the top 10% of the VIP scores. The data from the malate resonance bins at 2.515–2.573 ppm, shown in Fig. 4c, illustrate the extent of variation in the concentration of this metabolite when the two field experiments, PT1 and PT2, are compared. The malate concentration was approximately 5-fold higher in the former experiment. Such a large difference may be assumed to relate to overall climatic differences between experiments, that exclude water availability but may reflect a higher mean daily temperature throughout for PT1 compared with PT2 (Supplementary Fig. 4S). In addition, stomatal conductance measurements were higher for watered plants grown throughout PT1, compared with PT2 (data not shown).

Several unassigned chemical shifts contributed both to the separation of the D and W plants and the differentiation of experiments. These relate to compounds that have not been identified currently within these experiments. However, none of these metabolites featured among those making the most significant contributions to the PLS calculations resulting in the highest VIP scores, which were targeted for identification and have been presented here.

4 Discussion

This study has focussed on the determination of metabolic changes that may be induced in leaves under different

Fig. 4 Peak intensity plots of (a) PT1 and PT2 combined data showing the regions between 8.072 and 8.089 ppm, (b) GH data showing the resonance at 4.737 ppm (●, Drought-stressed plants; □, Well-watered plants) and (c) the region between 2.515 and 2.573 ppm (▲, PT1; ◇, PT2). The black lines on the plots indicate the means for every group represented. Data in (a) and (b) are from trigonelline, whilst that in (c) is from malate



climatic conditions. NMR spectroscopy has been used to demonstrate that the distribution of water-soluble metabolites in the leaves of *Pisum sativum* was affected significantly by drought-stress and additional environmental factors. In contrast, few if any significant leaf metabolic effects could be attributed to the drought-induced expression of a transgene, encoding a bacterial marker enzyme, in some of the lines used in these experiments. This validated the inclusion of such lines in a study of drought-stress responses. Previous studies have demonstrated that the effect of transgenesis on metabolism is specific to the function of the transgene (Defernez et al. 2004). Any assessment of the effects of the transgene on metabolism needs to be combined with an assessment of the effects of environmental factors, and there are reported cases where metabolomic data have proven not to be robust when other sources of variance were included in the study (Kirschenlohr et al. 2006). In the work reported here, an array of robust statistical methods has been employed to dissect the nature of the changes to NMR spectra, where reliance has not been placed on any one method.

The distinction of plants grown in different experiments according to their metabolome is most likely to relate to additional environmental factors, including temperature. For example, the mean daily temperature was higher throughout for PT1 compared with PT2 (Supplementary Fig. 4S). Despite this, the data derived from the different experiments reported here show a remarkable consistency in that the majority of the differences in NMR profiles were reproducibly attributed to drought-stress, under both greenhouse and field conditions. This is consistent with the relative extent to which the transgene and drought-associated dehydrin gene expression were induced in different experiments (Fig. 1). The analysis conducted here was based on total leaves harvested at the onset of flowering. In this way, the study has not dissected effects at different nodes but rather captured all the leaf responses prior to the major resource re-allocation that occurs during seed set and development (Schiltz et al. 2005; Larmure et al. 2005). Since the study was based on lines that were all derived from the cultivar Puget, the plants had a very similar habit, and very few agronomic differences regarding pod and

seed number were apparent within treatments (Supplementary Table 1).

The metabolites that differed significantly according to treatment, and made the most significant contributions to the PLS calculations, resulting in the highest VIP scores, were selected for compound identification (Table 3). The combined data for metabolites altered in response to drought-stress and/or between experiments indicate major environmental effects on primary and secondary metabolism in leaves. The primary metabolites identified in Table 3 indicate three points at which carbon may be diverted during glycolysis under drought-stress conditions, namely the pathways leading to the synthesis of: leucine, isoleucine and valine via phosphoenolpyruvate (PEP); asparagine, homoserine and threonine via oxaloacetate; and glutamate, proline and GABA via 2-oxoglutarate. As the last two involve TCA intermediates, differences in malate concentration may determine the extent of differences in flow through the TCA cycle. However, in this work, the large differences in malate concentration (Fig. 4c) occurred between experiments but not directly as a consequence of drought-stress, reflecting the greater influence of additional environmental factors on this metabolite. A central role for malate in controlling the activity of enzymes of primary metabolism, including PEP carboxylase and NADP-malate dehydrogenase, in plants is clear. For example, the over-expression of malate feedback-insensitive forms of PEP carboxylase leads to major changes in many seed biochemical pathways (Radchuk et al. 2007) and, in leaves, rapid fluctuations in temperature can lead to changes in the activity of ribulose biphosphate carboxylase/oxygenase, reflecting redox status and NADP-malate dehydrogenase activation (Schrader et al. 2007). Malate has also been associated with reduced stomatal conductance (Patonnier et al. 1999) and the accumulation of malate may reflect controls on leaf transpiration. In the experiments reported here, however, relative malate content was correlated positively with stomatal conductance values determined for PT1 and PT2 (see above; conductance values recorded for watered plants were significantly higher in PT1 than in PT2). The relatively large concentration of malate reported here, associated more with environmental factors other than water availability, may reflect the enhancement of a malate-oxaloacetate shuttle (the malate valve) to prevent over-reduction of photosynthetic electron chain components, leading to the accumulation of malate in vacuoles (Scheibe 2004). The ability of plants to store malate is likely to reflect genetic variation in key pathways, offering potential advantage under a range of adverse growth conditions. Finally, the accumulation of compounds such as GABA, malate and the amino acids may reflect disruption of export of these compounds or their precursors from the leaf under stress conditions, since both organic and amino

acids are common constituents of sap exudates in diverse plant species (Bialczyk and Lechowski 1995; Nakamura et al. 2008).

A higher myoinositol concentration is indicative of changes in the metabolism of glucose and/or oligosaccharides that can exert an osmoprotective effect under conditions of severe water loss. This is consistent with the accumulation of so-called compatible solutes to maintain a positive leaf osmotic potential. Other such reported compounds include mannitol, proline and glycinebetaine (Zhang et al. 1999; Verslues et al. 2007; Morison et al. 2008). Glycinebetaine and mannitol could not be detected in the NMR data reported here, whilst elevated concentrations of proline were evident in the drought-stressed plants. The absence of glycinebetaine and mannitol may reflect the different plant species being studied and/or different mechanisms induced as a response to drought among plant species. For example, trigonelline (nicotinic acid betaine) was increased in concentration as a result of drought-stress in this study and may fulfil an analogous role to that of glycinebetaine in other species. Trigonelline is a nitrogenous compound present in coffee beans, and its synthesis in mungbean seedlings has been hypothesised to detoxify excess nicotinic acid and nicotinamide products of the NAD cycle that are known to act as growth inhibitors (Zheng et al. 2005). Such a function may extend to drought-stress metabolism where the effects of some metabolites may be to depress growth.

5 Concluding remarks

We have identified several key plant metabolites that are elevated in concentration in drought-stressed plants under both simulated field and glasshouse conditions, some of which have not previously been associated with such responses. These metabolites include proline, leucine, isoleucine, valine, threonine, GABA, homoserine, myoinositol and trigonelline and are indicative of major effects on primary, as well as, secondary metabolism. Further metabolites (malate, glutamic acid, asparagine and GABA) have been identified that were altered between experiments; of these, malate concentrations differed dramatically. The relative increases in amino acids may relate to a higher investment in nitrogen accumulation and protein synthesis, as has been demonstrated for some environmental responses in peas (de Sousa-Majer et al. 2004; Larmure et al. 2005), and an elevated concentration of some amino acids has been linked to higher seed nitrogen content in genetic variants of peas (Vigeolas et al. 2008). Changes in the amounts of all of the metabolites identified here, and their precursors, indicate that the composition of crops can be expected to vary as a function of climate. Some of these metabolites will impact

on the end-uses of crops, influencing, for example, taste and flavour compounds as is the case with trigonelline, and may have consequences for sustainable food production in a changing climate. Natural genetic variation in the metabolites identified here and in those produced constitutively should be investigated in relation to habitat to identify novel sources of drought tolerance for crop improvement.

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