Planta (2004) 219: 884–893
DOI 10.1007/s00425-004-1287-5

ORIGINAL ARTICLE

**Samia Djennane · Isabelle Quilleré
Marie-Thérèse Leydecker
Christian Meyer · Jean-Eric Chauvin**

Expression of a deregulated tobacco nitrate reductase gene in potato increases biomass production and decreases nitrate concentration in all organs

Received: 10 December 2003 / Accepted: 31 March 2004 / Published online: 9 June 2004

Abstract We investigated the physiological consequences for nitrogen metabolism and growth of the deregulated expression of an N-terminal-deleted tobacco nitrate reductase in two lines of potato (*Solanum tuberosum* L. cv Safrane). The transgenic plants showed a higher biomass accumulation, especially in tubers, but a constant nitrogen content per plant. This implies that the transformed lines had a reduced nitrogen concentration per unit of dry weight. A severe reduction in nitrate concentrations was also observed in all organs, but was more apparent in tubers where nitrate was almost undetectable in the transgenic lines. In leaves and roots, but not tubers, this nitrate decrease was accompanied by a statistically significant increase in the level of malate, which acts as a counter-anion for nitrate reduction. Apart from glutamine in tubers, no major changes in amino acid concentration were seen in leaves, roots or tubers. We conclude that enhancement of nitrate reduction rate leads to higher biomass production, probably by allowing a better allocation of N-resources to photosynthesis and C-metabolism.

Keywords Nitrate reductase · Nitrogen metabolism · Plant transformation · *Solanum* · Tuber

Abbreviations DAP: Days after planting · Gln: Glutamine · NR: Nitrate reductase · WT: Wild type

I. Quilleré · M.-T. Leydecker · C. Meyer (✉)
Unité de Nutrition Azotée des Plantes, INRA,
Route de St-Cyr, 78026 Versailles, France
E-mail: meyer@versailles.inra.fr
Tel.: +33-1-30833067
Fax: +33-1-30833096

S. Djennane · J.-E. Chauvin
Station d'Amélioration de la Pomme de Terre et des Plantes à Bulbes, INRA, Domaine de Kéraïber, 29260 Ploudaniel, France

Introduction

Potato is one of the most important crops in the world. This is due to its high productivity and nutritive value in human alimentation and animal feeding, and to the possibility of cultivating this plant in a large number of areas. Nitrogen availability directly influences potato yield, and the role of nitrogen in potato nutrition has already been the subject of several studies. For instance, it has been shown that nitrogen favours canopy development during the early stages of culture but, later, favours tuber initiation and swelling (Van Kempen et al. 1996). With the intensive agricultural methods and the increasing use of fertilizers, nitrate may become an ecological problem in areas where potatoes are intensively produced (Richards et al. 1990; Levallois et al. 1998; Peralta and Stockle 2002). Because yield increases with nitrogen supply, fertilizer application can reach very high values, up to 300 kg ha^{-1}. Since potato is a poor user of available nitrate (less than 60% of the supply), leaching may be high and lead to groundwater contamination. Moreover, high use of N-fertilizers results in poor potato tuber quality if the nitrate content in this organ exceeds the acceptable human intake limit (Gravoueille et al. 1992; Marin et al. 1998). Nitrate reductase (NR; EC 1.6.6.1.), the first enzyme of the nitrate assimilation pathway, catalyses the reduction of nitrate into nitrite using the reducing power of NADH. The expression and activity of the NR gene are controlled by many endogenous and environmental factors (for a review, see Meyer and Stitt 2001) and are thought to be a major regulatory step in overall plant N metabolism (Beevers and Hageman 1969; Maldonado et al. 1996). Potato reduces nitrate mainly in the leaves and possesses a single NR gene per haploid genome (Harris et al. 2000). At high nitrate concentration, NR activity was detected not only in the shoots but also in the roots and stolons (Harris et al. 2000).

Genetic manipulation has allowed a modulation of NR expression in transgenic plants. For instance, com-

plementation of an NR-deficient mutant with a complete NR gene has produced plants with low amounts of NR activity (Vaucheret et al. 1990). Surprisingly, these plants grew as well as the wild type (WT). Indeed, they compensated for the loss of NR expression by having a higher activation state of the enzyme (for a review, see Meyer and Stitt 2001). Overexpression of the tobacco NR coding sequence has also been achieved by placing it under the control of the constitutive cauliflower mosaic virus 35S RNA promoter in transgenic tobacco (Dorhlac de Borne et al. 1994), *Nicotiana plumbaginifolia* (Quilleré et al. 1994; Foyer et al. 1993) and lettuce (Curtis et al. 1999). Transgenic *N. plumbaginifolia* plants again showed little change in their growth characteristics (Vincentz and Caboche 1991) or C-metabolism (Foyer et al. 1993) but accumulated glutamine (Gln), the end product of nitrate assimilation and the main form of transported N, and malate (Foyer et al. 1993; Quilleré et al. 1994). This suggests that the flux through the nitrate assimilation pathway was higher in these transgenic plants. The other major change observed was a significant decrease in leaf nitrate concentration, which was also reported in field-grown transgenic tobacco (Dorlhac de Borne et al. 1994) and in lettuce (Curtis et al. 1999). Finally, it appears that this decrease could be not only the result of a higher nitrate reduction rate but also of a lower nitrate uptake rate due to increased inhibition of uptake by reduced N (Gojon et al. 1998). Taken together, these results suggest that plants compensate for the introduced deregulation of NR expression by either accumulating Gln or by having NR in a more or less active form. These deregulations have ultimately had little impact on overall nitrogen metabolism and growth in the plants studied so far. But these plants (tobacco, lettuce) all lacked strong sink organs. Potato has highly developed storage organs, tubers, which accumulate mainly carbon but also some nitrogen. Whether tubers are capable of nitrate reduction remains rather controversial. Although Harris et al. (2000) did not find NR activity in tubers, it was found recently that NR activity in tubers from the Sava cultivar can contribute significantly to overall plant nitrate reduction, especially under low nitrate supply (Mäck and Schoerring 2002). Moreover, potato is known to be a poor user of available nitrate, but the reasons for this deficiency are so far unclear. It might be partly due to a superficial and often weak rooting system (van Loon et al. 1994) but also to a low N-use efficiency (Errebhi et al. 1998, 1999). When nitrate is provided in excess, potatoes can accumulate this ion in large amounts, up to 30% of total N in young leaves for instance, indicating that NR activity may be limiting (Millard and Marshall 1986). For the above reasons we introduced a deregulated NR gene into potato. This chimeric NR gene contains an N-terminal-truncated NR coding sequence and the 35S promoter (Nussaume et al. 1995). The N-terminal deletion has been shown to partially relieve the resulting NR enzyme from inactivation by phosphorylation, which normally occurs in the dark (Nussaume et al. 1995; Lillo et al. 1997). Indeed, the truncated NR enzyme has a higher activation state and seems less susceptible to proteolytic degradation (Lillo et al. 1997).

Transgenic *Solanum tuberosum* plants were first obtained via *Agrobacterium*-mediated transformation in several potato cultivars and studied for field behaviour. We first showed that the NR transgene was expressed in the transformed potato lines (Djennane et al. 2002a, 2002b). We then analyzed the growth characteristics and N-metabolite contents of two potato cultivars (92T.118.5 and 92T.110.29) grown in a greenhouse with two fertilization regimes. Thus, we were able to show that the deregulated expression of the truncated tobacco NR protein leads to highly reduced nitrate contents in tubers (Djennane et al. 2002a).

It was also found that nitrate concentrations decreased dramatically in the tubers of the all transgenic lines grown in the field with no differences in tuber yield (Djennane et al. 2002b). This reduction proved to be effective throughout the years and regardless of the nitrate supply (Djennane et al. 2002a, 2002b). In the present study we performed a more complete analysis of the physiological and biochemical consequences of the NR chimeric gene expression in another, early ripening, potato cultivar (Safrane) grown in more-controlled greenhouse conditions. Two transgenic lines from this cultivar were retained for this study and it was found that the expression of a deregulated NR leads not only to decreased nitrate accumulation but, contrary to what has been observed in other species, also to increased biomass accumulation.

Materials and methods

Plant material

Two independent transgenic clones (706.2 and 716.2) of potato (*Solanum tuberosum* L.), harbouring and expressing the deleted tobacco (*Nicotiana tabacum* L.) NR gene described by Nussaume et al (1995), were used for this study. These two clones were obtained by *Agrobacterium*-mediated transformation of the commercial potato cultivar Safrane (Djennane et al. 2002b). They were also selected after 2 years of field observations to make sure that they were not presenting additional phenotypic variations due to the process of potato transformation. The control plants in the present study were the untransformed Safrane cultivar.

Growth and sampling conditions

Tubers of the transformed and control (WT) potato lines (harvested after the 2000 field cultivation period) were cultured from March to June 2001 in greenhouse conditions at INRA (Versailles, France). The tubers were grown in individual pots (volume 5 l) of coarse sand. The plants were irrigated at least daily with

complete nutrient solution (Lesaint and Coïc 1983) containing 4 mM NO_3^- as the sole source of nitrogen. Temperatures varied between 15 and 18°C at night, and from 18 to 30°C during the day throughout the whole culture period. For the different genotypes, three plants were randomly sampled at each stage of development: i.e. 25, 50, 67, 81 and 109 days after planting (DAP). Plants were divided into different parts: aerial parts (including leaves, petioles and stems), roots, and tubers. Different biochemical analyses were then undertaken.

Biochemical analyses

For each sampling stage, the three different parts of the plants were weighed before and after freeze-drying for dry-matter determination. NR activity and NR activation state were measured on frozen fresh tissues as described earlier (Nussaume et al. 1995) on four different samples. The freeze-dried parts were ground to a fine powder, which was used for further analyses.

Aliquots of 7–9 mg of the powder were weighed in tin capsules and used for total nitrogen determination in a Fisons-Instrument (NA 1500 CN) analyser. The powder was also used for determination of other metabolites using extraction procedures derived from Rochat and Boutin (1989). Aliquots of 20–25 mg of freeze-dried powder were extracted successively with 1 ml of 80% (v/v in water) ethanol, 1 ml of 60% ethanol and finally with 1 ml water (1 h of extraction for each step) at 4°C. The three successive extracts were pooled and mixed. An aliquot (2×1 ml) of the supernatant was then evaporated in a Speed-Vac and resuspended in 1 ml water for nitrate and nitrite determination and in 1 ml citrate buffer (pH 2.2) for determination of free amino acids.

Nitrate and nitrite concentrations were determined by HPLC (Dionex DX-120 analyser; AS14 HPLC column; 3.5 mM Na_2CO_3 and 1 mM $NaHCO_3$ as eluent). Peaks were identified and quantified in an integrator (Peak-net station) by comparison with standard concentrations (10 μM to 1 mM for nitrate and 1 μM to 100 μM for nitrite).

Total free amino acid content was determined by the Rosen colorimetric method (Rosen 1957) with Gln as the reference compound. Then, the amino acids were separated on a Biotronik LC 5001 analyser by ion-exchange chromatography as described by Rochat and Boutin (1989).

Malate was determined colorimetrically using enzymatic kits of L-malate according to the procedure described by the manufacturer (Boehringer Mannheim).

Starch was quantified in the residue remaining after ethanol extraction and quantified only in tubers at harvest. It was determined after hydrolysis with α-amylase and amyloglucosidase. The resulting glucose was determined colorimetrically using enzymatic kits according to the procedure described by the manufacturer (Boehringer Mannheim).

Chlorophyll contents were determined on fresh tissues as described previously (Nussaume et al. 1995) by measuring the OD at 652 nm.

Statistical analysis

The differences between the lines were assessed by ANOVA (STAT-ITCF package, $P < 0.05$).

Results

In this study we used two independent transgenic lines of the potato cultivar Safrane. We have already shown that these two lines (named 706.2 and 716.2) express the tobacco NR transgene at the mRNA level and probably at the protein level (Djennane et al. 2002b).

Indeed, a tobacco-specific cDNA band was amplified by Reverse transcription–polymerase chain reaction (RT–PCR) in these lines, but no statistically significant difference in the amount of NR enzymatic activity was detected between the transgenics and the Safrane WT when the plants were grown in the field. Still, in vitro the transgenic lines showed a higher sensitivity to chlorate, a toxic analogue of nitrate, which suggests that the NR protein is differentially expressed in these plants. But significant NR activity was never detected in the transformed or control tubers (Djennane et al. 2002b).

Biomass production and dry-matter content

We first analysed the biomass production of the two transgenic lines at the different sampling times, measured as days after planting the tubers (DAP). Total dry-matter production of the two transformants was increased by 23–55%, depending on the sampling stage, when compared to the WT (Fig. 1). Although an increase in fresh weight was also seen for the transgenic lines (data not shown), the augmentation in dry weight was higher. Thus the transgenic lines seem to have a lower water content.

A more detailed analysis of the dry-matter variations over time among the different potato organs revealed that the dry-matter increase is not homogeneous. It appeared that the tubers made the major contribution to the higher biomass observed in the two transgenic lines during the later stages and at harvest (Fig. 2). Indeed, at harvest, biomass accumulation in the tubers was doubled for the transgenic line 706.2 and 1.5-fold higher for line 716.2, when compared to the untransformed control line. The number of tubers per plant was not statistically significantly different and the mean tuber number at the final harvest ranged from five to eight tubers per plant. Starch contents of the tubers were at this latest stage not statistically significantly different across the three lines, and were around 70% of dry weight.

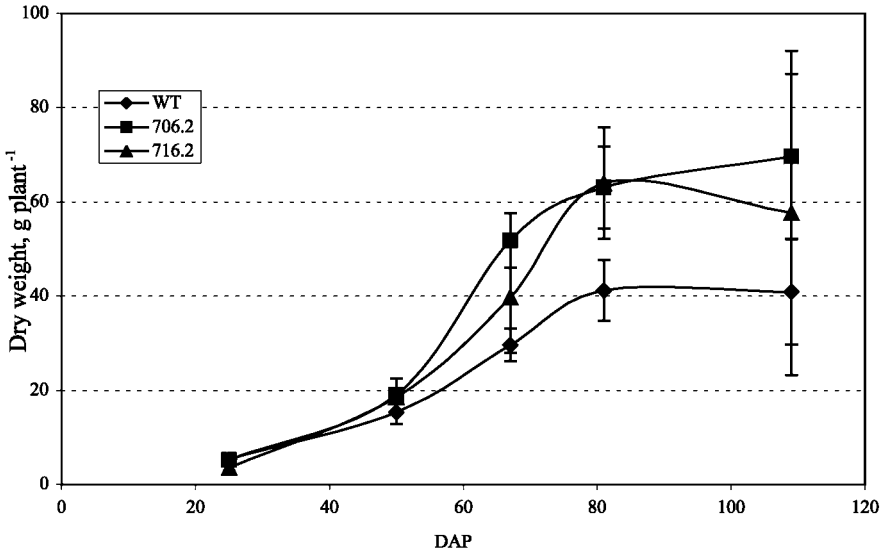

Fig. 1 Dry biomass accumulation during the time of culture for the two transgenic potato (*Solanum tuberosum*) lines expressing tobacco (*Nicotiana tabacum*) NR (*706.2* and *716.2*) and for the control Safrane cultivar (*WT*). Time is given in days after planting (*DAP*) and values as means ± SD ($n=3$)

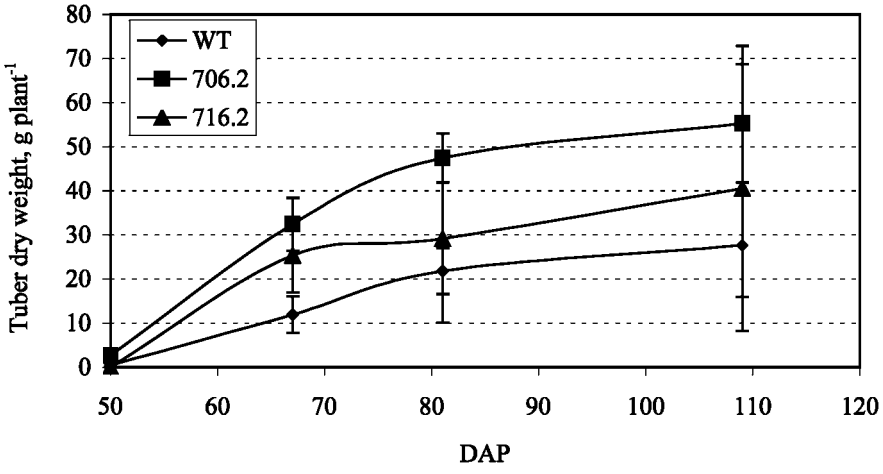

Fig. 2 Dry biomass accumulation in tubers during the time of culture for the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2*) and for the control Safrane cultivar (*WT*). Means ± SD ($n=3$)

The differences in root and shoot biomass were much less evident among the different lines. However, it should be noted that the variability among the three sampled plants was quite large, as illustrated by the high standard deviations. The ratio of shoot (aerial parts) to tuber dry weight was always smaller in the transformed lines, but the differences between the transgenic lines and the WT tended to decrease during the culture (from a ratio of 1.4 at 67 DAP to 0.45 at 109 DAP for the WT, from 0.56 to 0.25 for line 706.2, and from 0.53 to 0.41 for line 716.2). Conversely, the ratio of shoot/root dry weight was most of the time similar across the three lines (data not shown).

Total nitrogen and chlorophyll contents

Whatever the sampling stage, total nitrogen content per plant was never statistically significantly different between the two transgenic lines and the WT (Fig. 3). This implies that, considering the above results on biomass production, the transgenic plants absorb the same quantity of nitrogen as the WT but produce more dry matter. Thereby, the nitrogen content of the dry matter is constantly lower in the roots (data not shown), as well as in shoots and tubers, of the transgenic genotypes, and this is true at all sampling stages (Fig. 4). Conversely, the amount of total nitrogen accumulated in transgenic tubers is often higher than in WT tubers when measured per plant, whereas the amount of nitrogen stored per plant in the aerial parts is constantly lower in the transgenic lines (data not shown).

Chlorophyll content was measured in fresh leaf tissue at 50 DAP and was found to be slightly higher in the transgenics lines (1.81 ± 0.24, 1.95 ± 0.21 and 2.01 ± 0.29 mg chlorophyll g^{-1} FW for the WT, line 716.2 and line 706.2, respectively).

Nitrate content

Nitrate content in all organs is drastically reduced in the transgenic clones compared to the WT. For instance, shoot nitrate concentration decreases by about 62% in

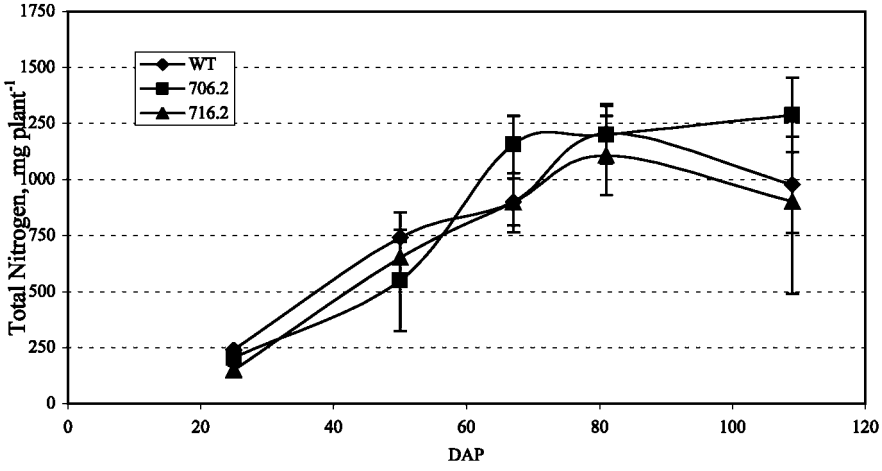

Fig. 3 Total nitrogen content per plant during the time of culture for the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2*) and for the control Safrane cultivar (*WT*). Means ± SD ($n = 3$)

both transgenic lines when compared to the WT (Fig. 5a). Furthermore, nitrate was almost undetectable in transgenic tubers at the last three sampling stages (Fig. 5b). The absence of nitrate in transgenic tubers was already apparent at the onset of tuberization. On the other hand, the decrease in nitrate concentration was much less marked in transgenic roots (data not shown). Nitrite was undetectable in all samples.

Rate of nitrogen reduction

We then examined overall nitrogen metabolism in the different potato lines by first calculating the percentage nitrogen reduction per plant, i.e. the ratio of reduced nitrogen to total nitrogen per plant (Table 1). The results clearly indicate that the percentage nitrogen reduction in the whole plant is always statistically significantly higher in the transgenic lines than in the untransformed control, with a mean increase of 13%. This is also in accord with the fact that the transgenic lines accumulate less nitrate than the WT whereas the total nitrogen content per plant is similar (compare Fig. 1 and Fig. 5).

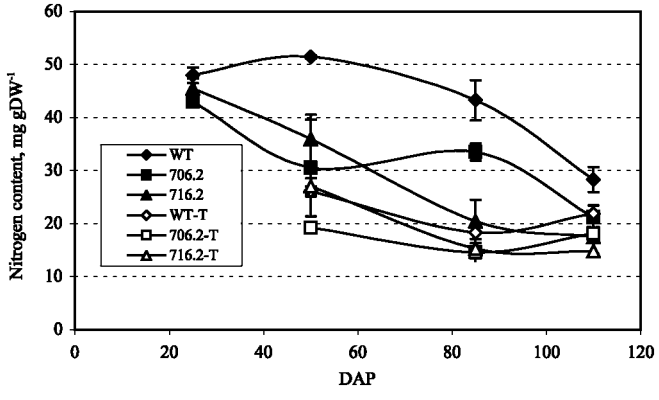

Fig. 4 Total nitrogen content per g of dry weight (*DW*) in aerial parts and tubers in the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2* for aerial parts, *706.2-T* and *716.2-T* for tubers) and in the control Safrane cultivar (*WT* for aerial parts and *WT-T* for tubers). Means ± SD ($n = 3$)

Free amino acid content

As the transgenic lines were showing a higher percentage nitrate reduction, we decided to investigate the amount of free amino acids in various organs at the different harvesting times. The total free amino acid content in leaves and roots was not statistically significantly different between the transgenic lines and the WT (data not shown). In contrast, tubers of the transgenic lines showed a slight but reproducible decrease in free amino acids at the two latest sampling stages (at 81 DAP, the free amino acid contents were 308 ± 23, 291 ± 21 and 246 ± 36 nmol mg^{-1} DW for the WT, line 706.2 and line 716.2, respectively; similar values were observed at 109 DAP). The level of the different amino acids was then analysed in the tubers. The contributions of the three major amino acids asparagine (Asn), Gln and proline (Pro) to the total amino acid content in tubers, together with the contents of some minor amino acids, are shown in Fig. 6. The percentage of Gln in the free amino acid pool increased in the transgenic lines, especially at 67 DAP (Fig. 6a). No clear trend in the variations of Asn percentage was detected, while Pro clearly made a lower contribution to the total amino acid pool in the transformed lines. Moreover, Pro was almost undetectable in the early stages of tuber formation and accumulated in statistically significantly amounts only at the final harvest (Fig. 6a). For the minor amino acids we chose to show the results of the amino acids that changed the most, together with a control, i.e. constant, amino acid tyrosine (Tyr), which represents well all other invariant amino acids (Fig. 6b). Percentages of glutamic acid (Glu), lysine (Lys), methionine (Met) and γ-amino-butyric acid (GaBa) in the free amino acid pool were always slightly higher in the transgenic tubers, with Lys showing the highest increase (from 130 to 300%). This statistically significant increase in Lys concentration, an essential amino acid for humans, could improve the nutritional value of these potato tubers.

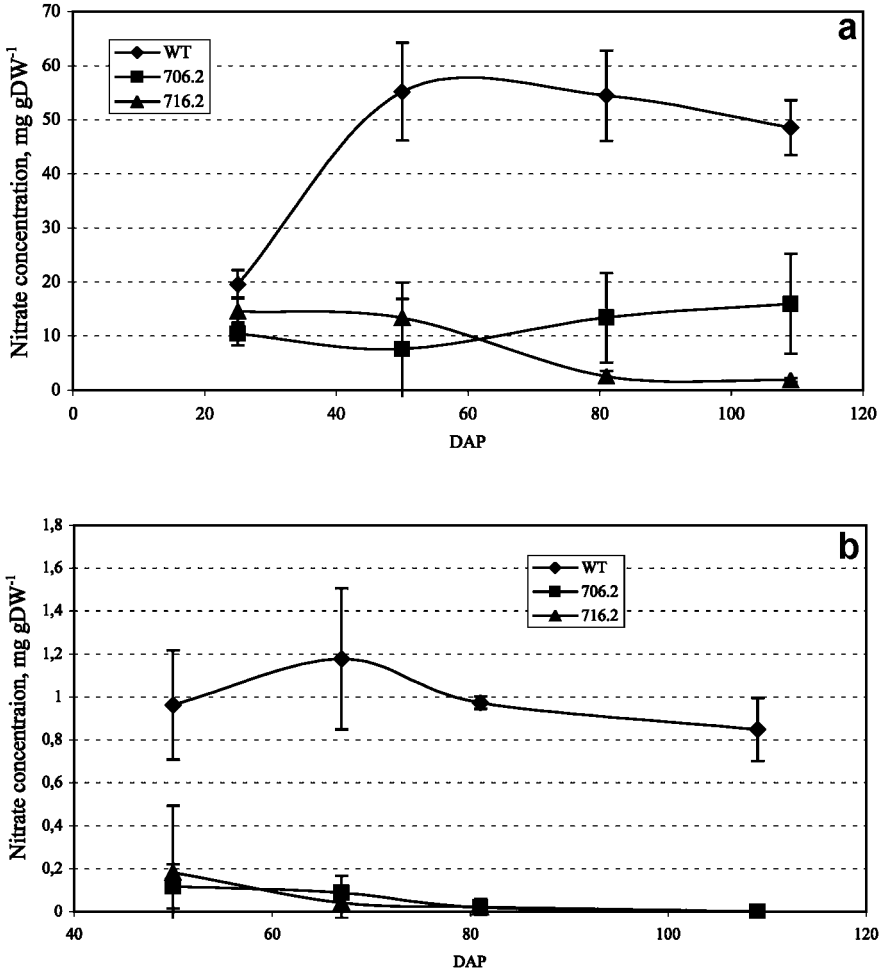

Fig. 5a, b Nitrate concentration in aerial parts (**a**) and tubers (**b**) during the time of culture for the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2*) and for the control Safrane cultivar (*WT*). Means ± SD ($n=3$)

Table 1 Percentage nitrogen reduction per potato plant (mean ± SD, $n=3$). Percentage nitrogen reduction is defined as the ratio of reduced nitrogen per plant (total nitrogen minus nitrogen in nitrate form) to total nitrogen absorbed by the plant, multiplied by 100. *DAP* Days after planting

Lines	25 DAP	50 DAP	67 DAP	81 DAP	109 DAP
WT	90.88 ± 1.06	76.69 ± 3.49	86.06 ± 3.67	81.12 ± 3.52	84.5 ± 5.49
706.2	94.62 ± 1.01	95.67 ± 4.91	93.35 ± 3.81	96.13 ± 2.47	96.19 ± 2.12
716.2	92.61 ± 0.76	91.95 ± 3.24	91.08 ± 6.79	98.32 ± 0.43	99.14 ± 0.29

Glu was the most abundant amino acid in the leaves across all genotypes, and Gln and Asn were much less abundant than in the tubers (data not shown). But we did not see any clear differences in amino acid content in leaves when comparing the three lines.

Malate content

Malate is often considered as a good indicator of the rate of nitrate reduction (Touraine and Gojon 1997). The transformed potato clones, which had a greater percentage of nitrogen reduction, accumulated more malate in the shoots and in the roots (Fig. 7). In contrast, malate content in tubers was not affected by the introduction of the tobacco NR gene (data not shown). In roots, malate concentration decreased during the culture in the transgenic lines whereas it increased in the WT after 50 days (Fig. 7b). At 80 DAP, the root malate concentration was even higher in the WT than in the transgenics. In the shoots, malate concentration was always higher in the transgenic lines but the differences in malate levels between transformed and control lines were comparable in roots and in shoots (Fig. 7, compare a and b). It should, however, be noted that the variability in malate concentration measurements was quite high.

NR activity

We previously observed that there were no significant differences in total NR activity when the Safrane transgenic potato lines were grown in the field (Djenn-

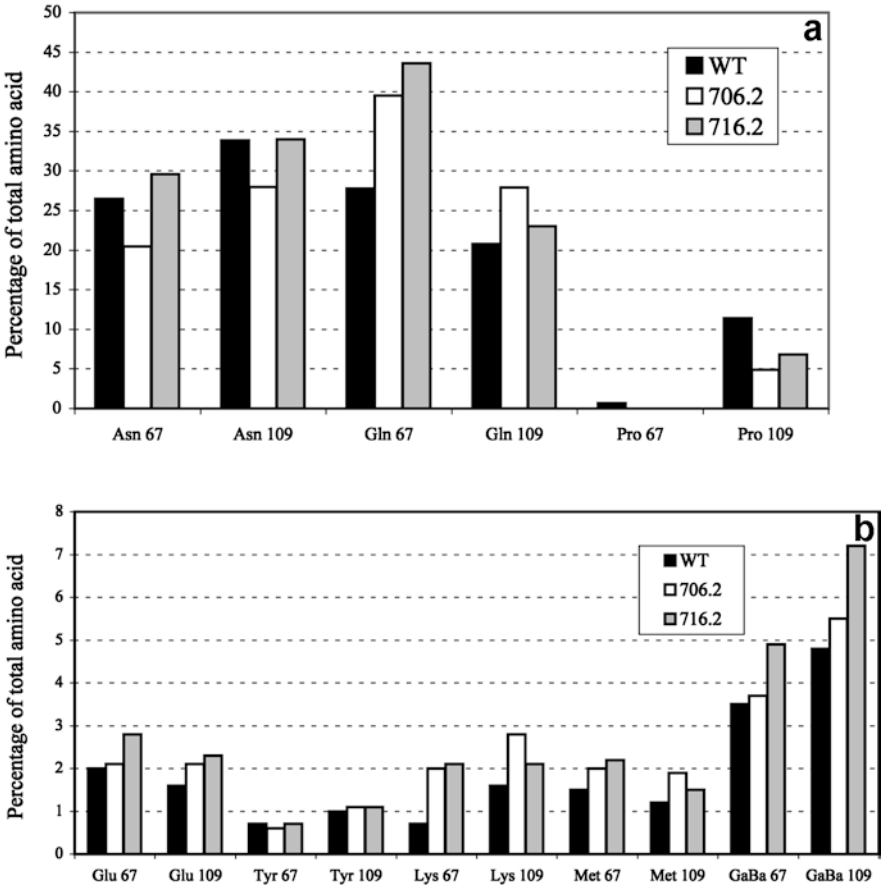

Fig. 6a, b Content of the three major amino acids (**a**) and some minor amino acids (**b**), as a percentage of total free amino acids, in tubers of the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2*) and of the control Safrane cultivar (*WT*). Samples were harvested 67 and 109 DAP. *Asn* Asparagine, *GaBa* γ-amino-butyric acid, *Gln* glutamine, *Glu* glutamic acid, *Lys* lysine, *Met* methionine, *Pro* proline, *Tyr* tyrosine

ane et al. 2002b). In the present study, we have compared the NR activity in leaves of the WT and the two transgenic lines at 50 DAP and we have again found no statistically significant differences among the genotypes (NR activities were 54.1 ± 34.2, 51.2 ± 27 and 67.2 ± 42.3 nmol nitrite formed min^{-1} g^{-1} FW for the WT, line 706.1 and line 716.2, respectively). The same result was observed at other sampling times and NR activity was always low in other plant parts (data not shown). As noted earlier (Djennane et al. 2002b), NR activity was very variable among replicates, which explains the high standard deviation.

Discussion

The introduction and expression of a deregulated tobacco NR gene in the potato genome leads to increased biomass production in the two Safrane transformants studied here. This trait is linked to the presence of the tobacco NR transgene, as we have shown earlier that the corresponding mRNA was expressed in these two transgenic lines (Djennane et al. 2002b). These lines also displayed a higher sensitivity to chlorate, which strongly suggests that transgene-derived NR is indeed expressed at the protein and activity levels, although NR activity showed no statistically significant increase in the transgenic lines. This biomass increase is mostly observed in tubers and, to a lesser extent, in aerial parts. Indeed, the difference in biomass accumulation between the transgenic lines and the WT became more evident after the onset of tuberization (Fig. 1). One explanation for this observation could be that the transformants are more precocious than the control line, which would then lead to a higher biomass accumulation at a given stage. Our results suggest that the rate of nitrate reduction is statistically significantly higher in the two transgenic lines. This higher N-use efficiency could allow the plants to invest more nitrogen in the photosynthetic apparatus, thus leading to the observed increase in biomass. Indeed, this augmentation of biomass is mainly due to a higher quantity of starch being stored in the transformants' tubers. This hypothesis is supported by the observed increase in chlorophyll level in the transgenic lines. In a recent study it was also shown that an increasing nitrate supply strongly enhances tuber yield (Mäck and Schoerring 2002). However, measurements of photosynthesis and of carbon fixation rates would be needed to verify this hypothesis. Lejay et al. (1997) have already demonstrated that ectopic expression of the N-terminal-deleted tobacco NR that we used in this study allows the plants to maintain nitrate reduction in low CO_2 concentrations. This could also contribute to a better efficiency of photosynthesis through a higher availability of reduced nitrogen when CO_2 becomes limiting. Furthermore, Nejidat et al. (1997) demonstrated that NR

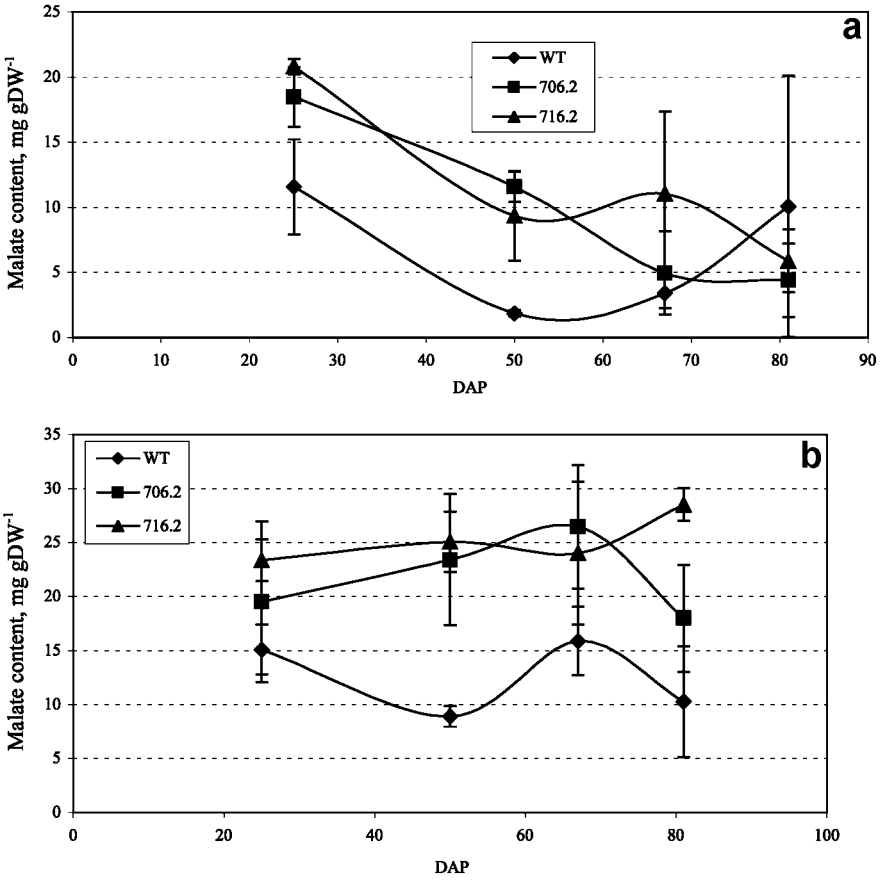

Fig. 7a, b Malate concentrations in roots (**a**) and aerial parts (**b**) during the time of culture for the two transgenic potato lines expressing tobacco NR (*706.2* and *716.2*) and for the control Safrane cultivar (*WT*). Means ± SD ($n = 3$)

overexpression in *Arabidopsis thaliana* leads to an increase in protein synthesis, particularly of Rubisco, a key enzyme for photosynthesis. In a previous study performed with other transformed potato cultivars grown in greenhouse conditions (Djennane et al. 2002a), we did not observe any modifications in biomass production. This difference can be attributed to the nature of the cultivars and/or to the experimental culture conditions. Indeed, in the present study an early ripening potato variety (Safrane) was used, whereas in the previous study late-ripening cultivars were employed (92T.118.5 and 92T.110.29). These differences could also be due to experimental conditions: the location of the experiment was different, the pot volume was much smaller in the previous experiment (1.2 l versus 5 l in the present work), and previously we also used a commercial nutrient solution containing 12 mM total nitrogen (19% $N\text{-}NH_4^+$ and 81% $N\text{-}NO_3^-$) while in the present study only nitrate (4 mM) was used as the nitrogen source (Djennane et al. 2002a). Moreover, this nitrate-based nutrient solution was provided continuously whereas the plants were watered by sub-irrigation in the previous experiment. In conclusion, the experimental conditions we used in the present work may be more suitable to reveal differences linked to ectopic NR expression, although a new experiment comparing these three cultivars would be needed to discriminate between a cultivar effect and an experimental conditions effect. NR over-expression has already been achieved in different species (including tobacco, lettuce and *A. thaliana*) but has never led to increased biomass production (Dorlhac de Borne et al. 1994; Quilleré et al. 1994; Nejidat et al. 1997; Curtis et al. 1999). Thus, to our knowledge, this is the first example of a higher biomass yield resulting from differential NR expression. The biomass increase observed in the transformants is not accompanied at any stage by an increase in total nitrogen content of the plant, which remains the same as in the WT. This implies that all these plants absorb the same total quantity of nitrogen. As a result, the transformants present a lower nitrogen content per dry weight unit compared to the WT and their C/N ratio is increased. In contrast, nitrate uptake was decreased in transgenic tobacco plants overexpressing the NR enzyme (Gojon et al. 1998). The authors ascribed this reduction in nitrate uptake to negative feedback regulation by the higher level of Gln present in these transgenic plants (Gojon et al. 1998). This is apparently not the case in potato where the level of Gln in the leaves of the transformed lines was no higher than in the WT, thereby indicating that nitrate uptake was not modified. In fact, the transformed potato plants could store their excess of reduced nitrogen in tubers rather than in leaves or roots, which would not affect nitrogen uptake. In turn, this would allow a better investment of N-resources in photosynthesis and an accumulation of biomass. In conclusion, the differences

in biomass accumulation between potato and other transgenic plants expressing tobacco NR could be simply due to the presence of a large sink organ in potato. Nitrate content was greatly affected in the transgenic plants. Indeed, nitrate decrease in the tubers was about 98% on average when compared to the WT. This result is in accordance with the observations in previous experiments, either in field or in glasshouse conditions (Djennane et al. 2002a, 2002b). Nitrate content was also highly reduced in aerial parts, whether it was measured in leaves, petioles or stems. In a previous study we measured nitrate contents in the petioles of other cultivars of transformed potato and, although a slight decrease in nitrate was sometimes observed, we did not record such a high decrease (Djennane et al. 2002a). It thus appears that, when using the Safrane cultivar, expression of the ectopic tobacco NR induces a statistically significant reduction in both circulating (petioles and stems) and stored nitrate (leaves). The diminution in the circulating nitrate pool could be the result of better NR expression, which would subsequently lead to an enhanced nitrate reduction in roots and/or vascular tissues. Indeed, the 35S promoter is more active in these tissues (Benfey et al. 1989). The extent of nitrate decrease seems to be much higher in potato plants than in other species expressing the same transgene. Nitrate content decreased by about 32–47% in transgenic tobacco plants (Dorlhac de Borne et al. 1994; Quilleré et al. 1994), and by about 16–48% in lettuce (Curtis et al. 1999). It seems that the available nitrate is reduced more quickly in the transgenic lines and that the reduced nitrogen produced is then diluted in a bigger quantity of (mainly tuber) biomass. This can in turn explain the increase in malate content. Indeed it has been suggested that malate is synthesized during nitrate assimilation as a counter-anion to replace nitrate and to prevent alkalinization of the cytoplasm, and is as such considered as a good indicator of the rate of nitrate reduction (Scheible et al. 1997a; Touraine and Gojon 1997). It has also been postulated that nitrate itself induces enzyme activities needed for malate synthesis (Scheible et al. 1997a). In transformed tobacco expressing the 35S NR transgene, higher accumulation of malate in the different tissues was also observed (Quilleré et al. 1994). Taken together these data suggest that it is the quantity of nitrate being reduced (or entering the cell) that modulates malate accumulation rather than nitrate itself since the transgenic lines accumulate less nitrate, presumably because they reduce it at a greater rate, and more malate. In conclusion, our results show that the expression of a deregulated NR enzyme in potato allows better N-use efficiency and higher biomass production through accelerated nitrate reduction. Modifications in normal source–sink communications and C/N ratios in aerial and subterranean parts may explain the higher biomass of the major potato sink organ, tubers. Indeed it was shown earlier that the shoot/root ratio is strongly and positively correlated with the leaf nitrate content and the level of nitrate supply (Mäck and Schoerring 2002), even in plants with very low NR activity (Scheible et al. 1997b), whereas the root sugar content was found to be inversely correlated with this ratio. In our study, the transformed potato plants had very low nitrate contents in the leaves, which could mimic an N-starvation signal resulting in enhanced growth of the subterranean parts (roots and tubers) through higher carbon allocation.

Acknowledgments We thank Yves Chupeau, who initiated this work, for his constant support and valuable discussions. We are grateful to François Gosse and Joël Talbotec for taking care of the plants, to Anne Krapp for critical reading of the manuscript, and to Jean-Pierre Boutin for his help in amino acid analysis.

References

Beevers L, Hageman RH (1969) Nitrate reduction in higher plants. Annu Rev Plant Physiol 20:495–522

Benfey PN, Ren L, Chua NH (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression pattern. EMBO J 8:2195–2202

Curtis IS, Power JB, De Laat AMM, Caboche M, Davey MR (1999) Expression of a chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Rep 18:889–896

Djennane S, Chauvin JE, Meyer C (2002a) Glasshouse behaviour of eight transgenic potato clones with a modified nitrate reductase expression under two fertilization regimes. J Exp Bot 53:1037–1045

Djennane S, Chauvin JE, Quilleré I, Meyer C, Chupeau Y (2002b) Introduction and expression of a deregulated tobacco nitrate reductase gene in potato lead to highly reduced nitrate levels in transgenic tubers. Transgenic Res 11:175–184

Dorlhac de Borne F, De Roton C, Delon R, Chupeau Y (1994) Etude du comportement agronomique de tabacs industriels transgéniques présentant une activité nitrate réductase élevée. Ann Tabac 26:19–37

Errebhi M, Rosen CJ, Lauer FI, Martin MW, Bamberg JB, Birong DE (1998) Screening of exotic potato germplasm for nitrogen uptake and biomass production. Am J Pot Res 75:93–100

Errebhi M, Rosen CJ, Lauer FI, Martin MW, Bamberg JB (1999) Evaluation of tuber-bearing *Solanum* species for nitrogen use efficiency and biomass partitioning. Am J Pot Res 76:743–151

Foyer CH, Lefebvre C, Provot M, Vincentz M, Vaucheret H (1993) Modulation of nitrogen and carbon metabolism in transformed *Nicotiana plumbaginifolia* mutant E23 lines expressing either increased or decreased nitrate reductase activity. Aspects Appl Biol 34:137–145

Gojon A, Dapoigny L, Lejay L, Tillard P, Rufty TW (1998) Effects of genetic modification of nitrate reductase expression on $^{15}NO_3^-$ uptake and reduction in *Nicotiana* plants. Plant Cell Environ 21:43–53

Gravoueille JM, Grolier N, Henry E (1992) Teneur en nitrates dans les tubercules de pomme de terre. La pomme de terre française 471:173–178

Harris N, Foster JM, Kumar A, Davies HV, Gebhardt C, Wray JL (2000) Two cDNAs representing alleles of the nitrate reductase gene of potato (*Solanum tuberosum* L. cv Désirée): sequence analysis, genomic organization and expression. J Exp Bot 51:1017–1026

Lejay L, Quilleré I, Roux Y, Tillard P, Cliquet JB, Meyer C, Morot-Gaudry JF, Gojon A (1997) Abolition of posttranscriptional regulation of nitrate reductase partially prevents the decrease in leaf NO_3^- reduction when photosynthesis is inhibited by CO_2 deprivation, but not in darkness. Plant Physiol 117:623–630

Lesaint C, Coïc Y (1983) Cultures hydroponiques. Editions Flammarion, Paris

Levallois P, Thériault M, Rouffignat J, tessier S, Landry R, Ayotte P, Girard M, Gingras S, Gauvin D, Chiasson C (1998) Groundwater contamination by nitrates associated with intensive potato culture in Québec. Sci Environ 217:91–101

Lillo C, Kazazaic S, Ruoff P, Meyer C (1997) Characterization of nitrate reductase from light and dark exposed leaves. Comparison of different species and effects of 14-3-3 inhibitor proteins. Plant Physiol 114:1377–1383

Mäck G, Schoerring JK (2002) Effect of nitrate supply on N metabolism of potato plants (*Solanum tuberosum* L.) with special focus on the tubers. Plant Cell Environ 25:999–1009

Maldonado JM, Barro F, Gonzales-Fontes A (1996) Nitrate assimilation and protein accumulation in tritordeum. Plant Physiol Biochem 34:721–726

Marin J, Zee JA, Levallois P, Desrosiers T, Ayotte P, Poirier G (1998) Consommation de pommes de terre et leur contribution aux apports alimentaires en nitrates et nitrites. Sci Aliments 18:163–173

Meyer C, Stitt M (2001) Nitrate reduction and signalling. In: Morot-Gaudry J-F, Lea PJ (eds) Plant nitrogen. INRA–Springer, Versailles, pp 37–59

Millard P, Marshall B (1986) Growth, nitrogen uptake and partitioning within the potato (*Solanum tuberosum* L.) crop in relation to nitrogen application. J Agric Sci 107:1–9

Nejidat A, Zhang G, Grinberg M, Heimer YM (1997) Increased protein content in transgenic *Arabidopsis thaliana* overexpressing nitrate reductase activity. Plant Sci 130:41–49

Nussaume L, Vincentz M, Meyer C, Boutin JP, Caboche M (1995) Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. Plant Cell 7:611–621

Peralta JM, Stockle CO (2002) Dynamics of nitrate leaching under irrigated potato rotation in Washington state: a long-term simulation study. Agric Ecosyst Environ 88:23–34

Quilleré I, Dufossé C, Roux Y, Foyer CH, Caboche M, Morot-Gaudry JF (1994) The effects of deregulation of NR gene expression on growth and nitrogen metabolism of *Nicotiana plumbaginifolia* plants. J Exp Bot 45:1205–1211

Richards JE, Milburn PH, Maclean AA, Demerchant GP (1990) Intensive potato production effects on nitrate-N concentrations of rural New Brunswick well water. Can J Eng 32:189–196

Rochat C, Boutin JP (1989) Carbohydrates and nitrogenous compounds changes in the hull and in the seed during the pod development of pea. Plant Physiol Biochem 27:881–887

Rosen H (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67:10–15

Scheible W-R, Gonzales-Fontes A, Lauerer M, Müller-Röber B, Caboche M, Stitt M (1997a) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell 9:783–798

Scheible W-R, Lauerer M, Schulze E-D, Caboche M, Stitt M (1997b) Accumulation of nitrate in the shoot acts as a signal to regulate shoot–root allocation in tobacco. Plant J 11:671–691

Touraine B, Gojon A (1997) Integration of nitrate uptake in the whole plant. In: Morot-Gaudry J-F (ed) Nitrogen assimilation by plants. INRA, Versailles, pp 109–128

Van Kempen P, Le Corre P, Bedin P (1996) Phytotechnie. In: Rousselle P, Robert Y, Crosnier J-C (eds) La pomme de terre. INRA, Versailles, pp 363–414

Van Loon CD, Bedin P, Reust W (1994) Vers une production de pomme de terre respectueuse de l'environnement. Proceedings of the 12th EAPR Triennial Conference, Paris, pp 36–56

Vaucheret H, Chabaud M, Kronenberg J, Caboche M (1990) Functional complementation of tobacco and *Nicotiana plumbaginifolia* nitrate reductase deficient mutants by transformation with wild types alleles of the tobacco structural genes. Mol Gen Genet 220:468–474

Vincentz H, Caboche M (1991) Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants. EMBO J 10:1027–1035