Exogenous supply of glutamine and active cytokinin to the roots reduces NO$_3^-$ uptake rates in poplar

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ABSTRACT

The present study shows for the first time the influence of exogenously applied amino acids and cytokinin on the physiological and molecular aspects of N metabolism in poplar trees. In a short-term feeding experiment, glutamine or trans-zeatin riboside (tZR) was added directly to the nutrient solution. NO$_3^-$ net uptake declined significantly in response to both treatments. Feeding with glutamine brought about an increase in concentrations of different amino compounds in the roots (glutamine, glutamate, alanine, γ-amino butyric acid (GABA) and NH$_4^+$, which negatively correlated with the net NO$_3^-$ uptake. The plants showed a reduction of cytosolic glutamine synthetase 1 (GS1) transcript level in the roots. In addition, glutamine feeding changed the root-to-shoot distribution on N assimilation in favour of the leaves and plant internal N cycling. tZR treatment resulted in expansion of zeatin-type (Z-type) cytokinins in the roots and increased nitrate reductase (NR)–mRNA level. The results indicate that both particular amino acids and active cytokinins are involved in the feedback regulation of N uptake and metabolism in poplar. We propose that inhibition of N uptake by cytokinins in poplar is more complex than that mediated by amino compounds, and other effectors are involved in this regulation.

Key-words: amino acids; N cycling; trans-zeatin riboside.

INTRODUCTION

The N assimilation of higher plants is dynamically regulated by the N requirement for growth and development, environmental variations and the plant N status at the whole-plant level. The mechanisms involved in this regulation include induction and repression of N uptake by the roots. NO$_3^-$, the most common inorganic source of N, acts both as a nutrient and as a signal molecule in control of its own assimilation pathway (Crawford 1995). Expression of NO$_3^-$ carriers in the plasma membrane is directly induced by the presence of a substrate. Plants respond to changes in NO$_3^-$ concentrations in the soil by activating low- (LATS) or high-affinity transport system (HATS) (Glass & Siddiqi 1995). The low-capacity HATS plays a major role at low external NO$_3^-$ concentration, below approximately 200 µM. One subclass of high-affinity NO$_3^-$ transporters (HANTS) is expressed constitutively [constitutive HATS (cHATS)]; the other is induced by prolonged exposure to NO$_3^-$ [inducible HATS (iHATS)]. The LATS is of importance at higher external NO$_3^-$ concentrations, above 250 µM and exhibits linear (non-saturable) response to the substrate (Crawford & Glass 1998; Crawford & Forde 2002). Furthermore, NO$_3^-$ induces mRNA accumulation and enzyme activities of the N reduction pathway, such as nitrate reductase (NR) (Lin et al. 1994; Fan, Tang & Rengel 2002), glutamine synthetase (GS) and glutamate synthase (GOGAT) (Redinbaugh & Campbell 1993).

Besides nutrient availability, other environmental factors (e.g. atmospheric CO$_2$, temperature in the rhizosphere) represent major effectors of N uptake. In addition to these external factors, internal signals communicating the N status of the plant are of great importance as they coordinate root N uptake with the actual N demand of the whole organism (Imansande & Touraine 1994). Reduced N compounds, which are able to cycle between shoot and root via xylem and phloem transport, can signal the N demand of the shoot to the roots and/or exert direct feedback regulation on N uptake in the roots (Cooper & Clarkson 1989; Muller & Touraine 1992). An exogenous supply of particular amino acids results in a significant decrease in NO$_3^-$ uptake in different species (Gessler et al. 1998a; Zhuo et al. 1999; Aslam, Travis & Rains 2001) and glutamine seems to play a dominant role in this process (Nazoa et al. 2003).

In addition to reduced N compounds, cytokinins are also involved in the transduction of N signals. It is generally accepted that an accumulation of cytokinins in the roots and their transport via xylem to above-ground parts of the plant are modulated by inorganic N availability (Samuelson &
In leaves, the cytokinin signal is transduced via a histidine kinase receptor leading to an increased accumulation of mRNA for response regulator proteins and \( \text{NO}_3^- \) assimilating enzymes (Sheen 2002). However, because cytokinins can be produced also in the leaves (Singh et al. 1992) and transported basipetally in the phloem (Collier et al. 2003a), they might also act as basipetal signals indicating the \( \text{N} \) status of the plant to the roots (Emery & Atkins 2002). Cytokinins may, thus, support the amino acid-mediated communication of \( \text{N} \) demand from the shoot to the roots and participate in the regulation of \( \text{N} \) assimilation (Collier et al. 2003a; Gessler, Rennenberg & Kopriva 2004). The interaction between cytokinins and glutamine in regulation of \( \text{NO}_3^- \) uptake was demonstrated in \( \text{Fagus sylvatica} \) where it could be shown that an additional atmospheric \( \text{N} \) source resulted in the activation of cytokinins in the shoots, increased basipetal cytokinin transport via the phloem and accumulation of zeatin-type (Z-type) cytokinins in the roots (Collier et al. 2003a). Basipetal transport resulting in an enrichment of cytokinins in the roots was also demonstrated by feeding trans-zeatin riboside (tZR) into the phloem (Collier et al. 2003a). Both approaches caused a reduced \( \text{NO}_3^- \) net uptake and an increased level of particular amino acids. Similarly, feeding of glutamine via the phloem resulted in the down-regulation of \( \text{NO}_3^- \) uptake, higher non-protein–\( \text{N} \) level and enrichment in isopentenyladenine-type (iP-type) and Z-type cytokinins in the roots. In addition, tZR but not glutamine feeding caused an induction of mRNA for a HATS. From these observations, Collier et al. (2003a) hypothesized that phloem-translocated cytokinins initially induce \( \text{NO}_3^- \) transport by increased expression of HATS transporters in the roots and then, subsequently, the newly synthesized amino acids inhibit \( \text{NO}_3^- \) uptake. On the other hand, phloem-translocated amino compounds downregulate the \( \text{NO}_3^- \) transport directly. Because the enhanced cytokinin levels after glutamine feeding did not induce HATS, the cytokinin-mediated signal seems to act only when it is not repressed by amino compounds (Collier et al. 2003a).

The preliminary hypothesis drawn out from an experiment with beech provided a starting point for further analysis of amino acid and cytokinin signalling pathways in an integrated regulatory system for \( \text{NO}_3^- \) uptake. The major constraint of the work done by Collier et al. (2003a) is the fact that treatments could be only compared with controls after a particular incubation time without having the possibility to trace changes in uptake and related gene expression gradually over time. To overcome this constraint and to separate the particular effects of cytokinins and amino compounds on their concerted regulation of \( \text{NO}_3^- \) uptake, we analysed the time course of changes in \( \text{NO}_3^- \) uptake rates, accumulation of reduced \( \text{N} \) compounds in roots, concentration of cytokinins in the roots and the expression levels of enzymes of the \( \text{NO}_3^- \) assimilation pathway upon feeding the trees with glutamine or cytokinins. To characterize the local effects of the regulation of \( \text{NO}_3^- \) uptake of roots on \( \text{N} \) circulation at the whole-plant level, we applied a model describing \( \text{N} \) cycling in trees (Kruse et al. 2003). The experiments were performed with poplar (\( \text{Populus tremula} \times \text{Populus alba} \)), an increasingly used model for tree and plant molecular biology, enabling us to use the wealth of information obtained from the poplar genome project (Taylor 2002; Tuskan, Difazio & Teichmann 2004).

**MATERIALS AND METHODS**

**Plant material**

The experiments were performed with plants of the wild-type poplar hybrid \( \text{P. tremula} \times \text{P. alba} \), Institut National de la Recherche Agronomique (INRA) clone 717 IB4. The plants were micropropagated in *vitro* as previously described (Strohm et al. 1995), and transferred to pots with moistened balls (2–6 mm in diameter) of burned clay (Leca Ton, Leca, Lamstedt, Germany). They were further grown in a glasshouse with a 16 h light/8 h dark regime in a hydroponic culture [0.3 mM Ca(NO\(_3\))\(_2\), 0.4 mM KNO\(_3\), 0.025 mM CaCl\(_2\), 0.3 mM MgSO\(_4\), 0.05 mM KCl, 0.26 mM NaH\(_2\)PO\(_4\), 0.1 mM Fe\(_2\)(SO\(_4\))\(_3\), 0.1 mM Na–ethylenediaminetetraacetic acid (EDTA), 2 mM MnSO\(_4\), 10 mM H\(_2\)BO\(_3\), 0.2 mM CuSO\(_4\), 0.2 mM ZnSO\(_4\), 0.2 mM Na\(_2\)MoO\(_4\), and 40 mM CoSO\(_4\)] for 10 weeks. The nutrient solutions were changed every 2 d.

The poplar hybrid used in this study is naturally occurring in flood plain forests (Willmanns 1984), which are characterized by: (1) high nutrient input; and (2) high variability of nutrient concentrations, both as a consequence of periodic flooding. As a consequence, \( \text{NO}_3^- \) concentrations in soil- or groundwater solutions of wetland/flood plain forests are changing with time and space within a range that also covers the \( \text{NO}_3^- \) concentrations applied in this study (e.g. Bechtold, Edwards & Naiman 2003; Hefting, Bobbink & de Caluwe 2003; Hefting et al. 2004).

**Plant exposure**

Ten-week-old plants were transferred to 1 L glass pots containing 900 mL aerated nutrient solution [149 \( \mu \)M Ca(NO\(_3\))\(_2\), 3.6 \( \mu \)M FeSO\(_4\), 3.6 \( \mu \)M KH\(_2\)PO\(_4\), 50.9 \( \mu \)M KNO\(_3\), 57.5 \( \mu \)M MgCl\(_2\) and 40 \( \mu \)M Na\(_2\)SO\(_4\)]. Pre-incubation solutions were changed every 24 h. After 72 h of pre-incubation, glutamine or tZR was added to the nutrient solution to a final concentration of 10 mM glutamine or 4 \( \mu \)M tZR.

The control plants were kept in the same solutions as during the pre-incubation period. To get information about the time course of \( \text{NO}_3^- \) depletion in these three different treatments that could affect \( \text{NO}_3^- \) uptake, aliquots of 100 \( \mu \)L were taken from the media after 2, 5, 25 and 29 h. The \( \text{NO}_3^- \) concentrations were measured by ion-exchange chromatography. The \( \text{NO}_3^- \) uptake by the plants did not lead to a considerable \( \text{NO}_3^- \) deficiency during the incubation time in any treatment. After 29 h, 76, 57 and 60% of the initial \( \text{NO}_3^- \) supply was present in the glutamine, control and tZR treatment, respectively. From this observation, we assumed that during the experimental period, substrate deficiency and its interaction with net uptake can be excluded and, thus, changes in net \( \text{NO}_3^- \) uptake by the plants can be attributed to the incubation time and/or the glutamine/tZR.
treatment. Thus, root feeding treatments were performed for four different incubation periods (2, 5, 8 and 25 h).

Because natural diel changes as well as day-to-day variations [e.g. as a result of differences in photosynthetic photon fluence rate (PPFR)] of NO₃⁻ net uptake by roots (e.g. Macduff, Bakken & Dhanoa 1997) and of tissue amino acid concentrations (Gessler et al. 2002) had to be expected and, thus, to be taken into account, control plants were examined at each time point.

At the end of each treatment, the plants were transferred for 2 h into a fresh pre-incubation nutrient solution (i.e. glutamine and ZR free) with the same nutrient concentrations as shown previously in which all KNO₃ was ¹⁵N labelled. The plants were then divided into seven different tissues (fine roots, course roots, sink leaves, source leaves, bark, wood and xylem). The total fresh weights (FWs) as well as fresh-to-dry weight (DW) ratios were determined for all tissues. For xylem sap collection, the modified method of Scholander et al. (1965) described by Rennenberg, Schneider & Weber (1996) was used. The excised shoot was exposed to a raise in pressure at a rate of 0.1–0.2 MPa min⁻¹. During this procedure, the cut end was observed with a dissecting microscope with 10× magnification. The pressure at which the xylem sap first appeared was noticed as the actual shoot water potential. The first protruding solution was discarded to avoid contamination. Subsequently, the pressure was raised to 0.6 MPa over the shoot water potential. This pressure was kept constant for 2 min. Gessler et al. (1998b) showed that contamination of the xylem sap with cellular components was negligible under these conditions. The samples were stored at −80 °C until analyses.

**Determination of tissue N and ¹⁵N contents and calculation of NO₃⁻ uptake rates**

The enrichment in ¹⁵N, resulting from the 2 h root incubation in the medium containing K¹⁵NO₃, as well as the total N contents, was measured in different plant tissues (fine and course roots, sink and source leaves, bark, wood and xylem). For the determination of ¹⁵N abundance (atom percentage or δ¹⁵N) and total N, the tissue samples were oven-dried and homogenized. Aliquots of 1–2 mg were transferred into tin capsules (Type A; Thermo Quest, Egelsbach, Germany) and were injected into an elemental analyser (NA 2500; CE Instruments, Milan, Italy) coupled with a ConFlo II Interface (Finnigan MAT GmbH, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT GmbH). The specific ¹⁵N net incorporation (in micromoles per gram) was calculated using the following equation:

$$\text{¹⁵N incorporation} = \frac{(¹⁵\text{N}_\text{t}−¹⁵\text{N}_\text{c})−\text{[N]}×10^{4}}{\text{MW}}$$  (1)

where ¹⁵N and ¹⁵N₀ is the ¹⁵N abundance (atom percentage) in ¹⁵N-treated and control plants (without ¹⁵N application), [N] is the total N concentration (% N g⁻¹ DW) and MW is the molecular weight of ¹⁵N (g mol⁻¹). The calculation of total ¹⁵N incorporation per tissue was based on the specific ¹⁵N incorporation and the respective biomass; the total ¹⁵N incorporation per plant was computed by summing up the total ¹⁵N incorporation of all tissues. The NO₃⁻ net uptake rates were calculated as the sum of ¹⁵N accumulation in the plants over time and based on the FW of fine roots.

**Extraction and determination of amino compounds and NH₄⁺**

Soluble N compounds were extracted and analysed as described by Gessler et al. (1998b). Tissues (fine roots, source and sink leaves) were ground under liquid N with a mortar and pestle. Aliquots of 100 mg of the frozen powder were homogenized in a mixture of 1 mL methanol:chloroform (7:3) and 0.2 mL of N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (Hepes) buffer [5 mM ethylene glycol bis-2-aminoethyl ether -N,N',N''-tetraacetic acid (EGTA), 20 mM Hepes, 10 mM NaF (pH 7)]. Homogenates were incubated on ice for 30 min. Water-soluble amino compounds were extracted twice with 0.6 mL distilled water. The aqueous phases were combined and freeze-dried (Alpha 2-4; Christ, Osterode, Germany). The dried material was dissolved in 1 mL of 0.2 M lithium citrate buffer (pH 2.2) directly before analyses. Amino compounds were separated and detected by an automated amino acid analyser (BioChrom, Pharmacia LKB, Freiburg, Germany) described in detail by Gessler et al. (1998b).

**Extraction and analysis of NO₃⁻**

NO₃⁻ was extracted from leaf and root tissues by a modified method of Kruse et al. (2002). Aliquots of 50 mg of frozen material were mixed with 1.5 mL H₂O and 100 mg insoluble polyvinylpolypyrrolidone to remove phenolic compounds. Homogenates were shaken at 4 °C for 1 h, incubated at 95 °C for 5 min and centrifuged at 16 000 g for 10 min. Clear supernatants were stored at −20 °C until analysis. For NO₃⁻ analysis, aliquots of 100 μL extracts were injected into an ion chromatograph (DX 120; Dionex, Idstein, Germany). The xylem sap was analysed directly without further treatment.

**Measurement of leaf transpiration**

Transpiration was measured by the procedure described by Collier et al. (2003a). Measurements were conducted shortly before harvest, using a LCA4 infrared gas analyser (IRGA) (ADC, Hoddesdon, England). Firstly, transpiration rates of 3 fully expanded leaves per plant were measured. Secondly, the total plant leaf area was determined using an area meter (Delta-T Devices, Cambridge, UK). From these data, the transpiration rate of the whole plant (in millilitres per hour) was calculated.

**Expression analyses**

The total RNA was extracted from 80 mg powdered plant material using the Plant RNeasy kit, according to the
Cytokinin analysis

The procedure used for cytokinin analysis was a modification of the method described by Faiss et al. (1997). Freeze-dried root samples were extracted in ice-cold 70% ethanol (v/v) and deuterium-labelled standards were added, each at 5 pmol per sample to check the recovery during purification and to validate determination. The standards were [tH]trans-zeatin (tZ), [tH]tZR, [tH]tZ-9-glucoside (tZ9G), [tH]dihydrozeatin (DHZ), [tH]dihydrozeatin riboside (DHZR), [tH]dihydrozeatin-9-glucoside (DHZ9G), [tH]tJ, [tH]isopentenyladenosine (iPR), [tH]isopentenyl-adenine-9-glucoside (iP9G), [tH]tZ-O-glucoside (tZOG), [tH]tZR-O-glucoside (tZROG), [tH]dihydrozeatin-9-glucoside (DHZOG), [tH]dihydrozeatin-O-glucoside riboside (DZROG), [tH]benzyladenine (BA) and [tH]benzyladenosine (BAR) (Otechim Ltd., Olomouc, Czech Republic). The aromatic cytokinins were analysed using internal deuterium standards for BA and ortho-topolin (oT) ([tH]BA, [tH]BAR, [tN]oT). Thus, the values of these cytokinins may have an error load originating from insufficient internal standardization. After 3 h of extraction, the homogenate was centrifuged (15 000 g, 4 °C) and the pellets were re-extracted. The combined supernatants were concentrated to approximately 1.0 mL under vacuum at 35 °C, diluted to 20 mL with NH4 acetate buffer (40 mM, pH 6.5) and purified using a combined (diethylamino)ethyl (DEAE)–Sephadex (Sigma-Aldrich, St. Louis, MA, USA) (1.0 × 5.0 cm)–octadecylsilica (0.5 × 1.5 cm) column and immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against cytokinins (Faiss et al. 1997). This resulted in three fractions containing: (1) the free bases, ribosides and N-glucosides; (2) a nucleotide fraction; and (3) an O-glucoside fraction. The fractions from the IAC columns were evaporated to dryness and dissolved in 50 µL of the mobile phase used for high-performance liquid chromatography (HPLC) analysis. The samples were analysed by HPLC (Waters Alliance 2690; Milford, MA, USA) linked to a MicroMass ZMD 2000 (Waters, Milford, MA, USA) single-quadrupole mass spectrometer equipped with an electrospray (ES) interface [liquid chromatography (LC)(+)-ES–mass spectrometry (MS)] and photodiode array (PDA) detector (Waters PDA 996) under conditions described by Novák et al. (2003). Using a post-column split of 1:1, the effluent was introduced into an ES source (source block temperature: 100 °C, de-solvation temperature: 250 °C, capillary voltage: +3.0 V, cone voltage: 20 V) and PDA (scanning range: 210–300 nm, with 1.2 nm resolution), and quantitative analysis of the different cytokinins was performed in the selective ion recording (SIR) mode. All studied cytokinins were determined in aqueous methanol as dominant quasi-molecular ions of [M + H]+ (Novák et al. 2003). The limit of detection (LOD) was defined as the peak height being three times greater than the background noise. The ratio of endogenous cytokinins to appropriate labelled standards was determined and used to quantify the level of endogenous compounds in the original extract on the basis of known quantities of added internal standards (Tarkowski, Doležal & Strnad 2004).

Statistical analyses

The data obtained in the experiments were subjected to analyses of variance (ANOVA) and multiple range tests (Duncan) by ANOVA, and to Pearson product–moment correlation. All statistical analyses were performed with the statistical product and service solutions (SPSS) for Windows, version 12.0 (SPSS, Chicago, IL, USA).

RESULTS

NO3– uptake rates

Previous experiments revealed that feeding of glutamine and iZR via the phloem to beechn seedlings resulted in a reduction of NO3– uptake after 32 h (Collier et al. 2003a). To obtain information about the dynamics and mechanisms of this regulation, we measured the effects of glutamine and iZR treatments on NO3– uptake, concentration of reduced N compounds and cytokinins and accumulation of mRNA for proteins involved in NO3– reduction and assimilation in poplar during a time course of 25 h. The NO3– uptake rates were calculated from 15N accumulation in the whole plants. Figure 1 shows the NO3– uptake rates of the control and iZR- or glutamine-treated poplar plants harvested after 2, 5, 8 and 25 h of root feeding. The NO3– uptake rate of the control plants remained stable over the first 8 h and increased c. twofold after 25 h. In both glutamine- and iZR-treated plants, an incubation time of 2 or 5 h did not result in significant differences in NO3– uptake as compared to the
controls. After 8 h, however, the net NO$_3^-$ uptake rates declined in both glutamine- and tZR-treated plants, to 55 and 40% of controls, respectively, and were reduced further after 25 h, reaching 18 and 26% of the rate of control plants, respectively.

**Expression analysis**

To characterize the influence of an exogenous supply of glutamine and tZR on the enzymes involved in NO$_3^-$ reduction and assimilation, expression levels of NR and GS1 were measured by semi-quantitative PCR. Figure 2 shows the mean values of the transcript abundances in the roots of treated plants as the percentage of expression levels in the control roots. Twenty-five hours of glutamine treatment reduced the expression level of GS1–mRNA to 50% of that detected in the controls. The NR–mRNA accumulation was slightly repressed by the glutamine treatment in the plants harvested after 2 and 5 h of root incubation, and then increased to a steady-state level. The roots of plants supplemented with 4 µM tZR exhibited a strong induction of NR expression starting 5 h after the onset of the treatment and reaching 180% of the control after 25 h of root incubation. In contrast to NR, the GS1 transcript level was not affected by the cytokinin treatment.

Because the amino compound, glutamine, and the cytokinin tZR fed to the roots may be rapidly metabolized into other amino compounds and cytokinin derivatives, respectively, inside the roots, the effects on the net NO$_3^-$ uptake and on the transcription of enzymes involved in N assimilation observed cannot immediately be attributed to the compounds fed. In addition, Collier et al. (2003a) observed an enrichment in cytokinins in the glutamine treatment and increased amino acid contents as a result of cytokinin feeding. Therefore, the changes in amino N, NO$_3^-$ or NH$_4^+$ and cytokinin contents upon feeding of glutamine or tZR were determined.

**Figure 2.** Relative expression level of nitrate reductase (NR) (a) and glutamine synthetase 1 (GS1) (b) in the roots of glutamine-trans-zeatin riboside (tZR)-treated poplar trees as compared to controls. The expression level of controls was set to 100% at each time point. Data shown are means ± SE of three replicates. *, significant differences between controls and glutamine- or tZR-treated plants at $P \leq 0.05$. **, at $P \leq 0.01$.
Amino acid profiles in roots

The concentrations of soluble amino compounds in the roots were strongly affected by the glutamine treatment. The concentration of glutamine increased already after 5 h of pre-incubation and reached approximately 8 μmol N g⁻¹ FW after 25 h (i.e. equalling a sevenfold increase compared to the control) (Fig. 3). Mainly because of the increase of glutamine, the total amino N concentration in the roots was significantly higher compared to the control (Fig. 4).

The amounts of some amino acids [asparagine, γ-amino butyric acid (GABA), threonine, serine, citrulline, valine, leucine, lysine, histidine and tryptophan] in the roots were negatively affected by 2 h of glutamine feeding (data not shown). After longer incubation times (8 and/or 25 h), increased glutamine concentrations in the roots were accompanied by a significant enrichment of glutamic acid, alanine, GABA and NH₄⁺ (Table 1). In tZR-treated plants, small alterations in particular amino acid concentrations were observed. Threonine, serine, citrulline, leucine, GABA, lysine and tryptophan were reduced after 2 h; NH₄⁺ after 8 h; and isoleucine, β-alanine (BALA) and phenylalanine after 25 h of root incubation. The levels of aspartate and glutamic acid were increased because of 5 h of tZR treatment (data not shown). These changes did not influence the total amino N concentration, which remained comparable to the control at each point of time (Fig. 4).

Cytokinin concentrations in the fine roots

Cytokinin concentrations in the fine roots were determined for plants harvested after 2, 8 and 25 h of incubation with glutamine or tZR and compared to the controls. All cytokinin metabolites were tentatively identified by CapLC-QTOF (Waters, Milford, MA, USA) with exact mass determination (data not shown).

An exogenous supply of tZR caused a variable, incubation time-dependent expansion of the pool of Z-type cytokinins (Table 2). An 11-fold increase of tZR level after 2 h of treatment was accompanied by a 25-fold enrichment of its free base form, tZ, and a greater abundance of other tZ types ([ZOG, tZROG, tZR-5’-monophosphate (tZR5’MP)], which were low or not detectable in the roots of the control plants (Fig. 5b). The cis-zeatin types (cZ types) [cis-zeatin riboside (cZR) and cis-zeatin riboside O-glucoside (cZROG)] were found in low concentrations after 2 h of tZR treatment and were not different from the control. The DHZ-type cytokinin (DHZR) was detected in the roots of tZR-treated poplars, while the control plants did not contain any member of this fraction. The level of iP types was not altered by 2 h of tZR treatment (Table 2).

Table 1. The N concentration of particular amino compounds in the fine roots of control plants and poplars supplied with 10 mM glutamine for 25 h

<table>
<thead>
<tr>
<th>Amino compound (μmol amino N g⁻¹ FW)</th>
<th>Control</th>
<th>Glutamine treatment</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>0.97 ± 0.14</td>
<td>7.20 ± 0.84</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.64 ± 0.07</td>
<td>2.44 ± 0.23</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.27 ± 0.18</td>
<td>1.56 ± 0.19</td>
<td>P ≥ 0.05</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.32 ± 0.10</td>
<td>1.44 ± 0.29</td>
<td>P ≤ 0.01</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.42 ± 0.11</td>
<td>0.89 ± 0.04</td>
<td>P ≤ 0.01</td>
</tr>
<tr>
<td>GABA</td>
<td>0.19 ± 0.03</td>
<td>0.74 ± 0.08</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Serine</td>
<td>0.20 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>P ≥ 0.05</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.18 ± 0.03</td>
<td>0.15 ± 0.01</td>
<td>P ≥ 0.05</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>0.09 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>P ≥ 0.05</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>P ≥ 0.05</td>
</tr>
<tr>
<td>Total amino N</td>
<td><strong>4.74 ± 0.57</strong></td>
<td><strong>15.22 ± 1.27</strong></td>
<td>P ≤ 0.01</td>
</tr>
</tbody>
</table>

Values shown are means ± SE of four replicates.

FW, fresh weight; ANOVA, analysis of variance; GABA, γ-amino butyric acid.
Table 2. Changes in cytokinin content of poplar roots during the time course of trans-zeatin riboside (tZR) root treatment

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Control</th>
<th>tZR treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>tZ types</td>
<td>51.7 ± 6</td>
<td>20.4 ± 4</td>
</tr>
<tr>
<td>cZ types</td>
<td>2.3 ± 1.4</td>
<td>28.3 ± 6</td>
</tr>
<tr>
<td>DHZ types</td>
<td>nd</td>
<td>13.2 ± 5</td>
</tr>
<tr>
<td>iP types</td>
<td>0.7 ± 0.3</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>oT types</td>
<td>nd</td>
<td>101 ± 27</td>
</tr>
<tr>
<td>Total cytokinin</td>
<td>55 ± 8</td>
<td>168 ± 30</td>
</tr>
</tbody>
</table>

Values shown are means ± SE of four replicates (pmol g\(^{-1}\) FW).

*Z, trans-zeatin; cZ, cis-zeatin; DHZ, dihydrozeatin; iP, isopentenyladenine; oT, o-topolin; nd, not detected; FW, fresh weight.

Figure 5. Enrichment of total (a) and (Z-type) cytokinins (b) in poplar roots incubated in glutamine- (a) and trans-zeatin riboside (tZR)- (a & b) enriched media for 2, 8 and 25 h as compared to controls. Data shown are means ± SE of four replicate samples. *, significant differences between the control and the treated plants at \(P \leq 0.05\); **, at \(P \leq 0.01\); ***, at \(P = 0.001\). tZ, trans-zeatin; iZ, trans-zeatin riboside; tZOG, tZ, O-glucoside; tZROG, tZR O-glucoside; cZ, cis-zeatin; cZROG, cZR O-glucoside.
After 8 h of iZR feeding, iZ was the most dominant Z-type species, followed by iZR, iZR5′MP, iZROG and iZR5. The iZ types increased up to ∼20 pmol µL⁻¹ forming 4.5% of the Z-type fraction (Table 2). Because of the accumulation of iZ species (Fig. 5b), the total cytokinin content was threefold higher compared to the control (Fig. 5a). The Z types made up the major part of cytokinins, although they contributed less to the total cytokinin concentration (84%) than after 2 h of incubation (94%). The sum of iP types [iPR + iPR-5′-monophosphate (iPR-5′MP)] was significantly reduced in the roots of iZR-treated plants, compared to the control. In contrast to the plants harvested after 2 h of the experiment, oT types were found in the roots of both the control and the iZR-treated plants (Table 2).

The mean total cytokinin concentrations in the roots of iZR-fed plants were lowest after 25 h of incubation (Table 2). The Z types were still the most abundant fraction, but because of the high accumulation of oT types, they contributed to only 51% of the total cytokinins. However, the mean concentrations of almost all (except iZR5′MP) iZ species were much higher than in the control plants (Fig. 5b), in which the trans forms made up only 17% of the Z types. In addition, particular DHZ types (DHZ and DHZHR) were more abundant in the treated plants. The contents of other cytokinins (oT and iP types) were not significantly affected by 25 h of iZR treatment (Table 2).

In the roots of glutamine-treated plants, the total cytokinin concentrations did not differ significantly from the controls for any incubation time (Fig. 5a).

### Plant internal N cycling

From the N and ¹⁵N contents in different tissues, the NO₃⁻ concentration in the roots, leaves and xylem as well as the leaf transpiration, the internal N cycling at the whole-plant level was calculated. For this purpose, the model described by Kruse et al. (2003) was modified and applied as described in detail in Appendix A. Changes in pool size and fluxes of different N forms (organic and mineral) were calculated as nanomoles of N accumulated or transported per plant and hour and expressed as the percentage of the NO₃⁻ taken up by the roots. Figure 6 shows the whole-plant cycling of newly taken up N in the control plants and poplars fed via the roots with iZR or glutamine for 25 h.

In the control plants, the majority of the N taken up from the medium was reduced (97%) and accumulated (74%) in the roots (Fig. 6a). Only ∼2% of the NO₃⁻ absorbed from the external medium was transported via the xylem to and reduced in the leaves. Because NO₃⁻ reduction in the roots exceeded the root N demand, the surplus of reduced N (23%) was loaded into the xylem and transported upstream to the shoot. Because of the low xylem transport of NO₃⁻, the N reduction in the leaves was not sufficient to fulfill the shoot N demand. Thus, part of the acropetally transported reduced N was unloaded in the leaves. The rest of the transported organic N (4%) was accumulated in the stem.

![Figure 6](image-url) Internal N cycling in poplar trees exposed to 10 mM glutamine or 4 µM trans-zeatin riboside (iZR) for 25 h. The values indicate pool sizes and fluxes of NO₃⁻ and reduced N as the percentage of N taken up by the plants.

The increments of NO₃⁻ in the shoot and roots were insignificant (< 1% of taken up N).

Despite its significant effect on the root NO₃⁻ uptake, the cytokinin treatment (25 h of root incubation in iZR-enriched medium) did not influence the relative distribution of N within the plant. Partitioning of NO₃⁻ assimilation among the leaf and root tissues as well as the relative accu-
mulation of NO\textsubscript{3}\textsuperscript{–} and reduced N forms in the leaves, roots and stem were similar to the control plants (Fig. 6b).

In poplars treated with glutamine, the NO\textsubscript{3}\textsuperscript{–} uptake declined significantly and amounted to 18% of the controls after 25 h of treatment (Fig. 1). However, absolute NO\textsubscript{3}\textsuperscript{–} transport via the xylem was not reduced and amounted to 32% of the total NO\textsubscript{3}\textsuperscript{–} taken up by the plants (Fig. 6c).

Consequently, more NO\textsubscript{3}\textsuperscript{–} was reduced and accumulated in the leaves compared to the control; still, the increment of NO\textsubscript{3}\textsuperscript{–} in the leaves was negligible. Root assimilation was reduced to 68% of the NO\textsubscript{3}\textsuperscript{–} taken up, and only 41% of reduced N was accumulated in this tissue. Thus, glutamine feeding significantly changed the root-to-shoot distribution of N assimilation and plant internal N cycling.

**DISCUSSION**

One of the most critical issues in any experiment that comprises artificial enrichment or depletion of metabolites for assessing regulatory functions – independent if based on feeding of particular compounds, use of inhibitors or application of transgenic lines – is the concentration of target substances in the plant tissue. Only variations within a ‘natural’ range (i.e. a range that could also be induced by fluctuations of growth conditions a plant is subjected to in its natural environment) allows realistic insights into complex antagonistic or synergistic interacting regulatory processes.

Even though there is not much information on amino acid concentrations in tissues of tree species under natural conditions, the variation induced here is clearly within the environmental range described in the literature. NH\textsubscript{3} fumigation of beech leaves with natural NH\textsubscript{3} concentrations (Gessler et al. 1998c) resulted in amino acid enrichment in the exposed tissues in the same order of magnitude as observed in this study. Adult spruce grown in an N-over-saturated forest (Schneider et al. 1996; Gessler et al. 1998b) showed up to fivefold higher phloem amino acid contents as compared to individuals from an N-poor environment (Weber et al. 1998). In addition, Rennenberg & Gessler (1999) showed that the total amino acid and glutamine concentrations in the fine roots of adult beech at a given stand could differ by a factor of 5 between years. The year-to-year variation was induced by changes in beech nut production and related changes in N demand. Foliar amino acid concentrations in field-grown poplar varied by a factor of 2 depending on the water supply (Gebre et al. 1998).

Collier et al. (2003b) revealed that the total cytokinin concentrations in the needles of field-grown Sitka spruce increased by a factor of >7 when the N supply was enhanced. NH\textsubscript{3} fumigation of beech leaves with natural concentrations (Collier et al. 2003a) resulted in cytokinin concentrations to rise by a factor of c. 5 in the fine root tissue. Because we observed only transiently higher, but after 5 and 25 h much lower, cytokinin enrichment we can conclude that we remained within the natural range of variation for cytokinins too.

**Regulation of NO\textsubscript{3}\textsuperscript{–} uptake and assimilation by glutamine**

To investigate the feedback mechanism for N uptake in poplar, we chose glutamine because: (1) it has frequently been reported as a down-regulator of N assimilation both in trees and herbaceous plants (Gessler et al. 1998a; Pal’ove-Balang & Mistrik 2002; Nazoa et al. 2003); (2) it is the most abundant N compound in the xylem sap of poplar (Table 3) (Sauter & Vancleve 1992; Escher et al. 2004); and (3) it can be exchanged in trees between xylem and phloem (Gessler et al. 2004). This indicates a role of glutamine in basipetal signalling and regulation of NO\textsubscript{3}\textsuperscript{–} uptake also in poplar roots. In the present study, the treatment of poplar plants with glutamine resulted in its accumulation in the roots, alteration in the composition of amino compounds in the roots, changes in the expression levels of GS1, a significant decrease in NO\textsubscript{3}\textsuperscript{–} uptake rates and an altered root-to-shoot distribution of N assimilation. Because of the short duration of the experiment, the effects of glutamine feeding on growth rates and, as a consequence, the potential effects of changing biomass accumulation on N uptake and assimilation can be excluded.

These results are consistent with previous observations with annual and perennial herbaceous as well as woody plants. In herbaceous plants, an additional supply of different amino compounds led to the identification of several feedback inhibitors of NO\textsubscript{3}\textsuperscript{–} uptake with glutamine as the most prominent compound (Nazoa et al. 2003). It may be expected that in trees, N nutrition may be subjected to a more complex regulation, because of their specific life form (longevity, size) that requires a regulation and adaptation not only to the current N demand, but also involves storage and mobilization processes (Millard 1996; Frak et al. 2002; Grassi et al. 2003). Still, glutamine seems to be the dominant amino compound interacting with NO\textsubscript{3}\textsuperscript{–} uptake in trees (Gessler et al. 1998a,c; Collier et al. 2003a).

Feeding with glutamine brought about an increase in the amino acid levels in the roots (Fig. 4), and the closest correlation was found between glutamine and glutamic acid

<table>
<thead>
<tr>
<th>Amino compound (µmol amino N g\textsuperscript{-1} FW)</th>
<th>Control</th>
<th>Glutamine treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>1.85 ± 0.53</td>
<td>3.75 ± 1.69</td>
</tr>
<tr>
<td>GABA</td>
<td>0.43 ± 0.14</td>
<td>1.28 ± 0.31</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.30 ± 0.14</td>
<td>0.94 ± 0.57</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.48 ± 0.20</td>
<td>0.45 ± 0.22</td>
</tr>
<tr>
<td>NH\textsubscript{4}\textsuperscript{+}</td>
<td>0.19 ± 0.05</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.16 ± 0.05</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>Serine</td>
<td>0.14 ± 0.07</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.17 ± 0.04</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Total amino N</td>
<td>4.23 ± 1.19</td>
<td>7.67 ± 2.72</td>
</tr>
</tbody>
</table>

Values shown are means ± SE of four replicates. FW, fresh weight; GABA, γ-amino butyric acid.
Table 4. Correlations between the levels of amino compounds and NO$_3^-$ uptake in the fine roots of plants supplied with 10 mM glutamine for 2, 5, 8 and 25 h

<table>
<thead>
<tr>
<th>Correlations (r)</th>
<th>Glutamine</th>
<th>Glutamic acid</th>
<th>NH$_4^+$</th>
<th>GABA</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine × N</td>
<td>–</td>
<td>0.864**</td>
<td>0.801**</td>
<td>0.794**</td>
<td>0.796**</td>
</tr>
<tr>
<td>N × N uptake</td>
<td>−0.611*</td>
<td>−0.702**</td>
<td>−0.789**</td>
<td>−0.809**</td>
<td>−0.755**</td>
</tr>
</tbody>
</table>

GABA, γ-aminobutyric acid.

*P ≤ 0.05

**P ≤ 0.01

concentration (Table 4). Increased levels of GABA, glutamic acid and alanine caused by glutamine treatment were also observed in *Lolium perenne* (Thornton 2004). In similar experiments with other tree species, glutamine treatment brought about enhanced levels of, for example, GABA, alanine and NH$_4^+$ (beech) (Gessler et al. 1998a) or glutamic acid, alanine and NH$_4^+$ (spruce) (Gessler et al. 1998a). Alanine strongly repressed NO$_3^-$ uptake in soy bean (Muller & Touraine 1992); glutamic acid in *Arabidopsis thaliana* (Zhuo et al. 1999; Nazoa et al. 2003), barley (Vidmar et al. 2000; Aslam et al. 2001), spruce (Gessler et al. 1998a) and *Brassica napus* (Beuver et al. 2004). Whereas in general, it is now assumed that assimilated organic compounds are the main factors regulating NO$_3^-$ uptake (e.g. Vidmar et al. 2000); NH$_4^+$ was suggested as a negative regulator of NO$_3^-$transporter 2 in roots of *A. thaliana* (Ntr2;1At) (Zhuo et al. 1999) and of NO$_3^-$ influx in barley (Kronzucker, Glass & Siddiqi 1999). In the present study, the concentration of glutamate, glutamic acid, alanine, NH$_4^+$ and GABA in glutamine-treated plants correlated negatively with NO$_3^-$ net uptake rates by the roots, and the highest correlations were found for GABA ($r = −0.809$) and NH$_4^+$ ($r = −0.789$; Table 4). Thus, the repression of N transport may be caused by the combined action of several amino compounds.

Recently, a role of GABA in N signalling and up-regulation of N uptake has been discussed (Beuver et al. 2004). It has been proposed that GABA acts contrarily to the negative regulation of N influx mediated by glutamate. A positive correlation between the GABA content in the phloem and NO$_3^-$ influx was found in short-term and long-term feeding experiments with *B. napus*. Furthermore, an exogenous supply of GABA resulted in the induction of N transport, mainly at the molecular level (Beuver et al. 2004). Thus, a potential role of GABA in the reduction of N uptake in poplar must be considered with caution. High levels of GABA in the roots after glutamine treatment can be explained as the consequence of an increased glutamic acid content ($r = 0.912$), which serves as a direct substrate for GABA synthesis (‘GABA shunt’). Root treatment of poplars with glutamine caused an enrichment of GABA also in the xylem (Table 3), as well as the sink and source leaves (data not shown). Thus, the negative correlation between GABA content and NO$_3^-$ net uptake might be explained by the close metabolic linkage between GABA and glutamine – a compound with the ability to down-regulate NO$_3^-$ uptake (e.g. Gessler et al. 1998c). It is, however, possible that GABA is not involved in the reduction of net uptake or even reduces the inhibitory effect of glutamine or other amino compounds.

The observed reduction of GS expression by glutamine treatment supports the view that glutamine and other amino acids play a role in regulating GS activity (Oliveira & Coruzzi 1999). In the present study, we chose the cytokinins form of GS, because it is dominant in non-photosynthetic tissues (Ireland & Lea 1999) and is not considerably induced by light (Oliveira & Coruzzi 1999), which was not controlled during our experiments. The decrease of GS–mRNA level observed by prolonged glutamine feeding brought about a large increase of NH$_4^+$ in the roots (Table 1). Apparently, either NH$_4^+$ originating from metabolic interconversions of glutamine does not affect GS expression in a similar way than NH$_4^+$ produced by NO$_3^-$ reduction or glutamine-mediated repression of GS overrules GS induction by NH$_4^+$.

At the whole-plant level (Fig. 6c), glutamine feeding resulted in a strong reduction of NO$_3^-$ assimilation and of accumulation of newly taken up N in the roots. As a consequence, the distribution of N reduction and assimilation was changed in favour of the shoot. Apparently, N nutrition strongly affects the root-to-shoot distribution of N reduction and assimilation. Such changes are considered a tissue-specific feature of regulation of N nutrition (Black, Fuchi-gami & Coleman 2002). The signalling cascade leading from glutamine feeding to changes in the whole-plant distribution of N reduction and assimilation remains to be elucidated. It should be mentioned that fluxes and accumulation rates shown in Fig. 6 might not represent a steady-state situation neither in controls nor in treated plants. The plants were kept under an 18/6 h light/dark period with natural fluctuations in light conditions, and thus, photosynthesis and transpiration. Changes in energy/carbon availability and in xylem transport rates over time may generally prevent plants to reach a steady state in N uptake, transport and accumulation. For the treated plants, it is possible (e.g. Figs 3 & 5) that a constant level of cytokinins or amino compounds has not been reached after 25 h. Thus, the change in N partitioning in the glutamine-fed plants might be transitory. Long-term experiments have to prove if any increase in amino compound concentration leads to
continuous change of root-to-shoot transport of newly taken up N.

Nevertheless, the results of this study show for the first time the effects of an additional supply of glutamine on the NO$_3^-$ uptake spanning the scale from the molecular over the physiological, to the whole-plant level in poplar. They, thus, not only support the results drawn for other model species (A. thaliana, barley), but expand the view from local regulation of N transport to the root–shoot partitioning of newly taken up N.

**Regulation of NO$_3^-$ uptake by iZR**

We also present here for the first time the effects of direct application of active cytokinin form to the nutrient solution on NO$_3^-$ uptake by the roots of poplar trees. The work is one of the first approaches in which the problem of potential up-/down-regulation of root N uptake mediated by this class of hormones is directly addressed (Trčková & Kamíněk 1999; Collier et al. 2003a). Metabolic conversions of cytokinins and related changes in NO$_3^-$ uptake by the roots and in expression of N assimilation enzymes are here observed continuously for the first time.

As shown in Fig. 1, the root incubation of poplar with 4 µM of iZR resulted in a reduction of NO$_3^-$ net uptake. This reaction was accompanied by a significantly increased level of Z-type cytokinins (trans-forms) in the roots (Fig. 5b). The expansion of cytokinin was observed already after 2 h and can be attributed to the direct absorption of iZR by the root cells. An accumulation of cytokinin in response to external N supply (Takei et al. 2001) can be excluded, because the NO$_3^-$ concentration in the medium was moderate and a reduction rather than an increase of iZ types was observed in the control roots (Table 2).

BA-derived cytokinins are known to be widespread in higher plants, but no BA and m-topolin compounds were detected in this study. On the other hand, oT types made up a larger proportion of the cytokinin complement in the poplar plants screened (Table 2). In poplar, there appears to be no N-glucoside conjugates. Most cytokinins are free bases and their corresponding O-glucosides and nucleotides. Furthermore, the most important cytokinin representatives of this plant (iZ and oT) occur usually at very high free-base levels being probably supplemented from the high O-glucoside pools.

The relationship between reduced N compounds and accumulation of cytokinin can be addressed by comparing the cytokinin profiles in the roots of the control and the glutamine-treated plants. Samuelson & Larsson (1993) suggest that NO$_3^-$-dependent zeatin riboside (ZR) response requires previous synthesis of the reduced N compounds. Indeed, in barley, ZR response to increased N supply was repressed by NR and GS activity inhibitors. Furthermore, the use of the protein synthesis inhibitor, cycloheximide (CHX), negatively affected the ZR accumulation in the barley roots (Samuelson & Larsson 1993). In addition, Collier et al. (2003a) reported a positive relationship between the total soluble non-protein N content (mainly free amino acids) and the cytokinin level in the roots of beech trees. However, in our study, an enrichment in glutamine and other amino acids as a result of glutamine feeding did not bring any significant changes in the total cytokinin level in the roots, compared to the control, at any particular time of harvest (Fig. 5a). In A. thaliana, a rapidly increased NO$_3^-$ supply activates the A. thaliana isopentenyl transferase 3 (AtIPT3) gene for isopentenyl transferase and, subsequently, the accumulation of cytokinins in the roots (Takei et al. 2004). The response occurs shortly after NO$_3^-$ re-supplement and is not suppressed by CHX (Miyawaki, Matsumoto-Kitano & Kakimoto 2004). These findings suggest that NO$_3^-$ by itself is not only a prerequisite, but also a sufficient signal in the NO$_3^-$-dependent cytokinin response.

While increasing information on N-specific cytokinin accumulation and transport is available (Takei et al. 2002), still little is known about the potential regulation of NO$_3^-$ uptake by the roots mediated by cytokinins. It is supposed that cytokinins act not only as signalling molecules in N-dependent responses of the leaves (Takei et al. 2001), but can also signal the N status from the shoot to the roots (summarized by Gessler et al. 2004). An expanded pool of cytokinin in the roots can participate in the regulation of genes involved in N transport and reduction. In wheat plants, spraying shoots of N-starved plants with cytokinin [N$^\text{\textsuperscript{-}}$-(meta-hydroxybenzyl)adenosine] increased NO$_3^-$ net uptake by up to 70% (Trčková & Kamíněk 1999). Different physiological responses were observed in trees. Similar to our results, reduced NO$_3^-$ assimilation was observed in studies with F. sylvatica, in which an increase in root cytokinins was induced by either the NH$_3$ fumigation of the leaves (Gessler et al. 1998c) or by loading iZR directly into the phloem (Collier et al. 2003a). Thus, it is likely that the cytokinin-mediated NO$_3^-$ uptake response differs between plant species. It is also probable that different cytokinins exert different roles in the regulation of N uptake by the roots.

Poplars treated with iZR showed, despite of a significant decrease in the net NO$_3^-$ uptake, an increased NR–mRNA levels (Fig. 2a). Induction of the NR activity and/or the transcription by feeding the plant with different cytokinins were observed also in, for example A. thaliana (Yu, Sukumar & Marton 1998), barley (Samuelson, Campbell & Larsson 1995) and wheat (Trčková & Kamíněk 1999). Thus, cytokinins are likely to coordinate light- and NO$_3^-$-dependent activation of NR (Saroop Sant Thaker, Chanda & Singh 1998). Moreover, experiments with beech revealed an elevated level of mRNA for a HATS because of the iZR treatment (Collier et al. 2003a). The authors of this study postulated that cytokinin enrichment in the roots resulted in a transient increase in NO$_3^-$ net uptake, which caused an accumulation in amino compounds. These compounds were supposed to exert in a further-step post-transcriptional reduction of NO$_3^-$ transport. The following observations in the present experiments support this hypothesis: (1) induction of NR expression in the roots because of the iZR treatment; and (2) reduced net NO$_3^-$ uptake after a prolonged cytokinin treatment. Other aspects of the
hypothesis of Collier et al. (2003a) were not confirmed for poplar. We did not observe a transient increase of NO$_3^-$ net uptake preceding its reduction. An elevated level of amino compounds in the roots after 8 or 25 h of cytokinin treatment, which could overrule a cytokinin-mediated signal, was not detected as well.

Interestingly, the concentrations of both iZR and the total Z-type cytokinins in the roots of cytokinin-treated plants correlated with: (1) NO$_3^-$ uptake ($r = 0.684$ and $r = 0.584$, for iZR and Z type, respectively); and (2) NO$_3^-$ concentration in the roots ($r = 0.933$ and $r = 0.753$). These provide circumstantial evidence that N uptake by poplar trees is dependent on the cytokinin concentration in the roots. The mechanisms underlying this regulation are, undoubtedly, more complex than feedback inhibition mediated by amino compounds, and thus, need to be further explored. At the whole-plant level, iZR treatment, different from glutamine exposure of the roots, did not affect the partitioning of N compounds and N assimilation in poplar plants.

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**APPENDIX A**

For the determination of internal N circulation and accumulation within the plant, the model described by Kruse et al. (2002) was modified and applied. All N pools and fluxes were calculated as nanomoles of N atom accumulated or transported in 1 h per plant, and then expressed as the percentage of the N taken up by the roots (*J*<sub>uptNO₃⁻</sub>). The sink and source leaves, as well as the fine and coarse roots, were combined to ‘leaf’ or ‘root’ fractions, respectively.

Accumulations of NO₃⁻ and reduced N forms were calculated as follows:

\[
(NO_3^-)_r = \left(\frac{[NO_3^-]_{root} \times 100\%}{N_{root}}\right) \times \Delta^{15}N_{root},
\]  
\[
(NO_3^-)_l = \left(\frac{[NO_3^-]_{leaf} \times 100\%}{N_{leaf}}\right) \times \Delta^{15}N_{leaf},
\]  
\[
(N_{red})_r = \left[\frac{(N_{leaf}-[NO_3^-]_{leaf}) \times 100\%}{N_{leaf}}\right] \times \Delta^{15}N_{leaf},
\]  
\[
(N_{red})_l = \left[\frac{(N_{root}-[NO_3^-]_{root}) \times 100\%}{N_{root}}\right] \times \Delta^{15}N_{root},
\]  

where (*NO₃⁻)<sub>r</sub> and (*NO₃⁻)<sub>l</sub> are the accumulations of NO₃⁻ in the roots and leaves, respectively; [NO₃⁻]<sub>root</sub> and [NO₃⁻]<sub>leaf</sub> are the NO₃⁻ amount (nmol N) in the roots and leaves, respectively; N<sub>root</sub>/N<sub>leaf</sub> is the total N amount (nmol N) of the root/leaves; \(\Delta^{15}N_{root}/\Delta^{15}N_{leaf}\) is the \(^{15}N\) accumulation in the root/leaves during 1 h (nmol \(^{15}N\) h<sup>−1</sup>); and (N<sub>red</sub>)<sub>r</sub>/(N<sub>red</sub>)<sub>l</sub> is the accumulation of reduced N in the roots/leaves. The N uptake (*J*<sup>pNO₃⁻</sup>) was calculated from the sum of \(\Delta^{15}N\) in different plant tissues, as described in Materials and methods. The xylem transport of NO₃⁻ was determined experimentally:

\[\text{\(J^p\)NO}_3^- = T \times [\text{NO}_3^-]_{xyl},\]

where *J*<sup>p</sup>NO₃⁻ is the transport of NO₃⁻ via the xylem, *T* is the transpiration rate of the whole plant (mL h<sup>−1</sup>) and [NO₃⁻]<sub>xyl</sub> is the amount of NO₃⁻ in the xylem (nmol mL<sup>−1</sup>).

The remaining fluxes were calculated as follows:

\[J_{red}^r = J^{pNO}_3^- - J^pNO_3^- - (NO_3^-)_r,\]

where *J*<sub>red</sub><sub>r</sub> is the amount of NO₃⁻ reduced in the roots;

\[J_{red}^l = J^{pNO}_3^- - (NO_3^-)_l,\]

where *J*<sub>red</sub><sub>l</sub> is the amount of NO₃⁻ reduced in the leaves;

\[J_{load}^s = J_{red}^r - (N_{red})_r,\]

where *J*<sub>load</sub><sub>s</sub> is the amount of reduced N loaded into the xylem;

\[J_{unload}^s = (N_{red})_l - J_{red}^r,\]

where *J*<sub>unload</sub><sub>s</sub> is the amount of reduced N unloaded from the xylem; and

\[(N)_s = J_{load}^s - J_{unload}^s,\]

where (N)<sub>s</sub> is equivalent to the organic N remaining in the stem. It is either reallocated in basipetal direction via the phloem or stored in the stem tissue.