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Poplar trees for phytoremediation of high levels of nitrate and applications in bioenergy

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Summary

The utilization of high amounts of nitrate fertilizers for crop yield leads to nitrate pollution of ground and surface waters. In this study, we report the assimilation and utilization of nitrate luxuriant levels, 20 times more than the highest N fertilizer application in Europe, by transgenic poplars overexpressing a cytosolic glutamine synthetase (GS1). In comparison with the wild-type controls, transgenic plants grown under high N levels exhibited increased biomass (171.6%) and accumulated higher levels of proteins, chlorophylls and total sugars such as glucose, fructose and sucrose. These plants also exhibited greater nitrogen-use efficiency particularly in young leaves, suggesting that they are able to translocate most of the resources to the above-ground part of the plant to produce biomass. The transgenic poplar transcriptome was greatly affected in response to N availability with 1237 genes differentially regulated in high N, while only 632 genes were differentially expressed in untransformed plants. Many of these genes are essential in the adaptation and response against N excess and include those involved in photosynthesis, cell wall formation and phenylpropanoid biosynthesis. Cellulose production in the transgenic plants was fivefold higher than in control plants, indicating that transgenic poplars represent a potential feedstock for applications in bioenergy. In conclusion, our results show that GS transgenic poplars can be used not only for improving growth and biomass production but also as an important resource for potential phytoremediation of nitrate pollution.

Introduction

Nitrogen (N) is a key structural component of many plant biomolecules and consequently an essential macronutrient required for plant growth and development. In spite that this element is highly abundant in nature, it is frequently a limiting factor for plant growth. Most part of N is present in the atmosphere as dinitrogen, a form of N that cannot be assimilated by plants except for those associated with N-fixing microorganisms. Nitrate and ammonium are usually the major forms of inorganic N available to be assimilated by plants but their relative abundance in natural soils is quite low. For that reason, plants have evolved efficient and highly regulated mechanisms for N acquisition and assimilation.

Intensive N fertilization in agriculture has increased food production worldwide in the last decades, but it has also provided an excess of inorganic N that is not used by crop production. The excess reduced N is rapidly converted to nitrate by nitrification and, under anaerobic conditions, it may be denitrified to gaseous N compounds such as nitrous oxides (NOx) and emitted to the atmosphere (Schlesinger, 2009). Therefore, the massive utilization of N fertilizers has led to environmental problems with harmful effects including contamination of ground and surface waters and N-induced eutrophication of terrestrial and aquatic systems (Galloway *et al.*, 2008). In Europe, where nitrate fertilization dominates, nitrate pollution is a common problem in many areas with extensive agriculture. Poplar species growing in riparian zones are frequently exposed to high levels of N nutrition, mainly as nitrate, coming from streams draining agricultural lands (Rennenberg *et al.*, 2010). The ability of poplar trees to filter nitrate and achieve substantial reductions in the concentration of nitrate in contaminated waters have been reported (O'Neill and Gordon, 1994). In fact, poplars are well adapted to nitrate acquisition (Min *et al.*, 1998) through high-affinity and low-affinity nitrate transporters which encoded by a large gene family (Bai *et al.*, 2013).

Nitrate is a major macronutrient but also acts as a signal molecule to regulate plant metabolism and development (Miller *et al.*, 2007). Nitrate transporters are rapidly down-regulated when plants become N replete reaching the appropriate N status (Miller *et al.*, 2007). This is particularly important for woody perennials such as poplars, which integrate acquisition of external N with the endogenous metabolic processes of seasonal N recycling to maintain the N economy (Cantón *et al.*, 2005; Rennenberg *et al.*, 2010).

Once taken up and transported, nitrate is reduced to ammonium and incorporated to the pool of organic molecules in the reaction catalysed by the glutamine synthetase (GS, EC 6.3.1.2). GS plays a central role in the complex matrix of plant N metabolism as the enzyme catalyses the ATP-dependent condensation of ammonium and glutamate to form glutamine, a precursor of all nitrogenous compounds required for plant growth (Lea and Ireland, 1999). In addition, ammonium assimilated by GS can come from various metabolic activities of N recycling such as photorespiration, deamination of phenylalanine, and the mobilization of proteins and nucleic acids during senescence or in response to pathogen attack (Cren and Hirel, 1999). A small family of nuclear genes expressed in photosynthetic and nonphotosynthetic tissues encodes GS polypeptides which are assembled into oligomeric isoenzymes located either in the cytosol or in the plastids (Bernard and Habash, 2009). In

poplar, the GS family is organized in four groups of duplicated genes, three of which code for cytosolic GS isoforms (GS1.1, GS1.2 and GS1.3) and one that codes for the plastidic GS isoform (GS2) (Castro-Rodríguez *et al.*, 2011). Recent results support that GS gene redundancy may contribute to the homoeostasis of N metabolism in functions associated with changes in glutamine use in multiple metabolic pathways (Castro-Rodriguez *et al.,* 2015).

Consistent with the central role of GS in N metabolism, increased growth was observed in transgenic poplars overexpressing constitutively a pine *GS* gene (Fu *et al.*, 2003; Gallardo *et al.*, 1999; Jing *et al.*, 2004). Furthermore, enhanced GS expression in poplar resulted in enhanced efficiency in N assimilation (Man *et al.*, 2005) and altered wood chemistry (Coleman *et al.*, 2012).

In this study, we report assimilation and utilization of luxuriant levels of N by transgenic poplar overexpressing a pine cytosolic GS. Furthermore, transgenic poplars accumulate enhanced levels of cellulose in the aerial part of the plant. Poplar species are well suited for use in phytoremediation of a variety of environmental pollutants such as heavy metals, pesticides and waste products (Yadav *et al.*, 2010). Our results strongly support that GS poplars have improved potential for environmental remediation of polluted areas and at the same time they can be used as feedstocks for the biofuels or fibre markets. Field trials are necessary to assess the extent of this potential.

Results

Vegetative growth of transgenic poplars under high nitrate levels

Transgenic hybrid poplar *(P. tremula* × *P. alba)* plants exhibited higher growth than untransformed controls (WT) under adequate (10 mM) and luxuriant (50 mM) levels of nitrate, especially in the aerial part (Figure 1; Table 1). A total of 12 plants were analysed per treatment. During the 3-month growth period, plants grown under high nitrate (50 mM) showed greater vegetative growth; the fresh weight in the aerial part increased by 85.5% in controls and 193% in transgenic plants; meanwhile, dry weight (DW) increased by 53.5% in controls and 171.6% in the transgenics (Table 1). Transgenic plants exhibited root biomass increases under high nitrate supplementation of 44.5% in fresh weight and 27.9% increases in DW; in WT plants, main roots showed an 80% increase in fresh weight when were grown under high nitrate, whereas root fresh weight decreased (approximately 20%) in response to adequate 10 mM nitrate supply (Table 1).

Total N and C levels in transgenic plants reflect enhanced ability to assimilate nitrate

To further study the increased growth of transgenic plants under high nitrate, total N and C contents, nitrogen-utilization efficiency (NUtE) and nitrogen-uptake efficiency (NUpE) were examined in the same samples in which the biomass analyses were performed. Analyses were performed for three regions of the aerial part of the plants (L1/S1, L2/S2 and L3/S3) and two regions of the roots (R1 and R2) (Figure 2). Similar N and C contents were observed in WT and transgenic plants growing under 10 mM nitrate. In contrast, higher levels were observed in the transgenic plants at 50 mM nitrate. These increases were mainly observed in young leaves. Increases of eightfold in N content and sixfold in C content were observed in young leaves of transgenic poplar at 50 mM nitrate.

The parameter NUtE illustrates the use of N to produce biomass during growth under different N regimes (Good *et al.*, 2004). Thus, N availability differentially affected biomass accumulation in WT and transgenic plants. Transgenic plants accumulated more biomass than the WT in nearly all tissues, especially in young leaves. Significant differences between WT and transgenic plants under 10 mM nitrate were observed in young leaves, stems and secondary roots. In WT plants, increased NUtE at high nitrate was only observed in young leaves, whereas values remained unaltered in other plant regions.

The parameter NUpE showed the differences in N uptake and N metabolism between WT and transgenic plants under adequate and high N levels (Figure 2). Transgenic plants exhibited greater ability to remove N excess from soil and move it to leaves, particularly to young leaves. Enhanced N allocation was also observed in L2 (intermediate leaves) and L3 (mature leaves). WT plants under high nitrate showed limited ability to remove N excess when compared to the transgenics. Interestingly,

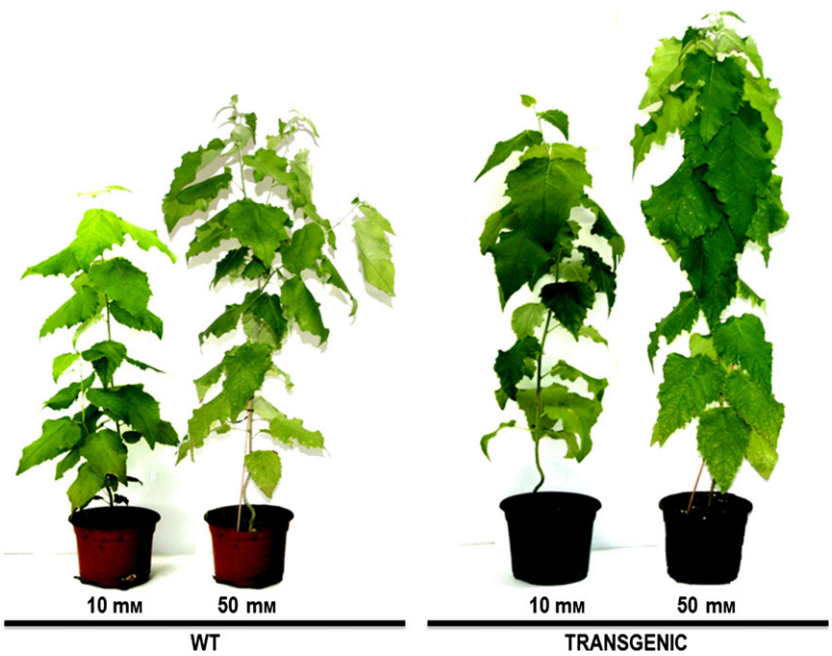

Figure 1 Phenotypes of 3-month-old untransformed controls (WT) and transgenic poplars irrigated with a solution containing adequate (10 mM) or high (50 mM) nitrate concentration.

Table 1 Biomass accumulation in control and transgenic poplars under nitrate nutrition

		WT			Transgenic		
Sample	Nitrate (mM)	Length (cm)	FW (g)	DW (g)	Length (cm)	FW (g)	DW (g)
Aerial Plant	10	31.84 ± 0.70	5.40 ± 0.12^{cde}	2.28 ± 0.07^{cd}	39.16 ± 1.03	7.61 ± 0.19^{bc}	3.10 ± 0.10^{bc}
Main Roots			2.10 ± 0.28	0.78 ± 0.10		2.36 ± 0.98	1.04 ± 0.43
Lateral Roots			6.32 ± 1.47^{cd}	1.33 ± 0.42		4.72 ± 0.97	1.50 ± 0.59
Aerial Plant	50	51.62 ± 1.09	10.02 ± 0.30^{b}	3.51 ± 0.16^{b}	63.63 ± 1.20^{a}	22. 35 ± 1.12^{a}	8.42 ± 0.27^{a}
Main Roots			3.78 ± 0.65	0.82 ± 0.30		4.00 ± 0.82	1.71 ± 0.50
Lateral Roots			5.31 ± 0.44^{cde}	1.12 ± 0.50		6.23 ± 1.44^{cde}	1.54 ± 0.46

The parameters length, fresh weight (FW) and dry weight (DW) were compared between WT and transgenics grown under different concentrations of nitrate. Means with different lowercase letters are significantly different, $P < 0.01$ (ANOVA and Tukey's test).

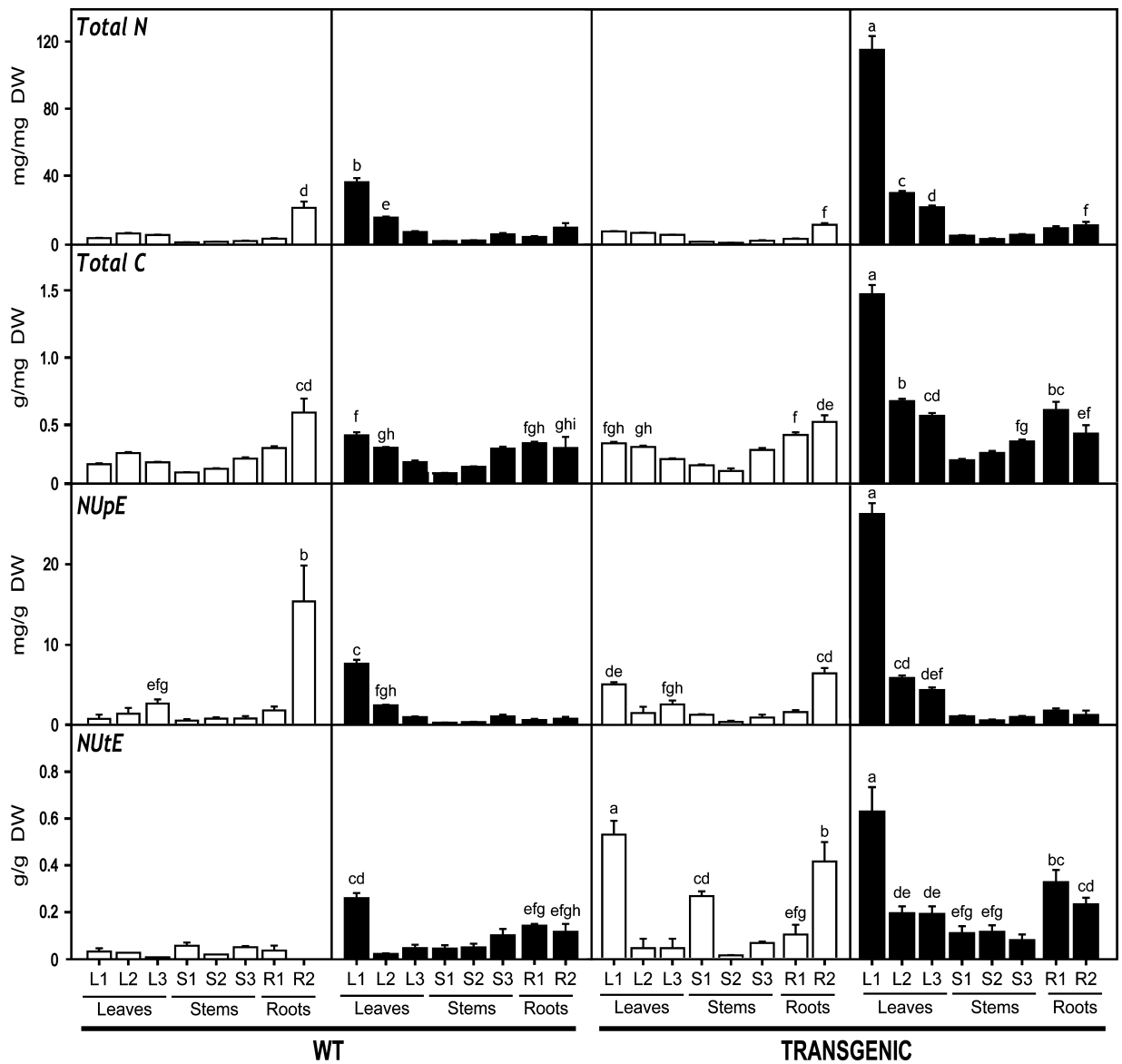

Figure 2 Spatial distribution of total N, total C, NutE and NupE in WT and transgenic poplars growing at different nitrate levels. Open bars correspond to poplars irrigated with a solution containing 10 mM of nitrate. Closed bars correspond to poplars irrigated with a solution containing 50 mM of nitrate. Values are means ± SD of three independent plant samples. Different letters indicate significant differences between samples at $P < 0.001$. The same notation is applicable to legends of Figures 3, 7 and 8.

enhanced N-uptake ability was observed in secondary roots of WT plants under adequate N availability.

Changes in soluble protein and chlorophyll contents in response to N excess

To further characterize the response of transgenic poplar to nitrate excess, protein and chlorophyll contents were determined (Figure 3). Compared with 10 mM nitrate conditions, both WT and transgenic plants grown on 50 mM nitrate exhibited greater levels of protein per fresh weight with the higher values exclusively found in young leaves and stems. Interestingly, young leaves of transgenic plants grown under nitrate excess accumulated significantly higher protein levels than WT plants grown under the same conditions. When compared to untransformed controls, transgenic plants also accumulated greater contents of chlorophylls a and b in their leaves under high nitrate. The accumulation of chlorophylls was significantly higher in young leaves of transgenic plants.

Changes in the transcriptome in response to N availability

The above data showed that the most significant changes that might explain the observed growth differences between WT and transgenic trees occurred in the young leaves under high N. To understand the molecular basis for these differences, transcriptomic studies in young leaves of plants grown at 10 and 50 mM nitrate were performed. Changes in the leaf transcriptome were more pronounced in the transgenics than in the WT (Figure 4, compare 50 mM WT vs 50 mM Transgenics and 10 mM WT vs 10 mM Transgenics; Table S2). Figure 4 also shows that 213 genes are in common between WT and transgenic plants in their response at high nitrate levels. Table 2 shows that a total of 1237 genes were a minimum of twofold differentially expressed in the transgenic leaves, 826 up-regulated and 411 down-regulated. In contrast, only 632 genes were differentially regulated in response to nitrate excess in untransformed controls, although a similar proportion of up-regulated and down-regulated genes were observed. Changes in the leaf transcriptome were also observed between untransformed and transgenic plants grown at high nitrate (Figure 4, 50 mM WT vs 50 mM Transgenics; Table S2). However, lower differences in the number of genes differentially expressed were observed when untransformed and transgenic plants were grown at adequate levels of nitrate (Figure 4, 10 mM WT vs 10 mM Transgenics; Table S2). Table 2 shows that 1346 genes were differentially expressed in the leaves of transgenics with respect to WT at nitrate excess. The proportion of up-regulated and down-regulated genes was similar to that of observed in the transgenics when grown at high nitrate. However, only 631 genes were differentially expressed in the transgenic plants when grown at adequate levels of nitrate with 391 up-regulated and 240 down-regulated genes (Table 2).

Validation of transcriptome analysis

To validate the transcriptome profiles in response to nutritional changes, we selected a number of representative genes for accurate determination of their expression levels using qPCR (Figure S1, Table S5). Overall, the expression analysis of the selected genes confirmed the microarray results. *PtPIP* (plasma membrane intrinsic protein), *PtNAC* (NAC domain protein), *PtMYB* (MYB transcription factor) and *PtFBA* (fructose-biphosphate aldolase) had enhanced expression in the transgenic plants under high N levels (Figure S1, panel 10 vs 50 mM Transgenics). In contrast, *PtFBox* (flavin-binding domain F-box protein), *PtWR* (wound-responsive protein-related), *PtPTR* (proton-dependent oligopeptide transport protein) and *PtTIP* (water channel tono-

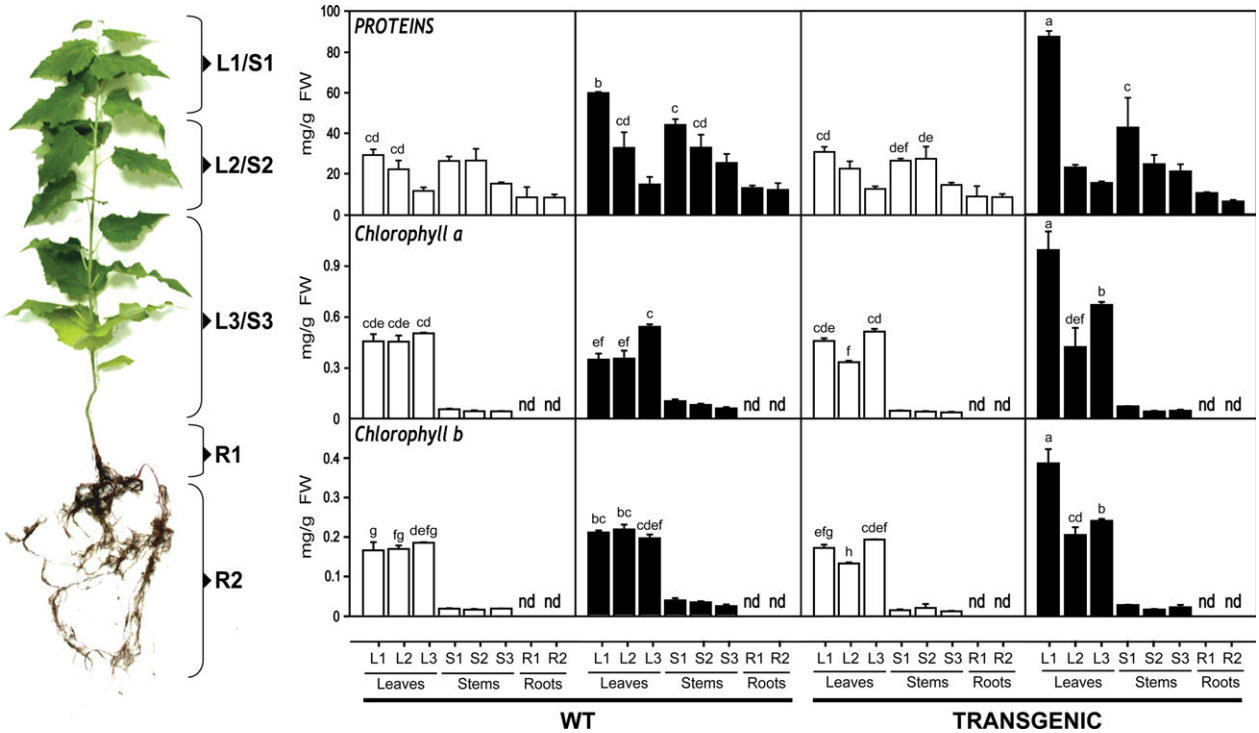

Figure 3 Spatial distribution of protein and chlorophyll contents in WT and transgenic poplars growing at different nitrate levels.

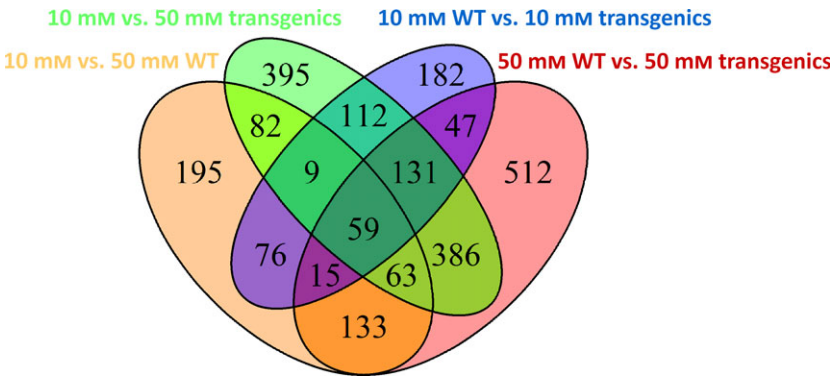

Figure 4 Changes in the transcriptome of WT and transgenic poplars grown at different nitrate levels. Venn diagram depicting the overlap between genes differentially expressed in 10 mM vs 50 mM WT, 10 mM vs 50 mM Transgenics, 50 mM WT vs 50 mM Transgenics and 10 mM WT vs 10 mM Transgenics.

Table 2 Differentially expressed genes in control and transgenic poplar leaves in response to high nitrate nutrition

Condition	Total	Up-regulated	Down-regulated
10 mM vs 50 mM WT	632	428	204
10 mM vs 50 mM transgenics	1237	826	411
50 mM WT vs 50 mM transgenics	1346	856	490
10 mM WT vs 10 mM transgenics	631	391	240

Effect of increased nitrate levels in WT and transgenic plants (10 mM vs 50 mM WT and 10 mM vs 50 mM Transgenics) and comparison of WT and transgenic plants under the same concentration of nitrate supply (50 mM WT vs 50 mM Transgenics and 10 mM WT vs 10 mM Transgenics).

plast intrinsic protein) had lower expression in the same samples. *PtFBox*, *PtPIP* and *PtWR* were expressed differently in WT plants under high nitrate (Figure S1, panel 10 vs 50 mM WT).

Functional enrichment analysis of the leaf transcriptome in response to N availability

In regard to the identity of the genes differentially regulated in response to nitrate excess, we first analysed those related to primary and secondary metabolism. Among the metabolic pathways that were activated in transgenic poplar in response to high nitrate were the biosynthesis of phenylpropanoids, terpenoids and flavonoids, the biosynthesis of the cell wall and the pathways involved in photosynthesis and carbon metabolism (Figures 5 and S2; Tables S1, S3 and S4). Genes involved in biosynthesis and transport of amino acids, aromatic compound synthesis, carboxylic and organic acid transport, glucosinolate, glucans and cellulose showed up-regulation (Tables S1, S3 and S4). However, transgenic plants grown under adequate nitrate levels had higher expression of ammonium transporters and TIP aquaporins. The regulation of transcription was altered in the transgenic plants growing at 50 mM nitrate with increased expression of a huge number of transcription factors (Figures S2 and S3; Tables S1, S3 and S4). There were a number of changes in genes involved in signalling, protein degradation and pathogenesis related (PR) triggering changes in the expression profiles (Figure 6) but not function enrichment in response to nitrate nutrition.

WT plants under adequate N displayed differential expression of genes involved in secondary metabolism mainly in flavonoid synthesis (Figure 5). In contrast, under high nitrate, WT plants enhanced expression of genes involved in regulation of carbon metabolism (as for example starch degradation) and in transcription factors (Figures 6, S2 and S3, and Tables S3 and S4). We identified changes in gene expression related to biotic and abiotic stresses, signalling and regulatory factors. Most of the signalling genes differentially expressed involved in the response to stress were up-regulated in high nitrate (Figure 6; Tables S1 and S2 and S4). Finally, there were a higher number of genes involved in proteolysis that were also up-regulated (Figure 6; Tables S1, S3 and S4).

Increased expression levels of secondary and minor CHO metabolism genes were found in transgenic plants when the WT and transgenic plants were compared (Figures S2 and S4; Tables S1, S3 and S4). In all cases, WT plants had higher expression levels of genes involved in central carbon metabolism (glycolysis and starch and sucrose metabolism) (Figures S2 and S4; Tables S1, S3 and S4). Nevertheless, in transgenic plants under 50 mM nitrate, photosynthesis-related genes were up-regulated (Figure S4). Nitrate nutrition had huge effects over the expression of genes involved in transcription, signalling and PR (Figures S2, S5 and S6; Tables S1, S3 and S4). At 10 mM nitrate, there were more transcription factors, signalling and PR genes up-regulated in WT plants, while at 50 mM this tendency was inverted being up-regulated this group of genes in the transgenic plants (Figures S2, S5 and S6; Tables S1, S3 and S4). The histones were a particular case. All differentially expressed histone genes were down-regulated at adequate nitrate levels whereas up-regulated in transgenic plants at high nitrate (Figure S6 and Table S4).

Transcript levels of poplar *GS* genes and *GS1a* in response to high nitrate

As N excess affected morphological and physiological traits of WT and transgenic plants, we were interested in determining to what extent the expression of endogenous *GS* genes and the transgene were also affected (Figure 7). In WT plants, higher levels of *PtGS1.1* transcripts were observed under high N, especially in leaves and young stems. In transgenic plants, high expression levels of *PtGS1.1* were observed in intermediate leaves (L2), older leaves (L3), and lateral roots (R2) independently of N availability (Figure 7). *PtGS1.3* expression was preferentially detected in the stems S1 of WT and transgenic plants, but transcript abundance was clearly enhanced in the stems of transgenic plants with a significant increase in S2 of transgenics under high nitrate (Figure 7). *PtGS2* was mainly expressed in young leaves of WT and transgenics, and the expression levels were not affected by the N availability (Figure 7). The pine *GS1a* transgene was expressed in all regions of the plants examined independent of N availability, but higher levels of *GS1a* were observed in leaves in comparison with other tissues analysed (Figure 7).

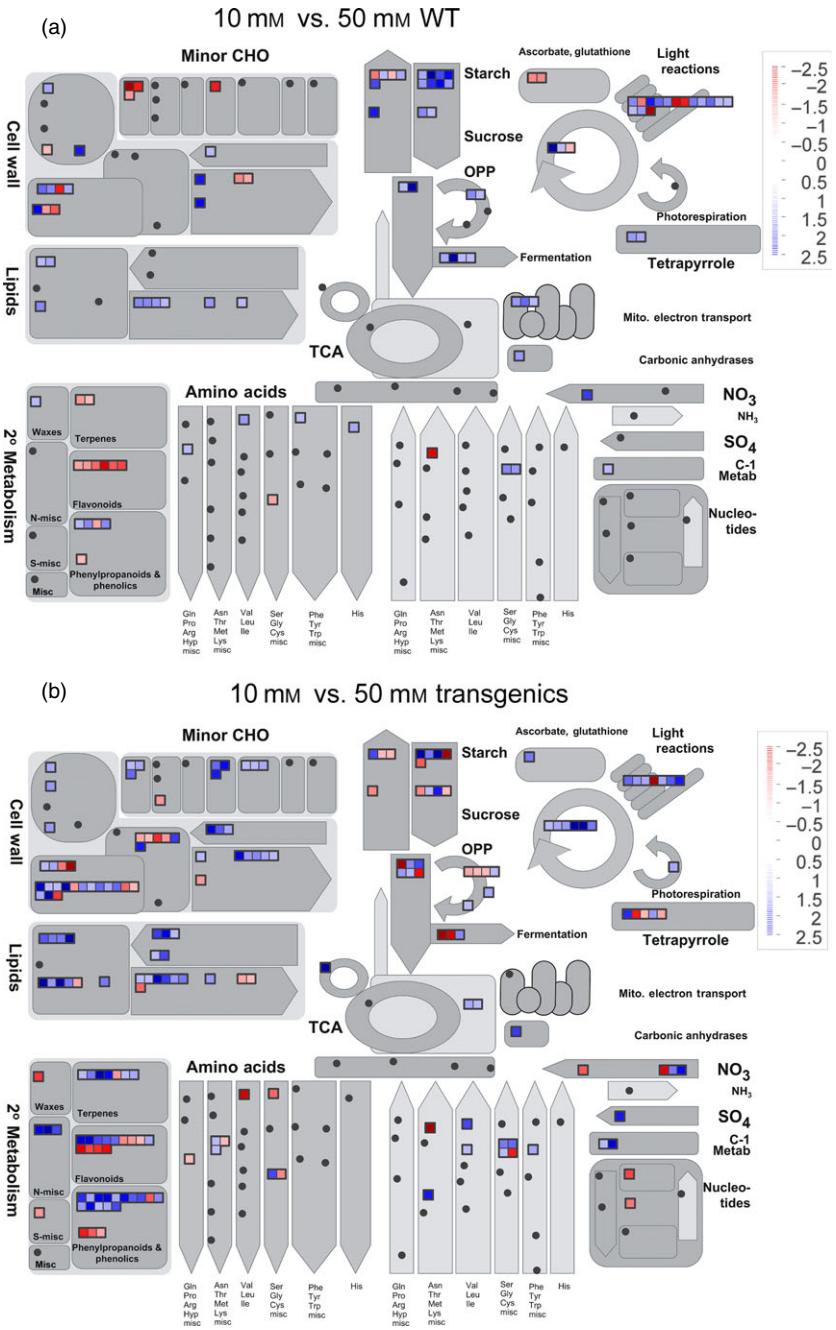

Figure 5 Mapman representation of a metabolism response overview. (a) Comparison of WT plants growing at 10 and 50 mM nitrate. (b) Comparison of transgenic plants growing at 10 and 50 mM nitrate. Blue scale indicates up-regulated genes at 50 mM. Red scale indicates down-regulated genes at 50 mM.

Changes in carbohydrates and lignin content in response to N excess

To understand the balance between N and C utilization, levels of carbohydrates and total lignin were examined in WT and transgenic plants (Figure 8). In general, glucose, fructose and sucrose profiles were similar for transgenic plants under all conditions. Glucose, fructose and sucrose profiles in WT plants grown under 10 mM nitrate were clearly lower than in plants grown under high nitrate nutrition. In transgenic plants, free sugars accumulated preferentially in leaves, and especially in young leaves under high N. This was particularly evident for glucose. Higher levels of starch were found in lateral roots of transgenic plants grown under high nitrate nutrition followed by WT plants grown under the same conditions. In fact, starch amounts were significantly higher in lateral roots grown under 50 mM nitrate than in roots grown under 10 mM nitrate. Maximum cellulose contents were observed in response to high N availability in transgenic plants; there was no significant effect on the WT plants grown under high nitrate nutrition. Cellulose contents showed significant increases in young leaves (L1 and L2) and in young stems (S1 and S2) mainly in S1. Total lignin showed similar profiles in all the plants increasing from the young leaves to the lateral roots (from L1 to R2). However, lignin contents were higher in the roots of plants grown at 50 mM nitrate. Furthermore, the transgenic plants at 50 mM nitrate exhibited the higher lignin content in the roots (R1 and R2) and in the middle stem (S2).

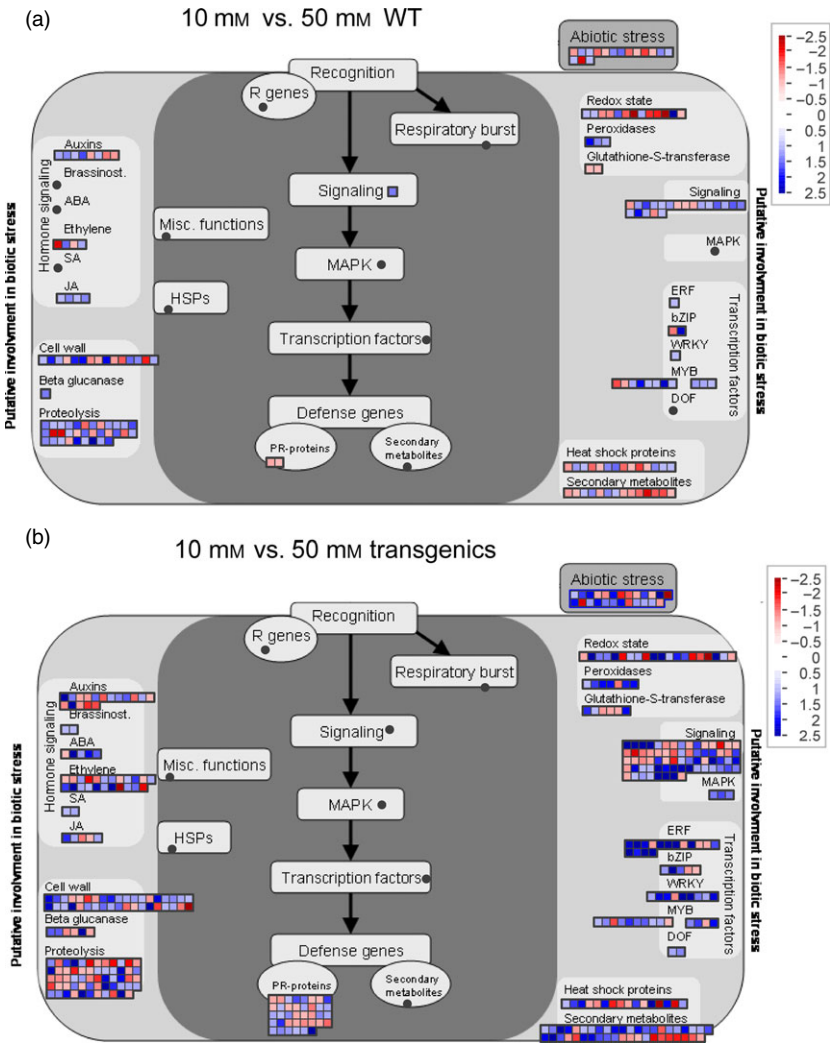

Figure 6 Mapman representation of a stress response overview. (a) Comparison of WT plants growing at 10 and 50 mM nitrate. (b) Comparison of transgenic plants growing at 10 and 50 mM nitrate. Blue scale indicates up-regulated genes at 50 mM. Red scale indicates down-regulated genes at 50 mM.

Discussion

The utilization of high amounts of nitrate fertilizers for crop yield leads to nitrate pollution of ground and surface waters. In Europe, application rates of N fertilizers vary from the lowest level of 42 kg/ha for agricultural land in Portugal to 243 kg/ha for grassland in the Netherlands (Erisman *et al.*, 2011). In this study, we report the assimilation and utilization of luxuriant levels of N by transgenic poplar overexpressing a pine cytosolic glutamine synthetase (GS1a) (equivalent to 5.3 t/ha N, much higher than the average N fertilizer application in Europe). The assimilation of these high levels of nitrate resulted in increased poplar biomass, growth and nitrogen-use efficiency (NUE). These results are consistent with previous reports of enhanced growth in young transgenic plants expressing the pine GS1a (Fu *et al.*, 2003; Gallardo *et al.*, 1999) and in a field trial of GS1a transgenic poplar (Coleman *et al.*, 2012; Jing *et al.*, 2004), and improved NUE of these plants (Man *et al.*, 2005).

Our data indicate that GS transgenic poplars are able to maintain balanced C and N assimilation even under luxuriant N levels. We have shown that GS1a transgenic plants provided with high N levels (50 mM nitrate) exhibit increased biomass (171.6%) compared with transgenic and WT plants grown under adequate N levels (10 mM nitrate). Total soluble proteins and contents of chlorophylls a and b also increased significantly in young leaves under luxuriant N levels. Previous analyses of GS1a transgenic poplars also observed reduced free ammonium amounts and increased glutamine levels in response to nitrate (Man *et al.*, 2005). We hypothesized that increased availability of organic nitrogen in the form of glutamine confers metabolic advantages to transgenic plants, resulting in improved growth and biomass (Cánovas *et al.*, 2006). Furthermore, it has been suggested that overexpression of cytosolic GS results in enhanced primary assimilation of ammonium and reassimilation of N released in photorespiration, protein remobilization and lignin biosynthesis (Cánovas *et al.*, 2007; Man *et al.*, 2005; Oliveira *et al.*, 2002). N assimilation and biomass production have a relationship with efficient N uptake and allocation into the plant. Previous studies suggest that leaf area and biomass are principal sinks under increased N availability in poplar trees (Cooke *et al.*, 2005).

Total sugars (glucose, fructose and sucrose) decreased in WT plants under luxuriant N levels, suggesting a shortage of carbon skeletons for N assimilation. However, their levels increased in the transgenics under these nutritional conditions, suggesting that GS-overexpressing plants have greater capacity to assimilate N. This likely reflects increased photosynthetic rates and carbon

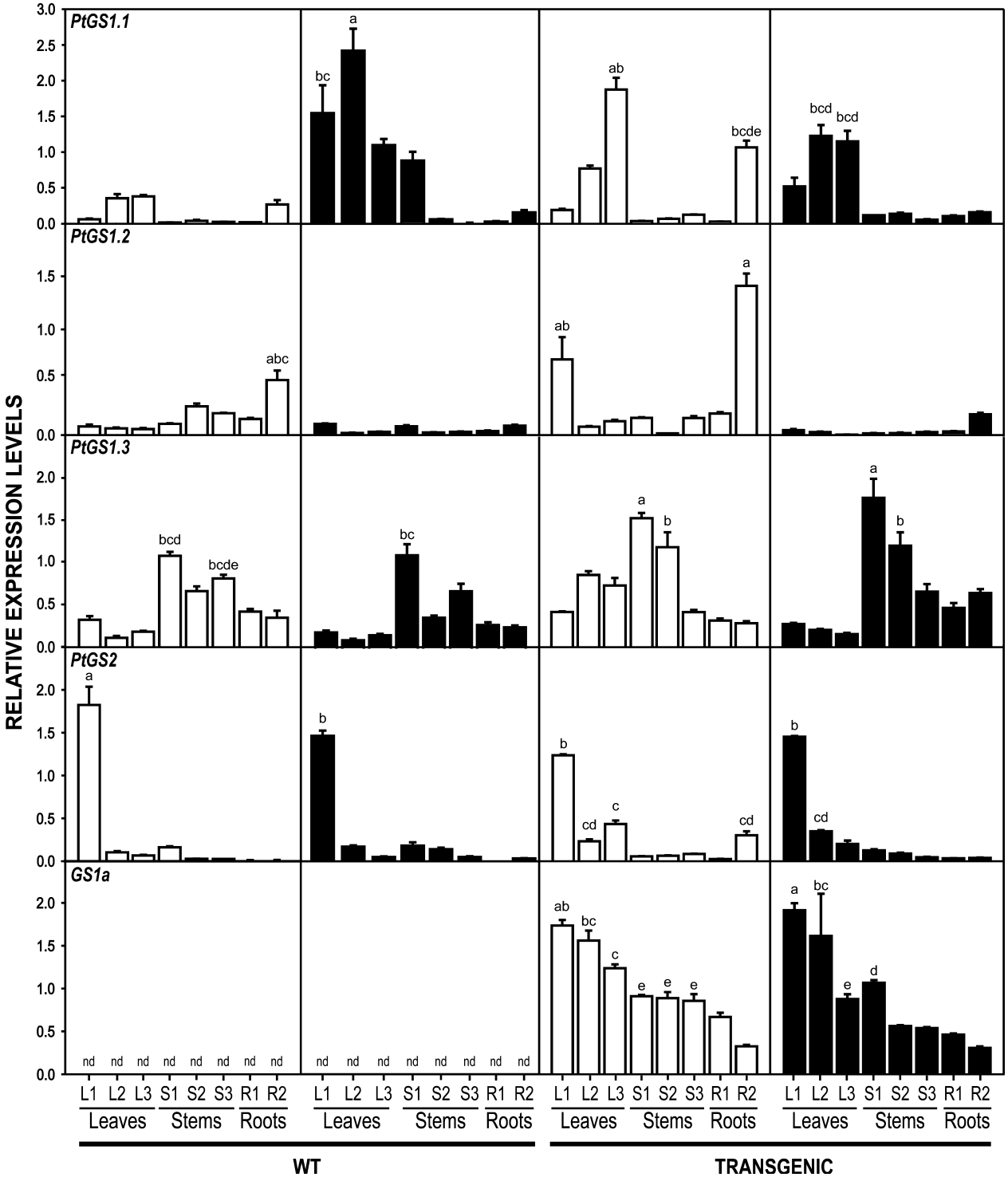

Figure 7 Spatial distribution of GS transcripts in WT and transgenic poplars growing at different nitrate levels.

fixation capacity (Cooke *et al.*, 2005; Man *et al.*, 2005). In fact, it was previously determined that net photosynthetic rates were higher in transgenic than in nontransgenic plants (El-Khatib *et al.*, 2004). GS transgenic poplar growing in field trials grew faster and produced more biomass in shorter time (Jing *et al.*, 2004), and composition of cell wall carbohydrates, including glucose, galactose, xylose and mannose in wood was increased (Coleman *et al.*, 2012). Starch accumulated in the poplar roots under luxuriant N levels, and some studies have proposed a negative correlation between starch concentration and plant N status (Wetzel *et al.*, 1995). In contrast, we have found starch accumulation in secondary roots of both transgenic and WT plants under high N, suggesting that the production of C skeletons (e.g. sucrose) in the aerial part of the plant is sufficient for N assimilation, and assimilated C excess could likely be stored in the roots. In fact, the transcriptomic data indicate that a β-amylase similar to *PtBAMY1*

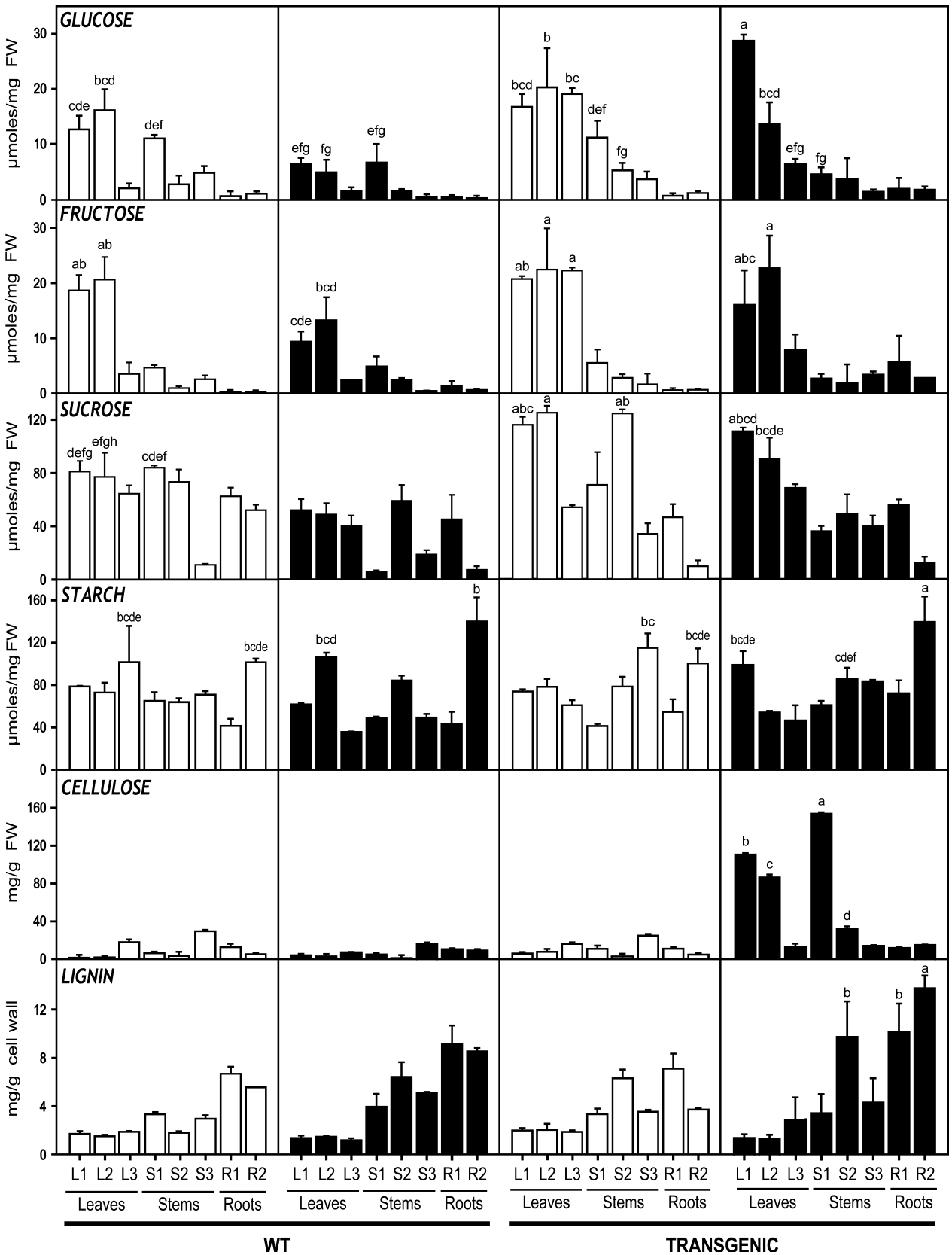

Figure 8 Spatial distribution of carbohydrates and lignin in WT and transgenic poplars growing at different nitrate levels.

(pt_40322) was up-regulated in young leaves of transgenic poplar under N luxuriant levels. These findings suggest that starch breakdown was activated providing C skeletons for N assimilation (Geisler-Lee *et al.,* 2006).

Differences in C and N allocation in poplar are correlated with different growth strategies during biomass formation and N usage (Li *et al.*, 2012a,b; Novaes *et al.*, 2009). Our data indicate that transgenic plants have greater capacity for N uptake and use even under luxuriant N levels. NUtE in transgenic roots was higher than in WT roots under adequate and luxuriant levels, and this is can be explained considering that in comparison with the WT, transgenic plants have a greater capacity to carry out primary N assimilation (from the soil) and, therefore, greater capacity to exploit mineral resources. Transgenics consequently display enhanced tolerance to high N levels. The highest NUtE and NUpE values were found in young leaves of transgenic plants under luxuriant N levels, the major organ where ammonium is assimilated by the glutamine synthetase/glutamate synthase (GS-GOGAT) cycle and then transferred to amino acids and other N compounds (Bernard and Habash, 2009). As previously suggested, poplar trees translocate most of resources to aboveground tissues (Cooke *et al.*, 2005). Interestingly, the NUpE was five times higher in the young leaves of transgenic plants under high N reflecting that N allocation and metabolism in transgenic plants are more efficient than in WT plants, confirming previous studies (Man *et al.*, 2005). Consequently, GS1 overexpression provides advantages for N assimilation in the leaves and improves the assimilation of carbon skeletons providing more biomass in the aerial part. NUpE involves different metabolic pathways, and N compounds allocated in the leaves could enhance the metabolic status of the plant and plant N use (Hirose, 2012). The high NUpE and low NUtE in the roots of WT plants under adequate N likely reflect that these plants are as efficient in N uptake but not in N use when compared to transgenics. During vegetative growth, leaves and roots are the principal sinks for inorganic N and the synthesis of amino acids for transport (Kant *et al.*, 2011).

The poplar transcriptome is altered during growth under high nitrate levels, resulting in changes in the expression of a range of genes involved in different processes (this paper, Cooke *et al.*, 2003, 2005). The number of genes differentially expressed in the leaves of GS transgenic plants was significantly higher than in the WT. In fact, 1346 genes were differentially regulated between transgenic and WT plants (minimum twofold) when plants were grown under luxuriant N compared with only 631 differentially expressed under N adequate levels. In transgenic plants, genes involved in phenylpropanoid, terpenoid and flavonoid biosynthesis were up-regulated under N luxuriant levels, consistent with enhanced carbon metabolism and N recycling. For example, the cellulose synthase like gene *PtCslD4* (pt_28112) was overexpressed only in transgenic plants under high N. This gene is mainly expressed in the shoot tips and is involved in poplar cell wall formation and modification (Suzuki *et al.*, 2006).

Lignin biosynthesis has been reported to decrease under high N availability in poplar (Novaes *et al.*, 2009; Pitre *et al.*, 2007), and our data show that there are lower lignin contents in the apical regions (young leaves and stems) likely reflecting the redirection of C skeletons for biosynthesis of other carbohydrates such as cellulose. This is consistent with the overexpression of *PtCslD4* and cellulose accumulation in young leaves. Under N luxuriant levels, young poplars increased shoot biomass and wood cellulose contents (Cooke *et al.*, 2005). N fertilization stimulates carbon allocation in shoots over roots and alters wood chemistry traits with increases in cellulose and hemicellulose levels and reduction in lignin content (Novaes *et al.*, 2009).

Furthermore, expression of genes for numerous physiological processes was modified in the transgenics including genes for biosynthesis and catabolism of amino acids and aromatic compounds and nutrient transport. Expression of genes for membrane transport proteins was also activated; for example, *PtAAP11* (pt_05342) has an important role during N transfer during xylem differentiation (Couturier *et al.*, 2010), and we have found that this amino acid transporter was up-regulated in transgenic and WT plants under high N, suggesting that this gene responds to N availability and could modify wood formation. A high-affinity nitrate transporter similar to *Arabidopsis* AtNRT2.7 (pt_00777) was up-regulated in transgenic plants under high nitrate. In contrast, *PtAMT3.1* (pt_32332), highly expressed in senescent leaves (Couturier *et al.*, 2007), was down-regulated under high N, suggesting that N availability repressed its expression.

The gene for *win-like VSP 425* protein (pt_ pt_34191) was highly expressed under N luxuriant levels in transgenic plants, suggesting that these vegetative storage proteins promote purine degradation and increase NUE through N remobilization from sink to source (Werner and Witte, 2011). These results are consistent with previous reports describing that *win4* and *bsp* respond to high N concentration levels (Coleman *et al.*, 1994; Cooke *et al.*, 2005).

Specific AP2 domain-containing transcription factors were up-regulated only in transgenic plants under high N. These genes are induced by external stimuli (Zhuang *et al.*, 2008), suggesting that these families of transcriptional regulators may play a crucial role in plant growth. Other transcription factors overexpressed in the GS transgenics under high N are *NAC*- (Li *et al.*, 2012a,b; Zhao *et al.*, 2014) and *MYB*-related proteins involved in wood formation in *Populus* (McCarthy *et al.*, 2010; Wang *et al.*, 2014; Zhong *et al.*, 2011). WRKY proteins implicated in abiotic stress responses (He *et al.*, 2012) showed similar profiles, and all these results are consistent with the observed tolerance of GS transgenics to different types of stress (Cánovas *et al.*, 2006). Overall, transcription factors were up-regulated in transgenic plants, particularly at high nitrate levels.

Expansins associated with woody tissues have an important role during xylogenesis (Gray-Mitsumune *et al.*, 2004). *PtEXLA1.1* (pt_03200), a gene mainly associated with developing wood tissues, was up-regulated under high N levels in transgenic poplar. When *PttEXPA1* was overexpressed in poplar, the transgenic plants increased expansion activity on cellulose–xyloglucan composites, resulting in stem elongation, leaf expansion and enhanced cell wall expansion (Gray-Mitsumune *et al.*, 2008). Genes implicated in cell wall growth, including expansin and xyloglucan, for example xyloglucosil transferase (pt_12600), were activated in response to N, as were genes involved in photosynthesis and Calvin cycle (Scheible *et al.*, 2004). This expression pattern confirms that for enhanced vegetative growth in transgenic plants under high N, it is necessary to have a preferential flux of carbon to the shoot and young leaves (Geisler-Lee *et al.,* 2006).

Transcripts for various genes implicated in DNA structure, repair and general process were up-regulated in WT plants under high N but down-regulated under adequate N nutrition. Rapid changes in histones produce specific DNA/chromatin modifications that play a crucial role in the regulation of responses to plant abiotic and biotic stress (Boycheva *et al.*, 2014). These findings together with the observed up-regulation of transcription factors suggest a general reorganization of the regulation of gene

expression induced by N nutrition, which affects differently to transgenic and WT plants.

Endogenous GS transcripts in *Populus* display organ-specific expression profiles (Figure 7) confirming previous studies (Castro-Rodríguez *et al.*, 2011). Interestingly, the expression of *PtGS1.1* increased more than fourfold in the leaves of WT plants under luxuriant N levels. *PtGS1.1* codes for a high-affinity ammonium enzyme that seems to play a crucial role in ammonium recycling in mature leaves (Castro-Rodríguez *et al.*, 2015). The ectopically expressed pine *GS1a* has an expression profile independent of N availability. The *GS1a* sequence is a putative target gene for *miR-3630-3p* miRNA. It has been reported that *miR-3630-3p* targeted nucleotide-binding leucine-rich repeat (NB-LRR) and F-box genes (Barozai *et al.*, 2012) are induced during abiotic stress (Li *et al.*, 2011). MicroRNAs are potential regulators of transgene targets throughout the plant, especially when their expression is associated with enhanced vegetative growth (Fu *et al.*, 2012; Zhang and Li, 2013). Considering these previous reports, *miR-3630-3p* could be important in the regulation of the pine transgene under high N.

Our results highlight the accumulation of biomass in aerial parts of *GS1a* transgenic plants in response to high N availability. Cellulose content in the leaves of transgenics increased fivefold in response to luxuriant N levels, whereas lignin content increased in basal stems and roots of plants grown under similar conditions. Previous studies have shown that increased biomass in trees is concomitant with reduced levels of lignin, reflecting a strong correlation between the balance in lignin biosynthesis and biomass accumulation in woody tissues (Novaes *et al.*, 2010). However, more lignin provides more structural support to growing plants with increased biomass (Donaldson, 2001). Our results indicate that wood formation in stems was regulated by increasing lignin content in the middle and basal section to support the biomass accumulation in apical regions. Analysis of wood chemistry of field-grown *GS1a* transgenics has shown enhanced levels of hemicellulose-associated polymers (galactomannans) and decreased lignin contents (Coleman *et al.*, 2012). Similar results were reported for poplar exposed to enhanced N fertilizer treatments (Pitre *et al.*, 2007). These data suggest that *GS1a* transgenic poplars grown under high nitrate levels may be of significant value in pulp and biofuels applications because they have improved pulping traits (high S/G ratios) and enhanced fibre characteristics (Coleman *et al.*, 2012). Taken together, our results strongly support the use of GS1a transgenic poplars as potential phytoremediation tools for environmental control of polluted areas and as feedstocks for the biofuels or fibre markets. Field trials will be required to assess these potential applications.

Experimental procedures

Plant material, culture conditions and sampling

Untransformed (WT) hybrid poplar (*Populus tremula* × *P. alba*, clone INRA 7171-B, INRA) and a transgenic line (line 4-29) overexpressing a glutamine synthetase cytosolic pine (GS1a) under the control of cauliflower mosaic virus 35S promoter were produced and maintained *in vitro* as previously described (Fu *et al.*, 2003; Gallardo *et al.*, 1999). The line 4-29 was identified as one of the superior performing lines in the growth studies performed in the greenhouse and under natural conditions (Fu *et al.*, 2003; Jing *et al.*, 2004). Rooted shoots of transformed and WT plants were maintained in plant growth chambers with a photoperiod of 16 h light with light intensity of 295 μmol m^2/s, a constant temperature of 24 °C and 80% humidity. Plants were cultivated in plastic pots containing a potting mix and vermiculite in proportions 1:1 and watered with distilled water supplemented with macro- and micronutrients for optimal growth (Gallardo *et al.*, 1999; Jing *et al.*, 2004). After 8 weeks, plants with similar height (approximately 60 cm) were divided by genotype: WT and transgenic poplars (24 individuals per genotype) and each group of plants were then subdivided randomly into two groups. One of the groups was irrigated with a nutrient solution enriched with 10 mM potassium nitrate, considered as adequate for poplar vegetative growth according to the previous data reported by Man *et al.* (2005). The remaining group of plants was supplied with 50 mM nitrate, a luxuriant concentration of nitrate for poplar according to Cooke *et al.* (2003). The plants were watered with the nutrient solution once a week by flooding, for a total of 4 weeks. Three-month-old *P. tremula* × *P. alba* plants were harvested and samples taken from eight different sections from the shoot apex to the root tip.

Growth measurements were carried out taking into account differences in height growth of the plants (12 plants were analysed per treatment). The aerial regions of the plants were divided into three parts: the 1st, 2nd, 3rd, 4th and 5th apical leaves (L1) and the corresponding stem (S1); the intermediate region with the 6th, 7th, 8th, 9th and 10th leaves (L2) and the corresponding stem (S2); and the more basal region including 11th, 12th, 13th, 14th and 15th leaves (L3) and the corresponding stem (S3). The root was sectioned into R1, main root and R2, secondary root. Harvested leaves, stems and roots were immediately frozen in liquid nitrogen. The samples were ground into fine powder in liquid nitrogen with a mortar and pestle and stored at −80 °C until processing. Frozen powder (100 mg) from the samples of each plant was dried at 70 °C for 48 h to determine the fresh to dry mass. For further studies, a fine powder was prepared from leaves, stems or roots of plants subjected to the two nutritional treatments. Extractions were performed from each plant per triplicate.

Determination of proteins and chlorophylls

Soluble proteins were extracted from poplar samples with a mortar and pestle according to the following procedure: 1 g of tissue, 1 g of fine sand and 1 mL of extraction buffer [0.175 M Tris pH 8.8, 0.1% (w/v) SDS, 15% (v/v) glycerol, 0.3 M mercaptoethanol] were homogenized (Castro-Rodríguez *et al.*, 2011) and the resulting extract was centrifuged at 10 000 ***g*** 4 °C for 30 min. The supernatant was used for the determination of soluble proteins using the assay described by Bradford (1976).

The extraction of total chlorophylls was performed from 100 mg of frozen samples on liquid nitrogen and 80% (v/v) acetone, and the content was determined according to Graan and Ort (1984).

Determination of soluble sugars

Sugars were extracted from frozen powder (100 mg) of leaves, stems and roots following the procedure described by de la Torre *et al.* (2014). Sucrose, glucose and fructose levels were measured enzymatically following the reduction of $NADP^+$ at 340 nm after successive addition of the coupling enzymes: glucose-6-P-dehydrogenase (4 units/mL), hexokinase (10 units/mL), phosphoglucose isomerase (5 units/mL) and invertase (Sekin, 1978). Starch was measured as described by Smith and Zeeman (2006). All enzymes were obtained from Roche Diagnostics, Mannheim (Germany).

Determination of cellulose and lignin

Cellulose content was determined by the anthrone method (Updegraff, 1969) following exactly the protocol previously described (de la Torre *et al.*, 2014). Total lignin quantification was performed following the thioacidolysis method described by Lange *et al.* (1995) according to the modifications specified by de la Torre *et al.* (2014).

Determination of total C and N content, NUtE and NUpE

Fine powder (100 mg) was dried in an oven at 70 °C for 48 h. The percentages of N (%) and C (%) of the triplicate samples were determined by an elemental macro-analyser Leco truSpec CHNS (Leco Corporation, St. Joseph, MI) at the Atomic Spectrometry Unit, University of Málaga. The NUtE was calculated using the increase in biomass DW of leaves, stems and roots divided by the total N supplied (g per plant) for each plant in the treatment (10 and 50 mM) (Good *et al.*, 2004). The NUpE was calculated using the observed increase in total N content of leaves, stems and roots divided by the N supplied (g per plant) (Good *et al.*, 2004).

Expression analysis of *GS* genes

RNA was isolated from leaves, stems and roots as described by Canales *et al.* (2010), and all traces of genomic DNA were removed by digestion with Dnase I (Promega Corporation, Madison, WI). RNA concentration and purity were quantified using a NanoDrop© ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). cDNA synthesis was performed as described previously (Canales *et al.*, 2010). The qPCR was carried out in a thermal cycler CFX384 (Bio-Rad, Hercules, CA) under the following conditions: 3 min at 95 °C (1 cycle), 1 s at 95 °C and 5 s at 60 °C (40 cycles) and a melting curve from 60 to 95 °C to verify the reaction specificity. Primers for *PtGS1.1, PtGS1.2, PtGS1.3, PtGS2*, previously described in Castro-Rodríguez *et al.* (2011), and *PpGS1a* (Forward: 5′-AATTACA AAGTGGAGGCCAG; Reverse: 5′-AGCCCTCGCCATATTACAA GT) were used. *Actin2* and *Ubiquitin* were used as reference genes (Brunner *et al.*, 2004). PCR reactions were performed in triplicate and relative expression levels were calculated using the R package qPCR (Ritz and Spiess, 2008). The raw fluorescence data from each reaction were fitted to the MAK2 model, which requires no assumptions about the amplification efficiency of the qPCR assay (Boggy and Woolf, 2010). The initial target concentrations (DO parameter) for each gene were deduced from the MAK2 model using the qPCR package and normalized to the geometric mean of the reference genes.

Microarray analysis

RNA was isolated as previously described, and RNA quality was assessed using the RNA Pico Assay for the 2100 Bioanalyzer (Agilent, Santa Clara, CA). Gene expression analyses were performed with the Agilent *Populus* whole genome array (4 × 44 k) (Tsai *et al.*, 2011). Microarray labelling, hybridization and washing were performed according to Agilent Low Input Quick Amp Labeling Kit-two color protocol (Agilent Technologies; cat# 5190–2306). For each amplification and labelling reaction, 200 ng of total RNA was employed. cRNAs with dye-specific activities (pmol Cy3 or Cy5 per μg cRNA) higher than 6 were used for the hybridizations. For each field of hybridization, 825 ng of a specific Cy3-labelled cRNA and 825 ng of a specific Cy5-labelled cRNA were used. Two technical and two biological replicates were performed. Microarray slides were scanned using GenePix 4100A scanner with a resolution of 5 μm/pixel. Data were extracted from the scanned images using the GenePix v6.0 software (Molecular Devices, Sunnyvale, CA).

Normalization and differential expression analysis of the data were performed using *limma* package in the R environment (Smyth, 2004). Normalization was carried out using quantile method and eBayes statistics to determine the differential expression between the comparisons (Smyth *et al.*, 2005). Statistical significance was corrected for multiple testing using the Benjamini–Hochberg procedure. The differentially expressed genes were selected with a significance *P*-value <0.05 and log2FC >1 and <−1.

Overall changes of the genes involved in metabolic pathways of plants, or in response to stress have been represented by Mapman tool and analysed by the functional Mapman categories (Thimm *et al.*, 2004). Mapman functional categories for differentially expressed genes are presented in Table S4. An overview of the Mapman functional categories including the differentially expressed genes is shown in Figures S7 and S8.

The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series Accession Number GSE61801 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62449).

Microarray validation

The differential expression of selected genes in the microarrays was tested using qPCR in the same samples as described above. Sequences of specific primers are listed in Table S5; all primers used the same annealing temperature and time (60 °C, 5 s).

Statistical analysis

Statistical variables were analysed by two-way ANOVA test and Tukey's multiple comparisons with a significance of $P < 0.001$. The identification of homogenous groups significantly different, and ANOVAs were performed in the R environment with the *agricolae* package using the *HSD.test* command.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Comparison between qPCR and microarray expression data to validate the microarray hybridizations.
Figure S2 Function enrichment analysis of the Mapman categories in the four analysed comparisons.
Figure S3 Mapman representation of the transcription related genes including differentially expressed genes.
Figure S4 Mapman representation of the metabolism response overview, including differentially expressed genes.
Figure S5 Mapman representation of the stress response overview, including differentially expressed genes.
Figure S6 Mapman representation of the transcription related genes including differentially expressed genes.
Figure S7 Overview of the Mapman functional categories including the differentially expressed genes.
Figure S8 Overview of the Mapman functional categories including the differentially expressed genes.
Table S1 Differentially expressed genes table.
Table S2 Limma analysis data for all differentially genes.
Table S3 Enrichment analysis of the Mapman functional categories.
Table S4 Table including all the Mapman Bin annotations for differentially expressed genes in each sample comparison.
Table S5 Genes primers for microarray validation.