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Cold stress in white clover - An integrated view of metabolome and transcriptome responses

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Abstract. We have studied metabolic and gene expression responses of two white clover genotypes differing in foliar basal flavonol and anthocyanin levels subjected to moderate cold in a controlled climate chamber experiment. The main flavonols and anthocyanins accumulating in white clover leaves are conjugated to galactose and xylose. Concentrations of flavonoids and sugars including starch significantly increased during cold treatment and a strong induction of flavonoid pathway genes was observed in both genotypes. Specific sugars like glucose, fructose, and starch were significantly higher in the genotype with low basal flavonoid levels. By combining metabolic and gene expression analysis we have identified two genes probably involved in the conjugation and deconjugation of flavonoids, a flavonoid galactosyltransferase and a β-galactosidase. The selected flavonoid galactosyltransferase was up-regulated by cold treatment and was also expressed at higher levels in the red leaved genotype. The β-galactosidase studied here was down-regulated by cold and expressed less in the red leaved genotype. These results indicate a tight link between primary and secondary metabolism in clover and demonstrate the value of combined metabolome and transcriptome analysis for the identification of specific gene functions.

Introduction

Trifolium repens is the main forage legume in New Zealand pastures (Caradus *et al.* 1996) but winter conditions can lead to a loss of *Trifolium repens* in mixed pastures and a reduced regrowth potential (Guinchard *et al*. 1997). The acclimation of forage plants to chilling and freezing results in a wide range of physiological responses e.g. the accumulation of sugars, sugar alcohols or proline (Guy 1990). Most studies have focused on the analysis of primary metabolites in forage plants exposed to cold. However, other important stress related compounds, especially secondary metabolites like flavonoids, have, to our knowledge, not been studied in this context. Flavonoids are known to be involved in general stress tolerance of plants (Dixon and Paiva, 1995). One very well known feature is the up-regulation of anthocyanin biosynthesis in red autumn leaves as a response to cold (Ougham *et al*. 2005). Flavonoids are stored and accumulate within a plant as glycosides, as the aglycones are not stable, and a direct link between the biosynthesis and accumulation of flavonoids and sugars has been suggested by several authors (Mita *et al.* 1997).

Global gene expression studies on cold responses of plants have shown that cold regulates a plethora of genes involved in a wide range of biochemical pathways, physiological processes and regulatory networks (Hannah *et al*. 2005). These studies usually involve model plant species like *Arabidopsis thaliana*, for which the whole genome has been sequenced and gene expression profiling on microarray chips is very advanced. AgResearch (NZ) in collaboration with Agriculture Victoria (Australia) has developed microarray slides based on clover EST databases, offering exciting new opportunities for the analysis of genome wide responses of clover to a wide range of environmental changes.

Several white clover lines that accumulate high levels of anthocyanins and flavonols resulting in red leaved varieties have been selected at AgResearch. We were interested in the responsiveness of these clover plants to cold compared to responses of green leaved clover plants with very low levels of flavonoids. This study focuses on effects of cold treatment on flavonoid metabolism, both on metabolite and gene expression levels and sugar accumulation and we provide evidence for a tight link between sugar metabolism and flavonoid accumulation. These data are valuable for the understanding of the molecular basis of stress responses and to help breeders tackle complex traits like e.g. cold tolerance.

Material and methods

Plant material

Two white clover genotypes (35A, low flavonoids; 38H, high flavonoids) were selected from various breeding lines (kindly provided by D. Woodfield, AgResearch). Plants were grown in two controlled environment chambers (HortResearch) at 25^{o}C (14 hours light, 10 hours dark). After 6 weeks one chamber was set to 10^{o}C under the same light conditions. Leaves were harvested in both chambers after 0, 3 and 5 days of the start of the cold-treatment, immediately frozen and ground in liquid nitrogen; material for subsequent analysis was stored at -80^{o}C.

Metabolite analysis

Frozen ground material (0.5 g FW) was extracted with 80% acetone, extracts were centrifuged and the acetone removed under a stream of nitrogen. The residue was re-dissolved in 1 mL 80% methanol (0.1 % acetic acid), centrifuged, the supernatant transferred to a glass vial and stored at -20^{o}C until analysis. Flavonols and anthocyanins were analysed by HPLC using UV-PDA detection.

Polar metabolites were extracted, separated by partitioning, derivatised with MSTFA and analysed by GC-MS as described by Roessner *et al.* 2001.

ANOVA of metabolites was performed using Genstat 8.11; flavonoid data were log transformed prior to analysis. Correlation analysis was performed using Minitab 14.

Gene expression analysis

The microarray slide contained 14,678 white clover unigenes and approx. 500 negative control spots. Total RNAs were isolated from frozen, ground plant material using TRIzol® reagent (Invitrogen) and further purified using RNeasy columns (Qiagen). A total of 20 microarray slides were hybridised, scanned, and spot intensities measured with ImaGene (BioDiscovery). Two-colour spotted microarrays were normalised (Baird *et al.* 2004) and differential expression levels expressed as $\log_2$ ratios.

For quantitative RT-PCR, RNAs were reverse transcribed into cDNAs using the ThermoScript™ RT-PCR System (Invitrogen). Primers suitable for qPCR were designed for three clover specific genes, encoding flavonoid-galactosyltransferase, galactosidase, and actin. The cDNAs were mixed with iQ™ SYBR® Green Supermix (Bio-Rad), primer pairs and water, data points were collected at the annealing temperature and measured as fluorescence.

Results and discussion

The main flavonols accumulating in the leaves of white clover genotypes used in this study are kaempferol- and quercetin-3-*O*-galactosides (Kg, Qg) and K- and Q-3-*O*-

galactosyl-xylosides (Kgx, Qgx) which have been isolated and identified previously (Hofmann *et al.* 2000). We also identified three anthocyanins: delphinidin-, cyanidin-, and pelargonidin-3-*O*-galactosyl-xyloside (Dgx, Cgx, and Pgx) accumulating in red leaved white clover genotypes, by LC-MS (data not shown). All of these flavonols and anthocyanins were significantly higher in the red leaved genotype 38H (table 1A, ACs include all 3 anthocyanins). Cold treatment resulted in significantly higher levels of the flavonols in both genotypes after 3 days; anthocyanins were significantly increased after 5 days only.

GC-MS analysis of various sugars (table 1B) in the leaves of cold treated white clover plants revealed that most sugars including starch were strongly increased in all cold treated samples. "Other" sugars listed in table 1B comprise arabinose, galactose, ribose, xylose, raffinose and an unidentified dimeric sugar. Interestingly, we also found significant differences for some carbohydrates between the two genotypes; the red leaved clover 38H had lower levels of fructose, glucose and starch, indicating a reduced capacity to accumulate carbohydrates in these flavonoid accumulating plants.

The expression levels of selected genes possibly related to flavonoid biosynthesis (Fig. 1) in cold treated white clover leaves are presented in Fig. 2. Enzymes involved in flavonoid aglycone biosynthesis are chalcone synthase (CHS), chalcone isomerase (CHI) and several hydroxylases (flavanone 3-hydroxylase, F3H; flavonoid 3'-hydroxylase, F3'H; and flavonoid 3',5'-hydroxylase, F3'5'H) (Winkel-Shirley, B., 2001), which have been well characterised in a range of plant species. Subsequently, flavonols are synthesised by flavonol synthase (FLS) and anthocyanidins by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). Genes encoding homologues of CHS, CHI, F3'H, and FLS were up-regulated in both genotypes upon cold stress, which is in good accordance with the cold induced increase of flavonols. F3'5'H was not regulated by cold, which probably reflects the lack of 5'-hydroxylated flavonols (myricetins) in white clover leaves. DFR and ANS, which are specific for anthocyanin biosynthesis, were also up-regulated. Thus under cold stress there is a strong correlation between the coordinated up-regulation of genes for flavonoid aglycone biosynthesis and increased accumulation of flavonoid glycosides.

On formation, both flavonols and anthocyanidins are subsequently conjugated to carbohydrates by various glycosyltransferases and sequestered into vacuoles. Glycosidases in turn are involved in the turnover of flavonoid glycosides and a down-regulation of those would maintain high levels of conjugated flavonoids. No details are known about the specific enzymes involved in these processes in white clover, and homology searches identified a range of candidate glycosyltransferases and glycosidases. We identified one flavonoid galactosyltransferase (FGalTF, AGalTF) that was up-regulated and one galactosidase (Galase) that was down-regulated in the cold treated plants (Fig. 2B). Quantitative RT-PCR was used to verify the expression pattern from the microarray analysis of these genes (Fig. 3) and results were in good accordance with each other. A correlation analysis of flavonoid glycoside levels and gene copies showed a significant positive correlation between galactosyltransferase and flavonoid levels (Pearson correlation coefficient (Pcc): Flavonols 0.89; Anthocyanins 0.9) and a significant negative correlation between galactosidase and flavonoid levels (Pcc: Flavonols -0.86; Anthocyanins -0.8). Flavonoid galactosyltransferase levels were also higher and galactosidase levels lower in the untreated red leaved genotype 38H, reflecting the significantly higher basal levels of flavonoids compared to 35A.

Conclusions

Cold treatment induces several biochemical pathways in white clover including carbohydrate and flavonoid biosynthesis. A coordinated investigation of these processes at the gene and metabolite level has shown the coupled up-regulation of flavonoid aglycone biosynthesis with up- and down-regulation of genes involved in flavonoid glycosylation. These conjugation reactions provide a direct link between primary and secondary metabolic pathways within the metabolic network.

This research demonstrates how the development of whole genomics tools like microarrays and metabolome analytical techniques is providing a much deeper understanding of responses to environmental changes in forage plants like white clover which will open up new opportunities for cultivar improvement by combining molecular and conventional breeding technologies. Successful breeding depends on broad understanding of the genetic architecture of relevant traits. Genes with major effects and genes contributing to the expression of quantitative traits have both a role in controlling abiotic stress tolerance (Humphreys *et al.* 2005). Genetic markers are now developed for forage plants including white clover to be used for marker-assisted selection programmes (Barrett *et al.* 2004). Knowledge about specific genes involved in abiotic stress responses can help to identify "perfect" markers, such as structural or regulatory genes involved in stress tolerance.

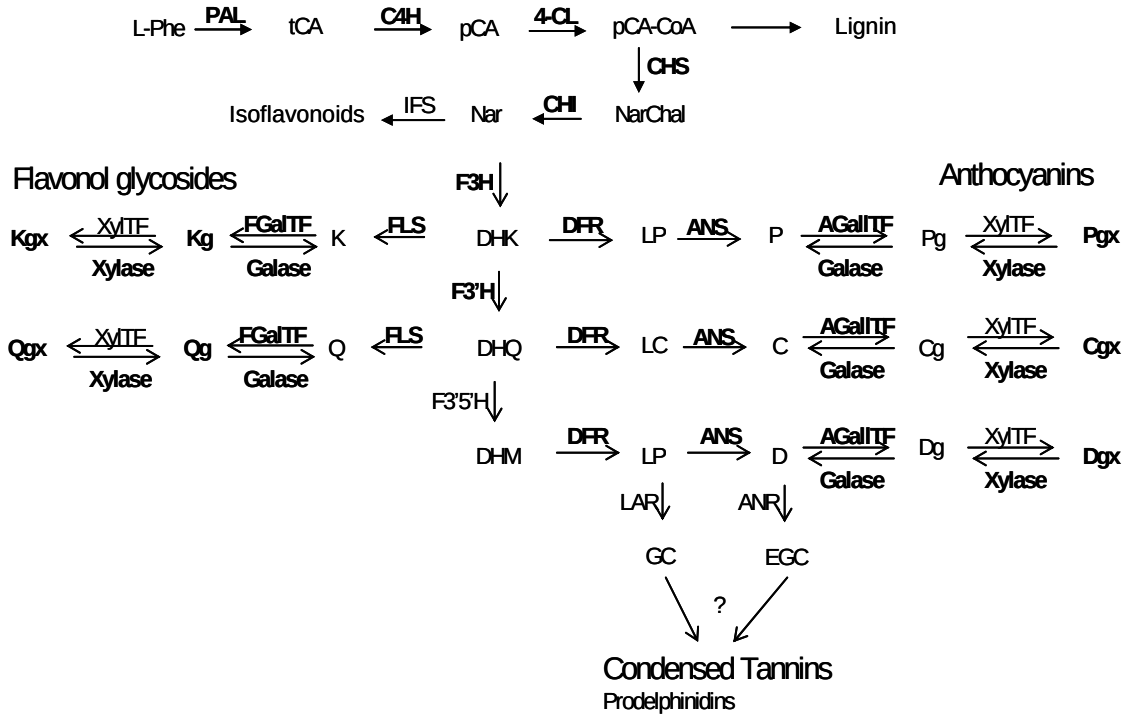
Table 1: Concentrations of A: flavonols (μg g^{-1} FW) and anthocyanins (rel. peak areas) and B: sugars (μg g^{-1} FW) in *T. repens* leaves of two genotypes with low (35A) and high (38H) levels of flavonoids grown at 25°C and 10°C, respectively.

	35A				38H			
	3 days		5 days		3 days		5 days	
	25°C	10°C	25°C	10°C	25°C	10°C	25°C	10°C
A:								
Kg[1]	3.6	14.1*	4.8	34.4*	37	45*	38	61*
Kgx[1]	10.5	17.4*	10.6	21.1*	152	177*	137	220*
Qg[1]	0.6	31.5*	0.6	78.3*	374	410*	315	560*
Qgx[1]	6.0	38.0*	4.6	106.5*	1008	1074*	916	1314*
ACs[1]	8.1	5.8	10.1	144.5*	9894	10194	11707	16510*
B:								
Fru[1]	1656	3139*	1053	3003*	964	1734*	702	1883*
Glc[1]	1278	2593*	759	2134*	550	1207*	412	1282*
Suc	1367	3489*	1389	3437*	2120	3565*	1793	3203*
Other	416.31	687.9*	518.69	683.94*	518.07	678.3*	530.91	688.6*
Starch[1]	13312	15179	15000	36406*	8024	12185*	4783	14079*

* denotes significant increase in cold treated plants ($P<0.01$)

[1] denotes significant genotypic differences ($P<0.01$)

Fig. 1**:** Schematic diagram of the flavonoid pathway in *T. repens* leaves. Metabolites and genes regulated by cold are presented in bold. For abbreviations see text.



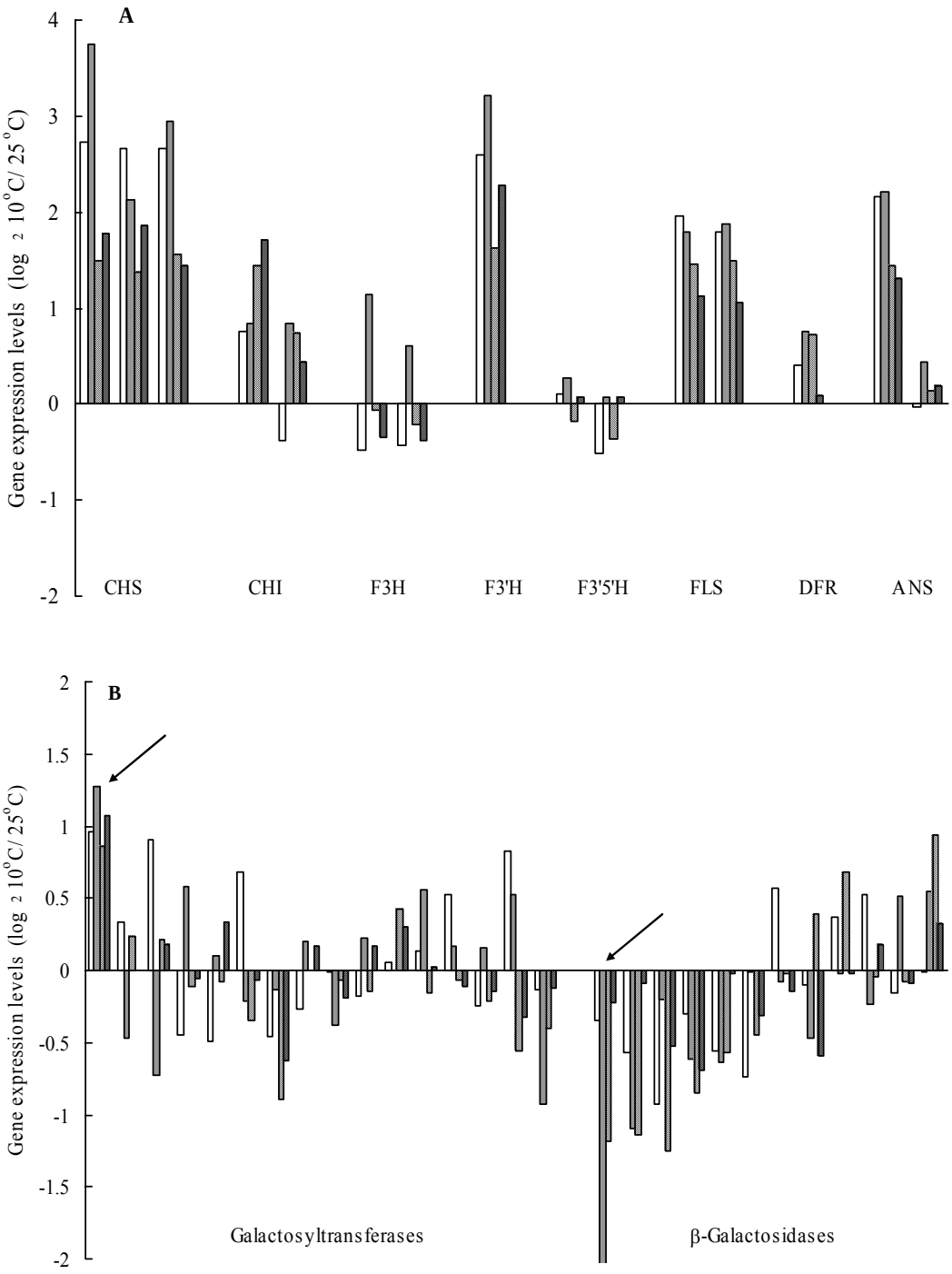
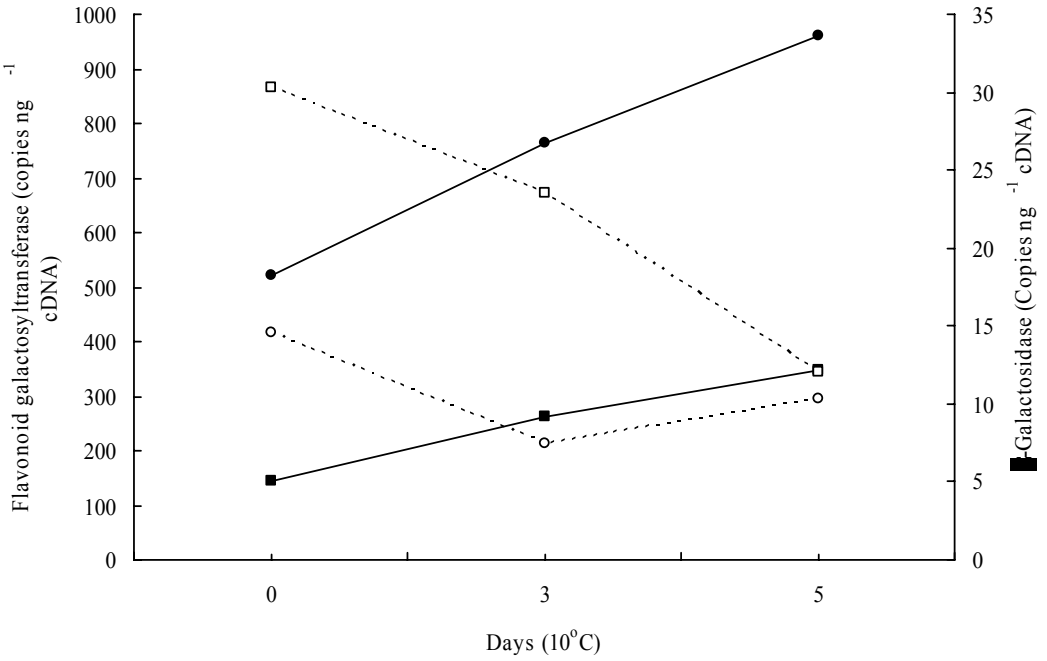


Fig. 2: Changes (log_2 ratios) in gene expression levels (cDNA microarrays) of flavonoid pathway genes (A) and putative flavonoid (de)conjugating genes (B) in *T. repens* leaves grown at 25°C and 10°C, respectively. White bars - 35A, log_2 10°C/25°C, 3 days; Grey bars - 35A, log_2 10°C/25°C, 5 days; Striped white bars - 38H, log_2 10°C/25°C, 3 days; Striped grey bars - 38H, log_2 10°C/25°C, 5 days.

Fig. 3**:** Quantitative RT-PCR of flavonoid galactosyltransferase and galactosidase in *T. repens* leaves grown for 3 and 5 days at 10^{o}C, respectively. Data are expressed as gene copies ng^{-1} cDNA and have been normalised to actin. (—) - Flavonoid galactosyltransferase, (■) - 35A, (●) - 38H; (---) - β-Galactosidase, (□) - 35A, (○) - 38H.



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