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TESI DI DOTTORATO DI RICERCA

UPTAKE OF NITROGEN FORMS IN *ZEA MAYS*

A molecular and physiological study of urea transport

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Summary

By 2050, the global population is expected to be 50% higher than at present and global grain demand is projected to double. Under current model of intensive agriculture, the productivity of crops is sustained by applying to cultures high amounts of industrially synthetized nitrogen fertilizer. As a consequence, the wide use of chemical inputs has led to negative impacts on environment and on farmer economies. In addition the nitrogen use efficiency (NUE) of cereal crops has declined in the last 50 years, being currently estimated to be around 33%. Based on these considerations, crop yield needs to be improved in a more cost-effective and eco-compatible way. This goal could be achieved by increasing the NUE of cereals and optimizing the acquisition of native and applied nitrogen.

Since urea is the worldwide most used nitrogen fertilizer, a research topic of great interest concerns the capacity of crop plants to use urea *per se* as a nitrogen source for their development and growth. However, to date only a limited number of studies focused on the uptake systems and assimilation of urea by roots.

This thesis focuses on the characterization of the urea acquisition mechanisms in maize roots at physiological and molecular level. Moreover, in an agricultural soil, plant roots are in contact with more than one source of nitrogen, i.e. urea, ammonium and nitrate, which may derived from the hydrolization of urea, and other organic nitrogen containing molecules; particular attention was paid to the influence of different nitrogen sources (N-sources) on the urea uptake system.

Maize plants (*Zea mays*, L.; 5-day-old) were grown in hydroponic conditions. The root capacity to take up urea by a high-affinity transport system was evaluated at physiological level. In addition the influence of organic (such as urea and amino acids) and inorganic (such as nitrate) N-sources on the mechanisms involved in urea acquisition were investigated. This was performed by exposing for a short time (up to 24 hours) the roots to different nitrogen forms (N-forms) and analyzing the time-course of changes in the uptake rates.

To evaluate a possible link between the physiological and transcriptional response, variations in the gene expression were detected using two different techniques. A genome-wide microarray analysis of maize roots exposed to urea and/or nitrate was performed. Concerning genes involved in urea and nitrate transport and metabolism, the transcriptional data were confirmed and expanded to other growth conditions and tissues by real time RT-PCR analyses. Thus time-course gene expression experiments were performed on root and leaf tissues of plants exposed for short time to different N-

forms, as urea and/or glutamine or nitrate. Besides physiological and transcriptional analyses, the molecular characterization of urea transporter was performed cloning the *ZmDUR3*-ORF sequence from maize roots, expressing it in heterologous systems, i.e. a yeast mutant, *X. laevis* oocytes and tobacco protoplasts and studying its urea uptake capacity and membrane localization.

This thesis work reports, for the first time, the physiological characterization of urea uptake in roots of intact maize plants. Results indicated that at micromolar urea concentrations (up to 300 µM urea), maize roots were able to take up this nitrogen source, using a high-affinity and saturable transport system, as also reported for Arabidopsis and rice. Moreover, the urea high-affinity transport system appeared to be regulated by urea itself, showing the induction of the urea influx after short (few hours) root exposition to the molecule.

In soil, plant roots are constantly exposed to a mix of different N-sources, such as organic and inorganic ones. Thus, to mimic field conditions, the influence of nitrate or glutamine on the capacity of maize roots to take up urea was studied. Interestingly, both N-forms exerted negative effects on urea acquisition, since low influx rates were detected when glutamine or nitrate were applied to the external medium.

At least under the experimental conditions employed in this work, the increase capacity to take up urea due to urea treatment did not appear to involve variations in the transcriptional level of the high-affinity urea transporter gene *ZmDUR3*. Indeed, analysis of the transcriptomic profile revealed that the presence of urea in the external medium as the sole N-source, determined the up-regulation of only four genes. Nevertheless, as also corroborated by Real time RT-PCR, intriguing data were obtained when urea was applied with nitrate. Under this treatment maize roots increased further the overexpression of genes induced by nitrate alone, especially those involved in nitrogen assimilation, indicating a positive interaction between nitrate and urea on transcript abundance .

In the last part of the present thesis, the isolation of *ZmDUR3*-ORF from maize roots allowed to functionally characterize its urea transport activity and its subcellular localization by expression in heterologous systems, such as in the *dur3* yeast mutant, *X. laevis* oocytes and tobacco protoplasts. In particular the ability of ZmDUR3 to phenotypically complement the yeast mutant YNVWI (Δura3, Δdur3) by restoring the capacity to growth in presence of urea as sole N-source (≤3mM), is an undoubted proof that ZmDUR3 transports urea.

The results reported in this work of thesis will help clarifying the relative contribution that uptake of urea can give to the overall nitrogen acquisition in maize roots and could provide a key to improving the use efficiency of nitrogen fertilizers in crop plants.

1 Introduction

1.1 Bioavailability of soil nitrogen

1.1.1 The biogeochemical nitrogen cycle

Nitrogen is an essential element for life as component of central importance in the formation of biological compounds, such as amino acids and nucleotides (Taiz and Zeiger, 2006). Nitrogen, along with hydrogen, carbon, and oxygen, is the most abundant element in plants and for this reason it is taken up in the greatest amounts from the soil.

In natural environment, nitrogen occurs for the most part in the atmosphere as molecular nitrogen gas (N_2) accounting for over the 75% by volume. However, in this form, nitrogen is not directly available for plant nutrition but has to undergo transformations, known as nitrogen fixation. In the biogeochemical cycle (Figure 1) molecular nitrogen is naturally converted in chemical reactive forms by two main reactions: i) *lightning and photochemical reactions,* that they account for 10% of the fixed nitrogen (ranging between 0.5 and 30 X 10^{12} g nitrogen per year; Miller and Cramer, 2004) and are responsible for the conversion of N_2 into the form of nitric acid (HNO_3), which subsequently falls to Earth with rain; ii) *biological nitrogen fixation*, that it consists in the fixation of N_2 into ammonium by bacteria or blue-green algae, accounting for 90% of fixed nitrogen (ranging between 45 and 330 X 10^{12} g nitrogen per year; Miller and Cramer, 2004). An example of mutualistic nitrogen fixing bacteria is represented by *Rhizobia*, which live in symbiosis with legumes, while *Azotobacter* is known as being free-living nitrogen fixing bacteria. On global scale, in the 1990s the natural biological nitrogen fixation on land accounted for 110 Tg nitrogen per year while in the ocean was around 140 Tg nitrogen per year (Gruber and Galloway, 2008).

Nitrogen in soil is mainly present as organic forms derived from animal and plant residues or from waste products. In general the organic compounds are considered to be a nitrogen source for microbial populations rather than plants. Indeed no evidence in the literature shows that plants are able to access directly complex organic matter, such as proteins, while soil microorganisms are able to break down proteins releasing peptides or single amino acids (Rentsch et al., 2007). Thus, depending on the rate of decomposition of the organic matter, the compounds, such as amino acids, nucleic acids, urea, and uric acid, may undergo to ammonification processes contributing to release ammonium. The bacteria and fungi that accomplish this process are called ammonifying organisms. The subsequent conversion of ammonium into the oxidized forms nitrite and nitrate is called *Nitrification process*. In a first step, ammonium and oxygen are converted into nitrite by two groups of organisms: ammonia-oxidizing bacteria, *Nitrosomonas* sp., and ammonia-oxidizing archaea. In the second step nitrite is oxidized to nitrate, mainly, by bacteria of the genus *Nitrobacter* sp (Taiz and Zeiger, 2006). Under normal soil conditions, the bacterial oxidation of ammonium to nitrite is much slower than nitrite to nitrate. Therefore, very little nitrite is normally found in soil. As a consequence of these equilibria, in aerated soils, nitrogen is available for plant nutrition mainly in form of ammonium and nitrate (Gessa and Ciavatta, 2005) and as organic compounds, such as urea, peptides and amino acids depending on the degradation rate of the organic matter.

However, in agricultural soils, the concentrations of these nitrogen forms are subjected to great fluctuations since agronomic practices, as fertilization events, may increase their bioavailability, especially that of nitrate, ammonium or urea. The inductrial production of the fertilizers is a further example of the atmospheric nitrogen fixation, since by Haber-Bosch process the gaseous nitrogen is fixed into ammonium and then is converted in the more oxidized forms of fertilizers, as nitrate or urea.

Besides reactions that contribute to increase the soil nitrogen bioavailability other processes can occur in the biogeochemical cycle leading to nitrogen losses from the soil, such as denitrification, volatilization, nitrate leaching or erosion.

Denitrification consists of the microbial conversion of nitrate to gaseous forms, such as nitrous oxide (N_2O) and molecular nitrogen (N_2), which go back to the atmosphere:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The denitrifying bacteria have the ability to switch their respiration from oxygen to nitrate. Since respiration is more efficient using oxygen, these bacteria will only switch to nitrate if oxygen is absent, such as in waterlogged soils. Therefore, denitrification becomes significant when soils become saturated. Other environmental factors that favour denitrification include carbon availability (crop residues), warm soil, and neutral to alkaline pH.

Nitrogen can be converted in gaseous form also by volatilization, leading to a physical loss of ammonia to the atmosphere. All ammonium-based fertilizers are subjected to volatilization when broadcastly applied on the surface of soils with high pH, surface lime salts, low organic matter, warm temperatures and dry conditions; the presence of water favours the formation of the ionic form ammonium, which is not susceptible to volatilization. This is the reason why it is so important to incorporate fertilizers such as anhydrous ammonia or urea into the dry soil.

Other processes, such as soil erosion and nitrate leaching can contribute to limit nitrogen bioavailability. Estimates of total nitrogen losses by leaching from ammonium-based fertilizers range between 10 and 150 Kg N ha^{-1} (based on data of International Fertilizer Industry Association: www.fertilizer.org/ifa/statistics.asp; Miller and Cramer, 2004).

Moreover, in the soil, nitrogen bioavailability may be temporary limited by microbial immobilization. Indeed, microbes can also utilize inorganic nitrogen for their metabolism, this action resulting in a depletion of nitrogen availability to the plants when elevated carbon levels are provided (e.g. by addition of crop residues with high carbon/nitrogen ratio like straw) to support the microbial biomass accumulation. Anyway, the competition between plant and microorganisms for nitrogen is complex, due to multiple pathways through which nitrogen cycles at variable rates and in varying amounts and it is further complicated in case of plant-microorganism symbioses (Hodge et al., 2000).

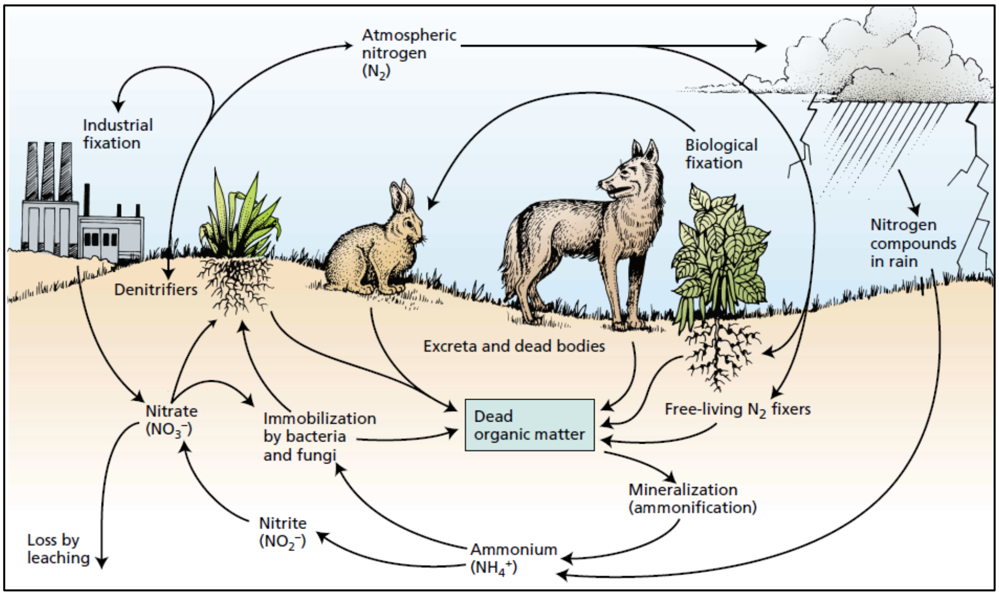


Figure 1. Biogeochemical nitrogen cycle. Nitrogen cycles through the atmosphere as it changes from a gaseous form to reduced ions before being incorporated into organic compounds in living organisms. Some of the steps involved in the nitrogen cycle are shown (Taiz and Zeiger, 2006).

1.1.2 Nitrogen fertilization

Despite the great abundance in atmosphere, nitrogen is commonly deficient in agricultural soils where it is present mainly in form of nitrate and ammonium.

As a consequence of the ready use of nitrate by plants and micro-organisms and its leachability, nitrate concentrations in the soil solution are usually very variable, typically in a range between 0.5 and 10 mM (Reisenauer, 1964), but it could also increase of three orders of magnitude (Crawford and Glass, 1998). On the other hand, ammonium is generally present in much lower amounts ranging from 20 to 200 µM (Owen and Jones, 2001); generally only 10% of the cation is found in solution remaining bound as an exchangeable cation to the negative soil surfaces. In soil it is also present an organic nitrogen fraction corresponding to 0.1- 50% of total soil nitrogen (Barber, 1984); however, not all this fraction is available for root uptake since plants absorb preferentially amino acids and urea. The amino acids concentration in agricultural soils generally ranges between 1 and 100 µM (Owen and Jones, 2001), while urea is reported to occur up to 70 µM (Gaudin et al., 1987).

So in aerated soils, nitrate is the dominant nitrogen form available for plant nutrition (Novoa and Loomis, 1981), although its concentration is very variable. It was estimated that most non legume plants require 20-50 g of nitrogen taken up by their roots to produce 1 Kg of dry biomass (Robertson and Vitousek, 2009). As consequence, the natural concentration of nitrogen in most agricultural soils represents a limiting factor for the development and growth of cultivated plants, especially for cereals such as maize, mainly because of their low Nitrogen Use Efficiency (NUE) (Raun and Johnson, 1999). Nitrogen use efficiency (NUE) can be defined as the ratio between grain yield and the total nitrogen taken up by the plant. It has been reported that more than one third of the total nitrogen applied for cereal production in the world, is removed in the grain, corresponding to a worldwide NUE around 33% (Raun and Johnson, 1999). In 1999, the cost of the remaining 67% accounted for 16 billion dollars (Raun and Johnson, 1999).

Three cereals, wheat, rice and maize, provide 60% of human food and have become the three most abundant plants on Earth (Tilman et al., 2002). Concerning maize, its cultivation has acquired great relevance in north Italy, especially in Friuli-Venezia-Giulia being the most cultivated crop plant in the last years (ISTAT 2011, Table 1).

Table 1. Crop production in Friuli-Venezia -Giulia for maize, rice, sorghum and other cereal species (ISTAT 2011: http://agri.istat.it). Data were collected between Nov-2011 and Apr-2012 depending on the culture.

	Maize (Nov-2011)	**Rice** (Apr-2012)	**Sorghum** (Dic-2011)	**Other cereals** (Dic-2011)
Area (ha)	91,404	9	181	30
Harvested production (Kg)	806,378,000	3,500	756,600	90,000

In order to increase the concentration and the bioavailability of nitrogen sources, a common agronomic practice is to supply nitrogen fertilizers to soil. These fertilizers can be produced by natural or synthetic process and are mainly divided in organic and inorganic ones. The commonly used fertilizers include a different collection of compounds with nitrogen supplied in form of nitrate, ammonium, urea or combination of two of them. During 2011, world demand of nitrogen fertilizer was evaluated to be around 105 million tons (FAO, 2011). Since cereal yields are highly responsive to supplemental nitrogen, the worldwide maize cultivation lead to annual application of an estimated 10 million tons of nitrogen fertilizer (FAO 2004; Moose and Below, 2008). Referring to Region of Friuli-Venezia-Giulia, during the 2010 the consumption of nitrogen fertilizers used to sustain the cultivations, such as maize, has been estimated to be around 37,000 tons where urea accounted for over 60% (22,742 tons urea, ISTAT 2010: http://agri.istat.it).

Nitrogen fertilizers enable farmers to achieve the high yields that drive modern agriculture. In the last decades, the large consumption of fertilizer for cereal production was due to a correlation between cereal yields and nitrogen application rates which prevailed especially in the early of 1990s (FAO, 2000). As reported by Dobermann and Cassman (2005) the NUE, as the broadest measure of nitrogen use efficiency, is a ratio of yield to the amount of applied nitrogen (kg grain yield per kg nitrogen applied). Defined in this way, it appears evident as in the last 50 years the large increase in nitrogen use resulted in a steep decrease in the efficiency to use nitrogen in the following decades, reaching constant values between 1980s to 2000. Since, NUE is a ratio, it has decline from large values at small nitrogen application rates, in 1960s, to small values of NUE at high nitrogen application rates, as to date situation (Figure 2). To date this relationship is no more sustainable since the increase in the amounts of applied nitrogen to cultures doesn't leed to great benefits in terms of yields.

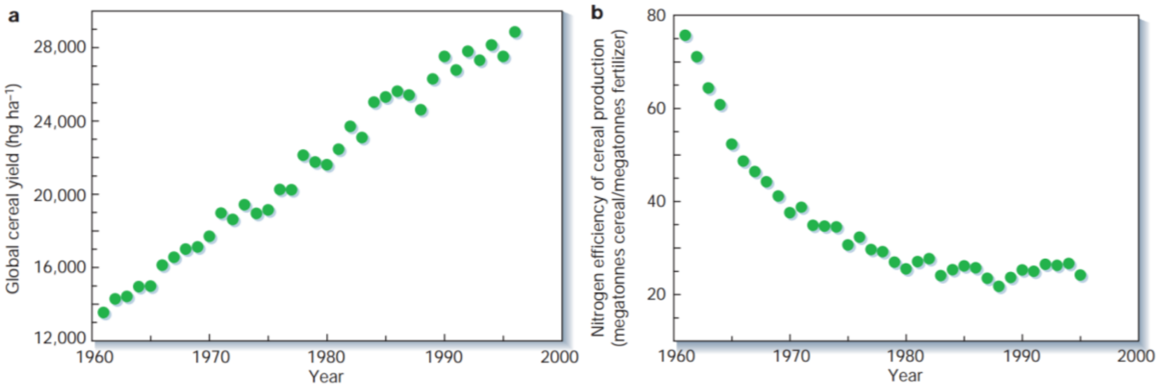


Figure 2. Trends of Global cereal yield and NUE of cereal production. Diminishing returns of fertilizer application imply that further applications may not be as effective at increasing yields. a: trends in average global cereal yields; b: trends in the nitrogen-fertilization efficiency of crop production (annual global cereal production divided by annual global application of nitrogen fertilizer) (Tilman et al., 2002).

Moreover in the next years it has been estimated an increase in the consumption of nitrogen fertilizers for agricultural use. By 2050 world population will increase reaching 9.15 billion (Alexandratos and Bruinsma, 2012) and as consequence of this demographic growth, the increase in food demand will require to be sustain by higher cereal production (about +38% by 2025 according to Dobermann and Cassman, 2005). In this way, especially if losses of cereal cropping area will continue at the rate of the past 20 years (-0.33% per year) and the NUE of cereals will not be increased substantially, in the future the cereal demand will lead to a 60 % increase of the global nitrogen use (Dobermann and Cassman, 2005). In the future wheat, rice and maize continue to dominate the demand for fertilizer into the next century. Nevertheless it has been estimated that the fertilizer use in maize production will surpass that of rice by 2030, as consequence of greater demand for meat in developing countries (FAO, 2000).

It is obvious that a similar inputs of chemical compounds will not be ecologically and even economically sustainable requesting new agronomical strategy for a better use of nutrients. As pointed out by Good et al. (2004) plant scientists have long recognized the need to improve the nitrogen use efficiency of cereals developing crops that absorb and use nutrients in a more efficient way. Essential knowledge to improve the NUE of cultivated plants will request a deep comprehension of soil-plant relationships and of plant mechanisms involved in the nitrogen uptake and utilization. In addition the new agronomical practices should be contribute to maintain the soil fertility minimizing as much as possible the nitrogen losses from the soil profile as consequence of volatilization or nitrate leaching processes.

1.1.3 Use of urea as nitrogen fertilization

As consequence of the dramatic increase of nitrogen fertilizers, these chemical inputs have a negative impact in the environment, affecting the biodiversity in the soil, air and water quality and also contributing to global climate change. Moreover It should be consider the economic sustainability, since the production of fertilizers is an energy-intensive process which depends by the fuel cost. Reducing the amount of supplemental nitrogen used in maize production will have significant positive economic and environmental benefits to world agriculture. In particular a combined lowering fertilizer and breeding plants with better nitrogen use efficiency is one of the main goals of research in plant nutrition (Hirel et al., 2007).

Urea is the most frequently used nitrogen fertilizers in the world (Figure 3) with annual amount of over 50 million tons accounting for more than 50% of the world nitrogen fertilizer consumption (www.fertilizer.org, 2008). The incredible increase in urea fertilizer use during the last decades is mainly due to its competitive price and the high nitrogen content (46% of mass), that allow reducing transport and distribution costs (Miller and Cramer, 2004).

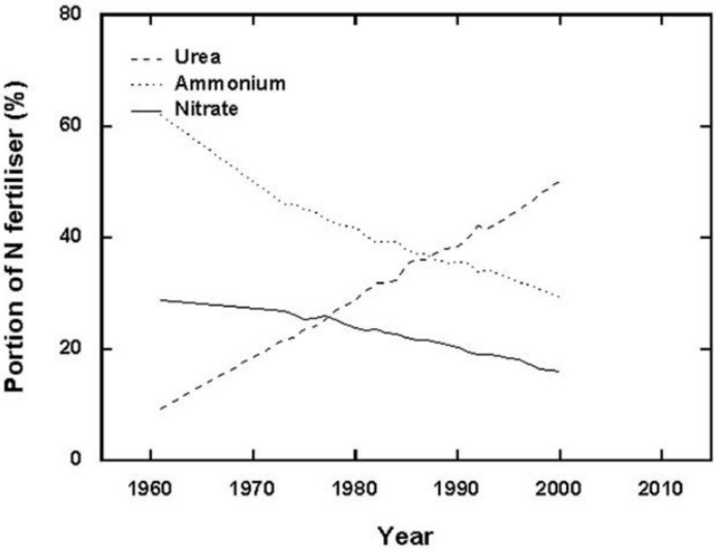


Figure 3. World fertilizer consumption: the proportion of total nitrogenous fertilizer applied containing urea, ammonium and nitrate or combinations of these (data from the International Fertilizer Industry Association, www.fertilizer.org/ifa/statistics.asp, Miller and Cramer, 2004).

Although experimental evidence reported the ability of plants to use urea *per se* when supplied through leaf application (Wittwer et al., 1963; Nicolaud and Bloom, 1998; Witte et al., 2002), a common agronomic practice is to supply urea to the crops by soil fertilization. In this way, at least for a short time, plant roots are exposed to urea as well to its hydrolysis products, such as ammonium and nitrate (Mérigout et al., 2008b).

The stability of urea in the soil is dependent on the activity of the microbial urease, a nickel-dependent enzyme ubiquitously expressed in microorganisms and released into soil (Watson et al., 1994). This enzyme catalyzes the hydrolysis of urea into ammonium and its activity is proportional to the microbial biomass, which in turn depends on the organic matter amount and the water content of the soil. Moreover the extremely stable urease activity persists in the soil also after the decay of the microorganisms (Watson et al., 1994). So a part of the urea applied into agricultural fields is converted into ammonium, which could remain in this form as exchangeable cation or volatilized in form of ammonia. Moreover the ammonium could be also substrate of nitrification process being transformed into nitrate. Thus, urea fertilization may result in a simultaneous exposition of plant roots to urea, ammonium and nitrate, at least for short periods of time (Mérigout et al., 2008b).

The efficiency of urea uptake could be affected by the use of urease inhibitors. Slowing the hydrolysis of urea, these molecules avoid a rapid and massive production of ammonium and nitrate; furthermore they allow the diffusion of urea far away from the application site favoring its uptake by the plant roots. The most promising and tested soil urease inhibitor is the N-(n-butyl) thiophosphoric triamide (nBTPT, trade name "Agrotain"), where the urease inhibitory activity is associated with the formation of its oxygen analogue (Watson, 2005). It is not unusual the marketing of urease inhibitors in combination with urea (Watson, 2005). However, in general little information is available on the urease inhibitors, whose activity could be affected by environmental factors such as pH (Hendrickson and Douglass, 1993), temperature (Hendrickson and O’Connor, 1987), and moisture content (Sigunga et al., 2002; Clough et al., 2004). In particular, an aspect that has not yet been elucidated concerns the effect of urease inhibitors on the capacity of roots to take up nitrogen sources, such as urea and nitrate. The comprehension of molecular mechanisms of urea uptake in cultivated plants could also be an important way to understand possible interference of nBTPT on the uptake and assimilation of nitrogen in plants.

1.2 Nitrogen acquisition in plants

A lot of papers have reported the ability of the higher plants to take up nitrogen as inorganic or organic forms (Nacry et al., 2013; Xu et al., 2012, Figure 4). Nitrate and ammonium are the main inorganic forms absorbed by plants and the mechanisms of their acquisition have been well characterized (Crawford and Glass, 1998; Crawford and Forde, 2002; Miller and Cramer, 2004). More recent studies concern the capacity of plants to acquire nitrogen also in form of organic compounds, such as peptide, single amino acids or urea (Rentsch et al., 2007). Only in the last decade, the molecular mechanism of urea transport in plants has been investigated (Kojima et al., 2006), by cloning and characterizing the urea transporters in Arabidopsis and rice (Liu et al., 2003a; Wang et al., 2012).

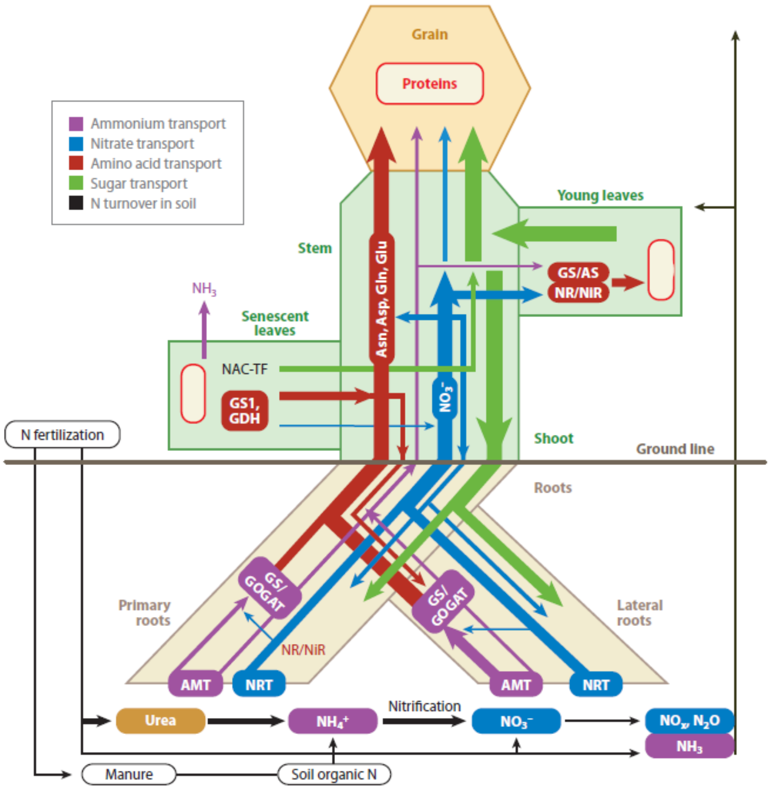


Figure 4. Schematic routes of N uptake from the rhizosphere. The source of N-fertilizer to be acquired, mainly in the form of ammonium and nitrate by roots, are included; transportation, assimilation and remobilization inside the plant are also indicated. The thicknesses of the arrows represent the relative amounts of nitrogen and sugar inside the plant. Abbreviations: AMT, ammonium transporter; AS, asparagine synthetase; Asn, asparagine; Asp, aspartate; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; GOGAT, glutamine-2-oxoglutarate aminotransferase; GS, glutamine synthetase; NAC-TF, certain transcription factors belonging to the NAC family; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter (Xu et al., 2012).

1.2.1 Inorganic nitrogen uptake and assimilation

In a typical aerobic agricultural soil, nitrate and ammonium are both present, as main inorganic nitrogen forms available for plant nutrition. Compared to ammonium, nitrate is the major form present in the soil solution, as described by Wolt (1994), who reported a study of 35 agricultural soil samples where the mean soil solution concentration of nitrate was around 6 mM, that is 10 times more concentrated than ammonium.

1.2.1.1 Nitrate uptake

From soil solution nitrate is actively transported across plasma membranes of epidermal and cortical cells of roots, then following different fates. In the cytosol nitrate can be reduced by the nitrate reductase as first step of the reductive assimilation pathway that leads to the synthesis of ammonium. The surplus of nitrate can be stored in the vacuole or translocated to the shoot. Moreover, the net uptake rate of nitrate is a consequence of two components, which contribute in opposite directions: by influx, nitrate is actively transported inside the cells using a symport with two protons; while, by efflux, nitrate is released outside the cells, in the rhizosphere, by specific transporters that act as an electrically driven passive uniport (Crawford and Glass, 1998).

Root cells present at least three nitrate transport systems (Glass and Siddiqi, 1995; Forde and Clarkson, 1999), characterized by distinct kinetic parameters (Table 2). Operation of the different systems depend on nitrate concentration in the root external solution: the low affinity transport system (LATS) becomes relevant under high nitrate concentration (over 0.25 mM), while the high affinity transport systems (HATS) account for the uptake when nitrate is at micro- molar concentration. LATS and HATS do not seem to be equally distributed along the root system, indeed as reported by Bothe et al. (2006) and Varanini and Pinton (2007), LATS is mainly present in the apical part of the roots while HATS in older root parts.

A property of the HATS is the coexistence of two different kinds of transport components: an inducible (iHATS) and a constitutive one (cHATS).

Table 2. Kinetic parameters of the nitrate transport systems as described by Crawford and Glass (1998).

cHATS	K_m 6-20 µM; V_{max} 0.3-0.82 µmol g^{-1} h^{-1}
iHATS	K_m 20-100 µM; V_{max} 3-8 µmol g^{-1} h^{-1}
LATS	Linear kinetic (up to 50 mM)

cHATS is characterized by very low value of K_m and V_{max}, corresponding to a low capacity pathway for nitrate absorption. However, this system is constitutively active in plants and it might be almost threefold upregulated by the exposure to nitrate (Aslam et al., 1992; Kronzucker et al., 1995). So, this system allows the plants to absorb nitrate also when it is present at very low concentration in the soil; in this way, the nitrate taken up in root cells allows the activation of iHATS, the inducible system. Usually the induction of iHATS leads to overshoot the plant demand for nitrate uptake and after the initial exposition to nitrate, the uptake system is rapidly down regulated (Glass and Siddiqi, 1995; Forde and Clarkson, 1999). As pointed out by Crawford and Glass (1998) the maximum uptake capacity of nitrate by iHATS is reached after hours or days of exposition to the anion, depending on its concentration in the external solution and on the plant species. So, iHATS is induced by nitrate while is feedback regulated at physiological and molecular level by downstream nitrogen metabolites, as ammonium and amino acids (Quesada et al., 1997; Krapp et al., 1998; Forde, 2000). In particular the induction by nitrate was demonstrated in maize by Santi et al. (2003), who reported that the root exposition to nitrate induced higher uptake rates of the anion and the overexpression of a high affinity transporter gene (*ZmNRT2.1*).

At molecular level, HATS and LATS are encoded by two different gene families: *NRT1* and *NRT2*. Both these families code for nitrate and protons co-transporters, whose expression can be induced by nitrate itself. In Arabidopsis several *NRT1* and *NRT2* genes have been characterized: seven *NRT2* genes (Orsel et al., 2002; Okamoto et al., 2003) and eleven *NRT1* genes (Tsay et al., 2007). These two families appeared to be involved in different transport systems. Most NRT1 family members characterized so far show low affinity nitrate transporters with the exception of NRT1.1, which operates with both high and low affinity (Xu et al., 2012). On the other hand, genes that belong to the *NRT2* family, code for transporters with high affinity; some of them require the association with a partner protein (NAR2) to transport nitrate (Yong et al., 2010). As regulators of nitrate acquisition, two genes encoding for nitrate-inducible kinases seem to be involved: *AtCIPK8* and *AtCIPK23* (c*alcineurin B-like interaction protein kinase*), which might regulate the dual affinity of NRT1.1 (Ho et al., 2009; Hu et al., 2009). Conditions of nitrogen starvation, as well as the presence of nitrate, nitrogen metabolites and pH variation, may influence the expression of the *NRT* genes (Krouk et al., 2010; Feng et al., 2011). Concerning the nitrate assimilation metabolites, it was demonstrated in maize a negative effect of glutamine on the expression of *ZmNRT2.1* (Vidmar et al., 2000). Thus it was suggested that while nitrate is responsible for inducing gene expression (Santi et al., 2003), nitrate assimilation products might be responsible for the down-regulation of nitrate uptake (Krapp et al., 1998; Filleur and Daniel Vedele, 1999; Lejay et al., 1999) (Figure 5). To drive the symport of nitrate with two protons across the plasma membrane, an electrochemical proton gradient must be generated by a proton-pumping ATPase (H^+-ATPase). In maize a correlation between the nitrate uptake capacity and the PM H^+-ATPase activity was reported and this correlation was confirmed at molecular level (Santi et al., 2003).

1.2.1.2 Ammonium uptake

In aerobic soils nitrate is the major nitrogen source available for plant nutrition, and as reported above plant roots possess specific transporter for the uptake of the anion. Other transporters, located on the plasma membrane, can mediate the uptake of ammonium. It was demonstrated that plants are able to take up this nitrogen form, even if the root exposition to ammonium as the sole nitrogen source did not allow a completely healthy development of plants, expecially of leguminous and cereals (Wilkinson and Crawford, 1993). Arabidopsis plants showed a better growth under ammonium nitrate than ammonium alone when applied as unique nitrogen source, possibly reflecting the adaptation to growth in aerobic soils (Helali et al., 2010).

Several ammonium transporters (AMT) have been identified in Arabidopsis (6 *AMT* genes) and rice (10 *AMT* genes). The *AMT* genes may be divided into two groups on the basis of sequences similarity: *AMT1* and *AMT2* (Shelden et al., 2001; Sohlenkamp et al., 2000). The expression of some *AMT1* genes in root hairs suggested their involvement in the ammonium uptake from the soil (Ludewig et al., 2002). Concerning the AMT1-type transporters it was observed also a correlation between ammonium uptake rate and the levels of gene expression (Kumar et al., 2003).

Analysis of concentration-dependent influx of ammonium into intact plant revealed biphasic kinetics of root uptake, indicating the presence of at least two distinct components of ammonium transport: a high affinity transport, which showed a saturable kinetic for external ammonium concentration <1 mM, and a low affinity transport, showing uptake rates increasing linearly for higher concentrations of the cation (Ullrichet al., 1984; Kronzucker et al., 1996).

The kinetic properties of the transport systems measured in the whole plant are highly variable and mainly dependent upon the nutritional status of the plant, which, in turn, is affected by environmental factors, such as light, temperature and previous external substrate availability. Unlike nitrate transporters, few days of nitrogen starvation resulted in an increased capacity of plants to take up ammonium (von Wirén et al., 2000). At molecular level, the expression of some *AMTs* genes is repressed by the presence of ammonium while the amounts of mRNA increase under nitrogen starvation (Yuan et al., 2007).

As described for nitrate, the expression of an *AMT* gene and ammonium influx were suppressed when plants were supplied with glutamine, suggesting a negative control by nitrogen assimilation metabolites (Rawat et al., 1999) (Figure 5).

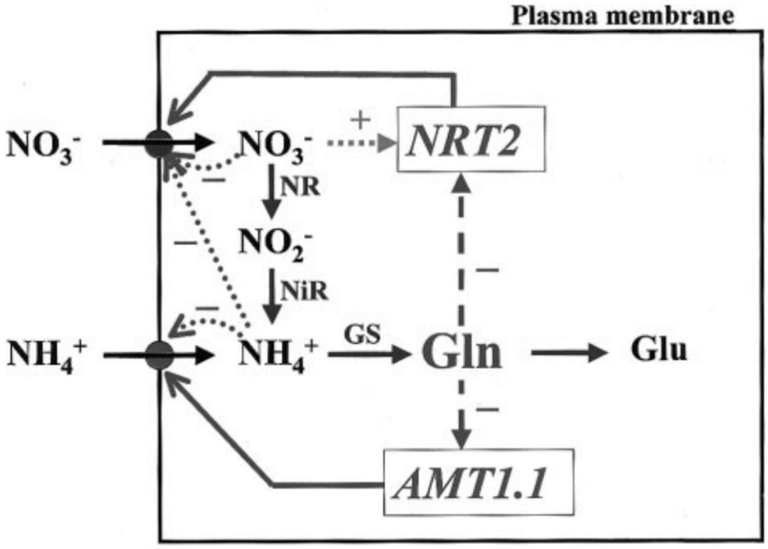


Figure 5. A model of proposed feedback processes involved in regulating nitrate and ammonium uptake. Glutamine (- - -) down-regulates the abundances of root *NRT2* and *AMT1* transcripts; cytosolic nitrate (NO_3^-) and ammonium (NH_4^+) concentrations (…) directly affect transporters activity. Induction of *NRT2* expression by nitrate is also indicated. Solid lines from *NRT2* and *AMT1.1* indicate the pathways of transcription and translation leading to accumulation of high affinity nitrate and ammonium transporters (circles) in the plasma membrane (from Glass et al., 2001).

1.2.1.3 Nitrate and ammonium assimilation

The inorganic nitrogen, in form of nitrate and ammonium, is assimilated by plants onto carbon skeletons to synthetize organic nitrogen compounds, such as amino acids, nucleotides and other secondary metabolite that are essential to plant growth. In the past years, the nitrogen assimilation pathway has been extensively studied in a large variety of plant species, and the enzymes involved in the assimilation reactions are well known (Figure 6).

Nitrate, absorbed by roots, is assimilated in either roots and shoots by the same pathway, where reductive reactions convert nitrate into ammonium. The first reaction is catalyzed by Nitrate Reductase (NR), which transfers two electrons from NAD(P)H to nitrate, producing nitrite:

$$\text{NR:} \qquad NO_3^- + NAD(P)H + H^+ + 2\,e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$$

NR is located in the cytosol and is composed of two identical subunits. In each subunit several redox centers and prosthetic groups are present, such as: Flavin adenine dinucleotide (FAD), heme (cytochrome 557) and a molybdenum-pterin cofactor (MoCo). As electron donor this enzyme can use NADH or NADPH, so depending on reductant source, different forms of NR occur in plants. The most common form of NR uses only NADH; in roots is also present a form of NR that uses either NADH or NADPH.

The expression of the genes encoding NR is tightly regulated (Crawford, 1995). In particular this enzyme is molecularly modulated by nitrate, in fact the transcription rate of *NR* gene increased in presence of nitrate (Ireland and Lea, 1999). Other factors, including light and carbohydrate, enhance the expression of *NR*, whereas reduced forms of nitrogen, in particular glutamine, down-regulated its expression (Cheng et al. 1992; Vincentz et al. 1993).

Subsequently, nitrite is translocated in plastids (or in chloroplasts for green tissues), where it becomes substrate for Nitrite Reductase (NiR). During this second reaction, nitrite is converted into ammonium by transfer of six electrons derived from ferrodoxin or its analogues. In maize, Suzuki et al. (1985) have described a ferrodoxin-like electron carrier (non-heme iron protein) for the transfer of electrons in non-green tissues, like in roots.

NiR: $NO_2^- + 6\ Fd_{red} + 8\ H^+ + 6\ e^- \rightarrow NH_4^+ + 6\ Fd_{ox} + 2H_2O$

Concerning the NiR, it was reported that its gene expression is also induced by light and nitrate, but not by carbohydrate and is reduced by the amino acids glutamate, glutamine, or asparagine (Back et al., 1991; Vincentz et al., 1993).

While nitrate can be translocated in the xylem and be assimilated also in shoots, the ammonium taken up by soil solution is assimilated directly in roots where, in form of organic compounds, is subsequently translocated to leaves. However, there are arguments in favour of a translocation of ammonium to leaves. Indeed Schjoerring et al. (2002) reported that when *Brassica napus* plants were exposed to ammonium as sole nitrogen source, the ammonium concentration in the xylem increased with increasing external concentrations and with time of expousure.

So, ammonium, as derived either from root absorption or generated through NO_3^- assimilation, is converted into glutamine by Glutamine Synthetase/Glutamine OxoGlutarate Aminotransferase (GS/GOGAT) cycle (Lea et al., 1992; Campell, 1999). GS catalyzes the ATP-dependent amination of glutamate to produce glutamine, than GOGAT (Fd- or NADH-GOGAT) transfers one amide group from glutamine to α-chetoglutarate producing two molecules of glutamate:

GS: $\text{Glutamate} + HN_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$

GOGAT: $\text{Glutamine} + \alpha\text{-chetoglutarate} + Fd_{red}/\text{NADH} + H^+ \rightarrow 2\ \text{Glutamate} + Fd_{ox}/\text{NAD}^+$

In plants, there are two GS isoforms: one localized in the cytosol (GS1) and one in the plastids or chloroplasts (GS2). Numerous studies have been carried out to define the roles of these isoforms in nitrogen assimilation and remobilization. In roots, both GS isoforms have been found: GS1 is more expressed in the vascular bundles to produce glutamine for intracellular nitrogen transport; whereas the GS2 generates amide nitrogen for local consumption. Concerning the GS2 in shoot chloroplasts it may be involved in the photorespiratory NH_4^+ re-assimilation (Lam et al., 1996).

In maize, at least five isoforms of GS1 have been identified and some of them are specifically expressed within the roots (Martin et al., 2006). Less information is available about GS2, which in maize was found to be expressed only in the early stages of plant development (Ueno et al., 2005). Light and carbohydrate levels may influence the expression of the plastid forms of the enzyme, but they have little effect on the cytosolic GS1 (Taiz and Zeiger, 2006).

There are two types of GOGAT that can use either NADPH or reduced Fd as the electron donor; both are usually located in plastids. In roots, especially root tips, Fd-GOGAT is the major form (Brugière et al., 2001). In Arabidopsis two genes encoding for Fd-GOGAT have been identified: *GLU1* and *GLU2* (Coschigano et al., 1998). *GLU1* expression is low in root tissues, and most abundant in leaves, while *GLU2* is constitutively expressed at low levels in leaves, and at higher levels in roots. NADH-GOGAT seems to be involved in the primary nitrogen assimilation in roots since its activity (from 2- to 25-fold lower than that of Fd-GOGAT) was found mainly in non–photosynthetic tissues, like roots (Ireland and Lea, 1999). Moreover, it was found that NADH-GOGAT is regulated by inorganic nitrogen forms, as it is induced by ammonium (Hirose et al., 1997) and also by nitrate (Wang et al., 2000).

Besides the GS/GOGAT cycle, it was proposed that ammonium might be directly incorporated into glutamate by Glutamate Dehydrogenase (NAD(P)H-GDH), a mithocondrial enzyme that catalyzes a reversible reaction. Using 2-oxoglutarate and ammonium as substrate, GDH catalyzes the synthesis of glutamate, which may be coverted into glutamine by cytosolic GS1. Studies on source-sink relations have shown that GDH is induced in old leaves when nitrogen remobilization is maximal (Srivastava and Singh, 1987; Masclaux et al., 2000). This led to the proposal that the physiological role of GDH is to synthesize glutamate for translocation in senescing leaves (Miflin and Habash, 2002). However, there is no evidence to discern a redundant or indispensable role of GDH and GOGAT for glutamate synthesis and nitrogen remobilization.

GDH: $\text{2-Oxoglutarate} + NH_4^+ + \text{NAD(P)H} \leftrightarrow \text{glutamate} + H_2O + \text{NAD(P)}^+$

Once assimilated in glutamine and glutamate, nitrogen may be transferred to other organic compounds such as the amino acids asparagine and aspartate by trans-amination reactions catalyzed by transaminases. Aspartate aminotransferase (AspAT) transfers the amino group of glutamate to the carboxyl atom of oxaloacetate to synthetize aspartate. This amino acid may become substrate for the Asparagine synthetase (AS), the enzyme that converts aspartate into asparagine. In most species glutamine, glutamate, aspartate, and asparagine are the main free amino acids translocated as nitrogen carriers from roots to shoots.

AspAT: Glutamate + oxaloacetate → aspartate + 2-oxoglutarate

AS: Glutamine + aspartate + ATP → asparagine + glutamate + AMP + PPi

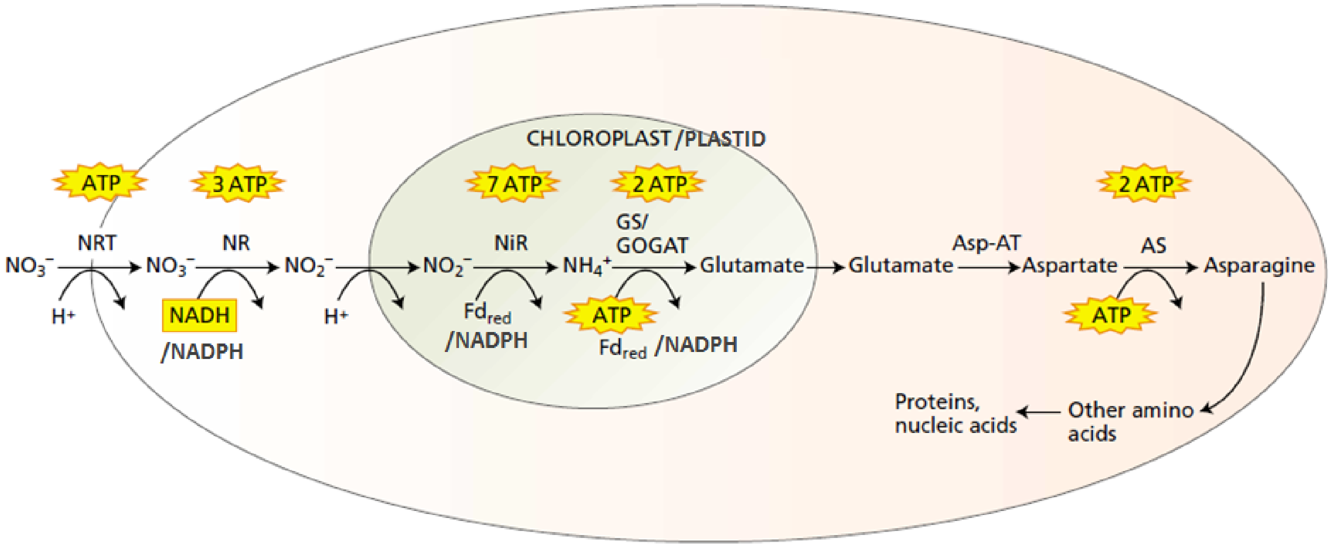


Figure 6. Schematic representation of nitrate and ammonium assimilation pathway in plant cells. Nitrate–proton symporters (NRT); nitrate reductase (NR); nitrite reductase (NiR); glutamine synthetase (GS) and glutamate synthase (GOGAT); aspartate aminotransferase (Asp-AT); asparagine synthetase (AS). The approximate amounts of ATP equivalents are given above each reaction (figure modified from Taiz and Zeiger, 2006).

In conclusion, as pointed out by Suzuki et al. (2001), in plants cells the nitrate assimilatory genes of *NR, NiR, GS* and *GOGAT* are under the regulation of light and metabolites. In particular, it was reported that nitrogen and carbon metabolites such as nitrate, glutamine and sucrose constitute the signal transduction pathway in gene expression: sucrose induces *Fd-GOGAT* (Coschigano et al. 1998), *NR* (Cheng et al. 1992) and chloroplast *GS* (Edwards and Coruzzi 1989); nitrate and glutamine induce (Cheng et al., 1992; Rastogi et al. 1993) or repress (Vincentz et al., 1993) *NR* and *NiR* (Figure 5).

1.2.2 Organic nitrogen uptake and assimilation

1.2.2.1 Amino acid uptake

Among the organic nitrogen forms available for plant nutrition, single amino acids and peptides can be taken up by the roots. Evidence have been provided that plants possess specific transporters for amino acids operating at low concentrations compatible with those of soil solution (Borstlap and Schuurmans, 1988; Bush, 1993; Soldal and Nissen, 1978). In maize Jones and Darrah (1994) reported that when inorganic nitrogen sources in soil were limiting, the uptake of amino acids could account for up to 90% the total nitrogen taken up by the roots. However, in agricultural soils, under normal conditions of high fertilizer inputs and low levels of organic matter in soil, the contribution of amino acids to plant nitrogen nutrition might still be expected to account for < 30 % of the total nitrogen taken up by the root system (Jones and Darrah, 1994).

The natural occurrence and concentrations of amino acids in soils is highly variable, ranging from 0.1 to 10 µM (Raab et al., 1996, 1999; Öhlund, 2004; Jones et al., 2005), although concentrations up to 100 µM have been recorded (Raab et al. 1996).

For uptake of amino acids by roots, high and a low affinity transport system have been predicted (Tegeder and Rentsch, 2010). In Arabidopsis several amino acid and peptide transporters are expressed in roots, however a direct role in amino acid uptake has only been demonstrated for two transporters: amino acid permease 1 (AtAAP1, Lee et al., 2007) and lysine histidine transporter 1 (AtLHT1, Hirner et al., 2006). As physiological substrate for AAP1 were identified the amino acids glutamate, histidine and neutral amino acids, including phenylalanine; whereas aspartate, lysine and arginine were not (Lee et al., 2007).

Although the direct uptake of amino acids from the soil solution by plant roots has been an ecologically critical issue, Lee et al. (2007) demonstrated the ability of AtAAP1 to contribute up to 50% of the amino acid uptake when organic nitrogen is applied at millimolar concentrations (2 and 10 mM).

Studies about AtLHT1 and AtLHT2 suggested a high affinity transport of neutral and acidic amino acids (Hirner et al., 2006).

1.2.2.2 Urea uptake and assimilation

1.2.2.2.1 Physiological evidence for the existence of urea transport in plants

Urea in natural environment is estimated between 0.1 and 3 µM (Cho et al., 1996; Mitamura et al., 2000a; Mitamura et al., 2000b), but in agricultural soils urea can reach higher concentration (up to 70 µM, Gaudin et al., 1987). In the past, only few studies investigated the possible mechanisms for urea uptake. Due to the presence of microbial ureases and the low urea concentrations in the soil, it was believed that urea contributed only in minimal part to plant nutrition. For a long time, it was considered that roots take up urea-nitrogen mainly in form of ammonium, a product of urea hydrolysis (Polacco and Holland, 1993; Marschner, 1995). At most, due to the neutral character and the low molecular weight, it was suggested that urea moves as intact molecule across the cellular membranes by simple diffusion (Galluci et al., 1971).

In the last decades the mechanism of urea acquisition in plants has become a research topic of great interest, mainly due to the use of urea as the most common nitrogen fertilizer for crop species in the world.

The first experiment that documented the protein-mediated uptake of urea as an intact molecule was performed by Wilson et al. (1988). Thereafter, as listed below, other papers supported the existence of mechanisms of urea transport in plants:

1988 Wilson et al.	*Protein-mediated urea uptake in algal cells (Chara australis)* Using short-term ^{14}C-urea influx assay and by electrophysiological voltage clamp It was demonstrated that urea uptake was dependent on the ATPase inhibitor DCCP or the protonophore CCCP, thus appearing to be coupled to the proton gradient across the plasma membrane. Moreover depending on urea concentration a multiphasic kinetic was observed, consistent with the presence of a high- and a low- affinity transport systems.
1999 Tyerman et al.	*Urea permeability in membrane vesicles of wheat roots* Endosomal membrane vesicles showed higher urea permeability (up to 3-fold) and mercure-sensitivity than plasma membrane vesicles.
1999 Gerbeau et al.	*Urea permeability in tonoplast vesicles from suspension cells of tobacco* A functional characterization of the aquaporins NtTIPa was performed by using *Xenopus* oocytes, confirming the transport specificity for water, urea and glycerol.
2003a Liu et al.	*^{14}C-urea influx in suspension cells of Arabidopsis* These authors confirmed that urea transport is energy-dependent, since the uptake was inhibited by the protonophore CCCP dissipating the proton gradient across the plasma membrane.

2007 Kojima et al. *First experimental evidence "in planta": short term ^{15}N-urea influx into Arabidopsis*

At low urea concentration (micro-molar range), the kinetic followed a Michaelis-Menten behavior. Using *dur3* Arabidopsis mutants (*atdur3-1* and *atdur3-3*), it was demonstrated the role of AtDUR3 as the major transporter for high-affinity urea uptake in Arabidopsis (Figure 7, a).

At high urea concentration (over 0.2 mM) a liner kinetic, typical for channel mediated urea transport, was observed. Aquaporins could be responsible for this type of transport as confirmed by Liu et al (2003b), who characterized AtTIP2;1 by heterologous expression in oocytes (Figure 7, b).

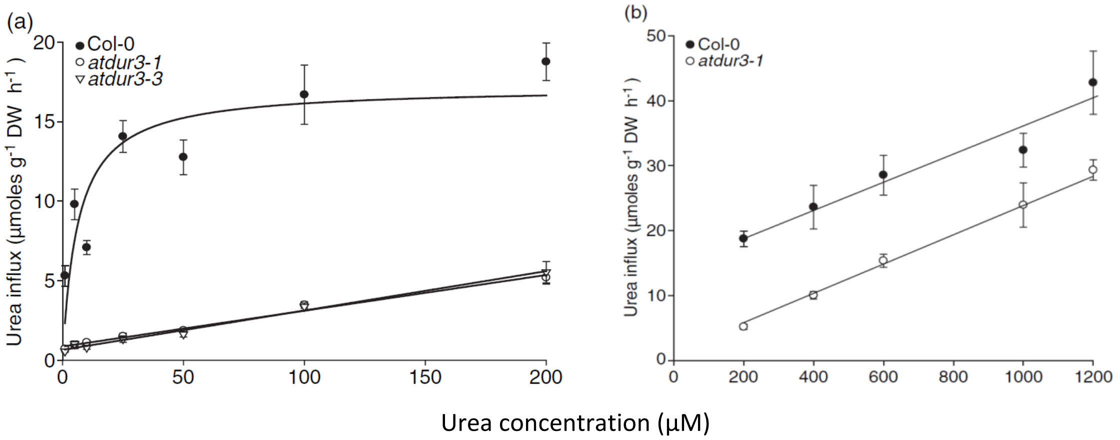


Figure 7. Concentration-dependent influx of ^{15}N-labeled urea into Arabidopsis roots. Disruption of AtDUR3 decreases the capacity for high-affinity uptake of ^{15}N-labeled urea. Plants of Col-0, atdur3-1 and atdur3-3 were cultured in nutrient solution containing 2 mM ammonium nitrate for 38 days before transfer to nitrogen deficiency for 4 days. (a) Urea influx into wild-type and *atdur3-1* or *atdur3-3* plants at an external supply of 3–200 μM urea. (b) Urea influx into roots of wild-type and atdur3-1 plants at an external supply of 200 – 1,200 μM urea. Symbols indicate means ±SD, n = 10 (Kojima et al., 2007).

Other reports corroborated the above experimental evidence that led to the identification of aquaporins as responsible for the low affinity urea transport system in higher plants (Siefritz et al., 2001; Liu et al., 2003b; Gaspar et al., 2003; Klebl et al., 2003). In particular to date not only members of *Tonoplast Intrinsic Protein* (TIP) family, but also *Plasma-membrane Intrinsic Protein* (PIP) and *Nodulin 26-like Intrinsic Protein* (NIP-like) aquaporins have been identified that allow urea permeation (Wang et al., 2008). However, for most of these aquaporins the specific localization in subcellular membranes remains unclear. Besides aquaporins, urea transport activity has been identified only for orthologs of ScDUR3, the urea tranporter of *Saccharomyces cerevisiae*. In plants, two *ScDUR3* orthologs have been isolated and functionally characterized: *AtDUR3* and *OsDUR3*, of Arabidopsis and rice, respectively. Thus, as pointed out by Kojima et al. (2007), in higher plants DUR3 homologs may be the major transporters responsible for the high-affinity urea uptake across the plasma membrane (Figure 8).

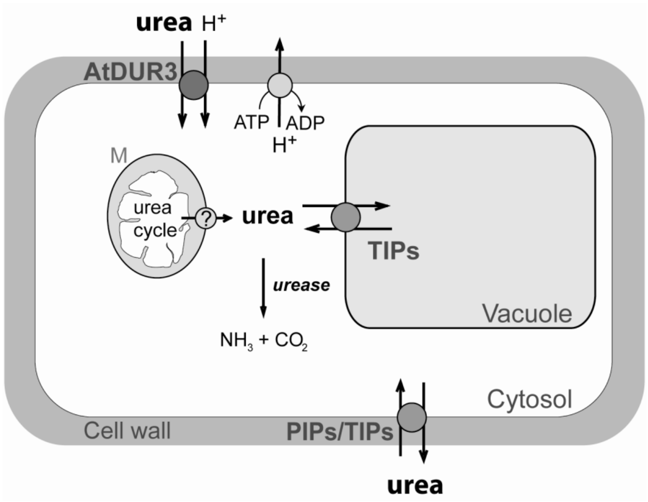


Figure 8. Model for protein-mediated urea transport pathways in plant cells. AtDUR3 mediates secondary active, high-affinity urea transport across the plasma membrane, while aquaporins of the PIP or TIP subfamilies mediate low-affinity urea transport. In particular TIPs might further transport urea across the tonoplast for transient storage in the vacuole or remobilization (Kojima et al., 2006).

1.2.2.2.2 Low affinity urea transporter

In addition to water transport, some plant *Major Intrinsic Proteins* (MIPs) were reported to mediate movement of small uncharged solutes, such as urea (Rivers et al., 1997; Dordas et al., 2000). Based on sequence homology, the plant MIPs have been classified in four subfamilies: *Plasma membrane Intrinsic Proteins* (PIPs), *Tonoplast Intrinsic Proteins* (TIPs), *Nodulin 26-like Intrinsic Proteins* (NIPs) and *Small basic Intrinsic Proteins* (SIPs). Concerning those MIPs that facilitate the urea permeation in plant cells, members belonging to the subfamilies of PIPs, TIPs and NIPs were identified. Although the cellular localization of most PIPs and TIPs aquaporins is still unclear, in general PIPs are referred to localize in the plasma membrane while TIPs localize in the tonoplast. However the classification of an acquaporin to TIPs, or PIPs subfamilies depends on sequence similarity rather than subcellular localization, since the name of these protein classes is linked to an earlier nomenclature (Gaspar et al., 2003). In particular, it was reported a localization of some PIPs and TIPs also in subcellular membrane fractions (Barkla et al., 1999; Schäffner, 1998). Moreover, the plasma membrane and the tonoplast vesicles isolated from wheat and tobacco showed different urea permeation characteristics. The urea permeability of tonoplast membrane vesicles was higher than the plasma membrane ones (Tyerman et al., 1999; Gerbeau et al., 1999).

In order to isolate putative genes encoding for urea transporters in plants, a molecular approach was used, based on yeast complementation. A *dur3* mutant *S. cerevisiae* strain, defective in the endogenous urea transporter ScDUR3 (ura$^-$ dur3$^-$, YNVWI), was unable to grow on medium containing < 5 mM urea as sole nitrogen source (Liu et al., 2003a). Thus YNVWI mutants were transformed with a cDNA library of Arabidopsis and screened on 2 mM urea. In this way four putative genes involved in the low affinity urea transport system were isolated: AtTIP1;1, AtTIP1;2, AtTIP2;1 and AtTIP4;1 (Liu et al., 2003b). All these genes encoded members of the TIPs family. Concerning the physiological characterization of AtTIP2;1 in *Xenopus* oocytes, a linear concentration dependency (up to 30 mM urea) was demonstrated, that is typically associated to channel-mediated substrate transport and commonly observed for low-affinity transporters (Liu et al., 2003b). Interestingly, by GFP localization in Arabidopsis protoplasts, these TIPs did not target only to the tonoplast, but a weak signal was also detected at the plasma membrane. So, a possible role of TIP/PIPs in the acquisition of urea from soil solution remains to be clarified.

Urea transport by some plant MIPs was also confirmed in other species, such as tobacco (NtTIPa, Gerbeau et al., 1999), zucchini (CpNIP1, Klebl et al., 2003) and maize (ZmPIP1-5, Gaspar et al., 2003; ZmNIP2-1, ZmNIP2-4 and ZmTIP4-4, Gu et al., 2012).

By phylogenetic analysis, most of the aquaporin sequences of maize have been identified (Chaumont et al., 2001). In particular, four maize aquaporins have shown to facilitate the urea transport: ZmPIP1-5b; ZmNIP2-1, ZmNIP2-4 and ZmTIP4-4. Furthermore, their transport activity was functionally characterized in *Xenopus* oocytes or in yeast mutant. Based on experimental evidence, the authors speculated on the putative roles of these aquaporins: besides the urea uptake from soil solution and translocation in plants, they might be involved in nitrogen homeostasis across the tonoplast (Gu et al., 2012). Concerning ZmPIP1-5 it was reported that the addition of nitrate to maize roots grown under nitrogen starvation, could induce the expression of *ZmPIP1-5* (Gaspar et al., 2003). The authors suggested that the overexpression of this aquaporin might be involved in the response to the osmotic stress due to nitrate accumulation in vacuoles (Gaspar et al., 2003). However, more investigations are required to understand the physiological contribution of aquaporins to the overall urea transport.

1.2.2.2.3 High affinity urea transporter

To investigate plant proteins involved in urea acquisition, the Arabidopsis genome was analyzed searching genes showing similarity to known sequences encoding urea transporters in other organisms, such as mammals and microbes. One singleton sequence (At5g45380, *AtDUR3*) was identified which showed a 40% similarity with the active urea transporter *ScDUR3* of *Saccaromyces cerevisiae*. Sumrada et al. (1976) described for the first time a yeast mutant strain carrying a defect in the active transport system of urea; the mutant locus was called "DUR3" (Degradation of URea-3). In yeast the expression of *ScDUR3* showed to be highly sensitive to nitrogen catabolite repression and also required a functional *ScGLN3* product (a yeast transcriptional activator of genes involved in the allantoin pathway).

Based on sequence similarities, putative *AtDUR3* homologous sequences were found in other plant species, such as in rice, maize, sorghum, poplar and tomato (Wang et al., 2008). However to date only the Arabidopsis *AtDUR3* (Liu et al., 2003a) and rice *OsDUR3* (GenBank ID AY463691, Wang et al., 2012) have been cloned and functionally characterized by yeast mutant complementation assay and ^{14}C- urea influx in *Xenopus* oocytes.

The Arabidopsis and rice *DUR3* genes code for 694 and 721 amino acids, respectively, sharing an amino acid identity of around 75%. A different number of predicted transmembrane spanning domains (TMSDs) were identified in DUR3 of Arabidopsis (14 TMSDs) and rice (15 TMSDs). Structurally, the plant DUR3 transporters belong to the *Sodium Solute Symporter* (SSS) superfamily. Various solutes (amino acids, nucleosides, vitamins, inositols and urea) have been identified to be transported by symport with Na^+ by the SSS proteins (Jung, 2002), even if only a closely related group of molecules is usually substrate of an SSS-transporter (Leung et al., 2000; Reizer et al., 1994). Despite this class of transporters share the greatest sequence similarity within the kingdoms, no close homologues to *AtDUR3* was found within the Arabidopsis SSS superfamily. So, to date the genome data corroborate the hypothesis that higher pants possess a single gene coding for urea transporter DUR3, as identified in Arabidopsis and rice (Wang et al., 2008).

Yeast complementation showed that both transporters (AtDUR3 and OsDUR3) restored the ability of mutants (strain YNVWI, Liu et al., 2003a) to grow on media containing 2 mM urea as the sole nitrogen source. Moreover AtDUR3 and OsDUR3 were also characterized kinetically by heterologous expression in *Xenopus* oocytes. The ^{14}C-urea uptake was observed to be concentration dependent (for concentrations up to 200 µM urea) following a Michaelis-Menten curve. The reported affinity constants (K_m) were around 3 and 10 µM urea for AtDUR3 and OsDUR3, respectively (Liu et al., 2003a; Wang et al., 2012). These values were in agreement with those reported for the high-affinity urea transport system *in planta*: Arabidopsis plants showed a K_m of 4 µM urea (Kojima et al., 2007; Figure 7, a) whereas in rice it was around 7 µM urea (Wang et al., 2012).

By heterologous expression in oocytes it was also possible to verify the substrate specificity of OsDUR3. Thiourea, a structural urea analog, inhibited competitively the urea uptake: in experiments with urea:thiourea=1:1 a decline in urea accumulation by over 50% was registered when compared to oocytes not exposed to thiourea. Uemura et al. (2007) analyzed the specificity of yeast urea transport and, besides urea, also the polyamine putrescine and spermidine were reported to be substrates for ScDUR3. However, concerning the rice transporter, a polyamine permeation was not supported by OsDUR3.

Since DUR3 transporters belong to SSS superfamily, the transport activity was tested in the presence of sodium and under different pH conditions. Data showed that urea uptake capacity in AtDUR3 injected oocytes was not affected by 3 mM sodium in the bathing solution (Liu et al., 2003a). On the other hand low pH values stimulated the urea import; particularly for OsDUR3 urea uptake was strongly reduced by pH values over 5.0 suggesting a H^+/urea symport.

GFP localization in Arabidopsis protoplast revealed that the fluorescent signals for both transporters, GFP-AtDUR3 and GFP-OsDUR3, were detectable at the plasma membrane. This data was consistent with the putative role of DUR3 as transporter of urea from outside into the cells (Wang et al., 2012) and corroborated the experimental evidence in heterologous organisms, such as oocytes and yeast. In addition, by gel blot analysis using antibodies against AtDUR3, it was possible to detect the signal in the plasma membrane enriched protein fraction extracted from nitrogen deficient roots of Arabidopsis. Since a weak signal was also detected in endosomal membrane fraction, the authors speculated that AtDUR3 is predominantly localized at the plasma membrane even if a little portion of AtDUR3 might reside in endosomal compartments, possibly reflecting the trafficking to or from the plasma membrane (Takano et al., 2005; Kojima et al., 2007).

The physiological role of AtDUR3 and OsDUR3 was also confirmed by restoration of the capacity of *dur3* Arabidopsis mutant to grow in the presence of urea as unique nitrogen source, ranging from 0.5 to 1 mM. This data, together with ^{15}N-urea kinetic assay in *dur3* Arabidopsis mutants (Figure 7), suggested that AtDUR3 also contributes to urea uptake in a millimolar concentration range. In fact, up to 1.2 mM urea, the influx of ^{15}N-urea in *atdur3-1* mutants was still significantly lower than in wild type plants (Figure 7; Kojima et al., 2007).

Finally, both genes, *AtDUR3* and *OsDUR3,* were found expressed in roots and in leaves of Arabidopsis and rice plants (Liu et al., 2003a; Wang et al., 2012). In particular the expression levels of both genes, *AtDUR3* and *OsDUR3*, significantly increased in root tissues after at least two days of nitrogen starvation (Liu et al., 2003a; Wang et al., 2012). Furthermore the expression levels of *OsDUR3* were inducible by substrate. Indeed the urea resupply after 2 days of N-starvation increased the amounts of *OsDUR3* transcript while the ammonium sulphate-resupply did not induce changes in the *OsDUR3*-mRNA levels (Wang et al., 2012).

1.2.2.2.4 Urea as a secondary metabolite in plants

Besides acquisition of urea from soil as nitrogen source, urea can also be accumulated in plant cells as a consequence of the secondary metabolism (Mérigout et al., 2008a). In particular, urea is produced as metabolic intermediate of nitrogen catabolism by two major biochemical processes: arginase action and the degradation of purines and ureides (Wang et al., 2008).

The first pathway is involved in the protein degradation and in nitrogen re-cycle from storage compounds, such as arginine (Figure 9(1)). This catabolic process is frequently called "urea cycle" for its similarity with the animal urea synthesis, and it takes place in the mitochondrial matrix. The arginine is firstly broken down by arginase in two compounds: urea and ornithine. Subsequently, ornithine is converted into glutamate, while urea is exported to the cytosol possibly involving an aquaporin as membrane transporter (Soto et al., 2010). At this point urea becomes substrate of the cytosolic urease, which catalyzes its hydrolysis releasing ammonium. Together with the arginine-derived glutamate, the ammonium is re-assimilated in the cytosol by the glutamine synthetase (GS). In this way all the nitrogen of arginine is re-cycled in form of glutamine for the synthesis of new proteins. An important role is performed by urease, which is required for the remobilization of half of the nitrogen stored in arginine. To date, the only other documented process that involves the ureolitic activity in plants, is the breakdown of urea taken up by roots.

Developing embryos/seeds contain large amount of arginine accumulated for nitrogen store and its biosynthesis is located in the plastids. Upon germination arginase activity rises (Cao et al., 2010; Flores et al., 2008) and arginine is translocated across mitochondrial membrane for its degradation. So, during the germination as well as senescence, the equilibrium among arginase and urease activities becomes a crucial point for the re-mobilization and re-cycle of nitrogen.

Furthermore, the synthesis of urea is connected to the polyamine metabolism and to the hydrolyzes of canavanine. In the polyamine pathway arginine is substrate of a cytosolic enzyme (arginine decarboxylase) that catalyzes the synthesis of agmatine and then, by agamtinase action, urea and putrescine are released (Figure 9 (2)). These reactions constitute an alternative way to the well-known ornithine degradation for the synthesis of polyamine (Kusano et al., 2007).

Canavanine is a non-proteinogenic amino acid that is accumulated in large amounts in seeds of leguminous species, such as in jack bean (Rosenthal, 1982). This secondary metabolite has a dual biological function: as nitrogen storage and also as defensive compound against herbivores. By canavanine hydrolase, canavanine is hydrolyzed to canaline and urea (Figure 9 (3)).

The ureides allantoin and allantoate are nitrogen-rich compounds derived from purine catabolism. The exact route of allantoate degradation in plants is still a matter of debate. It is uncertain whether the production of ammonia and CO_2 from allantoate is direct or by way of an urea intermediate. Allantoate is hydrolyzed into ureidoglycolate and subsequently into glyoxylate (Figure 9(4)).

In yeast this process occur in two steps catalyzed by the enzymes allantoate amidinohydrolase and ureidoglycolate lyase. In total, by this way, two molecules of urea are released from allantoate degradation (Yoo et al., 1985; Yoo and Cooper, 1991). A different pathway was identified in Arabidopsis, where the breakdown of allantoate involves three enzymes: allantoate amidohydrolase, ureidoglycine amidohydrolase and andureidoglycolate amidohydrolase (Todd and Polacco, 2006; Werner et al., 2008). All these enzymes catalyze the deamination of their substrates releasing ammonia and not urea. To date, among higher plants, the generation of urea from allantoate has been reported only in soybean and tentatively assigned to ureidoglycolate lyase activity. Putative ureidoglycolate lyases have been purified from legumes, but the corresponding gene has not yet been identified. So in soybean, both degradation pathways coexist, however ureides degradation via amidohydrolases seems of major importance (Faye et al., 1986; Fahmy et al., 1994).

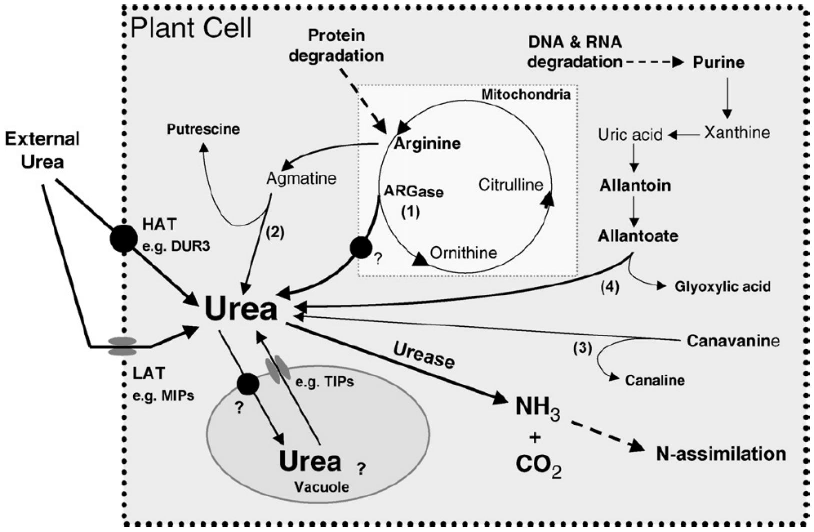
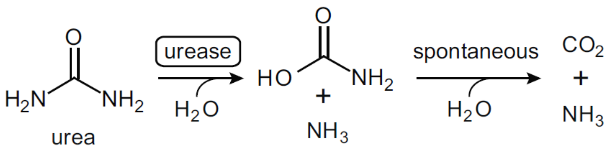


Figure 9. Simplified sketch showing urea generation, transport and degradation in plant cells. Urea is derived either from metabolism or is taken up from the environment through high-affinity transport (HAT) and low-affinity transport (LAT) systems located in the plasma membrane. Urea generating enzymes are (1) arginase, (2) agmatinase, (3) canavanine hydrolase, and (4) amidinohydrolases, namely allantoate amidinohydrolase and urea-lyase (for details, see Section 3). Urea could be compartmentalized between cytoplasm and vacuoles by tonoplast-targeted transporters, such as TIPs and active urea transporters (not yet identified). However, so far it is not clear if urea is stored in vacuoles. The pathway for urea movement out of mitochondria is unknown. Cytosolic urea can be hydrolyzed by urease to ammonia, which can be assimilated by glutamine synthetase (Wang et al., 2008).

1.2.2.2.5 Urea assimilation

In higher plants, the urea, derived either from root absorption or generated by secondary metabolisms, can be hydrolyzed by the cytosolic nickel-dependent urease. This metalloenzyme was found in most ureolytic organism, except few algae, fungi and bacteria where an alternative urease-independent pathway hydrolyzed urea through the intermediate allophanate (Kanamori et al., 2004).

Urease catalyzes the degradation of urea by one-step reaction that leads to ammonia and carbamate (Blakeley et al., 1969). By spontaneous and rapid decay, the carbamate is broken down forming a second molecule of ammonia and one of carbon dioxide (Carter et al., 2009) (sketch of urease reaction from Witte, 2011):



Structurally all ureases are made of three subunits (UreA, UreB and UreC); in plants they are fused in trimeric units that may associate giving an exameric structure (Balasubramanian and Ponnuraj, 2010). For the formation of the active enzyme, three accessory proteins are further required (UreD, UreF and UreG) (Witte, 2011). The affinity constant (K_m) for this enzyme range from 0.15 to 3 mM, even if more often is around 0.5 mM (Cao et al., 2010; Davies and Shih, 1984), a concentration comparable with that determined in Arabidopsis and rice tissues (up to 0.9 mM) (Mérigout et al., 2008a; Cao et al., 2010).

Urease activity was found in a wide variety of plants, including crop species like maize (Davies and Shih, 1984; Gheibi et al., 2009) and barley (Chen and Ching, 1988). Further studies performed in soybean and potato demonstrated that its activity was ubiquitously present in all plant tissues (Witte et al., 2001; Torisky and Polacco, 1990; Polacco and Winkler, 1984); in Arabidopsis it was also shown that the transcription of the urease gene occurs in all tissues (Witte, 2011).

Among higher plants, experimental data suggest that only one single gene codes for urease (as in Arabidopsis, rice, maize and in most other sequenced plants), while in soybean almost two isoforms were identified (Witte, 2011). Furthermore, in maize genome two putative sequences have been identified, that may encode for accessory proteins: UreF (EU953829.1) and UreG(EU956120.1) (from MaizeGDB: http://www.maizegdb.org); however their functionality is not yet characterized.

Despite a constitutive urease activity in roots, other studies reported urea accumulation in xylem sap or in leaves when urea was used as sole nitrogen source to roots (Hine and Sprent, 1988; Gerendas et al., 1998), indicating that at least part of the root absorbed urea is translocated to shoots before urea degradation. However, it remains unclear in which way urea is transported across intercellular membranes and also across intracellular compartments, as mitochondria and vacuole (Kojima et al., 2007).

Concerning the urease regulation, at both physiological and molecular level, is not clear if this enzyme is inducible by urea. Gheibi et al. (2009) analyzed the enzymatic activity in maize roots exposed for several weeks (up to 6) to different nitrogen sources. After two weeks of treatment, the urea-fed plants showed a 2-fold increase of the urease activity when compared to ammonium nitrate treated roots. Although this data were confirmed also by several other studies, as listed by Polacco and Holland (1993), other reports were in contradiction with this evidence. Witte et al. (2002) analyzed the effect of urea leaf fertilization in potato showing that in leaves the urease activity was not induced by urea.

At molecular level, urea doesn't seem to induce the transcription of urease gene. Indeed, Mérigout et al. (2008a) performed a microarray study on Arabidopsis plants that were exposed to urea as sole nitrogen source, and no transcriptional regulation by urea was reported for urease gene or genes coding for urea cycle enzymes. In a recent study performed in *Brassica napus*, it has been reported that in shoots and roots the expression of urease responded to nickel-deficiency (Arkoun et al., 2012a). After 7 days of urea treatment, nickel deficient plants showed higher amounts of urease transcripts than nickel sufficient plants.

1.2.3 Effect of nitrogen sources on their uptake system

Three main nitrogen forms are present in soil as native sources or as applied fertilizers: ammonium, nitrate and urea. Their relative amount and bioavailability under field conditions are obviously influenced by biogeochemical reactions; however, at least for a short time, plant roots are exposed to a combination of these three nitrogen sources (Mérigout et al., 2008b). Experimental evidence has demonstrated that higher plants are able to take up either nitrate, ammonium or urea when supplied as sole nitrogen source, and possess dedicated transmembrane transport systems in root cells for each form (Forde, 2000; Liu et al., 2003a).

The study of the interactions among different nitrogen sources may have great relevance in order to improve the comprehension and the optimization of nitrogen nutrition in plants. However, to date only a limited number of works have focused on the reciprocal influence of nitrogen sources on the root uptake (Bradley et al., 1989; Criddle et al., 1988; Mérigout et al., 2008b; Garnica et al., 2009; Arkoun et al., 2012b).

A preliminary study was performed by Kirkby and Mengel (1967), who measured the ionic balance in different tissues of tomato plants supplied with nitrate, ammonium, or urea in hydroponic conditions. The plant growth was strongly affected by the ammonium treatment, since, in comparison to nitrate, small leaves with very poorly developed stubby root system was observed. On the other hand, plants fed with nitrate grew better and were characterized by a great development of the root system. Intermediate size was observed in urea fed plants, even if the root system was quite well developed. Also in terms of dry weight, urea showed intermediate values between nitrate and ammonium. These data were also corroborated by Houdusse et al. (2005), who reported that in wheat and pepper ammonium nutrition and, to a lesser extent, urea nutrition determined reductions in plant growth in comparison to nitrate treatment.

Several authors have demonstrated that the root exposition to a combination of different nitrogen sources, led to positive effects on the nutritional status of crop plants (Houdusse et al., 2005; Britto and Krozucker, 2002; Garnica et al., 2009; Arkoun et al., 2012b). In particular the negative effects associated to ammonium or urea nutrition were corrected by the addition of nitrate to the nutrient solution. Anyway, to date the physiological and molecular mechanisms of this beneficial influence remain mostly unknown.

Garnica et al. (2009) performed experiments feeding wheat plants (up to 96 hours) with nutrient solutions containing ammonium (A), nitrate (N) or urea (U) (each source: 0.5 mM nitrogen) applied singularly or in combination (A, N, U, UA, UN, ANU). Uptake patterns observed under A, N, U,AU, UN and ANU treatments (Figure 10) showed that when the sources were applied singularly (A, N, U), the amount of nitrogen that was taken up by urea-fed plants was lower than that of nitrate- or ammonium-fed plants. Interestingly, the simultaneous presence of two or more nitrogen forms favored urea absorption: indeed when urea was supplied with nitrate or ammonium (UA or UN) its uptake rates increased. This effect was much more evident in UN-fed plants between 24 and 96 hours.

Further, Garnica et al. (2009), analysing shoots and roots urea content, observed that urea hydrolysis and ammonia assimilation were enhanced when nitrate was also present in the nutrient solution (UN treatment). This positive effect of nitrate on urea assimilation was prevented by ammonium (UAN treatment), suggesting that ammonium might regulate urease by feedback inhibition (Garnica et al., 2009).

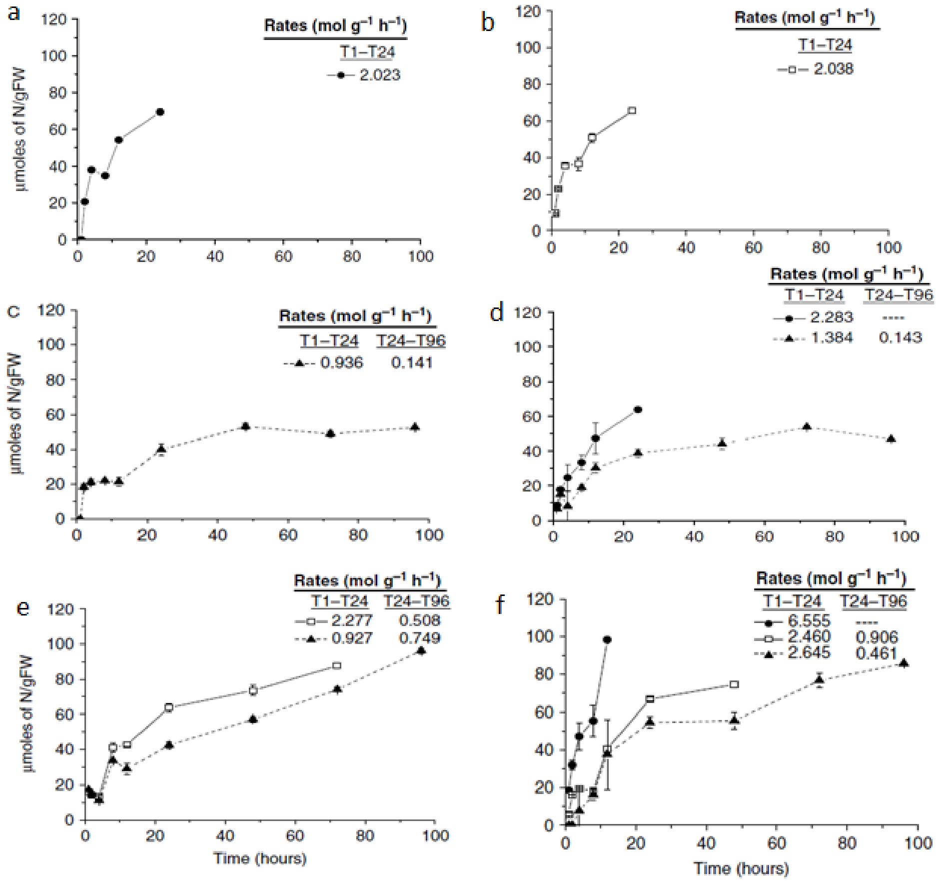


Figure 10. Time course of ammonium (-•-), nitrate (-□-) and urea (-▲-) uptake from the (a) A, (b) N, (c) U, (d) UA, (e) UN and (f) ANU treatments of wheat plants. Two uptake rates were noted during the treatment: a higher rate that takes place during the first 24 hours (1–24 h), and a lower rate after the first day of the treatment until the end of the period of time of the study (24–96 h) (from Garnica et al., 2009).

In a molecular and physiological study, Mérigout et al. (2008a) reported the influx rates of nitrate, ammonium and urea in Arabidopsis plants exposed for 7 days to different nitrogen sources (urea, (U), ammonium nitrate (AN), ammonium nitrate and urea (ANU)) or under nitrogen starvation (Ctr). Interestingly, the urea uptake was observed to be somehow regulated by urea itself since the urea influx reached the highest level when plants were fed with urea as sole nitrogen source (U); on the other hand, the root exposition to urea in conjunction with ammonium nitrate (ANU) determined uptake rates comparable to those observed in plants grown under ammonium nitrate (AN) or under conditions of nitrogen starvation (Ctr). Moreover urea, when supplied as unique nitrogen source, influenced positively also the uptake of ammonium showing influx rates intermediate between plants grown in nitrogen starvation (Ctr, the highest value) and plants exposed to ammonium nitrate (AN and ANU, the lowest values). On the other hand the presence of urea along with ammonium nitrate (ANU) did not exerted negative effect on nitrate uptake since influx was comparable to ammonium nitrate fed plants (AN) (Mérigout et al., 2008a).

However the use efficiency of nitrogen forms depends also on the plant species. For example Mérigout et al. (2008b) performed a study comparing the nitrogen use efficiency in maize and wheat plants. Plants were fed for three weeks with urea or ammonium nitrate or ammonium sulfate as sole nitrogen source and, after 7, 14, 21 days, the urea content was analyzed in roots and shoots. The authors observed a different behavior between maize and wheat to cope with urea. Maize growth was not strongly affected by the different nitrogen sources. In contrast the wheat growth was highest under ammonium nitrate and lowest under urea treatment, whereas urea enhanced the root development. The authors hypothesized that the limiting factor for the use of urea as a nitrogen source was its low absorption.

Arkoun et al. (2012b) measured in *Brassica napus* the accumulation of ^{15}N-fertilizer taken up by nitrogen-starved plants fed for 15 days with (as sole nitrogen source): urea (U), nitrate (N), urea and nitrate (UN) or ammonium and nitrate (AN); or maintained in nitrogen deficiency (Ctr). In this work the authors did not found a positive effect of nitrate on urea uptake as well on the uptake of ammonium, respectively under the UN and AN treatments. This data was in contradiction with that reported by Garnica et al. (2009) who showed in wheat plants a significant enhancement of urea and ammonium uptake rates when nitrate was supplied to the plants. However, as observed also in wheat by Garnica et al. (2009), in oilseed rape the presence of urea greatly delayed nitrate uptake.

These physiological results suggest that response to urea is dependent on plant species (wheat, maize, Arabidopsis or oilseed rape).

Thus, although Garnica et al. (2009) and Mérigout et al. (2008b), as well other several authors (Britto and Kronzucker, 2002; Cruz et al., 2003; Houndusse et al., 2005; Arkoun et al., 2012b), pointed out the importance of the combination of nitrogen forms supplied to the plant and, in turn, of the types of fertilizer that are applied to the soil for improving the NUE of a specific crop, further studies are needed to better understand the mechanisms that are involved in the uptake of the different nitrogen forms and of the physiological and molecular bases of their reciprocal interactions in different plant species. Particularly, due to its huge utilization, the comprehension of physiological and molecular bases of urea uptake and the influence of the other nitrogen sources on the mechanisms of its acquisition by plants might have great relevance to enhance the nitrogen use efficiency in crop species, such as maize.

1.3 Aim of the work

Beside the use of urea as the most worldwide nitrogen fertilizer, a research topic of great interest concerns the capacity of crop plants to use urea per se as a nitrogen source for their development and growth. However, to date only a limited number of works had focused on the uptake systems and assimilation of urea by roots, especially in maize plants. Thus, general aim of this thesis focuses on the comprehension of physiological and molecular mechanisms involved in urea uptake by maize roots.

In the first part of the study, an in vivo kinetic characterization of high-affinity urea uptake was performed, especially with the aim to ascertain the existence of a possible inducible process. Further analyses were conducted to determine if the concentration of urea in the external medium and the time of root exposition to the N-source could influence the uptake capacity of maize roots.

Since in the field roots are commonly exposed to a combination of different nitrogen forms, part of this thesis concerned also the influence of organic and inorganic nitrogen sources on the mechanism of urea acquisition in plants. Particular attention was dedicated to reciprocal influence of nitrate and urea on their own uptake systems.

In order to evaluate a possible link between the physiological response and variations at transcriptional level, the second part of the thesis focused on the effect of urea on gene expression in maize roots and leaves. A genome-wide analysis was performed in roots in order to investigate which transcripts could be induced by urea treatment when at physiological level roots showed the maximal uptake rates of this organic source. This analysis was conducted also using roots exposed to nitrate or to urea and nitrate applied simultaneously, thus trying to draw a more comprehensive picture of transcriptomic changes associated with the root response to the two main N-sources conceivably present in the soil. Expression of genes involved in urea and nitrate transport and metabolism was further analysed using a real time RT-PCR analysis of maize roots and leaves during the exposure to the different N-sources.

An important aim of this work was concerned the molecular identification and functional characterization of a putative high-affinity urea transporter of maize (ZmDUR3). This task was accomplished in the third part of the work by cloning the *ZmDUR3*-ORF from maize roots and by testing the capacity of ZmDUR3 to transport urea by expression in heterologous *Saccharomyces cerevisiae* mutant and in *Xenopus laevis* oocytes. Finally, to identify the subcellular localization of ZmDUR3, ZmDUR3/GFP phusion proteins were transiently expressed in *Nicotiana tabacum* protoplasts.

2 Materials and methods

2.1 Plant material and growth conditions

2.1.1 Maize culture

Maize seed (*Zea mays* L., cv. PR33T56, Pioneer Hybrid Italia S.p.A.) were germinated over aerated 0.5 mM $CaSO_4$ solution in a dark growth chamber at 25°C. After 4 days, the seedlings were transferred into an aerated hydroponic system (plastic posts) filled with 2 L of solution containing 0.5 mM $CaSO_4$ in a controlled climatic conditions: day/night photoperiod, 16/8 h; light intensity, 220 µE m^{-2} s^{-1}; temperature (day/night) 25/20°C; RH 70 to 80 %. After 24 hours, 5-day-old plants were transferred in a complete nitrogen-free nutrient solution containing (µM): KCl 5; $CaSO_4$ 500; $MgSO_4$ 100; KH_2PO_4 175; NaFe-EDTA 20; H_3BO_3 2.5; $MnSO_4$ 0.2; $ZnSO_4$ 0.2; $CuSO_4$ 0.05; Na_2MoO_4 0.05. The pH of solution was checked and adjusted to pH 6.0 with sodium hydroxide (NaOH).

Nitrogen was supplied in form of: urea (U treatment), calcium nitrate (N treatment), ammonium sulfate (A treatment), ammonium nitrate (AN treatment) or as single amino acids (glycine, Gly; arginine, Arg; aspartate, Asp; asparagine, Asn; glutamate, Glu; glutamine, Gln). Plants were exposed to these nitrogen forms supplied singularly or in combination, in a range of concentration from 1 to 4 mM of total nitrogen. As control plants were not exposed to any nitrogen source (Ctr).

It was also analyzed the effect on urea uptake rate of the urease inhibitor N(n-butyl)thiophosphoric-triamide (nNBPT, Apollo Scientific Ltd, UK). Thus, 0.897 µM nBTPT was added to urea treatments (Krogmeier et al., 1989).

Morphometric evaluation in long term experiments: 7 days of nitrogen treatments

To evaluate the effect of different nitrogen sources on root and leaf fresh weights and on the morphology of the root system, 5-day-old maize plants were exposed for 7 days to a nutrient solution containing a) as a sole nitrogen source: urea (U), ammonium (A), nitrate (N) or glutamine (Gln), 1 mM Ntot; or b) a combination of two nitrogen sources: urea and nitrate (UN), urea and ammonium (UA), urea and glutamine (UG) or ammonium and nitrate (AN), 2 mM Ntot. Control plants were fed with nutrient solution without the addition of any nitrogen form. At the end of the experiment (Figure 11, a) photos of root systems were taken and the upper- and lower-parts of 24 plants were weights.

Physiological and molecular studies in short term experiments: max. 24 hours of nitrogen treatments

- *Urea Kinetic, urea and nitrate uptake capacity and ^{15}N accumulation*

The urea and nitrate uptake assays were performed on 5-day-old maize plants exposed for a maximum of 24 hours to a nutrient solution containing a) as a sole nitrogen source: urea (U, from 1 to 4 mM Ntot), ammonium (A), nitrate (N) or single amino acids (glycine, Gly; arginine, Arg; aspartate, Asp; asparagine, Asn; glutamate, Glu; glutamine, Gln) ranging from 1 to 3 mM Ntot; or b) a combination of two nitrogen sources: urea and nitrate (UN), urea and ammonium (UA), urea and glutamine (UG) or ammonium and nitrate (AN), containing 2 mM Ntot. Control plants were fed with nutrient solution without the addition of any nitrogen form. After 2-4-6-8-10-12 and 24 hours of exposition to different nitrogen treatments Figure 11, b), the roots of intact seedlings were washed in 0.5 mM $CaSO_4$ and used to perform the urea and nitrate uptake assays. Another series of experiments were performed where^{15}N accumulation was measured in maize roots and shoots after 24 hours of exposition to different nitrogen treatments containing ^{15}N-labeled source (98 atom % ^{15}N; ISOTEC® Stable Isotopes, Sigma Aldrich, Milano, Italy): ^{15}N-ammonium (1 mM Ntot, $\underline{\text{A}}$), ^{15}N-nitrate (1 mM Ntot, $\underline{\text{N}}$), ^{15}N-urea (1 mM Ntot, $\underline{\text{U}}$), ammonium and ^{15}N-nitrate (2 mM Ntot, A$\underline{\text{N}}$) or ^{15}N-urea and nitrate (2 mM Ntot, $\underline{\text{U}}$N).

- *Microarray experiment*

Microarray analyses were performed on 5-day-old maize plants exposed to a nutrient solution containing as a sole nitrogen source: urea (U, 1 mM Ntot), nitrate (N, 1 mM Ntot) or in combination urea and nitrate (UN, 2 mM Ntot). Control plants were fed with nutrient solution without the addition of any nitrogen form. After 8 hours of exposition to different nitrogen treatments (nine hours after the beginning of light phase; Figure 11, c), the roots from six plants were pooled together, immediately frozen in liquid nitrogen and stored at -80°C until further processing. Microarray analyses are based on three independent biological replications.

- *Real time RT-PCR experiment*

Real time RT-PCR analyses were performed on 5-day-old maize plants exposed for a maximum of 24 hours to a nutrient solution containing a) as a sole nitrogen source: urea (U), nitrate (N) or glutamine (Gln), 1mM Ntot; or b) a combination of two nitrogen sources: urea and nitrate (UN) or urea and glutamine (UG), 2mM Ntot. Control plants were fed with nutrient solution without the addition of any nitrogen form. After 2-4-8-12 and 24 hours of exposition to different nitrogen treatments (Figure 11, b), the roots and leaves from six plants were pooled together, immediately frozen in liquid nitrogen and stored at -80°C until further processing. The collection was repeated in two independent experiments.

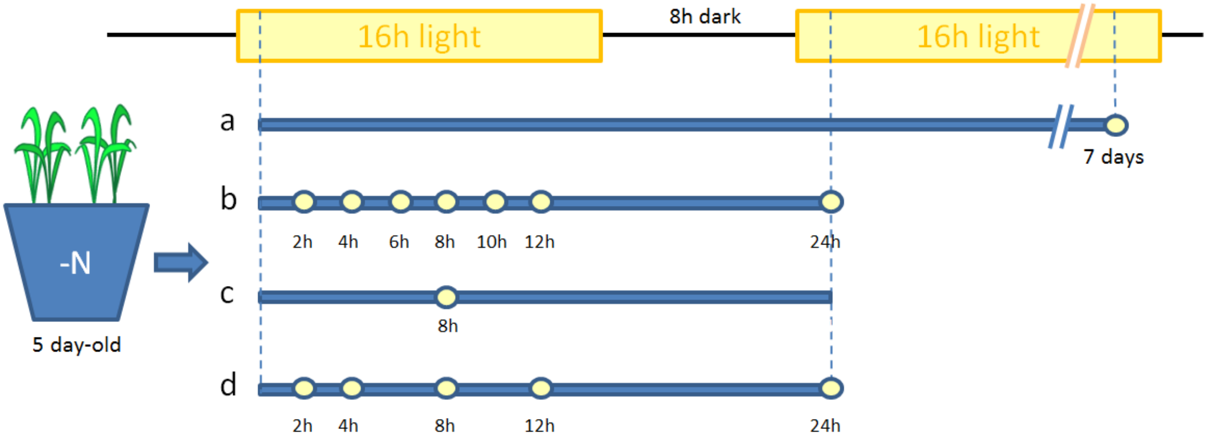


Figure 11. Schematic representation of the experimental plan for long term (a) and short term experiments (b, c and d). 5-day-old maize plants were grown hydroponically in 0.5 mM $CaSO_4$ in nitrogen starvation (-N). Then plants were transferred in nutrients solution containing different nitrogen treatments. Depending on exposition time to the nitrogen treatments, two type of experiments were performed: a) Long term experiment: 7 days of nitrogen treatments; b, c and d) Short term experiments: maximum 24 hours of nitrogen treatments. For the different analyses blue lines represent the time span and in yellow the harvesting times. All the experiments started 1 hour after the onset of light, thus "2h" samples were harvested after 3 hours from the beginning of the light phase.

a	Long term experiment (7 days of nitrogen treatments) for morphological and plant growth evaluations
b	Short term experiment (max 24 h of nitrogen treatments) for urea and nitrate uptake analyses and for ^{15}N accumulation.
c	Short term experiment (max 24 h of nitrogen treatments) for microarray analysis
d	Short term experiment (max 24 h of nitrogen treatments) for Real time RT-PCR analyses

2.1.2 Arabidopsis culture

To evaluate the contribution of single nitrogen sources on plant nutrition, Arabidopsis plants were treated with ^{15}N-labeled sources and the ^{15}N content in roots and shoots was analyzed in time course. So, Arabidopsis plants (*Arabidopsis thaliana* from the Columbia-0 ecotype) were grown under hydroponic culture conditions in a growth chamber with day/night photoperiod, 8/16 h; light intensity, 220 µE m^{-2} s^{-1}; temperature (day/night) 21/17°C; RH 70 to 80%. Seeds were sterilized and germinated at 4°C for 5 days in plastic tips filled with 1% w/v agarose gel. When two embryonic leaves appeared, plantlets were transferred in hydroponic conditions as described above for maize plants. For each plastic pots, 12 plants grew together exposed to 2 L of nutrient solution. The composition of the media was the same reported by Mérigout and coworkers (2008a). Thus, plants were grown with basic nutrient medium supplemented with 1 mM NH_4NO_3. Basic medium contained 1 mM $MgSO_4$, 1 mM KH_2PO_4, 2.5 mM K_2SO_4, 2.2 mM $CaCl_2$, 10 mM $MnSO_4$, 24 mM H_3BO_3, 3 mM $ZnSO_4$, 0.9 mM $CuSO_4$, 0.04 mM $(NH_4)_6Mo_7O_{24}$, and 10 mg L^{-1} Fe-EDTA. Nutrient solution was renewed every 2 days and, during the 2 first weeks, used at half-strength (pH 5.5 adjusted with KOH). At the age of 5 weeks, and 7 days before the experiment, most of the plants were transferred to basic medium without addition of nitrogen (complete nitrogen starvation, -N), while few plants were maintained in nitrogen sufficiency (by addition of 2 mM NH_4NO_3, +N). Nutrient solutions were renewed daily during these 7 days to ensure constant concentration nitrogen and pH stability. The day of the experiments (42 days after sowing), the nutrient solution was renewed and the nitrogen was supplied as [^{15}N]-labeled sources (98 atom % ^{15}N; ISOTEC® Stable Isotopes, Sigma Aldrich, Milano, Italy). The nitrogen sufficient plants were supplied with [^{15}N]-urea (1 mM Ntot) or [^{15}N]-nitrate(1 mM Ntot). 5 treatments were performed on nitrogen deficient plants, using as sole nitrogen source: [^{15}N]-ammonium (1 mM Ntot, $\underline{A}$), [^{15}N]-nitrate (1 mM Ntot, $\underline{N}$) or [^{15}N]-urea (1 mM Ntot, $\underline{U}$); or a combination of two nitrogen sources: urea and [^{15}N]-nitrate (2 mM Ntot, $U\underline{N}$) or [^{15}N]- urea and nitrate (2 mM Ntot, $\underline{U}N$).

After 2, 4, 8, 12 or 24 hours of exposition to the nitrogen treatments, plants were rinsed in $CaSO_4$ 0.5 mM solution and shoots and roots were weighed separately and dry at 60°C. For each nutritional condition, three plants were pooled and homogenized to a powder for the ^{15}N-analysis.

2.2 Physiological analyses

2.2.1 Measurement of net high-affinity urea uptake and calculation of kinetic parameters

Roots of intact seedlings were immersed in 40 ml of a constantly agitated on shaker (Janke & Kunkel KS 501D) and aerated solution containing 500 µM $CaSO_4$ and 200 µM urea. Net uptake was measured as urea depletion from the solution per unit of time, removing samples of solution (60 µl) for urea determination every 2 min for 10 min, span time during which uptake had a linear trend.

The urea was determined by diacetylmonoxime and thiosemicarbazide colorimetric assay (modified from Killingsbaeck, 1975). In order to analyze a great numbers of samples, the colorimetric reaction was performed using 96-well ELISA-microplates and the volumes of reagents were optimized. Thus, the samples, as aliquots of 60 µl, were mixed thoroughly with 120 µl of color development reagent, which consisted of 1:1 mixed color reagent [7% (v/v) 0.2 M diacetylmonoxime; 7% (v/v) 0.05 M thiosemicarbazide]: mixed acid reagent [20% (v/v) sulfuric acid (H_2SO_4); 0.06% (v/v) 74 mM ferric chloride hexahydrate in 9% (v/v) ortho-phosphoric acid]. The tubes were incubated for 15 min at 99°C (lid temperature: 105 °C) in thermocycler (Eppendorf Mastercycler DNA Engine Thermal Cycler PCR). The samples were cooled 5 min on ice and the urea concentration was determined spectrophotometrically by measuring the absorbance at 540 nm using a microplate reader (GENios Microplate Reader TECAN, Ges.m.b.H-Austria). Results were expressed as velocity of net-uptake of urea: µmol urea g^{-1} root fresh weight (FW) h^{-1}.

The same reaction was used to determined urea accumulation in Arabidopsis tissues by Kojima et al. (2007), who pointed out that the ureides allantoin, ornithine, arginine, and uric acid, did not interfere with urea determinations, although other ureides were not tested.

Kinetic parameters of the high-affinity urea uptake system (V_{max} and K_m) were calculated in the 5 - 300 µM concentration range. Uptake rates were measured as described above except that the uptake solution contained 5, 10, 25, 50, 100, 200 or 300 µM urea. The results were obtained using the linearization of Woolf–Augustinsson–Hofstee. These kinetic parameters are not to be attributed to a single transporter, but are related to the overlapping activities of different transporters.

To determine the direct effect of urease inhibitor on the urea transport system, the nBTPT was added to the uptake solution. So, roots of intact seedlings were immersed in 40 ml of solution containing 0.5 mM $CaSO_4$, 200 µM urea and 0.897 µM nBTPT. As described above, samples of 60 µl were removed every 2 min during the time span of 10 min and urea was determined following the same colorimetric method.

2.2.2 Measurement of net high-affinity nitrate uptake

Roots of intact seedlings were immersed in 40 ml of a constantly agitated on shaker (Janke & Kunkel KS 501D) and aerated solution containing 500 µM $CaSO_4$ and 200 µM KNO_3. Net uptake was measured as nitrate depletion from the solution per unit of time (Cataldo et al., 1975), removing samples (200 µl) for nitrate determination every 2 min for 10 min, span time during which uptake had a linear trend. Aliquots of 200 µl were mixed thoroughly with 0.8 ml of 5 % (w/v) salicylic acid in concentrated H_2SO_4. After 20 min incubation at room temperature, 19 ml of 2 M NaOH was added. Samples were cooled to room temperature and nitrate concentration was determined spectrophotometrically by measuring the absorbance at 412 nm.

2.2.3 ^{15}N analysis

To performed the analyses of ^{15}N accumulation in plants of maize and Arabidopsis, approximately 1 mg of dried samples of leaf and root tissues were weighed in a tin capsule for the measurement of $d^{13}C$ and $d^{15}N$ in one run. The analysis was carried out using a Delta V isotope ratio mass spectrometer (Thermo Scientific, Germany) equipped with a Flash EA 1112 elemental analyser (Thermo Scientific, Germany). The isotope ratios were expressed in d‰ versus V-PDB (Vienna – Pee Dee Belemnite) for $d^{13}C$ and air for $d^{15}N$ according to the following formula:

$$d‰ = [(R_{sample} – R_{standard})/R_{standard}]\cdot 1000$$

where R_{sample} is the isotope ratio measured for the sample and $R_{standard}$ is the isotope ratio of the international standard. R is the abundance ratio of the minor, heavier isotope of the element to the major, lighter isotope (e.g. $^{13}C/^{12}C$). The isotopic values were calculated against international reference materials: L-glutamic acid USGS 41 and ammonium sulphate IAEA-N-2 (IAEA-International Atomic Energy Agency, Vienna, Austria).

2.3 Molecular work

2.3.1 RNA extraction

At harvesting times, root and leaf maize samples were collected, immediately frozen in liquid nitrogen and conserved at -80 °C until further processing. RNA extractions were performed using the Invisorb Spin Plant RNA kit (Stratec Molecular, Berlin, Germany) as reported in the manufacturer’s instructions (http://www.invitek.de/). 70 mg of maize tissue were homogenized in liquid nitrogen and the powder was mixed with 900 µl of DCT solution and dithiothreitol (DTT) according to the supplier's instructions. In order to verify the absence of genomic contamination, 1 µg of total RNA was analyzed electrophoretically running on 1% agarose gel and it was quantified by spectrophotometer Nanodrop 2000 instrument (Thermo Scientific, Wilmington, USA). For the microarray analysis the RNA quality and quantity were determined using a Bioanalyzer Chip RNA 7500 series II (Agilent, Santa Clara, CA).

2.3.2 Microarray analysis

The cDNA synthesis, labeling, hybridization and washing reactions were performed according to the NimbleGen Arrays User's Guide (www.nimblegen.com). Each hybridization was carried out on a NimbleGen microarray (Roche, NimbleGen Inc., Madison, WI), representing 59,756 transcripts predicted from the B73 reference genome version 1 (B73 RefGen_v1) (www.maizesequence.org). The chip probe design is available at the following URL: http://ddlab.sci.univr.it/FunctionalGenomics/.

The microarray was scanned using a ScanArray 4000XL (Perkin-Elmer) at 532 nm (Cy-3 absorption peak) and GenePix Pro7 software (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions. Images were analyzed using NimbleScan v2.5 software (Roche), which produces Pair Files containing the raw signal intensity data for each probe and Calls Files with normalized expression data (quantile normalization) derived from the average of the intensities of the four probes for each gene through RMA analysis.

Analysis of normalized data (Calls Files) was performed by the open source software of the Bioconductor project (Gentlemen et al., 2004; http://www.bioconductor.org) with the statistical R programming language (Ihaka and Gentleman, 1996; http://www.r-project.org). Differentially expressed probes were identified by linear models analysis (Smyth, 2004) using limma package and applying Bayesian correction, adjusted p-value of 0.05. All microarray expression data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

2.3.3 Real-time RT-PCR experiments

1 µg of total RNA was retrotranscribed in cDNA following the protocol to the total RNA (checked for quality and quantity using a spectrophotometer NanoDrop™ 1000 (Thermo Scientific), followed by a migration in an agarose gel). To 1 pmol of Oligo d(T)23VN (Sigma Aldrich, Milano, Italy), 15 U Prime RNase Inhibitor (Eppendorf, Hamburg, Germany) and 10 U M-MulV RNase H^- were added and keep at 42°C for 1 h (Finnzymes,Helsinki, Finland) following the application protocol of the manufacturers. After RNA digestion with 1 U RNase A (USB, Cleveland, USA) for 1 h at 37°C, gene expression analyses were performed by adding 0.16 µl of the cDNA to the realtime PCR complete mix, FluoCycleTM sybr green (20 µl final volume; Euroclone, Pero, Italy), in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). The analyses of real-time result were performed using Opticon Monitor 2 software (Biorad).

Specific primers (Tm = 58 °C) were designed to generate 80-140 bp PCR products (Table 3)and they were synthesized by Sigma Aldrich (Milano, Italy). Four genes were used as housekeeping to normalized the data: *ZmRPS4, ZmRPL17, ZmpolyUBI, ZmRPL30*; data were confirmed with all housekeeping genes, however *ZmRPS4* in roots and *ZmPolyUBI* in leaves were more constitutively expressed than the others and for this reason used for the relativization of the data. So, the mRNA levels of target gene were normalized with respect to the transcript level of the housekeeping gene (HK) using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,HK})_{Time\ x} - (C_{T,Target} - C_{T,HK})_{Time\ 0}$ (Livak and Schmittgen, 2001).

Table 3 **List of specific primers used to performed Real time RT-PCR analyses.**

Genes	**Protein description**	**GenBank Acc N°**	**Primer FOR (5'- -3')**	**Primer REV (5'- -3')**
ZmNRT2.1	High affinity nitrate transporter (NRT)	AJ344451	GATCGACGATCACCTATACCTC	GTGCTCCGTTGACATGAG
ZmNRT2.2	High affinity nitrate transporter (NRT)	AY659965	CCTACCTTTACGTGTATGCCTTG	GATGTGCCAACGATATTCATC
ZmNRT1.2	Low affinity nitrate transporter (NRT)	NM_001112455.1	GGTGTCGATGGTGCTCTTCT	ACGCGGTTGTCTATGGTCTC
ZmNAR2.2	Component for high affinity nitrate transporter (NAR)	NM_001112454.1	GCCGTCATGTTGTGTAGTGC	CAGGTCCAGCTTGTGACTGA
ZmNR1	Nitrate reductase (NR)	AF153448	CCAGCCGACTTGCCAGCGTAA	GCATGGCCTATGTTATCTGCTGCTC
ZmNR2	Nitrate reductase (NR)	U20450.1	GGTGAAGGTCAACGTGTGC	CGGTCTCGAGGTGCTTCT
ZmNiR	Nitrite reductase (NiR)	M23456.1	CTTCATGGGCTGCCTCAC	GTAGACGTCGGCCAGGTG
ZmGS2	Glutamine synthetase (GS)	EU963258.1	TGTGAAGCAGCTGAAGGATG	GAGCAGAGAGTCGCAAGACC
ZmFd-GOGAT	Fd-glutamine oxoglutarate aminotransferase (Fd-GOGAT)	NM_001112223	GGTGAAGGCGTTCTCTGAAG	GCAACAGCTTGGACATCTCA
ZmDUR3	High affinity urea transporter (DUR)	BQ164112.1	CCTCAATCTGGTGGGTGTCT	ATTGGCCTTTCTCCACAGC
ZmUrease	Urease	NM_001151384	ATGCTGATGGTTTGTCACCA	GCAATATGTCCTCAGCAGCA
ZmUreG	Urease associated protein (UreG)	EU956120	CAGGCCGATCTGTTGGTAAT	CACCTGTGCAAACACAAAGG
ZmArginase	Arginase	BT087608	ATTTGCTCCTCCACGCATAC	TTAGCACCCGAGGATCATTC
ZmPAL	Phenylalanine Ammonia Lyase (PAL)	NM_001254868.1	CGGTGAAGAACACCGTGAC	ATCAGCTCCTTCTCGCTGAA
ZmANR1	MADS box transcription factor: "Arabidopsis Nitrate Regulated-1" (ANR1)	HM994692.1	ATCAACCTGATTCGCCAAGA	TGCAAAGTTGAATGGAGTCG
ZmPIP4	Aquaporin, water and urea channel (PIP)	AJ271796	ACAGCAACCATGCAGCATAC	CGAGCACGCACCACTTACTA
ZmRPS4	ribosomal protein	AF013487	GCAACGTTGTCATGGTGACT	CTCCACGTGAATGGTCTCAA
ZmPolyU	Ubiquitin protein	S94466	GTACCCTCGCCGACTACAAC	ATGGTCTTGCCAGTCAAGGT
ZmRPL30	ribosomal protein	AF034949	AATGTCGACCTTGGAACTGC	TGCTCACCCGGTGTAGTCTT
ZmRPL17	ribosomal protein	EU975447.1	AAAGTCTCGCCACTCCAATG	ACGTCCAAGCCTTTCACATC

2.3.4 Isolation and functional characterization of *ZmDUR3*

2.3.4.1 RNA work

2.3.4.1.1 DNAse treatment and Reverse Transcription reaction

To the *ZmDUR3*-ORF, total RNA was extracted from root samples of 5-day-old maize plants exposed for 24 hours to 1 mM urea, as described above. To avoid contaminations by genomic DNA, the samples of total RNA were treated with DNAse. Thus, 10 µg of RNA were mixed with 5 U DNAse (RNAse-free, Fermentas); 2.5 µl 10 x DNAse-Buffer (Fermentas) in a final volume of 25 µl. The reaction was performed at 37°C for 1 h. The RNA was then precipitated for 1 h at -20°C by adding 2.5 volume of 99% EtOH and 1/10 volume Na-Acetate (3 M, pH 5.2). After centrifugation at 14,000 g for 30 min at 4°C, the pellet was washed with 70% EtOH and then resuspended in 9 µl of RNAse-free water.

The reverse transcription reaction (RT-reaction) was performed incubating in a thermocycler 2 µg of total RNA with 1 µl Oligo-dT23 (50 µM) or with 1 µl gene specific primer (2 µM; 192_R) at 75°C for 5 min for primer hybridisation and then cooled down at 50°C for at least 2 min. Samples were briefly centrifuged and the reaction mix containing 5x first strand buffer (Gibco BRL), 5 mM $MgCl_2$; 125 µM dNTP; 10 mM DTT; 16 U RiboLock™ Ribonuclease Inhibitor (Fermentas); 470 mM trehalose, were added in 20 µl of final volume. Afterwards 80 U of SuperScript® II Reverse Transcriptase (200 U μl^{-1}; GIBCO BRL) were added and the samples were incubate for 1 h at 50°C. The enzyme was then inactivated at 70°C for 15 min.

2.3.4.1.2 Polymerase Chain Reaction

The PCR reaction was performed in 50 µl of final volume with the addition of:

5 x GC Buffer for Phusion® High-Fidelity DNA Polymerase (NEB Labs);
0.2 mM ATP; 0.2 mM TTP; 0.3 mM GTP; 0.3 mM CTP
0.4 µM Primer Forward;
0.4 µM Primer Reverse;
10-100 ng Template

Before the addition of polymerase, the samples were incubated at 98°C for 5 min to denature the template and then 2 U Phusion® High-Fidelity DNA Polymerase (NEB Labs) were added to each samples. The specific forward and reverse primers are listed in theTable 4.

The samples were incubated in termocycler *T3000 Thermo Cycler* (Biometra, Göttingen, Germany) applying the following PCR profile:

Initial denaturation	98°C	30 sec	
Denaturation	98°C	10 sec	X 35 cycles
Annealing	58-68°C	30 sec	
Elongation	72°C	30sec -2min	
Final extension	72°C	10 min	

Table 4. List of primers used to amplify the *ZmDUR3*-ORF. Restriction enzyme recognition sites (RE site) are underlined.

ATG_EcoRI_F (RE site for *EcoRI*)	5'-CGGAATTC**ATG**GCCGCTGGCGGCGCCGGC-3'
166_F	5'-TTCTTCGCGCTCTTCACCTC-3'
192_R	5'-CAGGAATGAGGTGAAGAGCGCGAAGAAGGCGC-3'
2196_BamHI_R (RE site for *BamHI*)	5'-CGCGGATCCTTAAGCTAGCGAAAGATTATCTTCATC-3'
ATG Long Primer (RE site for *EcoRI* and overlap with *Second Long Primer*)	5'-CGGAATTC**ATG**GCTGCTGGTGGTGCTGGTGCTTGTCCTCCACCAGGTCTAGG TTTTGGTGGTGAATATTATTCTGTTGTTGAT*GGTGCTTGTAGTCGTGATGG* -3'
Second Long Primer (overlaps with *ATG Long Primer* and with *Second Fragment*)	5'-*GGTGCTTGTAGTCGTGATGG*TAGCTTTTTTGGCGGTAAACCAGTTCTAGCTCA AGCTGTTGGTTATGCTGTCGTTCTTGGTTTTGGTGCT*TTCTTCGCGCTCTTCACCTC*-3'
ATG_SpeI_F (RE site for *SpeI*)	5'-ATAACTAGT**ATG**GCTGCTGGTGGTGCTGG-3'
2196_BglII_R (RE site for *BglII*)	5'-ATAtAGATCTGCAGCTAGCGAAAGATTATCTTCATCG-3'
ATG_XbaI_F (RE site for *XbaI*)	5'-ATATCTAGA**ATG**GCTGCTGGTGGTGCTGG-3'
2196_NsiI_R (RE site for *NsiI*)	5'-ATAATGCATTTAAGCTAGCGAAAGATTATCTTCATCG-3'

Agarose Gel Electrophoresis

For the isolation and purification of PCR products, the DNA samples were applied on agarose gels. The electrophoretic mobility of DNA fragments mainly depends on the fragment size and to concentration of agarose used as well as applied voltage and electrophoresis buffer used. In order to purify the fragments a low agarose concentration was used: 0.6% agarose. So, 0.6 mg of agarose were dissolved in 100 ml of 1X TAE buffer and mixed with ethidium bromide (final concentration 0.5 µg ml^{-1}) . The electrophoresis was carried out at 90 mV voltage in TAE 1X buffer. In order to illuminate the ethidium-bromide stained DNA, the fragments were then visualized by UV-light and the desired band was removed with a razor blade. The desired DNA fragment was then purified from the sliver of gel by EconoSpin® mini spin column (Epoch Life Science) following the manufacturer's instructions.

10X TAE Buffer (1 liter)

48.4 g	Tris base [tris(hydroxymethyl)aminomethane]
11.4 ml	Glacial Acetic Acid (17.4 M)
20 ml	Na_2EDTA (0.5 M, pH 8.0)

2.3.4.2 *ZmDUR3"Native"*-ORF amplification

In the putative *ZmDUR3*-ORF sequence a high content of GC was observed, especially at the beginning of the ORF where very high values of GC% were reached (in the first 100 bp the GC-content was around 80%). Maybe for this intrinsic peculiarity the direct amplification of the whole sequence was difficult to perform and only aspecific amplifications were obtained.

So, the *ZmDUR3*-ORF (called *ZmDUR3"Native"*) sequence was amplified by two independent PCR-reactions which led to amplify two distinct fragments with an overlap of 20 bp.

-*First fragment*: covered the first part of the ORF sequence starting from +1 ATG (192 bp)

-*Second fragment*: covered most part of the ORF sequence, from +172 up to +2196 (2024 bp)

Subsequently to obtain the whole sequence, these two fragments were linked by PCR reaction based on the approach of Assembly PCR (Figure 12).

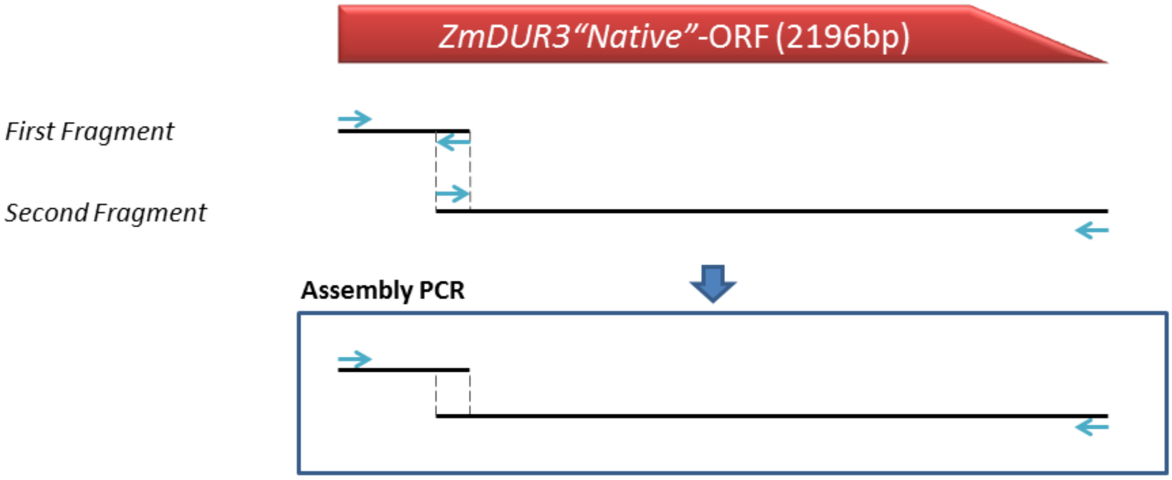


Figure 12. Schematic representation of the approach used to amplify the *ZmDUR3"Native"*-ORF. The whole sequence was obtained amplifying two distinct fragments. *First Fragment* and *Second Fragment* showed an overlapping of about 20 bp which allowed the subsequent assembled of the whole sequence called *ZmDUR3"Native"*.

First Fragment Amplification

The amplification of the *First Fragment* was performed by PCR reaction using as template the RT-reaction product obtained by the gene specific primer 192R. So, 50 ng of specific cDNA were amplified by forward primer, ATG_EcoRI_F (having the restriction site for *EcoRI*), and by reverse primer, 192_R. The melting temperature (Tm) was 68°C and the time of elongation was of 30 sec at 72°C.

Second Fragment Amplification

The amplification of the *Second Fragment* was performed by PCR reaction using as template the RT-reaction product retrotranscribed by the Oligo-dT23. So, 100 ng of total cDNA were amplified by forward primer, 166_F, and by reverse primer, 2196_BamHI_R (having the restriction site for *BamHI*). The melting temperature (Tm) was 58°C and the time of elongation was of 2 min at 72°C.

Assembly PCR for ZmDUR3"Native"-ORF

The *First Fragment* and *Second Fragment* of *ZmDUR3*-ORF were assembled using the approach of the Assembly PCR. So, the PCR reaction was carried out with 10 ng *First Fragment* and 10 ng *Second Fragment,* as template; while the forward and reverse primers were ATG_EcoRI_F (restriction site for *EcoRI*) and 2196_BamHI_R (restriction site for *BamHI*). The reaction was performed with a Tm of 62°C and 1 min 30 sec of elongation time at 72°C .

2.3.4.3 *ZmDUR3"Modified"*-ORF amplification

In order to reduce the GC content and to favour the expression of *ZmDUR3* in heterologous systems (such as yeast, oocytes or other plant species), a second version of the sequence was obtained.

Since, the highest GC content of *ZmDUR3"Native"*-ORF occurs in the first part of the sequence corresponding to the first 166 bp, only 47 nucleotides in this region were modified (Figure 13). These modifications are all synonymous substitutions occurring only at the third base of the codons (codons were modified to match the codon-usage preference of yeast according to http://www.kazusa.or.jp/codon/) and the resulting ORF version was called *ZmDUR3"Modified"*-ORF. In this way the GC content was reduced from 75% in *ZmDUR3"Native"*-ORF to 47% in *ZmDUR3"Modified"*-ORF. However the differences between the two ORF versions were only at the nucleotide level, while no any difference occurred at amino acid sequence of the protein.

ZmDUR3"Native"-ORF

5'-<u>ATG</u> GCC GCT GGC GGC GCC GGC GCG TGC CCT CCG CCG GGG CTG GGC TTC GGC GGG GAG TAC TAC TCG GTG GTG GAC GGC GCG TGC AGC CGC GAC GGC AGC TTC TTC GGC GGG AAG CCG GTG CTG GCG CAG GCC GTT GGG TAC GCC GTC GTC CTT GGC TTC GGC GCC TTC TTC GCG CTC TTC ACC TCA TTC CTG…-3'

ZmDUR3"Modified"-ORF

5'-<u>ATG</u> GC**T** GCT GG**T** GG**T** GC**T** GG**T** GC**T** TG**T** CCT CC**A** CC**A** GG**T** CT**A** GG**T** TT**T** GG**T** GG**T** GA**A** TA**T** TA**T** TC**T** GT**T** GT**T** GA**T** GG**T** GC**T** TG**T** AG**T** CG**T** GA**T** GG**T** AGC TT**T** TT**T** GGC GG**T** AA**A** CC**A** GT**T** CT**A** GC**T** CA**A** GC**T** GTT GG**T** TA**T** GC**T** GTC GT**T** CTT GG**T** TT**T** GG**T** GC**T** TTC TTC GCG CTC TTC ACC TCA TTC CTG…-3'

Figure 13. Nucleotide differences occurring in the *ZmDUR3"Modified"*-ORF. Bold letters are referred to the 47 modified nucleotides occurring in the *ZmDUR3"Modified"*-ORF.

The modified region of *ZmDUR3"Modified"*-ORF was obtained assembling two long primers, *ATG Long Primer* and *Second Long Primer* (about 100 bp each) which were synthetized *in vitro* (Microsynth AG, Balgach, Switzerland).

Two consecutive Assembly PCR reactions were performed to add the long primers to the *Second Fragment* which was previously isolated. In order to allow the Assembly PCR, *ATG Long Primer* was designed to overlap for 20 bp the *Second Long Primer*; in the same way also *Second Long Primer* showed an overlapping with the *Second Fragment* (Figure 14).

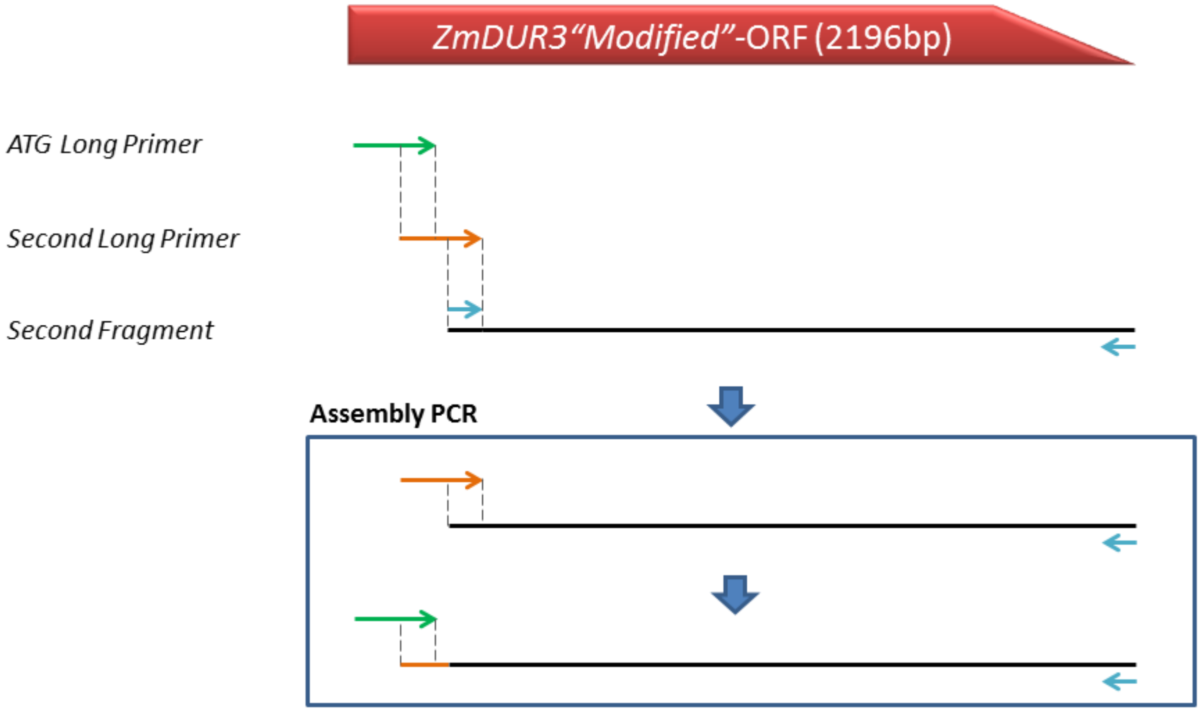


Figure 14. Schematic representation of the approach used to amplify the *ZmDUR3"Modified"*-ORF. The whole sequence was obtained amplifying the *Second Fragment* with *Second Long Primer* as forward primer of a PCR-Assembly. Subsequently, a further reaction of PCR-Assembly was performed using as forward the *ATG Long Primer*.

Assembly PCR for ZmDUR3"Modified"-ORF

The *Second Long Primer* and *Second Fragment* were assembled by PCR. So, 10 ng of *First Fragment* were used as template; while *Second Long Primer and* 2196_BamHI_R (restriction site for *BamHI*) were used as forward and reverse primers. The reaction was performed with a Tm of 62°C and 1 min 30 sec of elongation time at 72°C .

The PCR product was directly purified using EconoSpin columns (Epoch Life Science, following the manufacturer's instructions). So, 10 ng of purified PCR product were used as template for the consecutive PCR with forward and reverse primers: *ATG Long Primer* (restriction site for *EcoRI*) and 2196_BamHI_R (restriction site for *BamHI*). The reaction was performed with a Tm of 62°C and 1 min 30 sec of elongation time at 72°C.

2.3.4.4 Cloning

2.3.4.4.1 pDR197-ZmDUR3"Native" and pDR197-ZmDUR3"Modified"

For the yeast complementation assay, both versions of *ZmDUR3* (*ZmDUR3"Native"* and *ZmDUR3"Modified"*) were cloned into the vector pDR197 (Doris Rentsch, unpublished; Figure 15) using restriction enzymes: *EcoRI* and *BamHI* (Fermentas, *FastDigest*®) yielding the plasmid pDR197-ZmDUR3"Native" and pDR197-ZmDUR3"Modified". pDR197, a modified version of pDR195 (Rentsch et al., 1995), consists of 6.3 kb and contains the following Multiple-Cloning Site (MCS): *Xho*I-*SmaI*-*PstI*-*EcoRI*-*EcoRV*-*HindIII*-*SalI*-*XhoI*-*Acc65I*-*KpnI*-*Bam*HI.

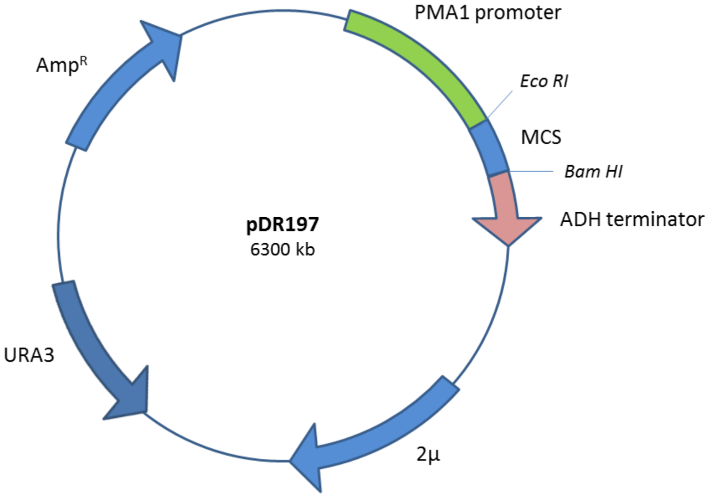


Figure 15. pDR197 plasmid map.

2.3.4.4.2 pBF1-ZmDUR3"Native" and pBF1-ZmDUR3"Modified"

For the functional characterization in *Xenopus* oocytes, *ZmDUR3*-ORF sequence was cloned into the plasmid pBF1 (Baukrowitz et al., 1999) (Figure 16), in which the sp6 promoter allows the *in vitro* transcription of the insert as cRNA. Thus, both versions of *ZmDUR3* (*ZmDUR3"Native"* and *ZmDUR3"Modified"*) were cloned into the vector pBF1. The inserts were excised from pDR197-ZmDUR3"Native" and pDR197-ZmDUR3"Modified" using the restriction enzymes *EcoRI* and *BamHI* and cloned into the *EcoRI* and *BglII* sites of pBF1.

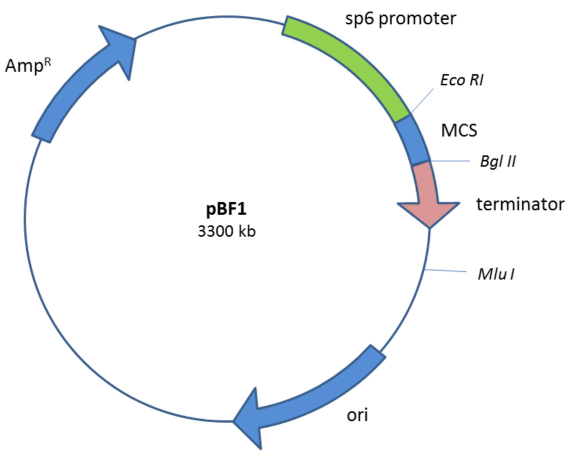


Figure 16. pBF1 plasmid map.

2.3.4.4.3 pUC18-ZmDUR3"Modified"-Sp-GFP6 and pUC18-GFP5T-Sp-ZmDUR3"Modified"

For the transient expression of *ZmDUR3"Modified"* in tobacco protoplasts, two plasmids harboring a sequence for the Green Fluorescent Protein (GFP) were used: pUC18-Sp-GFP6 and pUC18-GFP5T-Sp (Komarova et al., 2012). Both plasmids derived from pUC18 which was modified by addition of the gene encoding the GFP: in pUC18-Sp-GFP6, the *GFP* sequence is located up-stream of MCS; while in pUC18-Sp-GFP5T*, GFP* is down-stream the MCS.

pUC18-ZmDUR3"Modified"-Sp-GFP6

The amplification of the insert *ZmDUR3"Modified"* was performed by PCR reaction using as template pBF1-ZmDUR3"Modified". So, 20 ng of pBF1-ZmDUR3"Modified" plasmid were amplified by forward primer, ATG_SpeI_F (having the restriction site for *SpeI*), and by reverse primer, 2196_BglII_R (having the restriction site for *BglII*). The melting temperature (Tm) was 58 °C and the time of elongation was 1 min and 45 sec at 72 °C. The amplified fragment was purified and cloned into pUC18-Sp-GFP6 (C-terminal GFP) using the restriction enzymes *SpeI* and *BglII*.

pUC18-GFP5T-Sp-ZmDUR3"Modified"

The amplification of the insert *ZmDUR3"Modified"* was performed by PCR reaction using as template pBF1-ZmDUR3"Modified". So, 20 ng of pBF1-ZmDUR3"Modified" plasmid were amplified by forward primer, ATG_XbaI_F (having the restriction site for *XbaI*), and by reverse primer, 2196_ NsiI_R (having the restriction site for *NsiI*). The melting temperature (Tm) was 58 °C and the time of elongation was 1 min and 45 sec at 72 °C. The amplified fragment was purified from agarose gel and digested by the restriction enzymes *XbaI* and *NsiI.* In this way compatible ends for the restriction sites of *NheI* and *PstI* were respectively produced. So, *ZmDUR3"Modified"* was cloned into the *NheI*/*PstI* site of pUC18-GFP5T-Sp (N-terminal GFP).

2.3.5 Transfomation of electrocompetent *Escherichia coli*

To prepare electro competent cells, a single colony of *E.coli* (SURE, Stratagene, Heidelberg) was used to inoculate 20 ml of SOB medium. This starter culture was incubated overnight at 37°C with shaking and 5 ml aliquots were used to inoculate 500 ml of SOB medium (1 in 100 dilution). This culture was incubated at 37°C with shaking until the culture density had reached an OD of 0.6 at 600 nm. Then, the culture was chilled on ice for 30 min and transferred to ice-cold sterile 500 ml centrifugation bottles. Cells were harvested by centrifugation at 2,800 g for 15 min at 4°C, the supernatant was removed and the pellet was washed two times with ice-cold 500 ml of 10% glycerol (Washing buffer). The centrifugations were performed under same conditions after every wash. Finally the pellet was resuspended in 2 ml of Washing buffer and aliquots of 200 µl were stored at -80˚C. All equipment and solutions were maintained at 0°C to 4°C throughout the procedure.

A 100 µl aliquot of electrocompetent *E. coli* cells was mixed 1-5 µl (containing 0.02 µg to 1 µg) of desalted plasmid DNA derived from DNA mini- or maxipreps or from a ligation reaction) and transferred to a pre-chilled 1 cm gap-width electroporation cuvette. The cuvette was placed in the Electro Cell Manipulator 600 (ECM600) electroporation system from Biotechnologies and Experimental Research Inc. (BTX) and electroporated at a voltage of 2.4 kV, a capacitance of 25 µFD and a resistance of 200 Ω. Following electroporation, 1 ml of pre-warmed SOC medium was added to the cells and the entire volume was transferred in a 2.0 ml Eppendorf tube. Cells suspension were incubated at 37°C for one hour with shaking and a 200 µl aliquot was spread on a LB-agar plate, supplemented with ampicillin 100 µg/ml. Plates were incubated at 37°C overnight and the grown colonies were screened by colony PCR for the desired recombinant DNA constructs. Colonies that were tested positive in the PCR screen were used to inoculate cultures for plasmid mini and /or maxipreps.

SOB Medium (1 liter)

20.0 g	Tryptone
5.0 g	Yeast extract
0.5 g	NaCl

Add deionized H_2O to a final volume of 1 liter and then autoclave
Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ prior to use

SOC Medium (100 ml)
2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose
SOB medium (autoclaved) to a final volume of 100 ml
Prepare immediately before use

LB Agar (1 liter)

10 g	NaCl
10 g	Tryptone
5 g	Yeast Extract
15 g	Agar

Add deionized H2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH and then autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB-Ampicillin Agar (1 liter)

1 liter of LB agar, autoclaved and cooled to 55°C
10 ml of 10 mg ml^{-1} filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

2.3.6 Transfomation of competent cells of *Saccharomyces cerevisiae*

In order to demonstrate the transport function of ZmDUR3, a complementation assay was performed using YNVW1 (*Δdur3, Δura3;* Liu et al., 2003a*),* a mutant strain of yeast *(Saccharomyces cerevisiae*). As previously described by Liu et al. (2003a), the strain 23346c (Mat a, *Δura3*; Grenson, 1969) was used to generate a mutant defective in urea uptake since the *ScDUR3* gene was substituted for a marker gene by PCR-based gene disruption (Liu et al., 2003a). The resulting mutant YNVW1 (*Δdur3*, *Δura3*) was defective in urea uptake and growth on <5 mM urea as a sole nitrogen source.

The *dur3* mutant YNVW1 was transformed by pDR197 harboring *ZmDUR3"Native"* or *ZmDUR"Modified"* or by the empty vector alone. Transformants were selected on uracil-deficient yeast nitrogen base medium containing ammonium sulfate (0.5% w/v) before a single colony was chosen for growth complementation tests with urea as a sole nitrogen source.

2.3.6.1 Yeast growth, transformation and selection

To prepare competent yeast cells, a single colony of YNVW1 yeast mutant was inoculated in 5 ml of YPD medium and grown at 28°C overnight with shaking. An aliquot of 1 ml of this starter culture was used to inoculate 100 ml of YPD medium (1 in 100 dilution). This culture was incubated at 28°C while shaking until OD_{600} reached values around 0.5 - 0.8 ($2x10^{-7}$ cell ml^{-1}). Then, yeast cells were harvested by centrifugation at 2,500 g for 5 min, the supernatant was removed and the pellet was washed with 20 ml of Solution A (Washing buffer). Finally the pellet was resuspend in 2 ml of Solution A and aliquots of 100 µl were stored at -80˚C.

The yeast transformation was performed mixing 100 µl of competent cells with 200 ng plasmid DNA (pDR197 harboring *ZmDUR3"Native"* or *ZmDUR"Modified"* or empty vector) and the addition of 5 µl 1 M Histamin and 50 µg of denaturated Salmon Sperm DNA. After incubation at 37°C for 5 min, 1 ml of Solution B was added to the cells followed by 1 h of incubation at 28°C while shaking. Then, the cell samples were centrifuged at 2,500 g for 5 min and the pellet was resuspend in 800 µl of Solution C. After further centrifugation, the pellet was resuspended in 100 µl of Solution C and plated on SD medium, an uracil-deficient yeast nitrogen base without amino acid medium (Difco) containing ammonium sulfate (0.5% w/v). The plates were incubated for 2-3 days at 28°C.

The complementation test was performed by streaking single colonies of yeast transformed with the different constructs on MM medium plates containing ammonium sulfate (0.5% w/v) or urea (1, 2 or 3 mM) as sole nitrogen source. Yeast transformed with the empty vector was used as a negative control.

Solution A

10 mM	BICINE (Serva)
1 M	Sorbitol
3% v/v	Ethylene glycol (Roth)

pH adjusted at 8.35 with KOH.

Solution B

200 mM	BICINE (Serva)
40% w/v	PEG 1000 (Roth)

pH adjusted at 8.35 with KOH.

Solution C

10 mM	BICINE (Serva)
150 mM	NaCl

pH adjusted at 8.35 with KOH.

YPD medium (1 liter)

10 g	Yeast Extract
20 g	Glucose
20 g	Peptone

SD medium (1 liter)

1.7 g	Yeast Nitrogen base (without amino acids and ammonium sulfate)
5 g	Ammonium sulfate
20 g	Glucose
20 g	Bacto Agar

MM medium (1 liter)

1.7 g	Yeast Nitrogen base (without amino acids and ammonium sulfate)
20 g	Glucose
20 g	Oxoid Agar

2.3.7 Expression in *Xenopus laevis* oocytes

To characterize the functionality of ZmDUR3, *Xenopus laevis* oocytes were injected with 50 ng (50 nl) of *ZmDUR3"Native"* or *ZmDUR3"Modified"* cRNA, as previously described by Dietrich et al. (2004) and Meyer et al. (2006).

cRNA Synthesis

10 µg of both constructs (pBF1-ZmDUR3"Native" and pBF1-ZmDUR3"Modified") were linearized by incubation with the restriction enzyme *MluI* (Fermentas) for 3 hours at 37°C. Subsequently the linearized DNA-fragments were purified by adding 1 volume of phenol:chloroform: isoamylalcohol (25:24:1, pH 8) followed by centrifugation for 15 min at 14,000 g and 4°C. The supernatant was transferred to a new eppendorf tube and the DNA was percipitated by adding 1/10 volume of 3 M NaAcetate and 2.5 volumes of Ethanol 100%. After incubation at 4°C for 20 min, the samples were centrifuged (20 min at 14,000 g) the supernatant was removed and the pellet was washed with ethanol 70%. The pellet was resuspended in 20 µl of RNAse-free water. To detect the quantity and quality of the purified DNA fragment, the samples were analyzed by gel electrophoresis and quantified using the *Nanodrop 2000* spectrophotometer instrument (Thermo Scientific, Wilmington, USA).

1 µg of both linearized constructs were used to synthetized cRNA using AMBION T7 mMESSAGE mMACHINE- Kit (Ambion, Austin, USA) following the manufacturer's instructions. cRNAs were quantified by spectrophotometer and visualized by electrophoresis performed in denaturating conditions (Figure 17).

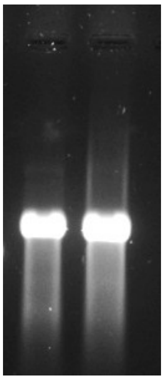


Figure 17. *ZmDUR3"Native"* (left) and *ZmDUR3"Modified"* (right) cRNA samples visualized using EtBr in denaturating 1% agarose gel.

Preparation of Oocytes and RNA injection

Stage V and VI female *Xenopus* oocytes were surgically removed and defoliculated by incubation in Barth's solution containing 50 mg collagenase for 1.5-2 hours at room temperature. Subsequently, oocytes were washed in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES pH 7.4) containing 1 mg ml^{-1} BSA.

After defoliculation and washing oocytes were injected with 50 ng (50 nl) of *ZmDUR3"Native"* cRNA or *ZmDUR3"Modified"* cRNA or water as control and stored in Barth's solution containing 50 µg ml^{-1} of gentamicin, 100 µg ml^{-1} of streptomycin, and 100 U ml^{-1} of penicillin. The injected oocytes were incubated at 16°C for 4-7 days in Barth's solution before two electrode voltage clamp experiments were performed. The experiments were performed at 21 ± 1 °C.

Two Electrode Voltage Clamp Experiments

Oocytes injected with 50 ng (50 nl) cRNA of *ZmDUR3"Native"* cRNA or *ZmDUR3"Modified"* were tested for urea transport by two electrode voltage clamp experiments at an external pH of 5.5. Two electrode voltage clamp experiments were performed as described by Hammes et al. (2010), Voltage pulses were applied for 150 ms and the currents were filtered at 5000 Hz. The analysis was performed exposing the oocytes to "Na-Ringer modified" solution buffered at pH 5.5 with or without the addition of 10 mM urea (the necessary pH adjustment were made).

Urea Uptake Assay

Since under these experimental conditions ZmDUR3 injected oocytes showed no significant induction of current in response to the addition of 10 mM urea to the solution, radiolabeled uptake experiments were performed using ^{14}C-labeled urea as substrate. The transport capacity was evaluated on single oocyte after 4 days from the injection.

For these experiments oocytes were transferred to "modified Na-Ringer" solution buffered with MES at pH 5.5 for 5 minutes in and then incubated for 1 hour in the same solution containing 5-10-50-100-200 µM of radiolabeled urea (20% of ^{14}C-labelled urea, 54 mCi $mmol^{-1}$; ARC American Radiolabeled Chemicals Inc.). Single oocytes were rinsed once with modified Na-Ringer solution (pH 5.5), two times with Na-Ringer modified solution (pH 5.5) containing 20 mM of unlabeld urea and two times with modified Na-Ringer (pH 7.5). Solutions used for washing of the oocytes after the incubation in radiolabeled urea were kept at 4°C. After washing oocytes were transferred to scintillation vials and lysed with 10% sodium dodecyl sulfate (SDS). Then, 4 ml of scintillation cocktail ULTIMA GOLD^{TM} XR (PerkinElmer Life Sciences) were added to the lysed oocytes and the radioactivity was determined using a *LS6500 scintillation counter* (Beckman Coulter, High Wycombe, UK).

Na-Ringer modified solution

115 mM	NaCl
2 mM	KCl
1.8 mM	$CaCl_2$ $2H_2O$
1 mM	$MgCl_2$ $6H_2O$

Buffered with MES or HEPES:

5 mM	MES-NaOH pH5.5
5 mM	HEPES-NaOH pH7.5

pH was adjusted with NaOH.

2.3.8 Transient expression in *Nicotiana tabacum* protoplasts

For the subcellular localization of urea transporter, a transient expression of GFP-fusion proteins in tobacco protoplasts was done as described by Komarova et al. (2012). So, protoplasts of tobacco (*Nicotiana tabacum*) were isolated and transformed with 10 µg of pUC18-ZmDUR3“Modified”-Sp-GFP6 or pUC18-GFP5T-Sp-ZmDUR3“Modified” or with empty vector pUC18-Sp-GFP6. Diameter of tobacco protoplasts was approximately 40 µm. Samples were examined with a SP2 AOBS confocal microscope (Leica Microsystems). Filter settings were 500 to 520 nm for GFP and 628 to 768 nm for chlorophyll epifluorescence detection.

For colocalization experiments, pUC-PTR1-Sp-EYFP (Komarova et al., 2012) was used as marker for plasma membrane. So, tobacco protoplasts were co-transformed with 7.5 µg of pUC18-ZmDUR3“Modified”-Sp-GFP6, or pUC18-GFP5T-Sp-ZmDUR3“Modified”, and 7.5 µg pUC-PTR1-Sp-EYFP. As reported by Kromarova et al. (2012), samples were excited with an argon ion laser at the wavelength of 458 nm for GFP and 514 nm for YFP. Fluorescence was detected at 492-511 nm for GFP and at 545–590 nm for YFP.

3 Results

Most of researches on nitrogen nutrition in higher plants have focused on the physiological and molecular characterization of nitrate and ammonium uptake systems. On the other hand, only few works have provided evidence of urea acquisition and on root mechanisms involved in the process, particularly in crop species. In particular, in maize the root absorption of molecular urea was demonstrated by Coïc et al. (1961) and Mérigout et al. (2008a).

The aim of the present work was to characterize the transport system involved in urea acquisition in maize.

Moreover it is well known that, in soil solution, fertilization with urea may result in a simultaneous exposure of plant roots to different nitrogen sources, such as urea, nitrate and ammonium at least for short time (Mérigout et al., 2008b). In addition the natural occurrence of peptide and aminoacids in soil solution and the rhizosphere (Jämtgård et al., 2010) could led to a coexistence of more organic and inorganic nitrogen sources. Thus uptake of urea was studied with respect to the presence of different nitrogen sources.

In order to limit the urea degradation, maize plants were grown in hydroponic solutions; net uptake of urea was determined measuring its depletion from the solution bathing the roots.

^{15}N-labeled nitrogen sources were also used to monitor root uptake and tissue distribution of nitrogen.

Experiments were also conducted using the most common urease inhibitor (nBTPT), which is often applied in the agricultural soils together with urea fertilizers.

3.1 Study the effect of different nitrogen sources on urea uptake

3.1.1 Morphometric evaluation of maize roots exposed to different nitrogen sources

Preliminary experiments were conducted in order to verify the growth performance of plants supplied with different nitrogen sources: 5 day old maize plants were fed for 1 week with nutrient solution containing as a sole nitrogen source: urea (U), nitrate (N), ammonium (A) or glutamine(Gln), 1mM of total nitrogen (Ntot). Moreover, in order to better simulate soil conditions, plant roots were exposed also to a combination of two nitrogen sources, such as ammonium and nitrate (AN), urea and nitrate (UN), urea and ammonium (UA) or urea and glutamine (UG), 2 mM Ntot. At the end of the treatment the plants were harvested and marked differences among the treatments were observed (Figure 18). In particular the exposition to a specific nitrogen form induced variations in terms of biomass distribution between shoots and roots (Figure 18, A) and changes of root architecture (Figure 18, B.2), while no great differences were noticed in shoot morphology (data not shown).

The supply of U or N (singularly or in combination) allowed a good development of maize plants, although changes in root morphology and biomass production could be highlighted depending on the N-source supplied. In comparison to nitrate, the presence of urea in the extra-radical solution promoted the biomass production and especially stimulated the root development. The beneficial effect of urea was mainly observed as a greater lateral root proliferation on primary and also on seminal roots. In addition to a high density of lateral roots, urea also favoured the elongation of whole root system (primary, seminal and lateral roots). Whereas, under nitrate treatment (N), the density of lateral roots was lower than that induced by urea and was mainly restricted to primary axis.

The beneficial effect of urea on the root development was also appreciated when roots were exposed to a combination of urea and nitrate (UN). In particular, under UN treatment, the positive effect of urea on the primary and seminal root elongation was much more evident than when this organic source was applied singularly (U); while the density and elongation of lateral roots were comparable to those detected in U-fed plants. Interestingly, under UN treatment, it was recorded the greatest biomass production mainly due to higher values in shoot fresh weight than U or N fed plants.

Completely different growth was noticed under ammonium (A) treatment. In agreement with published evidence (Kirkby and Mengel, 1967), A fed plants showed lower leaf and root weight accumulation than all the other treatments. Moreover the leaves were dark green and very small, while root system was very poorly developed and stubby. In general, ammonium exerted a negative effect on the elongation of roots (primary- and seminal-) and led also to a strong suppression of lateral root elongation. Similar root morphology was also observed when ammonium and urea were applied together (UA), in particular the presence of urea was not sufficient to contrast the negative effect of ammonium, since UA fed plants showed drastic limitation in secondary root development.

On the contrary, the nitrate supply reduced strongly the negative effect of ammonium, since ammonium and nitrate fed plants (AN) showed a fresh weight accumulation similar to U or N treatments. Previous evidence about the beneficial effect of nitrate in relieving ammonium effects on plant development was reported also in pepper (Houdusse et al., 2005) and wheat (Garnica et al., 2010).

Glutamine (Gln) has an intermediate effect with respect to A or N treatments and no great differences were noticed when plants where exposed to a combination of glutamine and urea (UG). Interestingly, under both treatments (Gln and UG) the elongation of lateral roots close by root tips resulted to be enchoraged by glutamine. Concerning the UG treatment, the supply of urea did not induced a higher proliferation of lateral roots than Gln fed plants, even if was appreciated a higher elongation of lateral roots throughout the seminal axis.

These results provide clear evidence that, under the experimental conditions employed, nutrient solution-grown maize plants are able to use urea as a sole nitrogen source sustaining the root development and the biomass accumulation in a similar way as when they are fed with nitrate.

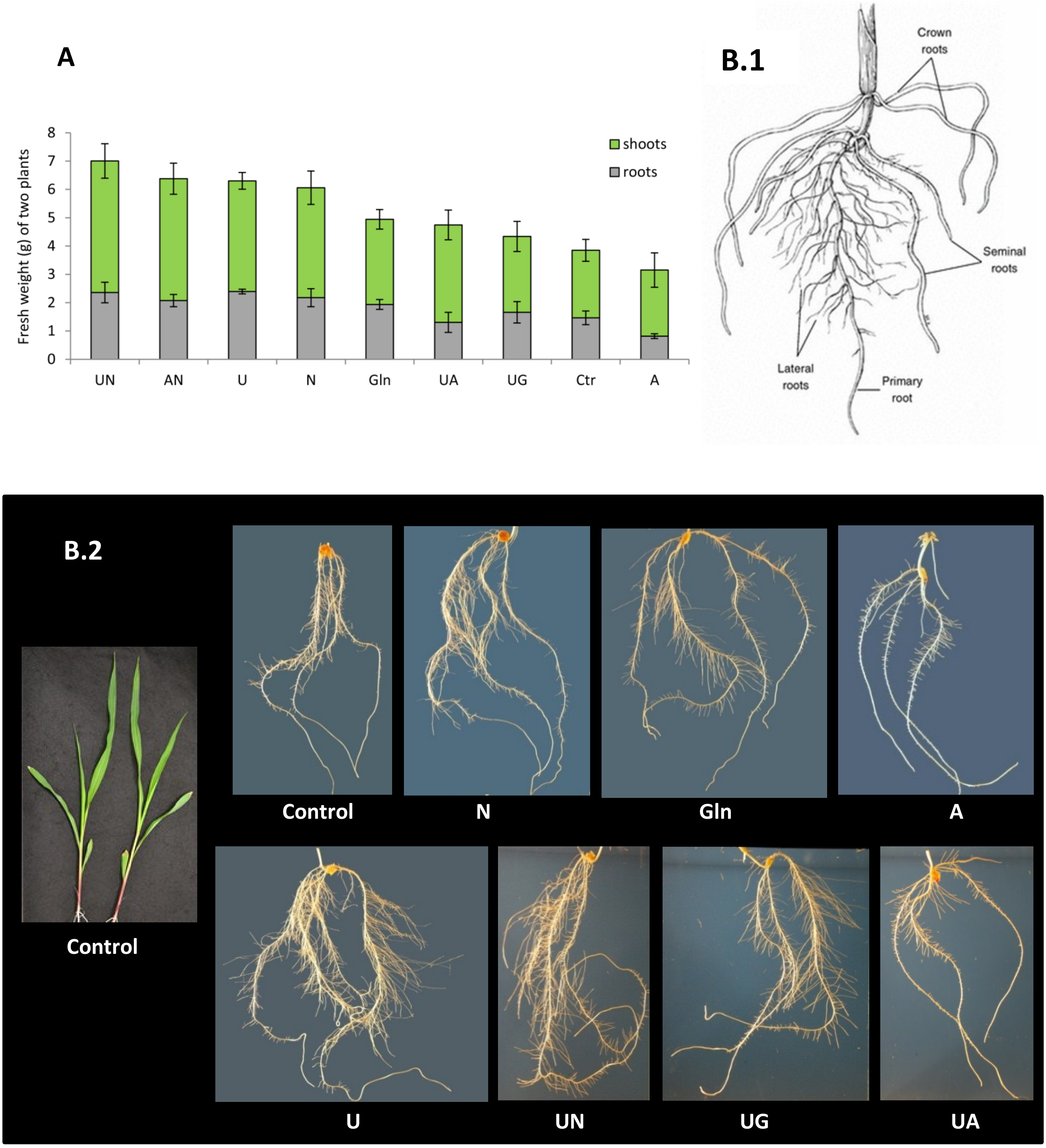


Figure 18. Effect of different nitrogen sources on the fresh weight accumulation of shoots and roots (A) and on the morphology of the root system (B.2) in maize plants by long term experiment (7 days of nitrogen treatments). (B.1) Schematic representation of maize root system (Hochholdinger, 2009). 5 day old maize plants were grown hydroponically for 7 days in nutrient solution containing a) as a sole nitrogen source: urea (U), ammonium (A), nitrate (N) or glutamine (Gln), 1mM Ntot; or b) a combination of two nitrogen sources: urea and nitrate (UN), urea and ammonium (UA), urea and glutamine (UG) or ammonium and nitrate (AN), 2mM Ntot. Control plants were fed with nutrient solution without the addition of any nitrogen form (Ctr). After 7 days of treatment fresh weight of leaf and root tissues were evaluated (the values are means ± SD, n=12).

3.1.2 Dynamics and kinetics of urea uptake in maize roots

3.1.2.1 Kinetic characterization of urea uptake in intact roots

To verify the capacity of maize roots to take up urea, a concentration dependent net-influx analysis was performed.

The experimental procedure involved 5 day-old maize plants grown hydroponically without added nitrogen. Before the uptake experiment, plants were exposed for 4 hours to a nutrient solution containing urea (1 mM) as a sole nitrogen source (urea treatment) or without (Control). Net uptake rate was measured by depletion approach from a solution containing 5 to 300 µM urea (Figure 19).

In control plants rate of urea uptake by maize roots showed a typical saturation kinetic corresponding to the Michaelis-Menten model. These results are compatible with the presence of a high affinity transport system and allow the definition of the kinetic parameters (K_m and V_{max}) for urea transport.

Interestingly, the exposition of roots to urea before the uptake assay induced modifications in kinetic parameters. Indeed the net influx of urea in roots of pre-treated plants was 2.5 fold higher as compared to control plants, with V_{max} values of 20 and 8 µmol urea g^{-1} FW h^{-1}, respectively. The urea treatment also affected the K_m value, which increased in treated plants more than 6 times with respect to untreated control plants (27 and 4 µM, respectively). These results indicate that, in maize roots, urea induces its own uptake causing a modification of the kinetic parameters in the high affinity concentration range.

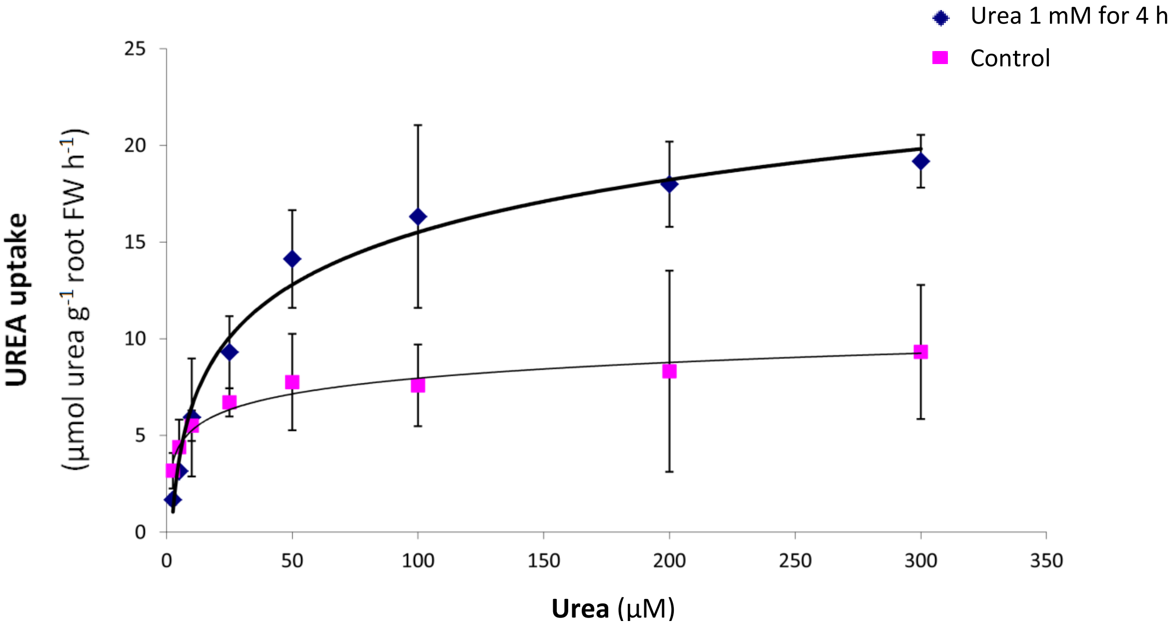


Figure 19. Kinetic assay of urea net uptake by maize roots. The concentration-dependent uptake was carried out using 5 day-old maize plants exposed for 4 h to a nutrient solution supplied with urea 1 mM as a sole nitrogen source or not (Control). Afterwards roots were incubated for 10 minutes in the assay solution containing urea at different concentrations (5-10-25-50-100-200-300 µM). Values are means ± SD (n=3).

3.1.2.2 Effect of urea concentration treatment on its uptake

Based on previous results, a further experiment was planned with the aim to assess if the uptake capacity could be modulated by urea pre-treatment, depending on incubation time and concentration of the molecule. For this purpose maize plants were exposed for up to 24 hours to nutrient solutions containing urea at different concentrations (0.5-2 mM urea). Every two hours a group of six plants from each treatment was transferred in the uptake assay solution containing 200 µM urea (Figure 20), where the net uptake rate was measured by depletion approach.

Results corroborated previous observation whereby plants exposed to urea treatment increased their ability to acquire this organic nutrient in comparison to untreated plants. In particular during the incubation time, the uptake capacity was modulated following a bell-shape curve. Plants fed with 1 mM urea rapidly increased their uptake capacity, which reached the peak after 4 hours of treatment (15 µmol urea g^{-1} root FW h^{-1}): afterwards the rate decreased showing, after 24 hours, values lower than those registered for untreated plants. On the other hand, during the 24 hours period no significant change of the net uptake rate was observed in untreated plants, which maintained a constant value of 5 µmol g^{-1} root FW h^{-1}. These data revealed that (high affinity) urea transport system in maize roots is a substrate-inducible process, possibly involving retro-regulation mechanisms. Moreover the comparison between urea treatments revealed that plants exposed to higher urea concentration before the uptake assay reached the highest level of induction in a time shorter than plants fed with lower concentrations of urea. For example plants treated with 2 mM urea (4 mM Ntot) reached the peak of uptake after 2 h of exposition to urea, while those treated with 0.5 mM urea (1 mM Ntot) reached the maximum induction 6 hours later.

These data suggest that in maize roots the induction of urea uptake system is dependent on the concentration and time of exposure to this organic N-source.

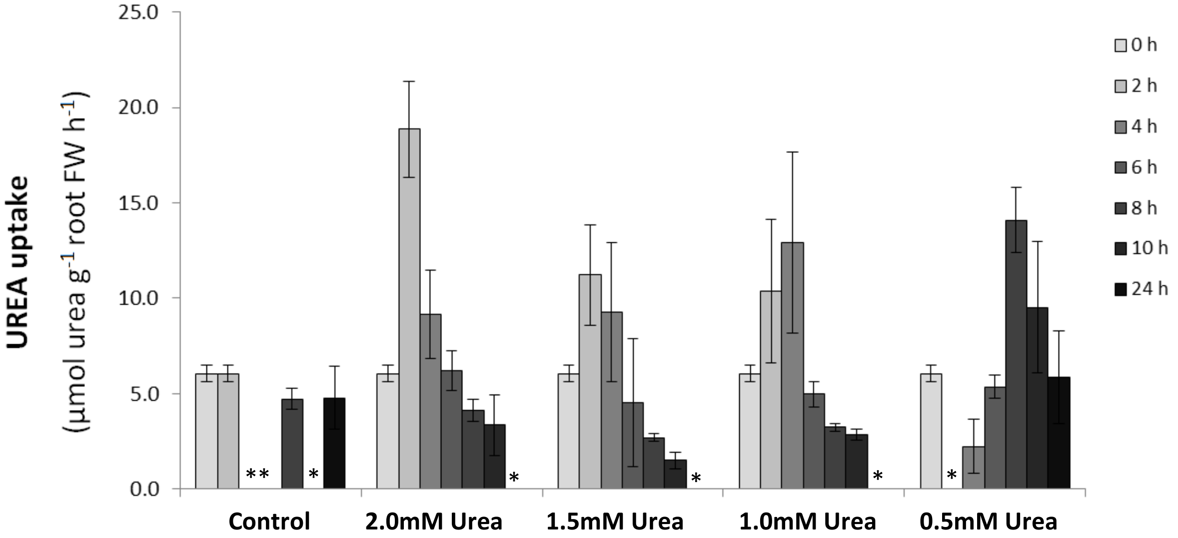


Figure 20. Time course of urea uptake in maize roots. 5 day-old maize plants were exposed up to 24 hours to a nutrient solution supplied with urea at different concentrations (0.5-1-1.5-2 mM urea) or without addition of nitrogen sources (Control). Every two hours groups of six plants from each treatment were transferred in the assay solution containing 200 µM urea. The values are means ± SD (n=3); (*) missing sample.

3.1.3 Effect of different nitrogen sources (organic and inorganic) on the net urea uptake capacity by maize plants

From previous experiments it was well evident that the exposition of plants for long periods (7 days) to different nitrogen sources induced changes in root morphology and fresh weight accumulation in shoots and roots (Figure 18). Furthermore it was demonstrated that maize roots are able to take up urea from a low-concentration solution suggesting the involvement of a high affinity transport system which is induced by urea itself (Figure 19). However in soil solution roots are normally exposed to a pool of nitrogen inorganic (ammonium and nitrate) and organic (mainly aminoacids, peptides and urea) sources; furthermore interactions with respect to uptake and assimilation of different nitrogen sources have been reported (Garnica et al., 2009; 2010). Aim of this part of the work was to determine the effect that the presence of these N-sources in the root external solution could have on urea uptake and, on the other side, to ascertain if urea could influence the uptake of others N-sources, such as nitrate.

3.1.3.1 An overview on the influence of nitrogen sources on urea uptake

In a preliminary set of experiments, it was investigated the effect of a large pool of nitrogen sources (such as ammonium, nitrate or amino acids) on the urea uptake system. In particular these inorganic and organic sources can occur in soil solution, even if their bio-availability for plant nutrition and their concentrations are subject to great fluctuations (Gaudin et al., 1987; Miller and Cramer, 2004; Jämtgård et al., 2010). Plants were fed with nutrient solution containing as unique nitrogen source: urea (U) or ammonium (A) or nitrate (N) (2 mM Ntot) or single aminoacids (Glycine, Arginine, Aspartate, Asparagine, Glutamate or Glutamine, 1mM). In three treatments plants were fed with a combination of two nitrogen sources (4 mM Ntot): urea and ammonium (UA), urea and nitrate (UN) or ammonium and nitrate (AN). Uptake capacity was measured in plants pre-treated for 4 hours with the appropriate N-source(s) and then transferred to the assay solution containing 200 µM urea (Figure 21).

Consistent with previous results, the exposition of plants to urea induced its own uptake reaching values around 25 µmol urea g^{-1} root FW h^{-1}. All the other treatments were able to induce only a limited urea uptake; urea uptake rates of glutamine-treated plants were very close to those recorded for control untreated plants. In general the rate levels detected in plants treated with different amino acids were quite similar, with the exception of glutamine. Indeed, the lowest uptake rate was registered under glutamine treatment.

Interestingly the presence of urea in combination with the inorganic N-sources (UA and UN treatments) did not lead to uptake rates comparable to those observed when urea was supplied alone; data rather resembled those registered for sole nitrate or ammonium treatments, with AN treatment causing even net influx rates lower than those observed when the two inorganic sources were applied singularly (A or N treatments).

These results suggest that the induction of urea uptake was a specific response to the presence of urea itself in the external solution and that the contemporary presence of the two most common inorganic sources during the induction period can limit its subsequent uptake.

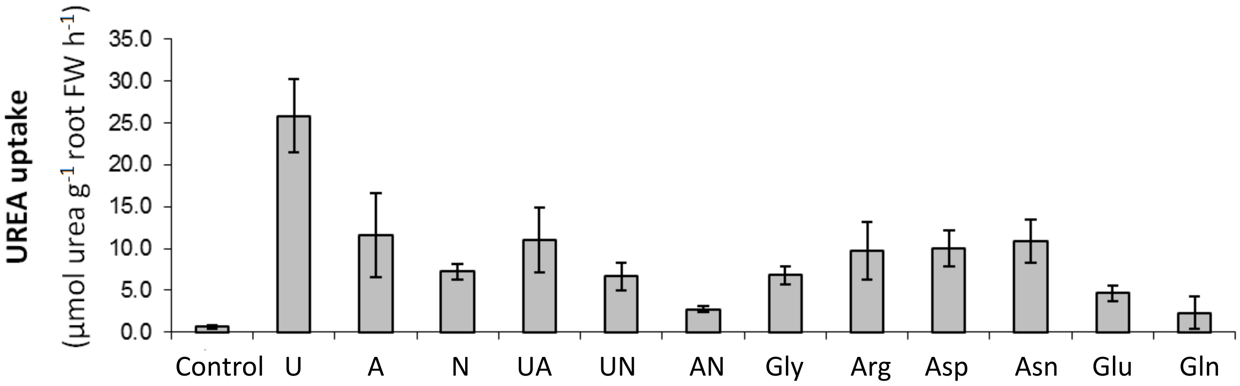


Figure 21. Urea uptake by roots of maize plants pre-treated with different N-sources. 5 day-old maize plants were exposed for 4 hours to a nutrient solution containing as a sole N-source: urea (U, 2 mM Ntot); ammonium (A, 2 mM Ntot); nitrate (N, 2 mM Ntot) or single amino acidc (glycine, Gly; arginine, Arg; aspartate, Asp; asparagine, Asn; glutamate, Glu; or glutamine, Gln; at concentration of 1 mM). As control some plants were exposed to nutrient solution without addition of nitrogen sources (Control). Combinations of different N-forms were also used: urea and ammonium (UA), urea and nitrate (UN) or ammonium and nitrate (AN), 4 mM Ntot. After 4 hours, groups of six plants from each treatment were transferred to the assay solution containing 200 µM urea. The values are means ± SD (n=3).

3.1.3.2 Reciprocal influence of nitrate and urea on their acquisition systems

The interaction at the level of root uptake between the two main nitrogen forms used in agricultural soils, urea and nitrate, was investigated exposing maize plants to a combination of these two N-sources for a definite period of time (up to 24 h). During this period the changes in the urea and nitrate uptake rates were measured from solutions containing each N-source (urea or nitrate) at 200 µM concentration. Interestingly 24 hours of exposition to the different nitrogen treatments determined small changes in lateral root development (Figure 22), but with no significant effect on root fresh weight accumulation (data not showed). This allowed a comparison among treatments with respect to the functionality of uptake systems without side effects of different growth rates.

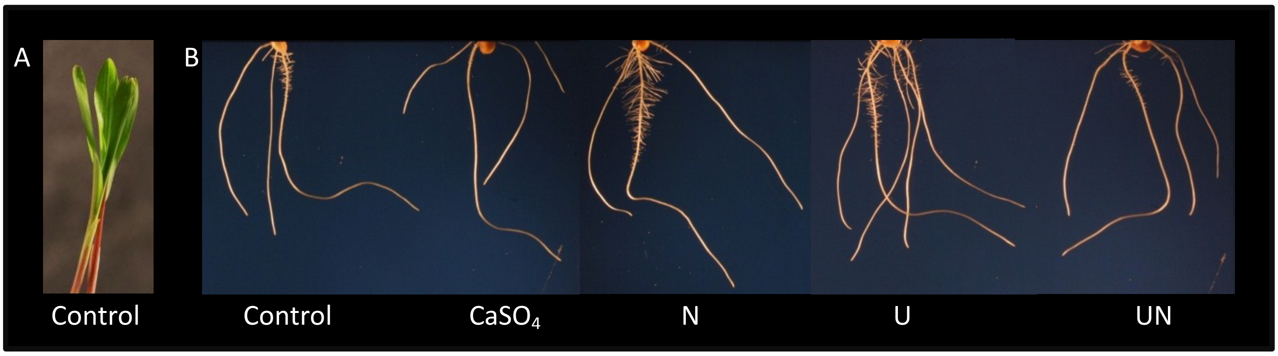


Figure 22. Effect of different nitrogen source on the root morphology of 5 day-old maize plants. Plants were exposed for 24 hours to a nutrient solution containing as a sole nitrogen source: urea (U, 1 mM Ntot), nitrate (N, 1 mM Ntot) or urea and nitrate together (UN, 2 mM Ntot). As control plants were grown in nitrogen deficient nutrient solution (Control) or in calcium sulfate solution (0.5 mM $CaSO_4$). A) upper parts of two control plants; B) root systems under different nitrogen treatments.

3.1.3.2.1 Effect of nitrate on the net urea uptake

Corroborating previous results, the exposition of maize plants to urea (U treatment, 1 mM Ntot) induced the high-affinity uptake system for the molecule, with a maximum influx recorded after 8 hours of treatment (14 µmol urea g^{-1}root FW h^{-1}). As previously reported, untreated plants (Control) showed a constant uptake rate ranging from 3 to 4 µmol urea g^{-1} root FW h^{-1}.

The induction of urea uptake was much reduced in plants treated with nitrate and urea (UN treatment, 2 mM Ntot), 7 µmol urea g^{-1} root FW h^{-1} after 8 hours, suggesting a control of urea uptake by nitrate availability (Figure 23).

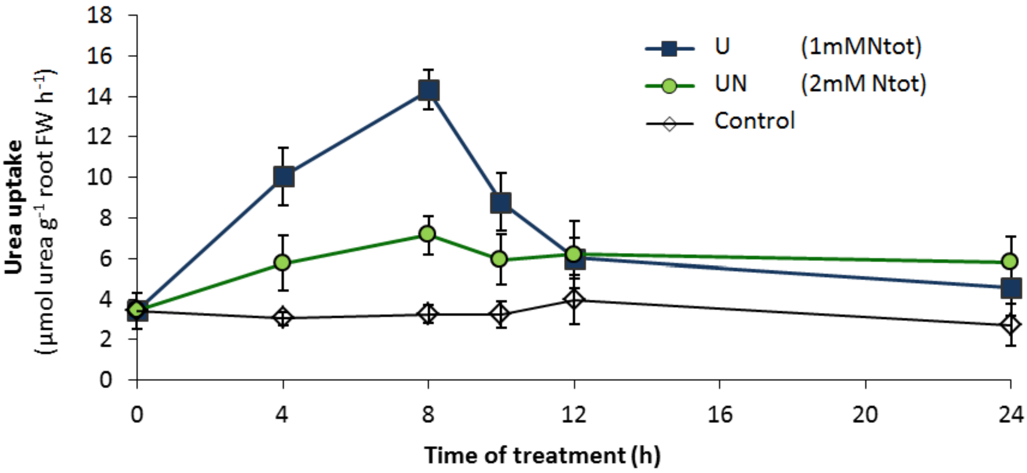


Figure 23. Time-course of urea uptake in maize roots. 5 day-old maize plants were exposed up to 24 hours to a nutrient solution without added nitrogen (Control) or supplied with 0.5 mM urea (U, 1 mM Ntot) or urea and nitrate (UN, 2 mM Ntot) or. After 4 - 8 - 12 and 24 hours of treatment, groups of six plants from each treatment were transferred to the assay solution containing 200 µM urea. The values are means ± SD (n=3).

3.1.3.2.2 Effect of urea on the net nitrate uptake

The high affinity transport system for nitrate has been extensively studied, showing that it is up-regulated by nitrate itself. The activity of this transport system typically exceeds plant demand for nitrate uptake, so that after the initial exposure to nitrate it is rapidly down-regulated (Glass and Siddiqi, 1995; Forde and Clarckson, 1999). Furthermore, it has been demonstrated that pre-treatment of plants with ammonium can negatively affect the development of a higher nitrate uptake rate (Locci et al., 2001).

Experiments were performed using maize plants pre-treated for up to 24 hours with nitrate alone (N, 1 mM Ntot) or nitrate plus urea (UN, 2 mM Ntot) and compared with untreated plants (Control) with respect to their capacity to take up nitrate from a 200 µM solution (Figure 24).

Results confirmed the induction of nitrate uptake systems and highest rates were observed after 8 hours of exposure to the anion (15 µmol nitrate g^{-1} root FW h^{-1}). Interestingly in UN fed plants the presence of urea limited the development of a higher nitrate uptake capacity: after 8 hours the uptake rate was 9 µmol nitrate g^{-1} root FW h^{-1}. A general increase in the uptake activity was observed until 8 hours but subsequently it slowly decreased returning at the same level of control plants.

As expected untreated plants did not modulate their nitrate uptake rate during the 24-hours treatment showing a constant value around 4-5 µmol nitrate g^{-1} root FW h^{-1}. Results indicate that the presence of urea in the external medium can limit the response of the roots to nitrate.

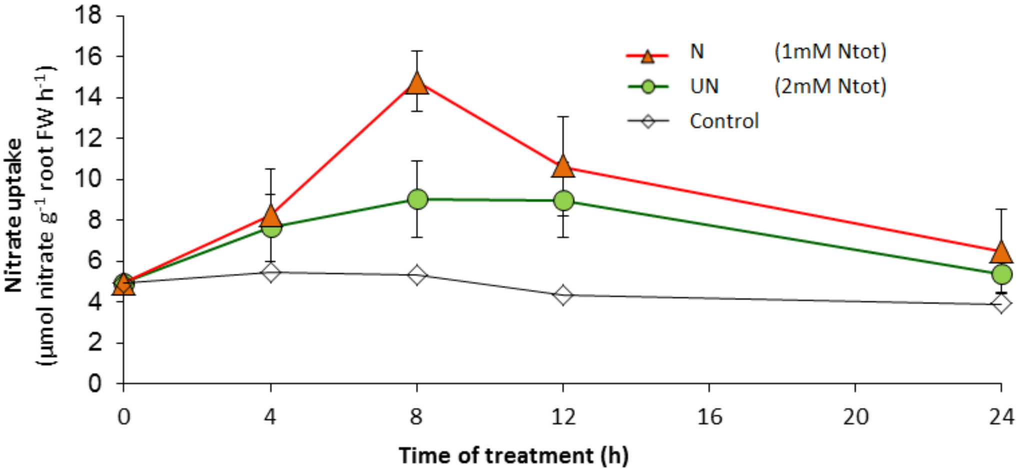


Figure 24. Time-course of nitrate uptake in maize roots. 5 day-old maize plants were exposed up to 24 hours to a nutrient solution without added nitrogen (Control) or supplied with nitrate (N, 1 mM Ntot) or urea plus nitrate (UN, 2 mM Ntot)r. After 4, 8, 12 and 24 hours of treatment, groups of six plants from each treatment were transferred in the assay solution containing 200 µM nitrate. The values are means ± SD (n=3).

3.1.3.2.3 Influence of nitrogen concentration on urea and nitrate uptake

In the above experiments the nitrogen concentration in the combined (UN) treatment was twice that those with a single N-source (U or N), for this reason additional experiments were performed exposing plants to treatments having the same nitrogen concentration (Figure 25). Thus maize plants were pre-treated with solutions containing only urea (U, 2 mM Ntot) or nitrate (N, 2 mM Ntot) or urea and nitrate together (UN treatment, 2 mM Ntot). Confirming previous data, the increase in urea concentration determined an earlier response of uptake system leading, in U plants, to a maximum induction after 4 hours of exposure to the organic N-source. The same behavior was relieved also in nitrate (N) fed plants corroborating published works which described the induction of nitrate high affinity transport system as concentration dependent (Crawford and Forde, 1998; Locci et al. 2001). However, as previously observed, in UN treatment no significant variation in the uptake rate of each N-source was registered during the 24-h treatment.

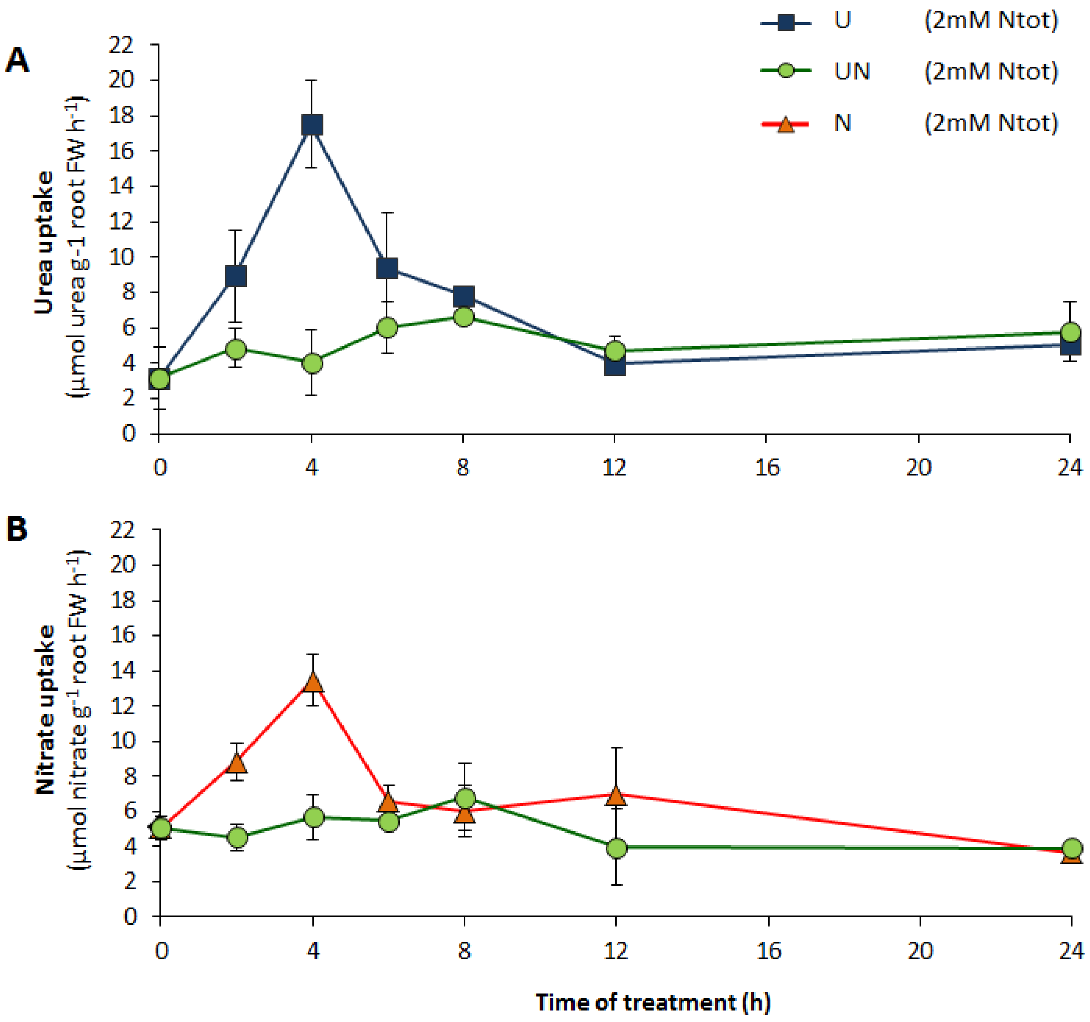


Figure 25. Time-course of Urea (A) and nitrate (B) uptake in maize roots. 5 day-old maize plants were exposed within 24 hours to a nutrient solution without added nitrogen (Control) or containing the same amount of total nitrogen (2 mM Ntot), namely 1 mM urea (U), 2 mM nitrate (N) or 0.5 mM urea and 1 mM nitrate (UN). After 2, 4, 6, 8, 12 and 24 hours of treatment, groups of six plants from each treatment were transferred to the assay solution containing 200 µM urea (A) or 200 µM nitrate (B). The values are means ± SD (n=3).

In summary these results (Figure 23-Figure 25) indicate that under the experimental conditions of this study, the presence of nitrate together with urea (NU treatment) limited the development of a higher uptake capacity by the high-affinity transport system of both nutrients.

3.1.3.3 Effect of organic nitrogen sources on the net urea uptake

In the following experiments it was studied the effect on urea uptake system exerted by another organic source which is known to have a regulatory role in nitrogen acquisition, the amino acid glutamine (Sugiharto et al., 1992, Stoelken et al., 2010). Thus, 5 day-old maize plants were fed for a maximum of 24 hours with a nutrient solutions containing as a sole N-source glutamine (Gln, 1 mM Ntot) or nitrate (N, 1 mM Ntot) or urea (U, 1 mM Ntot); furthermore combinations of two different N-sources were employed: urea and glutamine (UG, 2 mM Ntot) or urea and nitrate (UN, 2 mM Ntot). After 2, 4, 8, 12 and 24 hours of treatment, the urea uptake capacity of plants was measured by incubating the roots in the assay solution containing urea 200 µM (Figure 26).

During the exposition time, the urea influx was modulated by the external N-source. In particular, as observed above, the highest value in urea uptake was registered in U fed plants, while the lowest values were detected in Control plants, without significant changes with the time. Intermediate values were detected for nitrate treatment (N), reflecting the same trend observed in U fed plants. On the other hand, in Gln treated plants, the trend was quite similar to Control and values did not show great variation with the time of exposure, although a little increase was observed after 24 hours of treatment.

UG and UN fed plants showed almost the same pattern of urea uptake with values roughly comparable to those observed when nitrate was the sole N-source.

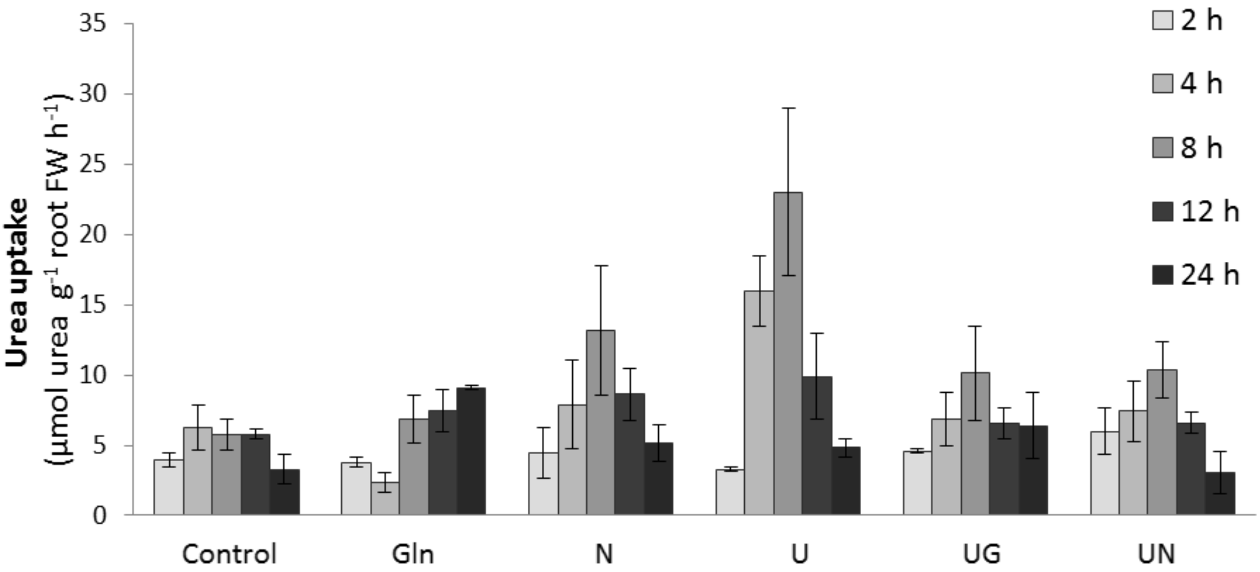


Figure 26. Time-course urea uptake in maize roots. 5 day-old maize plants were exposed up to 24 hours to a nutrient solution without added nitrogen (Control) or supplied with glutamine (Gln) or nitrate (N) or urea (U) (1 mM Ntot). Others two treatments containing urea together with glutamine (UG) or nitrate (UN), 2 mM Ntot. After 2, 4, 8, 12 and 24 hours of exposition, groups of six plants from each treatment were transferred to the assay solution containing 200 µM urea. The values are means ± SD (n=3).

3.1.4 Analysis of urea contribution to nitrogen accumulation in maize and Arabidopsis plants using ^{15}N-labeled sources

3.1.4.1 Urea absorption in shoot and roots of maize plants fed with ^{15}N-labeled nitrogen sources

Previous physiological experiments were performed in order to characterize the urea uptake systems in maize roots and the influence of organic and inorganic nitrogen sources on their activity. However this kind of analysis cannot provide an indication about the contribution of a specific source to the overall plant N-nutrition. Thus, nitrogen sources labeled with the stable isotope ^{15}N were also used to study urea absorption by root cells. The accumulation of ^{15}N analyzed within the plants can be regarded as being derived from external ^{15}N-urea absorbed by the roots. Nitrogen accumulation using ^{15}N-ammonium and ^{15}N-nitrate was also measured as compared to the urea treatment and to reveal possible effects of nitrate nutrition on the urea uptake system. Operatively, maize plants were fed with nutrient solution containing ^{15}N-ammonium (A), ^{15}N-nitrate (N), ^{15}N-urea (U), ammonium and ^{15}N-nitrate (AN), ^{15}N-urea and nitrate (UN) or, as a control, without any addition of nitrogen sources (Control). A, N, U -fed plants were exposed to 1 mM total nitrogen, while in AN and UN treatment the total nitrogen concentration was 2 mM. Plants were incubated with labeled sources for 24 hours, afterwards shoot and root samples were harvested and the ^{15}N-accumulation was measured (Figure 27).

The highest levels in shoot and root ^{15}N-accumulation were reached in ^{15}N-nitrate-fed plants (N and AN treatments); in particular its uptake was not affected by the contemporary presence of ammonium. Under both treatments (N and AN), the amounts of ^{15}N were almost equally distributed among shoots and roots. Compared to N treatment, much lower ^{15}N contents were found in ^{15}N-U-fed plants, although ^{15}N distribution pattern among the tissues was similar to that found feeding plants with ^{15}N-nitrate.

Concerning the total amounts of ^{15}N in plants, under ammonium treatment (A) an intermediate situation between N and U fed plants was reported, with significant differences in terms of allocation among root and leaf tissues.

In A fed plants the amount of ^{15}N accumulated in roots was almost 2-fold higher than that allocated in leaves, which showed a ^{15}N content comparable to that detected in leaves of U-treated plants.

Interestingly, confirming the physiological results reported above (Figure 23-Figure 25), urea uptake was reduced when this N-source was applied in combination with nitrate. In particular, as compared to U fed plants, UN shoots and roots showed 2-times lower ^{15}N accumulation, with essentially the same ^{15}N distribution among tissues.

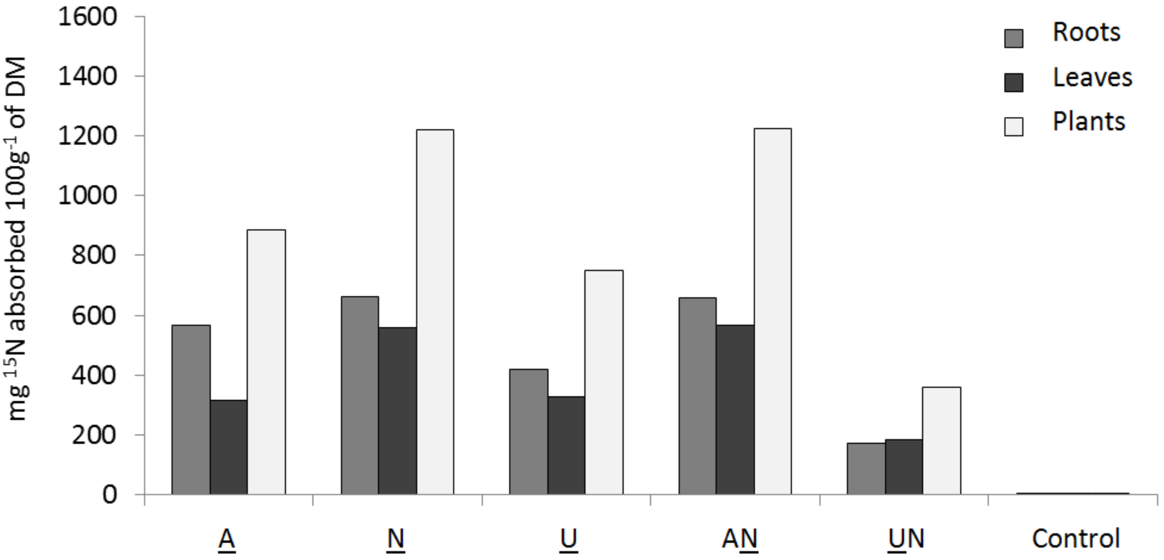


Figure 27. ^{15}N accumulation in maize plants fed with different ^{15}N-sources. Root and shoot ^{15}N accumulation was measured in 5-day-old maize plants fed for 24 hours with nutrient solutions supplied with different labeled nitrogen sources: ^{15}N-ammonium (A), ^{15}N-nitrate (N), ^{15}N-urea (U), ammonium and ^{15}N-nitrate (AN), ^{15}N-urea and nitrate (UN). As control plants treated with solution without any addition of nitrogen sources were used. The underlined letters are referred to the ^{15}N-labeled source. Nitrogen concentration in A, N and U treatments was 1 mM, while it was 2 mM in AN and UN treatments. The analysis was performed on six plants for each sample.

3.1.4.2 Time course of urea absorption in Arabidopsis plants fed with ^{15}N labeled nitrogen sources

At the time of analysis, Arabidopsis was the only plant species in which the high-affinity urea uptake system was already characterized. In particular experimental evidence of ^{15}N-urea influx in Arabidopsis roots was reported only in two works. Kojima et al. (2007) for the first time characterized *in vivo* the high- and a low-affinity systems for urea uptake. Later Mérigout et al. (2008a) performed a more thorough physiological characterization confirming the capacity of Arabidopsis roots to take up urea and demonstrating that urea uptake was regulated by the initial nitrogen status of the plants. These authors also showed that ammonium nitrate could influence urea influx, but without discriminating the effect of each inorganic source.

In the present work a time-course of ^{15}N accumulation analysis was performed in Arabidopsis plants pre-grown under different N-nutritional regimes and than treated with different ^{15}N-labelled N-sources. Thus, 5 week-old plants were grown for five weeks in hydroponic nutrient solution and than transferred for one week to N-sufficient or N-deficient conditions. At the end of the growing period leaves of plants grown without added nitrogen showed clear symptoms of starvation. For the uptake experiment, nitrogen sufficient (N-sufficient) and nitrogen deficient (N-deficient) plants were incubated for up to 24 hours in nutrient solutions containing different ^{15}N-labeled nitrogen sources: ^{15}N-ammonium (A), ^{15}N-nitrate (N), ^{15}N-urea (U), urea and ^{15}N-nitrate (UN), ^{15}N-urea and nitrate (UN); leaves and roots were harvested after 2, 4, 8, 12 and 24 hours for the ^{15}N determination.

In general, under all the treatments imposed, leaf and root tissues displayed a progressive increase during the incubation time in ^{15}N-labeled nitrogen contents (Figure 28, B and C).

3.1.4.2.1 Total ^{15}N accumulation in Arabidopsis plants

Results showed that under N-deficiency, $\underline{A}$ or $\underline{N}$ treated plants accumulated higher amounts of nitrogen in comparison to plants grown with an adequate nitrogen supply.

The $\underline{A}$ and $\underline{N}$ treatments were the most efficient nitrogen forms leading to the highest accumulation of ^{15}N in maize plants grown under N-deficiency (Figure 28, A). In contrast to inorganic sources, the urea was acquired at lower levels comparable to those found in nitrogen sufficient plants treated with labeled nitrate ($\underline{N}$).

About UN treatments (U$\underline{N}$ and $\underline{U}$N) two general observations should be considered: first, in UN total nitrogen concentration was 2-fold higher (2 mM tot) than in the single-source treatments (1 mM tot); second, to have an indication of the total nitrogen absorbed, the contribution from both labeled treatments, U$\underline{N}$ and $\underline{U}$N should be evaluated. Under N-deficiency the presence of nitrate didn't limit the urea-derived ^{15}N accumulation in maize plants. On the other hand urea showed a negative effect on ^{15}N-nitrate acquisition in Arabidopsis.

3.1.4.2.2 ^{15}N accumulation in roots

In general roots showed higher amounts of nitrogen than those found in leaves (Figure 28, B); moreover the accumulation patterns reflected those registered in whole plants with the exception of U$\underline{N}$ roots. In this case, after 12 and 24 hours, the amounts of ^{15}N were quite similar and the maximum accumulation (24 h) was significantly lower than that observed in $\underline{N}$ or $\underline{U}$ treated roots.

3.1.4.2.3 ^{15}N accumulation in leaves

Strong differences within the treatments were relieved in leaves (Figure 28, C). In particular, plants exposed to labeled nitrate ($\underline{N}$) led to the highest ^{15}N content in leaves either in N-sufficient of N-deficient leaves. In these latter plants comparable amounts of ^{15}N accumulation was also observed in U$\underline{N}$ -treated plants, while the values registered in $\underline{A}$ treatment were lower. Independently of the nitrogen status of the plants, ^{15}N-urea treatment ($\underline{U}$ and $\underline{U}$N) showed the lowest accumulation of labeled nitrogen as being derived from urea.

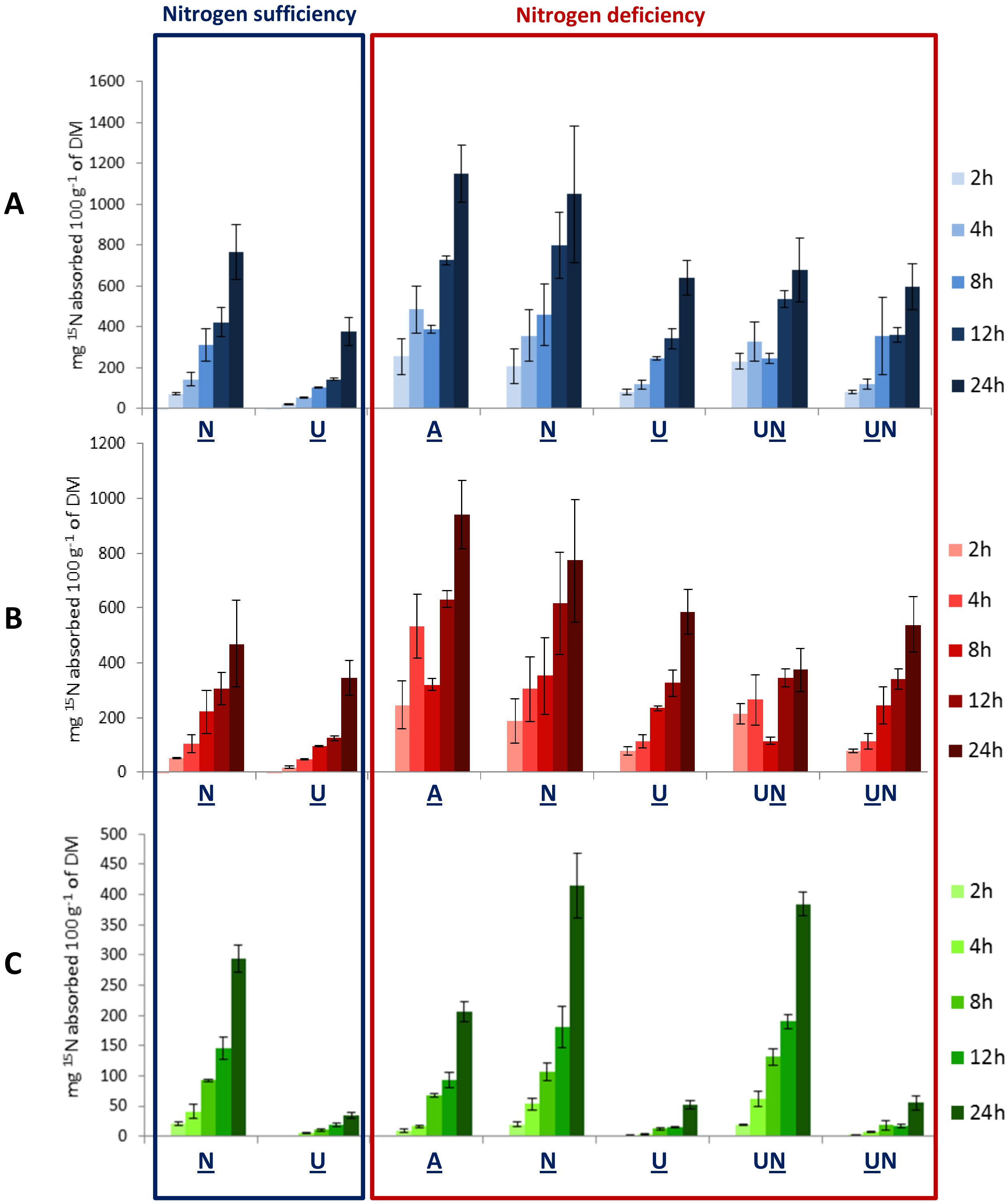


Figure 28. Time-course of ^{15}N accumulation in 6 week-old Arabidopsis plants (A), in roots (B) and in leaves (C). The growth conditions (complete nutrient solution) were the same for all the plants until the 5th week; afterwards some plants were maintained for 1 week in N-sufficiency (2 mM nitrogen, in the blue box on left side) while other were grown for 1 week without added nitrogen (N-deficiency, in the red box on right side). For the ^{15}N uptake experiment plants were exposed for up to 24 hours to nutrient solutions containing different ^{15}N-labeled nitrogen sources: ^{15}N-ammonium (A), ^{15}N-nitrate (N), ^{15}N-urea (U), urea and ^{15}N-nitrate (UN), ^{15}N-urea and nitrate (UN). The underlined letters are referred to the ^{15}N-labeled source. Nitrogen concentration in A, N and U treatments was 1 mM, instead two times (2 mM) in UN and UN treatments. Roots (B) and leaves (C) from three plants of each treatment were sampled and the ^{15}N accumulation was detected. The first histogram (A) represents the total ^{15}N absorbed in plants. The values are means ± SD of three replicates.

For the first time in maize, the effect of urea on the amounts of nitrogen taken up by the external nitrogen source was evaluated using ^{15}N-urea. On the other hand, previous works were performed on Arabidopsis (Mérigout et al., 2008a); thus further ^{15}N analyses on this model plant were done to evaluate the experimental conditions.

In summary, the ^{15}N accumulation data provided a valid indication that Arabidopsis and maize are able to take up urea and to translocate urea *per se* or urea-derived N-compounds to the shoot. Interestingly, in agreement with the urea uptake rates, UN-fed plants showed a lower accumulation of ^{15}N derived from ^{15}N-urea in both roots and shoots as compared to urea treatment (U). So, a hypothesis is that nitrate exerts negative effects on the urea uptake in plants; indeed nitrate limits the induction of the urea uptake system conceivably determining low amounts of nitrogen as being derived from urea. The reciprocal influence between urea and nitrate was analysed in more detailed in Arabidopsis plants. Interestingly, data clearly indicated that these two plant species react in a different way to nitrogen nutrition: in Arabidopsis nitrate did not exert adverse effect on the accumulation of ^{15}N-urea derived ^{15}N; thus urea contribution to nitrogen plant nutritrion under U and UN treatments was the same, in both roots and shoots.

On the other hand, in UN fed Arabidopsis plants, the presence of urea in the external medium limited the ^{15}N accumulation as being derived from ^{15}N-nitrate. This data is in agreement with the negative influence of urea on nitrate uptake reported by Mérigout et al. (2008a). Moreover, the low nitrogen amount was mainly due to a limited accumulation of the nutrient in roots more than in shoots since no significant variations were detected between N and UN treated leaves. These data suggest that the negative effect of urea on nitrate is related to root accumulation whereas the translocation of the nutrient to shoots is not influenced. A hypothesis could be a regulatory control of urea (or of related metabolites) on the mechanism of nitrate efflux in roots.

3.1.5 Stability of the treatment solution

In order to analyze the stability of the nutrient solutions, the amounts of urea, ammonium and nitrate were checked during the time span of the experiments. Nutrient solutions containing: urea (U, 1 mM Ntot), nitrate (N, 1 mM Ntot) or urea and nitrate (UN, 2 mM Ntot) were sampled after 2, 4, 8 and 24 hours of exposition to maize roots. During the time span of the experiments (24 hours) was observed a decreased in the concentrations of nitrate and urea in a range that was in main part attributed to the root uptake activity. In particular in the solutions containing urea, very few amounts of ammonium were detectable only after 24 hours of root incubation, suggesting that under the experimental conditions used no significant urea degradation took place. This observation was similar to that reported by Mérigout and coworkers (2008b) and by Wang and coworkers (2012).

3.1.6 Effect of urease inhibitor on the net urea uptake

Although under our experimental conditions we did not find a significant urea degradation, it is well accepted that in soil solution the stability of urea is completely different (Watson, 2005). It is subject to a rapid degradation due to the presence of microbial ureases, which are the most persistent enzymes in nature and almost ubiquitously expressed by most organisms (Polacco and Holland, 1993). The urease activity limits the efficiency of urea, since its hydrolys produces ammonium, which is rapidly volatilized as ammonia gas. So one of the most used strategies to reduce ammonia emissions from urea fertilizer is to apply urease inhibitors. The most promising and widely tested soil urease inhibitor is N- (n-butyl) thiophosphoric triamide (nBTPT), whose inhibitory activity is associated with the formation of its oxygen analogue. To date nBTPT is one of the few urease inhibitors that are commercially available and it is also marketed in combination with urea fertilizers (Watson, 2005).

Generally it has been demonstrated that nBTPT enhances efficiency of urea-containing fertilizers in soil, even preventing potentially toxic accumulation of urea degradation products (Watson, 2005); on the other hand, it has also been evidenced that the presence of the inhibitor can induce some phytotoxicity, possibly as a consequence of excessive urea accumulation in plants (Gerendàs and Sattelmacher, 1999).

To investigate the effect of this inhibitor on the urea uptake, an experiment was performed using the same growth and treatment conditions reported above. Thus net urea uptake rates were measured on the whole root system during 24 hours of contact with urea in presence or absence of 0.897 µM nBTPT. 5 day-old maize plants were grown in complete nutrient solution containing: urea (U, 1mM Ntot); urea +

nBTPT (U+nBTPT, 1 mM Ntot); urea and nitrate + nBTPT (UN+nBTPT, 2 mM Ntot); urea and ammonium + nBTPT (UA+nBTPT, 2 mM Ntot).

After 4, 8, 12 and 24 hours of treatment, the urea net influx was measured in an assay solution containing 200 µM urea.

Figure 29 shows that the presence of nBTPT altered the pattern of urea net uptake rates following exposure of roots to urea alone (U). In particular during the time span of the treatment with U+nBTPT, the induction of urea uptake was severely limited: constant values of uptake rates, not significantly higher than those detected in control plants, were found between 4 and 24 hours.

Similar patterns were also found in urea and nitrate or ammonium treatments in presence of the inhibitor (UN+nBTPT and UA+nBTPT treatments). In particular in urea and ammonium, after 24 hours, the uptake rate was lower than control plants maybe due to retro-regulatory events.

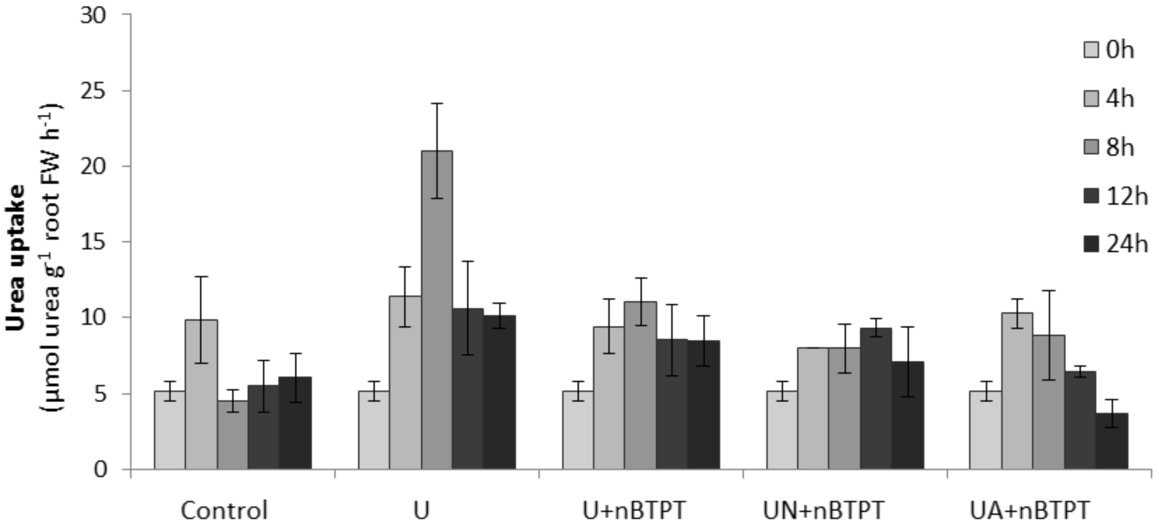


Figure 29. **Effect of the urease inhibitor (nBTPT) on the time-course of urea uptake in maize roots.** 5 day-old maize plants were exposed up to 24 hours to a nutrient solution without added nitrogen (Control) or supplied with urea (U, 1 mM Ntot); the effect of the urease inhibitor on urea uptake was tested using pre-treatment solutions containing: 0.897 µM nBTPT and urea (U+nBTPT, 1mM Ntot), or urea and nitrate (UN+nBTPT, 2 mM Ntot) or urea and ammonium (UA+nBTPT, 2 mM Ntot). After 4, 8, 12 and 24 hours of treatment, groups of six plants from each treatment were transferred to the assay solution containing 200 µM urea. The values are means ± SD (n=3).

A possible direct effect of the urease inhibitor on the urea transport system was also evaluated; to this purpose the net uptake rate was measured by adding nBTPT to the assay solution (Figure 30). Thus, plants were exposed for 8 hours to a nutrient solution with (U) or without (Control) urea and then transferred to an assay solution containing 200 µM urea with or without nBTPT. In this case plants were exposed to nBTPT only for a maximum of 10 minutes. Results showed that in U-fed plants the presence of nBTPT in assay solution limited the uptake capacity, determining a 30% decrease in uptake rates. On the other hand, the direct effect of nBTPT on control plants caused a 40% stimulation of urea uptake rate.

These data suggest that nBTPT might interfere with the mechanism of urea acquisition, possibly acting directly on high-affinity inducible urea transporters.

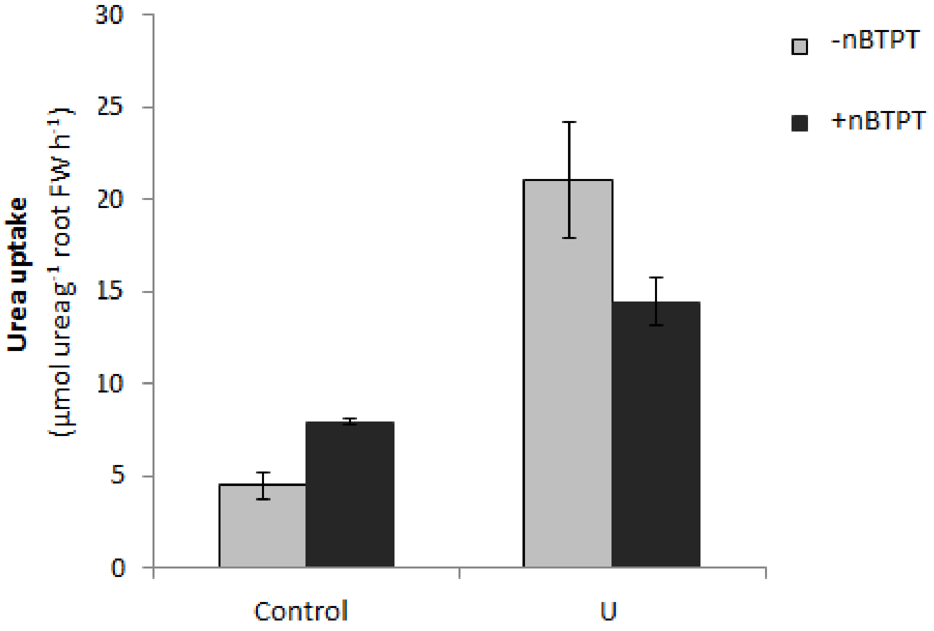


Figure 30. Direct effect of nBTPT on the urea transport system. 5 day-old maize plants were exposed for 8 hours to a nutrient solution with (U) or without (Control) urea and then transferred to an assay solution containing 200 µM urea with 0.897 µM nBTPT (+nBTPT) or without the urease inhibitor (-nBTPT). The values are means ± SD of three replicates of two plants each.

3.2 Transcriptomic aspects of urea and nitrate uptake in maize plants

3.2.1 Genome-wide gene expression analysis in maize roots

At physiological level (Figure 23, Figure 24), nitrogen treatments led to changes in the root uptake capacity of the sources. In experiment described previously, compared to control, urea or nitrate treated plants showed an increase in their respective uptake velocity, reaching the maximum value after 8 hours of exposition. To investigate the transcriptomic changes involved in this physiological response, a genome-wide gene expression analysis was performed using the same experimental conditions of above experiments. So, maize plants were grown for 5 days in nitrogen starvation and then were exposed for 8 hours to a nutrient solution under four nitrogen treatments: urea (U, 1 mM Ntot); nitrate (N, 1 mM Ntot); urea and nitrate (UN, 2 mM Ntot); without any addition of nitrogen source (Ctr) as control. Roots were sampled from three independent experiments and the total mRNA was extracted in order to perform the microarray analysis. Differences in root gene expression among the treatments were obtained using the maize chip 12 X 135K Arrays (http://ddlab.sci.univr.it/FunctionalGenomics) developed by NimbleGen (http://www.nimblegen.com, Roche NimbleGen, Inc.). This array allows the simultaneous analysis of all the samples, monitoring the expression of 59,756 transcripts predicted from the B73 maize reference-genome version 1 (B73 RefGen_v1, http://www.maizesequence.org).
Depending on nitrogen treatment, four transcriptomic profiles were obtained (Ctr, U, N, UN) and compared to each other. So, six comparisons were carried out (U *vs* Ctr, N *vs* Ctr, UN *vs* Ctr, N *vs* U, UN *vs* U and N *vs* UN) and statistical analyses were performed on data using Linear Models for MicroArray (LIMMA, adjusted p-value ≤ 0,05) (Table 5). Surprisingly, only few genes were found as differentially expressed by nitrogen treatments: the highest value was detected in UN *vs* Ctr (132 differentially expressed transcripts), the lowest in U *vs* Ctr (4 transcripts), and in N *vs* UN there were no significant variations in gene expression. Most of the detected transcripts showed a strong modulation with high values of fold change (FC), indeed, except very few transcripts, the fold changes (FC) were over |2|.
Results suggested that under these experimental conditions, in presence of urea alone, only few genes were differentially expressed (U *vs* Ctr). A completely different situation was registered under nitrate, since it resulted as responsible for the major transcriptional variation among the comparisons, especially in N *vs* Ctr and UN *vs* Ctr. This hypothesis is confirmed by N *vs* UN, where the presence of urea along nitrate did not influence significantly the gene expression profile, any significant change in expressed

transcripts was not identified by statistical analysis. This means that under N and UN treatments, roots express the same pool of transcripts with a similar level of expression.

As consequence of this similar transcriptome in U and Ctr roots, the comparison of nitrate *versus* urea (N *vs* U) or *versus* control (N *vs* Ctr) gave similar numbers of transcripts, same observation could be done for UN *vs* U. However in both case (N *vs* U and UN *vs* U) the numbers of transcripts were 40% lower than *versus* Ctr (N *vs* Ctr and UN *vs* Ctr), indicating that even if not significative there is a drift in the expression profiles of urea treated roots in direction of the profile of nitrate treated one in comparison to the control.

Moreover the identified transcripts were divided in positive and negative FC, which correspond respectively to over- and down- expressed genes. As reported in Table 5, in all comparisons the numbers of up-regulated genes are much higher than those downregulated.

Table 5. Number of gene differentially expressed by nitrogen treatments in the six comparisons. Data provided by microarray analyses performed on mRNA extracted from root of maize grown under different nitrogen treatment. Maize plants were grown for 5 days in nitrogen starvation and then were exposed for 8 hours to a nutrient solution under three nitrogen treatments: urea (U, 1 mM Ntot); nitrate (N, 1 mM Ntot); urea and nitrate (UN, 2 mM Ntot). Control plants were not exposed to any nitrogen source (Ctr). The root transcriptomic profiles were compared to each other, obtaining six comparisons: U *vs* Ctr, N *vs* Ctr, UN *vs* Ctr, N *vs* U, UN *vs* U, N *vs* UN. For each comparison, the number of differentially expressed transcripts is reported in the table: numbers in the first row are referred to the total number of gene differentially expressed; in second and third rows are reported the number of over- and down- expressed transcripts, respectively. Thus, for example, in UN *vs* Ctr were identified 132 differentially expressed genes: 113 over- and 19 down- expressed by the UN treatment in comparison to control roots. This experiment was performed using three independent biological replications and the data were statistical analyzed using Linear Models for MicroArray (LIMMA , Smyth, 2004) adjusted *p*-value≤ 0,05.

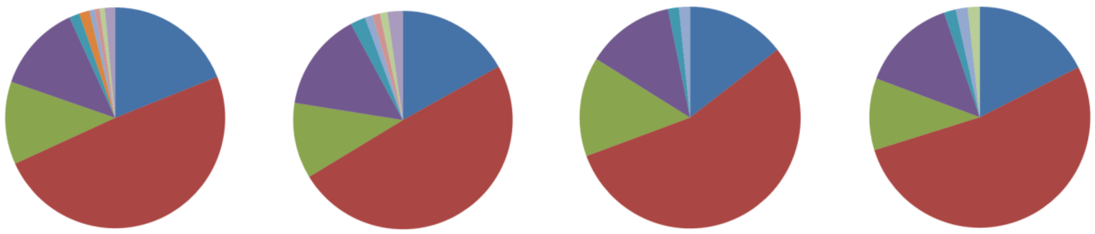
	UN *vs* Ctr	**N *vs* Ctr**	**UN *vs* U**	**N *vs* U**	**U *vs* Ctr**	**N *vs* UN**
Tot transcripts	**132**	**89**	**62**	**57**	**4**	**/**
Over expressed	+113	+79	+58	+53	+4	/
Down regulated	-19	-10	-4	-4	/	/

Manually curated annotation of the differentially expressed transcripts was performed by BLASTP analyses (UniProt Database: http://www.uniprot.org/?tab=blast) using the protein predicted by CDS (coding sequence, GenBank Database: http://blast.ncbi.nlm.nih.gov/Blast.cgi) of each maize transcript (Table 7 and Table 8). The annotated transcripts were then grouped in main functional categories according to biological process terms of Gene Ontology (GO) assigned to each transcript on the basis of BlastP results. The most representative functional categories were "metabolic process", "localization" and "biological regulation", while less abounded were "response to stimulus", "cellular component organization or biogenesis", "cellular process", "developmental process" and "carbon utilization" (Table 6). A part of transcripts were classified in "biological process" and "no hits found" categories. The first category concern transcripts encoding proteins involved in *unknown* biological process; and "no hits found" is referred to transcripts that do not show any homology to known proteins.

Although UN *vs* Ctr, N *vs* Ctr, UN *vs* U, N *vs* U show different numbers of differentially regulated genes (132, 89, 62 and 57 respectively), in each comparison the GO categories were represented by similar percentage (Table 6).

Table 6. Functional category distribution of differentially expressed transcripts in UN *vs* Ctr, N *vs* Ctr, UN *vs* U, N *vs* U comparisons. Distribution in main functional categories according to GO "Biological process" terms.

	Biological process GO classes:	**UN *vs* Ctr**		**N *vs* Ctr**		**UN *vs* U**		**N *vs* U**	
			%		%		%		%
🟦	Biological process (GO:0008150)	**25**	19	**15**	17	**9**	15	**10**	18
🟥	Metabolic process (GO:0008152)	**65**	49	**44**	49	**34**	55	**30**	53
🟩	Localization (GO:0051179)	**16**	12	**10**	11	**9**	15	**6**	11
🟪	Biological regulation (GO:0065007)	**17**	13	**13**	15	**8**	13	**8**	14
🟦	Response to stimulus (GO:0050896)	**2**	2	**2**	2	**1**	2	**1**	2
🟧	Cellular comp. org. or biogenesis (GO:0071840)	**2**	2						
🟦	Cellular process (GO:0009987)	**1**	1	**1**	1	**1**	2	**1**	2
🟥	Developmental process (GO:0032502)	**1**	1	**1**	1				
🟩	Carbon utilization (GO:0015976)	**1**	1	**1**	1			**1**	2
🟪	No hits found	**2**	2	**2**	2				
		132		**89**		**62**		**57**	



UN *vs* Ctr **N *vs* Ctr** **UN *vs* U** **N *vs* U**

3.2.1.1 Metabolic process (GO:0008152)

In Table 7, the transcripts from #29 to 97 belong to the GO category "metabolic process".

Most of the modulated transcripts are known to be regulated by nitrate exposure and overall they are involved in the nitrogen assimilation pathway. Hence my results confirm their modulation by nitrate as reported by Wang et al. (2000), who performed a microarray analysis to identify Arabidopsis genes that responded to nitrate at either low or high (up to 10 mM) nitrate concentrations. Generally, in my data these genes were found modulated by nitrate alone (N) as well by nitrate and urea together (UN) (comparisons N *vs* Ctr and UN *vs* Ctr).

Intriguing data were obtained in roots exposed to a combination of urea and nitrate (UN). In general, it was observed that the UN treatment determined a stronger variation in the gene expression levels than N treatment, suggesting a possible involvement of urea in facilitate the assimilation of nitrogen.

Moreover, comparing N and UN treatments to urea (N *vs* U and UN *vs* U), it was possible to discriminate among genes specifically modulated by the presence of urea source or of nitrate source, respectively.

3.2.1.1.1 Nitrogen assimilation pathway

The most overexpressed transcripts are involved in nitrate reduction, *i.e.* nitrate reductase, NR (#34 and 51); nitrite reductase, NiR (#30, 31, 33, 35, 36, 40); and indirectly uroporphyrinogen III methyltransferase, UPM (#29, 43, 50 and 55).

NR and *NiR* genes encode for two key enzymes in the nitrate assimilation pathway: nitrate uptake is firstly reduced by NR to nitrite, than NiR converts nitrite into ammonium. Previous works had demonstrated the nitrate induction of both enzymes, in particular in Crawford and Glass (1988), Wilkinson and Crowford (1991, 1993) and in Lahners et al. (1988). In all four comparisons, the most abounded transcript was UPM which is involved in a branch point step of the siroheme biosynthesis, an essential co-factor for NiR. In maize, UPM was observed strongly induced within 2 hours of 16 mM nitrate treatment (Sakakibara et al., 1996).

Nitrite reduction requires ferrodoxin (Fd) as reductant. In roots, Fd is reduced in plastids by ferrodoxin-NADP reductase (FNR), which uses NADPH. In agreement with Ritchie et al., 1994, both *Fd* (#66) and *FNR* (#49) genes were found to be induced by nitrate.

To support the production of reduced Fd in roots, NADPH is produced by the oxidative pentose phosphate pathway, which converts glucose 6-phosphate into ribose 5-phosphate and reduces two $NADP^+$ molecules (Bowsher et al., 1992). In 1998, Redinbaugh and Campbell observed that the activity of two enzymes in the pathway (glucose-6-phosphate dehydrogenase, G6PDH; 6-phosphogluconate dehydrogenase, 6PGDH) increase by at least a 10-fold factor in maize-root plastids after 24 hours of treatment with 10 mM nitrate. These data are consistent with the gene expression detected in my results: G6PDH (#42) was up-regulated in all four comparisons; in the other hand 6PGDH (#58) resulted differentially expressed only in N *vs* Ctr and UN *vs* Ctr.

As observed in Arabidopsis microarray analyses (Wang et al., 2003; Wang et al., 2004) glycolysis-related genes are positively modulated by nitrogen. Two genes encoding the glycolytic enzyme Glucose-6-phosphate isomerase (G6PI) were induced by N or NU treatments (#57, 68). Their modulation appeared to be closely related to the nitrate presence in extra-radical solution, since they were detected as differentially modulated in nitrate-treated roots in comparison to Control roots and even Urea-treated roots. G6PI catalyzes the isomerization reaction of Glucose 6-phosphate to Fructose-6-phosphate and it serves both glycolysis and the pentose phosphate pathways. These two catabolic pathways occur in cytosol and plastid compartments and are integrated, as pointed out by Dennis et al., 2000. It is possible that G6PI induction helps recycle carbon back to the pentose phosphate pathway and thus aids in the generation of NADPH by pentose phosphate oxidation.

Other genes that are known to be nitrate-induced, include those involved in ammonia assimilation, i.e. that encode for glutamine synthetase, GS (#32, 63, 76) and Fd-glutamate synthase, GOGAT (#79). These results are in agreement with the experimental evidence found by Redinbaugh and Campbell (1993), who demonstrated that maize roots induced Fd-GOGAT and GS within 30 min when exposed to 10 mM nitrate and within 2 hours with 10 µM nitrate. The ammonium could be substrate also of other enzyme which converts 2-oxoglutarate into glutamate, the 2-oxoglutarate-dependent dioxygenase (GDH). In my analyses the GDH transcript (#75) was found to be upregulated by nitrate or nitrate and urea treatments (UN *vs* Ctr and N *vs* Ctr comparisons) meaning that it is mainly modulated by nitrate, in agreement with the experimental data reported by Mérigout et al. (2008a).

An interesting transcriptional modulation was found for asparagine synthase (ASN), which converts aspartate into asparagine by deamination of glutamine. The strong up-regulation of two transcripts (#45 and 52) appeared strictly dependent on the urea presence in the extra-radical solution (UN *vs* Ctr, N *vs* U and U *vs* Ctr). The ASN induction is consistent with the Arabidopsis microarray data of Mérigout et al (2008a), who found a modulation of two isoforms of ASN as depending on urea.

Last group of known nitrate regulated genes includes those genes involved in organic-acid and starch metabolisms. Among the microarray data I had found two transcripts (#41 and 44) encoding for a subunit of pyruvate dehydrogenase (PDH). This enzyme catalyzes the oxidation of pyruvate into CO_2, NADH and acetic acid, which enters in the TCA cycle. The CO_2 released might be rapidly interconverted into bicarbonate or *vice versa* by the enzyme carbonic anhydrase, which transcript (#140 *Carbon utilization (GO:0015976)*) was found to be overespressed by a 5-fold factor in N *vs* Ctr, UN *vs* Ctr and N *vs* U comparisons.

3.2.1.1.2 Signaling and scavenging of ROS

SNF1-related protein kinase (SnRK) a serine/threonine protein kinase which is a crucial component in the transcriptional and metabolic regulation by integrating growth and development in response to nutritional status and stress conditions (Polge and Thomas, 2007). Among the SnRK, the subfamily SnRK3 is also designated as CIPK (*CBL-Interacting Protein Kinasis*) because they can interact with calcium sensor calcineurin B-like proteins (CBL) to form a network mediating calcium signaling and a complex array of environmental stimuli (Batistic and Kudla, 2004; Pandey, 2008). However, knowledge about SnRK genes is very limited. In 2011, Chen and coworkers had published a report about putative *CIPK* genes in maize. In this work, the authors observed that *ZmCIPK* genes transcriptionally responded to abiotic stresses, such as salt, drought, heat and cold stresses registering an upregulation of 24, 31, 20, and 19 *ZmCIPK* genes, respectively. My results revealed the overexpression of four CIPK (#38, 47, 48, 53) in four comparisons: UN *vs* Ctr, N *vs* Ctr, UN *vs* U and N *vs* U; while only in UN *vs* Ctr a transcript coding for SnRK2.4 (#56) was differentially expressed.

A fundamental protection mechanism against reactive oxygen species is through the ascorbate–glutathione cycle in which an important antioxidant, ascorbate, is utilized to convert harmful H_2O_2 to H_2O. The enzyme monodehydroascorbate reductase (MDHAR) maintains reduced pools of ascorbate by recycling the oxidized form of ascorbate. My results show that two transcripts (#37, 59) coding for a MDHAR were induced by N and UN in comparison to Ctr and U treatments (UN *vs* Ctr, N *vs* Ctr, UN *vs* U and N *vs* U). This enzyme is known to be involved in the scavenging of nitric oxide that can be produced by nitrate reductase (NR) when nitrite is used as substrate (Rockel et al., 2002; Igamberdiev et al., 2006). NO is a signaling molecule involved in many biochemical and physiological processes (Lamattina et al., 2003). In maize, it has been reported that nitric oxide plays a role as a mediator of nitrate dependent root growth (Zhao et al., 2007). Moreover Prinsi and coworkers (2009) reported that at a high external concentration of nitrate, maize roots reduce their endogenous level of nitric oxide with a concomitant protein accumulation of MDHAR. This experimental observation was consistent with the hypothesis that MDHAR activity might contribute in controlling nitric oxide levels in root tissues after exposition to nitrate (Igamberdiev et al., 2006; Rockel et al., 2002; Prinsi et al., 2009).

3.2.1.1.3 Shikimic pathway

The most interesting and novel founding in my data was the induction of genes that are involved in the shikimic acid pathway. This pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, lignin and secondary metabolites (Herrmann and Weaver, 1999; Tzin and Galili, 2010).

In our data, the first enzyme of shikimic pathway being modulated by nitrogen treatment was shikimate kinase (SK), which catalyzes the fifth reaction of this pathway, converting shikimate to shikimate 3-phosphate. Its transcript (#78) was found to be upregulated by the urea and nitrate treatment in comparison to control roots (UN *vs* Ctr); in all the other comparisons, the expression levels were not significantly different.

Same behavior was found for the transcript (#77) coding for the downstream enzyme arogenate dehydrogenase (TyrA) that catalyzes the conversion of arogenate to tyrosine. So under urea and nitrate (UN) it seems to induce the pathway that leads to the production of tyrosine. In *Arabidopsis* and in *Sorghum bicolor*, tyrosine is directly responsible for the feedback-inhibition of TyrA (Rippert and Matringe, 2002; Connelly and Conn, 1986). Tyr serves as precursor of several families of secondary metabolites, including, for example, tocochromanols (vit E), plastoquinones, isoquinoline alkaloids, several non-proteic amino acids and perhaps also some phenylpropanoids (Tzin and Galili, 2010).

My analyses revealed also an interesting modulation of two genes coding for ferulate 5-hydroxylase (FAH, #46 and 87). This enzyme catalyzes the conversion of ferulate (a phenylalanine derivate) to 5-hydroxyferulate, a precursor of sinapate which is a phenylpropanoid requested for lignin biosynthesis. One transcript (#46) was found to be strongly upregulated by UN and N in comparison to Ctr and even to U treatments, instead the other one (#87) was found to be downregulated in UN *vs* Ctr, N *vs* Ctr and N *vs* U, no significant modulation was observed in UN *vs* U. Thus, these results suggest that nitrogen treatments modulated in opposing way two genes putatively encoding for isoforms of the same enzyme which may indicate that they have a different role.

Probably another enzyme is involved in this pathway catalyzing a downstream reaction in the biosynthesis of sinapate ester. In UN *vs* Ctr, a 2-fold upregulation of a transcript (#81) coding for a putative serine carboxypeptidase which could convert the last precursor of sinapoyl-malate was found.

The induction of the phenilpropanoid pathway is usually correlated to a condition of nitrogen starvation, as observed by Fritz et al. (2006). They showed that in tobacco the nitrogen deficiency led to a marked shift from nitrogen-containing compounds (such as alkaloids) to carbon-rich products, such as phenylpropanoids which are synthesized from phenylalanine or tyrosine. The stimulation of phenylpropanoid metabolism is triggered by changes in nitrate concentration, rather than downstream nitrogen metabolites, and is mediated by induction of a set of enzymes in the early steps of the phenylpropanoid biosynthetic pathway. On the other hand as pointed out by Chikov and Batasheva (2012), when roots are exposed to nitrate concentration of nitrogen-containing compounds, the translocation from roots to leaves of a fraction of amino acids increases at first and then is also the turn for nitrate. In this condition, the regulation of these various processes in plants is not directly depending on either sugar or nitrate concentrations but on the ratio between them.

So, perhaps due to a change in the C/N balance, my nitrogen treatments induced the shikimic pathway leading to an increase in the aromatic-amino acid biosynthesis. In particular, under urea and nitrate, roots overexpressed the enzyme involved in Tyr biosynthesis, facilitating the nitrogen assimilation; but at the same time also the synthesis of Phe-derivates was induced, leading to increase the carbon compounds.

3.2.1.1.4 Amino acids biosynthesis

Among transcripts related to metabolic process, some are involved in the amino acid biosynthesis. In roots, the nitrogen deriving from nitrate or urea uptake is rapidly converted into ammonium; first enzymes to be involved in ammonia assimilation are GS, GOGAT, GDH and ASN as described above. However from my data, also the shikimic pathway for the synthesis of aromatic amino acids (Phe, Tyr, Trp) was found to be induced by nitrogen treatments. Other transcripts involved in the amino acid biosynthesis were found like aspartokinase/homoserine dehydrogenase (AK/HSDH), Alanine-glyoxylate aminotransferase (AGAT) and 5-adenylylphosphosulfate (APS) reductase.

In the N *vs* Ctr comparison, the nitrate treatment induced a 2-fold upregulation of a transcripts (#94) coding for a bifunctional aspartokinase/homoserine dehydrogenase (AK/HSDH). Enzymes with these kinds of activity could be involved in the synthesis of lysine, threonine, and methionine. These amino acids are synthesized from aspartate, and the first enzyme in the pathway, AK, catalyzes the phosphorylation of Asp to P-aspartyl phosphate; instead the third enzyme, HSDH, catalyzes the NADPH-dependent conversion of Asp 3-semialdehyde to homoserine. In 1992, Azevedo and coworkers reported biochemical evidences that in maize an AK-HSDH bifunctional enzyme exists and two years later Muehlbauer and coworkers (1994) characterized three maize genes that encode subunits of AK-HSDH.

Only in the UN *vs* Ctr comparison, data revealed a 2-fold downregulation of three transcripts (#86, 89, 93) coding for an alanine-glyoxylate aminotransferase (AGAT). This enzyme is involved in the C2 oxidative photosynthetic cycle which acts as a scavenger to recover fixed carbon lost during photorespiration by the oxygenase reaction of rubisco. In particular AGAT catalyzes a transamination reaction where glyoxylate is converted into glycine using as amino donor the amino acid alanine and releasing pyruvate. As pointed out by Igarashi and coworkers (2006) detailed regulation mechanisms of this biosynthetic pathway of glycine is unclear and moreover, in non-photosynthetic tissues (e.g. roots), Gly is synthetized mainly though glycolysis and the glyoxylate cycle.

Under urea and nitrate treatment (UN *vs* Ctr) the overexpression of a gene implicated in sulfur metabolism was detected. The transcript (#69), encoding for 5-adenylylphosphosulfate (APS) reductase, is involved in the assimilation pathway of sulphate, who leads to the synthesis of the amino acid cysteine. APS reductase transfers two electrons to APS producing sulfite.

Our results are in agreement with the Arabidopsis microarray data detected by Wang and coworkers (2003), who registered in roots an over-expression of APS reductase gene in presence of nitrate. In particular their data revealed that nitrate itself can induce sulfate uptake and metabolism genes, increasing sulfate assimilation.

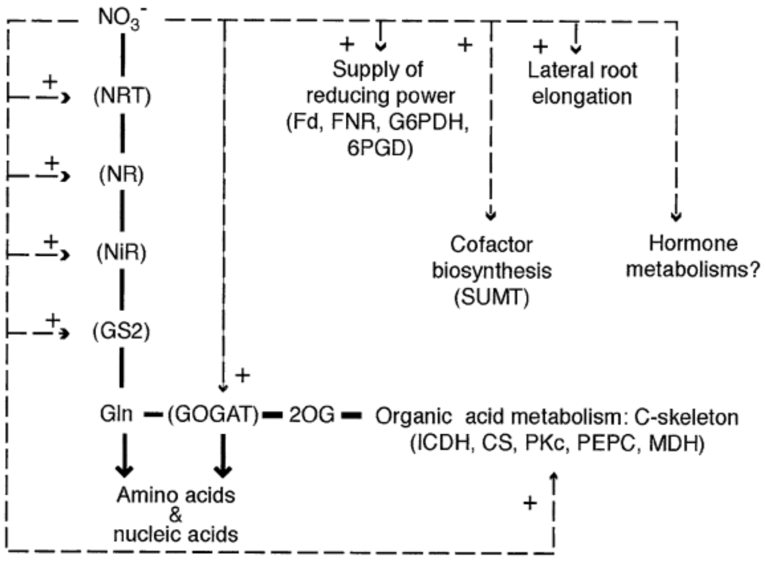


Figure 31. The nitrate-dependent gene regulation network. Solid lines represent metabolite fluxes, while dashed lines symbolize positive regulatory signaling by nitrate. Abbreviations in parentheses represent genes; NRT nitrate transporter, NR nitrate reductase, NiR nitrite reductase, GS2 plastid glutamine synthetase, GOGAT glutamate synthase, Fd ferredoxin, FNR Fd-NADP+ oxidoreductase, G6PDH glucose-6-phosphate dehydrogenase, 6PGD 6-phosphogluconate dehydrogenase, SUMT S-adenosylmethionine-dependent uroporphyrinogen III methyltransferase, ICDH isocitrate dehydrogenase, CS citrate synthase, PKc cytosolic pyruvate kinase, PEPC phosphoenolpyruvate carboxylase, MDH malate dehydrogenase (Sakakibara, 2003).

3.2.1.2 Localization (GO:0051179)

The other most representative functional category is the "localization" in which transcripts from #98 to 114 are clustered.

A strong modulation was registered for the gene coding a glucose-6-phosphate/phosphate translocator (GPT, transcripts #98 and 104). As observed before in "metabolic process", nitrogen treatments (N and UN) induced the pathway of glycolysis as well as the oxidative pentose phosphate pathway, which are localized in the cytosol and plastid respectively. The up-regulation of GPT gene is request for the translocation of cytosolic glucose 6-phosphate into plastid (Figure 32) where it is substrate for the glucose-6-phosphate dehydrogenase (G6PDH). The transcript #98 and 104 were detected strongly upregulated under urea and nitrate treatment (UN *vs* Ctr and UN *vs* U), in the other hand only #98 was observed to be also modulated by nitrate alone (N *vs* Ctr and N *vs* U). Moreover the oxidative pentose phosphate pathway is also connected to the shikimic acid pathway which is localized in the same compartment. The shikimic acid pathway converts phosphoenolpyruvic acid (a carbohydrate precursors derived from glycolysis) and erythrose-4 phosphate (from pentose phosphate pathway) to the aromatic-amino acids Trp, Tyr and Phe (Herrmann and Weaver, 1999). My analysis revealed that under UN treatment, a gene encoding for a phosphoenolpyruvate/phosphate translocator (PPT, Figure 32), transcript #105, was found to be overexpressed 3-fold in comparison to control roots. This protein could mediate the translocation of phosphoenolpyruvate from cytosol into plastid where it enters the shikimic acid pathway. The overexpression of these translocators is consistent with the induction of genes involved in the shikimic pathway.

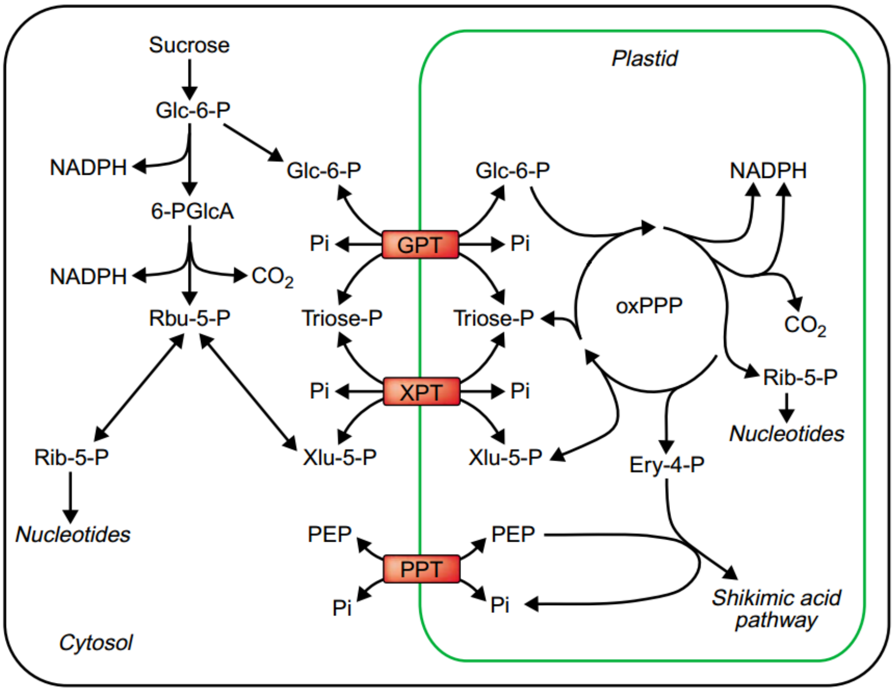


Figure 32. Exchange of oxidative pentose phosphate pathway intermediates by the plastid phosphate translocators in Arabidopsis. Glucose 6-phosphate (Glc-6-P) can enter plastids in exchange for triose phosphate or orthophosphate (Pi) via the Glc-6-P/phosphate translocator (GPT). Exchange of xylulose 5-phosphate (Xlu-5-P), triose phosphate (Triose-P) and Pi is catalysed by the XPT. In the absence of cytosolic transketolase and transaldolase, this activity facilitates further metabolism (within plastids) of pentose phosphates that are generated by the oxidative reactions in the cytosol, as well as the provision of pentose phosphates generated independently of NADPH production for nucleotide synthesis in the cytosol. The phosphoenolpyruvate/phosphate translocator (PPT) is required for the import of phosphoenolpyruvate into plastids for the biosynthesis of aromatic acids. The triose phosphate/phosphate translocator, which is expressed only in photosynthetic cells, is omitted for clarity (Kruger and von Schaewen, 2003).

Hemoglobin 2 and MDHAR (see "metabolic process") are known to be involved in the scavenging of nitric oxide. In particular, Prinsi and coworkers (2009) observed that the exposition of maize roots to high nitrate concentration (10 mM NO_3^-) in external medium determined an over-accumulation of the protein MDHAR as well as Hemoglobin 2. Furthermore nitrate have a positive influence on the transcription of the gene coding for Hemoglobin, since in a recent work Trevisan et al. (2011) registerd the over-expression of gene coding for Hemoglobin (GenBank: AF236080.1) in maize roots supplied with 1 mM nitrate, where the maximal induction was reached after 6 hours of exposition.

This effect of nitrate was also registered in the model plant Arabidopsis, where a genome-wide analysis revealed the over-expression of a non-symbiotic *hemoglobin 1* gene (*AtHB1*) (Wang et al., 2000). The

authors suggested that under nitrate treatment, the oxygen sensitiveness of NR and its molybdenum cofactor could request a reduction in oxygen concentration which could be mediated by hemoglobin itself (Wang et al., 2000).
Showing a similar behavior of *NR* and *MDHAR* genes, my analyses detected also a strong upregulation of *hemoglobin 2* (#99 transcript) in UN *vs* Ctr, N *vs* Ctr, UN *vs* U and N *vs* U comparisons.

A physiological and molecular linkage between sulfur and nitrogen metabolism has been known for many years (Reuveny et al., 1980; Vidmar et al., 1999; Prosser et al., 2001). In my data the transcripts encoding a sulfate transporter was detected to be overexpressed by a 4-fold factor (#102) in UN *vs* Ctr and N *vs* Ctr. These data are consistent with Vidmar and coworkers (1999), who reported that in nitrogen starved barley, the root exposition to nitrate or ammonium induced the gene encoding for the high affinity sulfate transporter and increased the sulfate uptake in roots. The authors suggested that a nitrogen metabolite may influence the gene expression of sulfate transporters.

Other interesting genes to be modulated by N and UN nitrogen treatments coded for tonoplast dicarboxylate transporters (DT, #101 and 108). The major carboxylate of vacuole is constituted by malate,which is accumulated in this compartment by specific channel protein or by DT, also known as malate transporter. In plant cells the activity of this transporter contributes to maintain cytosolic pH homeostasis and it has been identified in Arabidopsis (AttDT, Arabidopsis thaliana tonoplastic Dicarboxylate Transporter, Emmerlich et al., 2003). By transcriptomic analysis Hu et al. (2009) had reported the induction of *AttDT* gene by nitrate. The authors suggested that since the synthesis of amino acids from nitrogen as being derived from taken up nitrate led to a stechiometric production of OH^- ions (van Beusichem et al., 1985), under this conditions the synthesis of malate might contribute to maintain the pH homeostasis reducing the OH^- concentration in the cytosol (Davies, 1986). So the authors hypotyzed that the induction of *AttDT* expression was a cellular response to reduced the alkaline stress due to nitrate assimilation. In a similar way the microarray data reported in the present work could find a reasonable explanation. It is interesting observed as the ZmDT was overexpressed when plants were exposed to urea and nitrate (UN treatment), it is possible that in addition to nitrate also the assimilation of nitrogen as being derived from urea might contributed to increase the cytosolic pH, indeed in comparison to N treatment, the presence of urea in UN treatment determined an increase in the levels of *ZmDT* trancripts.

Interestingly, microarray data revealed a strong modulation in the amounts of a high affinity nitrate transporter transcript (#114, NRT2.5). When roots were exposed to urea as sole nitrogen source (U) the gene expression of *NRT2.5* was found upregulated in comparison to nitrate treatments (UN and N) showing higher values than those found when control was compared to nitrate treatments. So, overturning the comparisons as Ctr *vs* UN, Ctr *vs* N; U *vs* UN; U *vs* N, the following values are observed:

	Ctr *vs* UN	Ctr *vs* N	U *vs* UN	U *vs* N
NRT2.5 (#114)	/	8.28	8.38	14.57

Also Mérigout et al. (2008a) found in Arabidopsis the overexpression of NRT2.5 (At1g12940) as induced by urea when applied as unique source of nitrogen. In particular their transcript was found to be modulated in roots of urea-treated plants in comparison to nitrate-fed plants.

Table 7. List of transcripts modulated in maize roots in response to different nitrogen treatments, such as single sources urea (U) or nitrate (N), or a combination of them: nitrate and urea together (UN). As control roots of plants never exposed to any nitrogen source were used (Ctr). In the table are reported the Probe ID of the transcripts; the fold change values (FC) found in the five comparisons UN *vs* Ctr, N *vs* Ctr, UN *vs* U, N *vs* U and U *vs* Ctr; the description of the putative protein function with the UniProtID and e-value of the BLAST analysis. The bold transcripts are discussed in detail.

#	ProbeID	FC					Description	UniProtID	e-value
		UN *vs* Ctr	**N *vs* Ctr**	**UN *vs* U**	**N *vs* U**	**U *vs* Ctr**			
	Biological process (GO:0008150)								
1	GRMZM2G071704_T01	14.04	10.74	14.37	10.99		Putative uncharacterized protein	C5Y9Q9	7.0×10-93
2	GRMZM2G133684_T02	6.11	4.39	5.07	3.65		HPP	B6TMS3	1.0×10-169
3	GRMZM2G133684_T03	5.84	4.4	6.05	4.56		HPP	B6TMS3	2.0×10-46
4	GRMZM5G868296_T01	5.33					Putative uncharacterized protein	C4J6L4	8.0×10-51
5	GRMZM2G017319_T01	4.78	4.96				LOB domain protein 40, putative, expressed	Q75HN5	1.0×10-106
6	GRMZM2G474039_T01	4.78	4.15	6.53	5.67		Putative uncharacterized protein	B6U8K9	3.0×10-99
7	AC191113_2_FGT002	4.1	3.54				Loricrin-like protein	Q69JW8	0.0
8	GRMZM2G107226_T01	3.94	4.25	2.87	3.1		Putative uncharacterized protein	B6UAX1	7.0×10-69
9	GRMZM2G133684_T01	3.87					HPP	B6TMS3	1.0×10-169
10	GRMZM2G165914_T01	3.81					Putative uncharacterized protein	C6JS85	0.0
11	AC197340_3_FGT003	3.54					Putative uncharacterized protein	B6U9R4	5.0×10-27
12	GRMZM2G147787_T01	3.48					Putative uncharacterized protein	B4FLV4	1.0×10-105
13	GRMZM2G159732_T02	3.34	2.77	3.65	3.03		Nodulin-related protein-like	Q6YZD9	0.0
14	GRMZM5G856297_T02	2.98		3.21			Putative uncharacterized protein	B4FEU2	9.0×10-94
15	GRMZM5G803735_T01	2.61					Putative uncharacterized protein	C0PEG5	0.0
16	GRMZM2G144483_T01	2.54		2.94	2.54		Putative uncharacterized protein	B4FDB9	5.0×10-99
17	GRMZM2G342738_T01	2.4	2.01				Putative uncharacterized protein	B6TLE5	8.0×10-92
18	GRMZM2G038931_T02	2.19	1.96				Putative uncharacterized protein	B6T9I8	1.0×10-177
19	AC186512_3_FGT007	2.08					Putative uncharacterized protein	B6UGF5	1.0×10-137
20	GRMZM2G170137_T01	1.99	1.89				Protein binding protein	B6T6Z8	1.0×10-132

		UN *vs* **Ctr**	**N** *vs* **Ctr**	**UN** *vs* **U**	**N** *vs* **U**	**U** *vs* **Ctr**			
21	GRMZM2G101754_T01	1.92	1.87				Uncharacterized protein	C0P3Y9	0.0
22	GRMZM2G155746_T01	1.78	1.84		1.87		Putative uncharacterized protein	C5YV17	0.0
23	GRMZM2G563728_T01	-2.26					Os08g0116700 protein	Q69UI7	1.0×10-158
24	GRMZM2G098925_T01	-3.32	-3.33				Putative uncharacterized protein	C5XZ31	1.0×10-108
25	GRMZM2G388253_T04	-4.03					Putative uncharacterized protein	C5X873	6.0×10-44
26	GRMZM2G044902_T04		2.51				Seed specific protein Bn15D17A	B6UCF2	8.0×10-49
27	AC203257_2_FGT003			-2.36	-2.02		Putative uncharacterized protein	B6TT56	1.0×10-147
28	GRMZM5G806818_T01				2.61		Putative uncharacterized protein	C4J0G0	9.0×10-91
	Metabolic process (GO:0008152)								
29	GRMZM2G105604_T01	28.19	21.88	16.41	12.73		**Uroporphyrinogen III methyltransferase**	P93628	0.0
30	GRMZM2G079381_T01	14.54	12.84	9.96	8.79		**Ferredoxin--nitrite reductase**	B6SY01	0.0
31	GRMZM2G079381_T04	14.24	12.45	16.35	14.29		**Ferredoxin--nitrite reductase**	B6SY01	3.0×10-83
32	GRMZM2G098290_T03	13.97	11.95	7.18	6.14		**Glutamine synthetase**	B4FT28	0.0
33	GRMZM2G079381_T02	13.43	12.53	8.89	8.29		**Ferredoxin--nitrite reductase**	B6SY01	0.0
34	GRMZM5G878558_T01	13.33	13.29	24.2	24.12		**Nitrate reductase**	C5XTG6	0.0
35	GRMZM2G079381_T05	10.55	9.98	7.21	6.82		**Ferredoxin--nitrite reductase**	B6SY01	0.0
36	GRMZM2G102959_T01	10.23	9.04	6.38	5.65		**Ferredoxin--nitrite reductase**	B6SY01	0.0
37	GRMZM5G828229_T01	9.85	8.75	6.26	5.55		**Monodehydroascorbate reductase**	Q9SPM2	1.0×10-142
38	GRMZM2G181081_T02	9.34	7.35	8.68	6.83		**CIPK-like protein 1**	B6SY58	0.0
39	GRMZM2G127909_T01	9.29	9.29	7.92	7.91		Putative uncharacterized protein	C5Z610	5.0×10-55
40	GRMZM2G079381_T03	9.24	9.64	8.59	8.96		**Ferredoxin--nitrite reductase**	B6SY01	0.0
41	GRMZM2G043198_T02	9.04	8.23	7.9	7.19		**Pyruvate dehydrogenase E1 component**	B6TKX6	1.0×10-135
42	GRMZM2G177077_T01	8.27	6.46	7.87	6.15		**Glucose-6-phosphate 1-dehydrogenase**	B6SWV1	0.0
43	GRMZM2G000739_T02	7.97	6.48	6.61	5.38		**Uroporphyrinogen III methyltransferase**	P93628	5.0×10-79
44	GRMZM2G043198_T04	7.87	8.77				**Pyruvate dehydrogenase E1 component**	B6TKX6	0.0
45	GRMZM2G078472_T04	6.1				4.99	**Asparagine synthetase**	B6ETR6	0.0
46	GRMZM2G399530_T01	5.65	4.96	5.04	4.42		**Putative ferulate 5-hydroxylase**	C0KHM0	1.0×10-169

		UN *vs* Ctr	**N *vs* Ctr**	**UN *vs* U**	**N *vs* U**	**U *vs* Ctr**			
47	GRMZM2G181081_T03	4.87	4.08	5.24	4.38		**CIPK-like protein 1**	B6SY58	0.0
48	GRMZM2G181081_T04	4.86	4.66	4.19	4.02		**CIPK-like protein 1**	B6SY58	0.0
49	GRMZM2G058760_T01	4.37	4.59	4.43	4.65		**Ferredoxin--NADP reductase, root isozyme**	B6TRH0	0.0
50	GRMZM2G000739_T01	4.36	4.18	3.79			**Uroporphyrinogen III methyltransferase**	P93628	2.0×10-92
51	GRMZM2G568636_T01	4.2	4.15	7.33	7.25		**Nitrate reductase**	Q9XGW5	0.0
52	GRMZM2G078472_T02	3.93			-4.56	4.75	**Asparagine synthetase**	B6ETR6	0.0
53	GRMZM2G181081_T01	3.91	4.18	3.87	4.14		**CIPK-like protein 1**	B6SY58	0.0
54	GRMZM2G003426_T01	3.69	3.82	3.11	3.22		Polyphosphate kinase 2 family	Q3JLV8	2.0×10-5
55	GRMZM2G105604_T02	3.63	3.88	2.53	2.7		**Uroporphyrinogen III methyltransferase**	P93628	0.0
56	GRMZM2G110922_T04	3.43					**SnRK2.4**	D5FGN9	0.0
57	GRMZM2G076075_T01	3.37	3.49	2.79	2.89		**Glucose-6-phosphate isomerase**	C0PAU7	0.0
58	GRMZM2G440208_T01	3.26	3.12				**6-phosphogluconate dehydrogenase, decarboxylase**	B6TX10	0.0
59	GRMZM5G828229_T02	3.24	3.49	2.3	2.48		**Monodehydroascorbate reductase**	Q9SPM2	0.0
60	GRMZM2G139689_T01	3.05	2.68	2.65	2.33		L-aspartate oxidase 1	B6U0K6	0.0
61	GRMZM2G003023_T02	2.98	2.79				NTGP5	B6U3R7	4.0×10-78
62	GRMZM2G335709_T01	2.85					Putative wall-associated kinase 4	Q5VRE1	0.0
63	GRMZM2G046601_T01	2.73					**Glutamine synthetase**	B6UDS5	0.0
64	GRMZM2G404443_T01	2.67		2.39			Cytokinin dehydrogenase 6	E3T1X1	0.0
65	GRMZM2G061568_T02	2.62					Acid phosphatase ACP2	Q5N7Q9	1.0×10-159
66	GRMZM2G106190_T01	2.53	2.66		2.11		**Ferredoxin-6, chloroplastic**	P94044	1.0×10-102
67	GRMZM2G416875_T01	2.48	2.39	1.92			Cis,cis-muconate cycloisomerase-like	Q5ZC51	0.0
68	GRMZM2G076075_T02	2.46	2.37	2.52	2.43		**Glucose-6-phosphate isomerase**	C0PAU7	0.0
69	AC189750_4_FGT004	2.44					**Adenosine 5'-phosphosulfate reductase 1**	Q5EUC9	0.0
70	GRMZM2G003023_T01	2.43	2.26	1.89			NTGP5	B6U3R7	4.0×10-78
71	GRMZM2G443509_T02	2.42					Protein phosphatase 2C containing protein	B6T998	0.0
72	GRMZM2G094273_T01	2.37	2.42				Cis,cis-muconate cycloisomerase-like	Q5ZC51	0.0
73	GRMZM2G323830_T01	2.35					Cytochrome P450 family protein, expressed	Q10S02	0.0
74	GRMZM2G039757_T01	2.34					Protein tolB	G8A034	2.0×10-77

		UN *vs* **Ctr**	**N** *vs* **Ctr**	**UN** *vs* **U**	**N** *vs* **U**	**U** *vs* **Ctr**			
75	GRMZM2G046669_T01	2.28	2.19				**Putative 2-oxoglutarate-dependent dioxygenase**	Q7EYC8	2.0×10-99
76	GRMZM2G046601_T03	2.27					**Glutamine synthetase**	B6UDS5	0.0
77	GRMZM2G365961_T01	2.19					**Arogenate dehydrogenase isoform 2**	B6SS03	0.0
78	GRMZM2G004590_T04	2.1					**Shikimate kinase**	B6TNA7	0.0
79	GRMZM2G036609_T01	2.1					**Ferredoxin-dependent glutamate synthase**	P23225	0.0
80	GRMZM2G443509_T01	2.09	1.97				Protein phosphatase 2C containing protein	B6T998	0.0
81	GRMZM2G126541_T01	2.07					**Putative serine carboxypeptidase**	Q75I01	0.0
82	GRMZM2G064083_T01	2.06	2.19				Hydroxyproline-rich glycoprotein family protein	Q9M3G8	1.0×10-5
83	GRMZM2G036629_T01	2.03					Metallothionein-like protein 1	B6TQN8	5.0×10-54
84	GRMZM2G047995_T02	-1.89	-1.9				Lipase	B6U116	1.0×10-149
85	GRMZM2G047995_T01	-1.94	-1.73				Lipase	B6U116	1.0×10-166
86	GRMZM2G107739_T02	-2.08	-2.98				**Alanine-glyoxylate aminotransferase 2**	B6U6Q5	0.0
87	GRMZM2G007698_T01	-2.31	-2.56		-2.35		**Putative ferulate 5-hydroxylase**	C0KHM0	1.0×10-178
88	GRMZM2G164967_T01	-2.51		-2.49			Carotenoid cleavage dioxygenase	B6UEM5	0.0
89	GRMZM2G127350_T01	-2.52					**Alanine-glyoxylate aminotransferase 2**	B6U6Q5	0.0
90	GRMZM2G086088_T03	-2.62					Ubiquitin-conjugating enzyme E2-17 kDa 9	B6UG63	1.0×10-102
91	GRMZM2G154007_T01	-2.94					Alcohol dehydrogenase 1	B6TD57	0.0
92	GRMZM2G010460_T10	-3.36					Putative ubiquitin-conjugating enzyme E2	Q75GA0	5.0×10-95
93	GRMZM2G107739_T03	-3.92					**Alanine-glyoxylate aminotransferase 2**	B6U6Q5	0.0
94	GRMZM2G136712_T01		2.11				**Bifunctional aspartokinase/homoserine dehydrogenase**	P49080	0.0
95	GRMZM2G152390_T06		-1.93				Type I inositol-1,4,5-trisphosphate 5-phosphatase CVP2	B6SKV9	0.0
96	GRMZM2G471083_T01			2.01			Sucrose phosphate synthase	G3CM26	0.0
97	GRMZM2G319445_T01			-1.86			Terpene synthase 7	B6SYF3	0.0
	Localization (GO:0051179)								
98	GRMZM2G009223_T01	15.73	12.36	9.75	7.66		**Glucose-6-phosphate/phosphate translocator 2**	B6SRN7	0.0
99	GRMZM2G168898_T01	7.01	6.01	5.95	5.11		**Hemoglobin 2**	Q3S3T0	1.0×10-128
100	GRMZM2G049852_T01	6.5				7.95	**MATE efflux family protein, expressed**	Q8H0A7	0.0

		UN *vs* **Ctr**	**N** *vs* **Ctr**	**UN** *vs* **U**	**N** *vs* **U**	**U** *vs* **Ctr**			
101	GRMZM2G176430_T01	5.13	4.16	4.39	3.55		**Tonoplast dicarboxylate transporter**	B6T0F4	0.0
102	GRMZM2G304700_T01	4.64	4.04				**Sulfate transporter**	B6TU79	0.0
103	GRMZM2G135175_T01	3.45	3	2.72	2.37		MATE efflux family protein, expressed	Q8H0A7	0.0
104	GRMZM2G180720_T01	3.4		3.21			**Glucose-6-phosphate/phosphate translocator 2**	O64909	0.0
105	GRMZM2G066413_T01	2.9					**Phosphoenolpyruvate/phosphate translocator 3**	Q5VQL3	4.0×10-77
106	GRMZM2G344163_T01	2.67	2.22	2.14			Putative chloride channel	Q6ZDC2	0.0
107	GRMZM2G078781_T01	2.58					Cyclic nucleotide-gated ion channel 2	B6U9Q0	0.0
108	GRMZM2G060749_T01	2.38	2.12	2.2			**Tonoplast dicarboxylate transporter**	B6T0F4	3.0×10-24
109	GRMZM2G166976_T01	2.21	2.46	2.38	2.66		SPX domain-containing membrane protein	Q6EPQ3	0.0
110	GRMZM2G028129_T03	2.16					ATPase 2	B6SVY2	0.0
111	GRMZM2G180720_T02	2.03					**Glucose-6-phosphate/phosphate translocator 2**	O64909	0.0
112	GRMZM2G375116_T02	-1.8					Potassium transporter 25	Q6YWQ4	0.0
113	GRMZM2G024196_T01	-3.4	-3.62				Yellow stripe-like transporter 12	E3UJZ2	0.0
114	GRMZM2G455124_T01		-8.28	-8.38	-14.57		**High affinity nitrate transporter NRT2.5**	A0EXC0	4.0×10-82
	Biological regulation (GO:0065007)								
115	GRMZM2G004161_T05	11.35	8.68	6.28	4.81		BTB/POZ and TAZ domain-containing protein 1	Q9FMK7	9.0×10-77
116	GRMZM2G001205_T01	8.68	6.15			4.33	ZFP16-1	B6TTL8	1.0×10-150
117	GRMZM2G002915_T01	8.04	6.53	6.68	5.42		Homeobox-leucine zipper protein HOX25	A3BYC1	7.0×10-72
118	GRMZM2G124495_T01	6.51	5.8	5.37	4.78		Transfactor	B6SXM1	0.0
119	GRMZM2G437490_T01	4.07					Leucine zipper protein-like	Q5VP18	1.0×10-156
120	GRMZM2G004161_T01	3.53	3.34	3.02			BTB/POZ and TAZ domain-containing protein 1	Q9FMK7	2.0×10-95
121	GRMZM2G134759_T01	3.44					C2H2 Zn finger protein	C6YY76	1.0×10-61
122	GRMZM2G004161_T02	3.25	3.39	2.93	3.06		BTB/POZ and TAZ domain-containing protein 1	Q9FMK7	9.0×10-77
123	GRMZM2G004161_T03	3.14	2.81	2.95	2.63		BTB/POZ and TAZ domain-containing protein 1	Q9FMK7	2.0×10-95
124	GRMZM2G035370_T04	3.04	3.66	2.7	3.25		Transfactor	B6SXM1	0.0
125	GRMZM2G035370_T02	2.86	3.27	2.57	2.94		Putative Myb-like DNA-binding domain-containing protein	G9C2U8	1.0×10-143
126	GRMZM2G134759_T02	2.62					C2H2 Zn finger protein	C6YY76	1.0×10-61

		UN *vs* Ctr	**N *vs* Ctr**	**UN *vs* U**	**N *vs* U**	**U *vs* Ctr**			
127	GRMZM2G137532_T01	2.4					BZIP protein	B6UHB7	9.0×10-36
128	GRMZM2G319187_T01	2.16	2.42		1.92		Response regulator 4	Q9FRZ1	1.0×10-154
129	GRMZM2G020772_T02	2.1	2.09				MYB-type transcription factor	K4JRL7	0.0
130	GRMZM2G398055_T01	-1.62					G2-like transcription factor	K4JBW4	0.0
131	GRMZM2G006745_T06	-2.61	-2.59				DRE-binding protein 1c (Dehydration responsive factor)	C3UZ65	0.0
132	GRMZM2G002128_T02		2.12				MYB transcription factor	B7ZY89	0.0
	Response to stimulus (GO:0050896)								
133	GRMZM2G048205_T01	2.97	2.85	2.81	2.7		Endonuclease/exonuclease/phosphatase family protein	Q33A95	0.0
134	GRMZM2G391596_T01	1.9					SAUR11-auxin-responsive SAUR family member	B6TVX0	1.0×10-145
135	GRMZM2G013970_T01		1.97				22.3 kDa class VI heat shock protein	Q6AUW3	1.0×10-97
	Cellular component organization or biogenesis (GO:0071840)								
136	GRMZM2G122187_T01	2.96					ANTH domain containing protein, expressed	Q53M05	1.0×10-121
137	GRMZM2G417496_T01	-2.29					Formin-like protein 12OSJNBa0091C12.5	Q7XWS7	0.0
	Cellular process (GO:0009987)								
138	GRMZM2G047474_T01	2.62	2.48	2.09	1.98		TLD-domain containing nucleolar protein	Q9FKA3	1.0×10-150
	Developmental process (GO:0032502)								
139	GRMZM2G023811_T01	7.39	6.27				CLE family OsCLE602 protein	A8R3R0	6.0×10-14
	Carbon utilization (GO:0015976)								
140	GRMZM2G121878_T04	4.97	5.79		5.77		**Carbonic anhydrase**	B4F9E2	0.0
	No hits found								
141	GRMZM2G095090_T01	3.01	3.02				No hits found	/	/
142	GRMZM2G149788_T03	-1.94	-1.93				No hits found	/	/

3.2.1.3 Effect of urea on gene expression

In our experimental conditions, the exposition of maize roots to urea induced a similar pool of transcripts to those found in Control roots, except for 4 genes that were strongly modulated by this organic nitrogen form (Table 8).

Table 8. List of the genes differentially regulated by urea treatment in comparison toc roots (UvsCtr). In the first column are reported the transcript numbers referred to Table 7. In the other columns are reported: the ID codes (ID), the correspondent fold change (FC, U *vs* Ctr), the putative function of the transcripts according to blast analysis with the correspondent UniprotID, the functional class of Gene Onthology. The positive FC indicates that the transcripts are all specifically over-expressed in exposure to urea (adjusted p-value<0.05).

#	ProbeID	FC U *vs* Ctr	Description	UniprotID	GO category
100	GRMZM2G049852_T01	**7.946**	MATE efflux family protein, expressed	Q8H0A7	localization (GO:0051179)
45	GRMZM2G078472_T04	**4.988**	Asparagine synthetase	B6ETR6	metabolic process (GO:0008152)
52	GRMZM2G078472_T02	**4.748**	Asparagine synthetase	B6ETR6	metabolic process (GO:0008152)
116	GRMZM2G001205_T01	**4.328**	ZFP16-1	B6TTL8	biological regulation (GO:0065007)

3.2.1.3.1 MATE efflux family protein

The first transcript to show the strongest urea modulation codes for a transporter belonged to the MATE efflux family, the expression level detected in urea roots were 8-fold higher than in control roots (#100, corresponding to the EST: ZM_BFb0199N08.r). By Blast analysis it was found to share a 66% of amino acid identity (e-value of 1.0×10^{-117}) with an Arabidopsis MATE transporter, AT4g23030. Yazaki et al. (2008) reported a phylogenetic relationship of 56 MATE transporter genes presented in *Arabidopsis* genome, although their functions have been only elucidated for some members in recent years. In particular the sequence of AT4g23030 appeared to be closely related to a MATE transporter named AtFRD3 (*ferric reductase defective*) necessary for correct iron distribution throughout the plant (Green and Rogers, 2004). However recent data elucidated that AtFRD3 mediates the efflux of citrate to the apoplast and is not directly involved in iron transport (Durrett et al., 2007). In the phylogenetic tree AT4g23030 appeared also to be closely related to another MATE called AtEDS5 (identified in *enhanced disease susceptibility* mutants) which its expression was induced by pathogen attack and UV-C treatment (Nawrath et al., 2002). Yazaki et al. (2008) speculated on possible AtEDS5 substrates, such as organic molecules involved in the signal transduction cascade and precursors of salicylic acids. Anyway the molecular and biochemical function of the majority of these transporters are still to be characterized.

3.2.1.3.2 Asparagine synthetase

As discussed above the urea induced the modulation of two transcripts encoding for an asparagine synthase ASN which were found to be *ca.* 5 fold overexpressed by urea treatment (#45 and 52). In both case their expression was observed also in presence of nitrate in the extra-radical solution (UN *vs* Ctr), with the difference that only the second transcript (#52) is specifically regulated in response to urea since it was detected also in N *vs* U as up-regulated by urea itself.

As for the regulation of other genes involve in urea metabolism (Figure 33), my analysis did not detect any specific variation in gene expression linked to nitrogen assimilation that are modulated by the urea treatment. We can only suggest that when urea is taken up by roots, it is rapidly hydrolyzed by the endogenous pool of cytosolic urease. In this way the released ammonium becomes substrate for GS, GOGAT, GDH and other enzymes involved in the ammonium assimilation pathway, along with the released CO_2 that could be used by carbonic anhydrase since we had found it upregulated by UN treatment. Concerning the modulation of gene involved in urea degradation and its synthesis, I did not observe any transcriptomic variation, in agreement with the Arabidopsis genome-wide analysis performed by Mérigout et al. (2008a). They reported that, as far as the enzymes of the urea cycle were concerned, no transcriptional regulation by urea was observed for urease, arginase, Orn carbamyl transferase, arginosuccinate synthase, or arginosuccinate lyase.

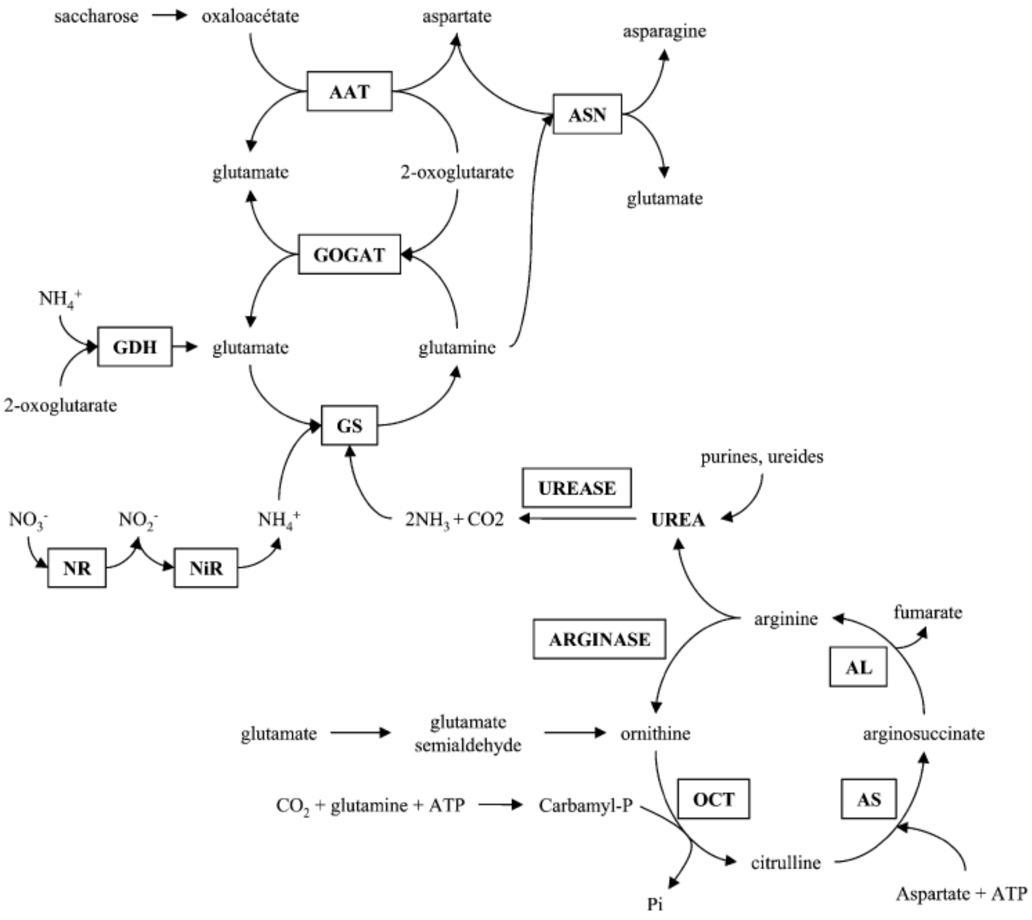


Figure 33. **Proposed urea assimilatory pathway in Arabidopsis.** Urea is synthesized inside mitochondria during Arg degradation via arginase in the Orn or urea cycle. Arginase-derived urea is then exported to cytoplasm and hydrolyzed by urease. Urea-derived ammonium is assimilated into Gln via GS; then, GOGAT catalyzes the formation of Glu via the GS-GOGAT cycle. AAT, Asp aminotransferase; AL, arginosuccinate lyase; AS, arginosuccinate synthetase; ASN, Asn synthetase; GDH, Glu dehydrogenase; NiR, nitrite reductase; NR, nitrate reductase; OCT, Orn carbamyl transferase (Mérigout et al., 2008a).

3.2.1.3.3 ZFP16-1

The urea source also modulated another transcript which was 4-fold more expressed in urea than control roots. Via Blast analysis the microarray sequence probe showed 100% of identity with a *Zea mays* mRNA NM_001157330 coding for a zinc finger protein (ZFP16-1, #116: NP_001150802.1/B6TTL8) belonged to the GO class of Biological regulation. The maize ZFP16-1 shows 69% of amino acid identity (e-value 3.0×10^{-84}) with the rice Cys2/His2-type zinc finger transcription factor (OsZFP16, AAP74357.1/Q7X9N8). The Cys2/His2-type zinc finger proteins constitute one of the largest transcription factor families in eukaryotes (Kubo et al., 1998) and in plants, they are induced in response to a large variety of stress conditions, in order to enhanced tolerance to salt, dehydration, and/or cold stresses (Sakamoto et al., 2000, 2004; Kim et al., 2001; Sugano et al., 2003). However, the roles of these transcription factors in plant stress responses are still not well understood (Sun et al., 2010).

3.2.2 Real time PCR: modulation of gene expression in time course experiment

Via microarray analysis, the transcriptomic modulation was measured after 8 hours of exposition to the different nitrogen sources. In order to confirm these data and to focus on gene expression level of transcripts involved in nitrogen uptake and metabolism, real time RT-PCR analyses on root and leaf tissues were performed on time-course experiments where maize plants were treated for 2, 4, 8, 12 and 24 hours with the different N sources. Maize plants were grown in the same conditions used for microarray experiments. Moreover the influence of a nitrogen acquisition metabolite, *i.e.* glutamine, was also investigate on the transcriptional regulation of enzymes involved in its synthesis. Thus, 5 day-old maize plants were exposed to a nutrient solution supplied with different nitrogen sources: urea (U, 1 mM Ntot), nitrate (N, 1 mM Ntot), glutamine (Gln, 1 mM Ntot), urea and nitrate (UN, 2 mM Ntot), urea and glutamine (UG, 2 mM Ntot). As control, few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Real time PCR data were confirmed in two independent biological replications, however the graphs are referred to only one representative experiment with mean values ± SD (technical replications: n=3). The gene expression values are relative to those found for the housekeeping gene (*ZmRPS4* in roots and *ZmPolyUbi* in leaves) and in all graphs the unit value (Relative gene expression = 1) is referred to the first bar on the left: 2 h control (Ctr).

3.2.2.1 Gene expression in maize roots

3.2.2.1.1 ZmNRT2.1-, ZmNRT2.2- and ZmNAR2.2-gene expression

The transcript amounts of the genes *ZmNRT2.1* and *ZmNRT2.2*, which encode two putative high-affinity transporters, were measured. In control plants (Ctr), no significant change in *ZmNRT2.1* transcript accumulation was measured during the experimental time span (Figure 34, a). In urea (U) or urea and glutamine (UG) treated plants, similar amounts of transcripts were detected in comparison to those found in control roots and within 24 hours no significant variations were observed. On the other hand, the root exposition to nitrate source (N treatment) induced a strong accumulation of *ZmNRT2.1* transcript already after 4 hours from the beginning of nitrate supply (8-fold with respect to the control) and after 8 hours the mRNA amount was similar to that registered in control roots.

Interestingly the presence of urea with nitrate (UN treatment) differently affected the expression levels of the gene. Under UN treatment the wideness of induction was lower than that observed in roots exposed to nitrate alone, nevertheless the up-regulation of the gene was significantly higher than in control roots and from 4 to 12 hours the expression values were maintained stable.

Glutamine treatment (Gln) did not show a significant accumulation in roots of *ZmNRT2.1*, but rather a decrease after 8 and 12 hours (less than 0,5 times the level measured in control roots at 2h). After 24 hours, the transcript level was higher up to 4 fold that measured at 2 h.

Like for *ZmNRT2.1*, the *ZmNRT2.2* expression profile was similar in control roots, remaining stable within 24 hours (Figure 34, b). Also *ZmNRT2.2* transcription was induced by nitrate when supplied together with urea or alone (N and UN treatments). After 4 hours of exposition the maximum expression levels was reached under both treatments, being higher in presence of urea (UN) than in its absence (N). After the peak of maximum induction and already after 8 hours of exposition a slow decrease of expression level was observed indicating that feedback regulation started rapidly.

Besides Ctr, also Gln showed comparable expression pattern to those detected for *ZmNRT2.1*: after 8 hours (and at least in the next three hours) the levels of *ZmNRT2.2* transcripts decreased remaining lower than control. U and UG treatments, instead, affected differentially the gene expression of this isoform. A gradual increase in *ZmNRT2.2* expression level was detected and after 24 hours it was up to 4 times the expression of control roots.

The transcript levels of *ZmNAR2.2* encoding for an accessory protein of NRT2s, was also examined (Figure 34, c). Interestingly, in roots treated with only nitrate (N), it was observed an early response in the modulation of this gene. After only 2 hours of exposition a peak value was registered accounting for over 12 times more than that of control. However this induction rapidly decreased since, already after 8 hours of treatment, the transcription values were comparable to control roots. Under the other treatments (Gln, U, UG, UN), the transcription of *ZmNAR2.2* was not significantly modulated by the nitrogen sources even if a barely higher expression was reported in presence of glutamine (Gln and UG).

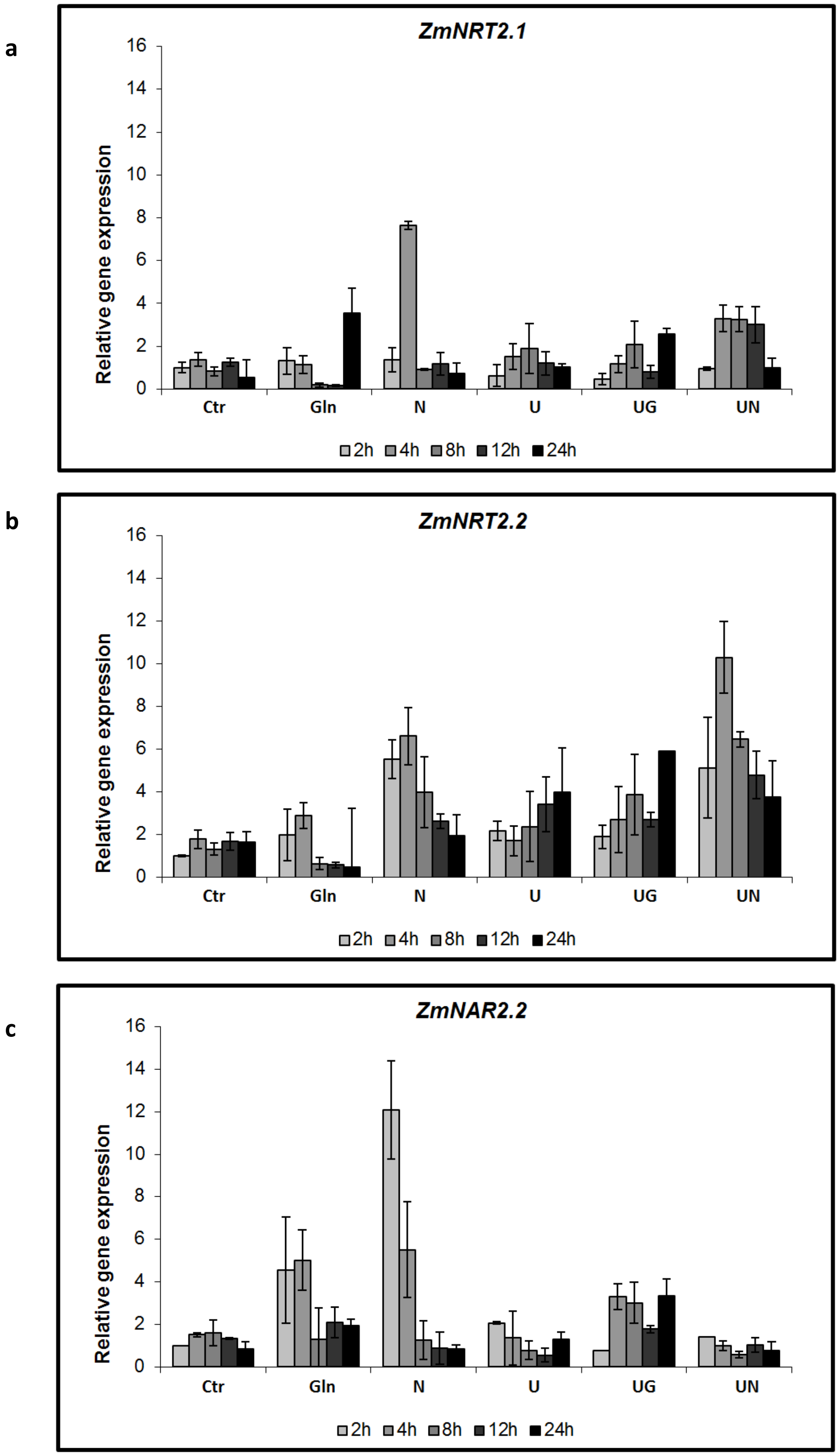


Figure 34. Real-time RT-PCR analyses of *ZmNRT2.1*-, *ZmNRT2.2*- and *ZmNAR2.2*-gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation,

exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). Analysed genes encode: a, b and c, high-affinity nitrate transporters (NRT2.1 and NRT2.2) and accessory protein of nitrate transporter (NAR2.2), respectively.

3.2.2.1.2 ZmNRT1.2 gene expression

By real time RT-PCR, the expression levels of *ZmNRT1.2* (NM_001112455.1) was also investigated (Figure 35). More information is available about its Arabidopsis homologous, *AtNRT1.2*. This gene was found expressed in root tip and in root epidermal cells and it is involved in nitrate uptake from soil into roots (Huang et al., 1999). AtNRT1.2 is solely a low affinity nitrate transporter and is responsible for the constitutive low affinity nitrate uptake capacity of roots, being expressed even if nitrate is not present (Huang et al., 1996; Huang et al., 1999).

Under our conditions, data suggest that in maize root the exposition to nitrate or glutamine (N or Gln treatments) down regulated the gene expression of *ZmNRT1.2*. On the other hand, after 24 hours, control roots induced its expression up to 3 fold, similar induction was also observed in roots treated with urea and glutamine (UG treatment). In fact, in UG roots the amount of *ZmNRT1.2* mRNA increased gradually until 8 hours when the peak value was registered. Interestingly, after 12 hours the transcription of this gene was rapidly down-regulated: at 12 and 24 hour a very low level of relative expression were detected. No significant modulation was observed in roots exposed to urea or to urea and nitrate, where the transcript levels were around 1 of relative gene expression or less.

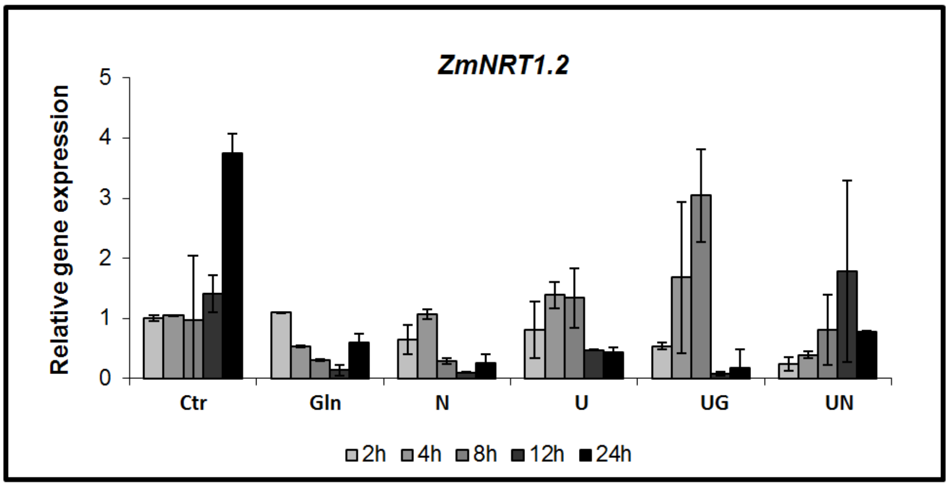


Figure 35. Real-time RT-PCR analyses of *ZmNRT1.2* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmNRT1.2* gene encodes for the low-affinity nitrate transporter (NRT1.2).

3.2.2.1.3 ZmNR2, ZmNiR and ZmGS2 gene expression

Concerning the nitrogen metabolism, the expression levels of genes encoding for enzymes involved in the nitrate reductive steps and ammonium assimilation pathway, such as nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) were investigated (Figure 36, a, b and c). The nitrogen treatments affected in the same way the transcription profile of all these genes (Z*mNR2*, *ZmNiR* and *ZmGS2*), which clearly responded to nitrate, under both N and UN treatments.

In particular under N treatment, *ZmNR2* and *ZmNiR* were strongly upregulated after 8 hours of exposition, while lower induction was reported for *ZmGS2*. These genes were also up-regulated in presence of nitrate with urea (UN). Compared to N treatment, the expression of *ZmNR2* in UN roots was induced earlier, reaching a peak already after 4 hours and was followed by a gradual decrease. On the contrary, the maximum up-regulation of *ZmNiR* by UN was reached two hours later (12 hours) than in N roots. The comparison between UN and N roots revealed a positive influence of urea on *ZmGS2* induction, since higher amounts of transcript were detected during all the experimental time span. Under other nitrogen treatments (Gln, U, UG), the expression levels were comparable to those observed in control roots. Further GS isoforms were also analyzed, such as ZmGS1-5 (GenBank: X65930.1), but no significant variations were registered among the different nitrogen treatments (data not shown).

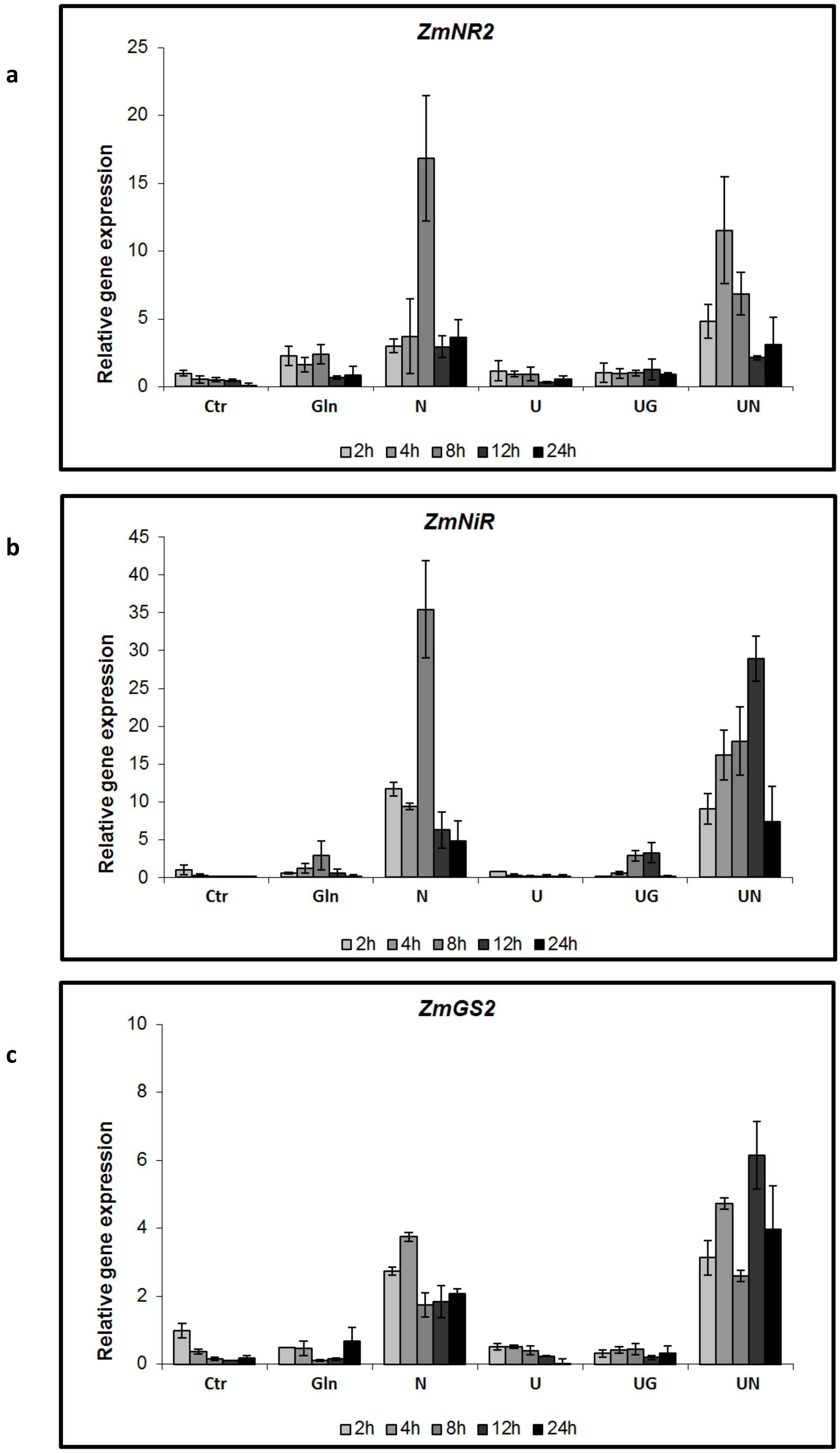


Figure 36. **Real-time RT-PCR analyses of *ZmNR2*, *ZmNiR* and *ZmGS2* gene expression levels in maize roots.** 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2

mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). Analysed genes encode: a, b and c, nitrate reductase (NR2), nitrite reductase (NiR) and glutamine synthetase (GS2).

3.2.2.1.4 ZmFd-GOGAT gene expression

The expression of a maize gene encoding for the enzyme Fd-GOGAT, which (together with GS) is involved in the GS-GOGAT cycle for the ammonium assimilation was also investigated (Figure 37). For this gene, the expression levels were referred to 4h of control roots, since no detectable levels were observed after 2 hours with the control treatment. Within 24 hours, control roots gradually increased the expression levels of Fd-GOGAT up to 4 times at the end of the experiment.

Under Gln, U and UN treatments its expression was significantly upregulated during the light phase reaching peak after 4 h of treatment, or already after 2 hours in UN roots, and later it declined showing the lowest values after 12 hours of exposition. These data may suggest a circadian regulation of *ZmFd-GOGAT* expression, since after 24 hours the amounts of transcript returned to be almost comparable, or higher, than those found at 4 hours under these treatments.

Different behavior was measured in N and UG roots. Under nitrate (N), the expression levels of *ZmFd-GOGAT* were up-regulated after 2 and after 12 hours of exposition to nitrate. In UG treated plants, a gradual reduction in transcript amounts was measured during the experimental timespan, after 24 hours the transcript amount was comparable to that measured after 4 hours in control roots.

In conclusion, the transcriptional regulation of *ZmFd-GOGAT* seems to be differentially modulated by the nitrogen treatments, nevertheless the highest amount of transcript was reached already after 2 hours of exposition to urea and nitrate (UN) treatment.

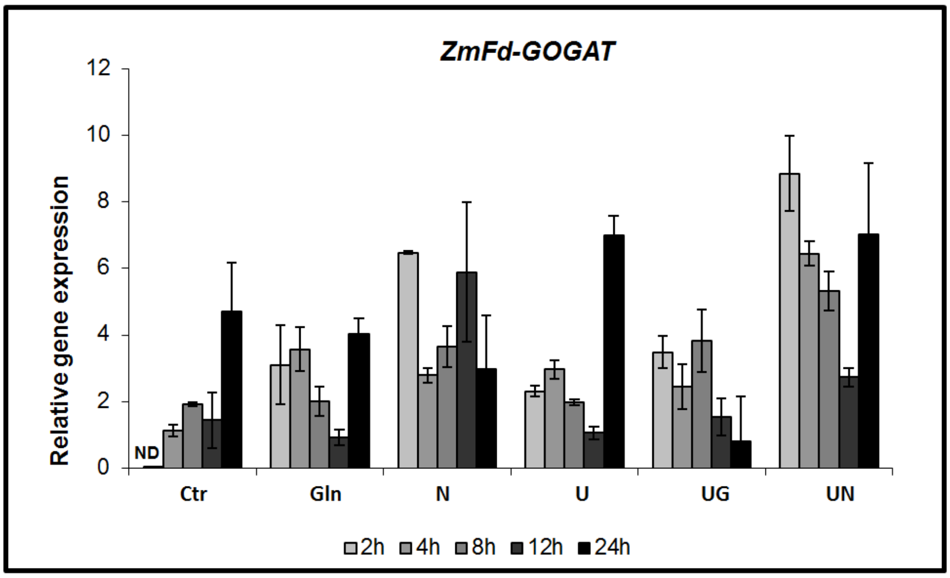


Figure 37. Real-time RT-PCR analyses of *ZmFd-GOGAT* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 4 hours (Relative gene expression=1), since after 2 h the expression level was not detectable (ND). *ZmFd-GOGAT* gene encodes for an enzyme involved in ammonium assimilation: glutamine oxoglutarate aminotransferase ferrodoxin dependent (Fd-GOGAT).

3.2.2.1.5 ZmDUR3, ZmUrease, ZmUreG gene expression

Genes involved in the uptake and assimilation of urea were analysed. *ZmDUR3* encode for the putative high affinity transporter of urea in maize, while *ZmUrease* and *ZmUreG* encode for the enzyme Urease and for UreG, respectively, an accessory protein that is required for the functionality of the apoenzyme urease.

Control roots showed a stable expression level of *ZmDUR3* ranging from 1 to 0.5, so during the experiment the mRNA amounts were not modulated meaningfully. The nitrogen treatments did not positively influenced the transcriptional regulation of this gene, indeed the *ZmDUR3* expression was down regulated by the different N sources. Most of the transcriptional patterns showed lower transcript abundance at any time span of the experiment as compared with values recorded in control roots. The only exception was reported for UG treatment, where after 24 hours the transcript amount was comparable to those found in control plants (Figure 38, a).

The urea, as being derived from soil uptake or from endogenous metabolism, is assimilated in plants via enzymatic degradation, that involve urease and UreG for the breakdown of urea into ammonium and CO_2.

ZmUrease expression was not influence by the presence of the different nitrogen sources, since control and treatment patterns were mostly similar showing stable expression levels during all the experimental time span. Nevertheless, a significant up-regulation of *ZmUrease* was induced after 24 hours of root exposition to urea source (U treatment). Also in UN roots exposed for 24hours to urea and nitrate, the gene expression was positively regulated, however in this case there was a great variation among the technical replications that led to high value in standard deviation (Figure 38, b).

In general, the expression levels of *ZmUreG* did not show any significant regulation of this gene. The relative amounts of transcript were comparable to that measured in control after 2 hours of exposition (around 1 of relative gene expression), except few samples, after 24 hours in control and glutamine treated roots (Ctr and Gln), in which the expression levels reached 2 of relative gene expression (Figure 38, c).

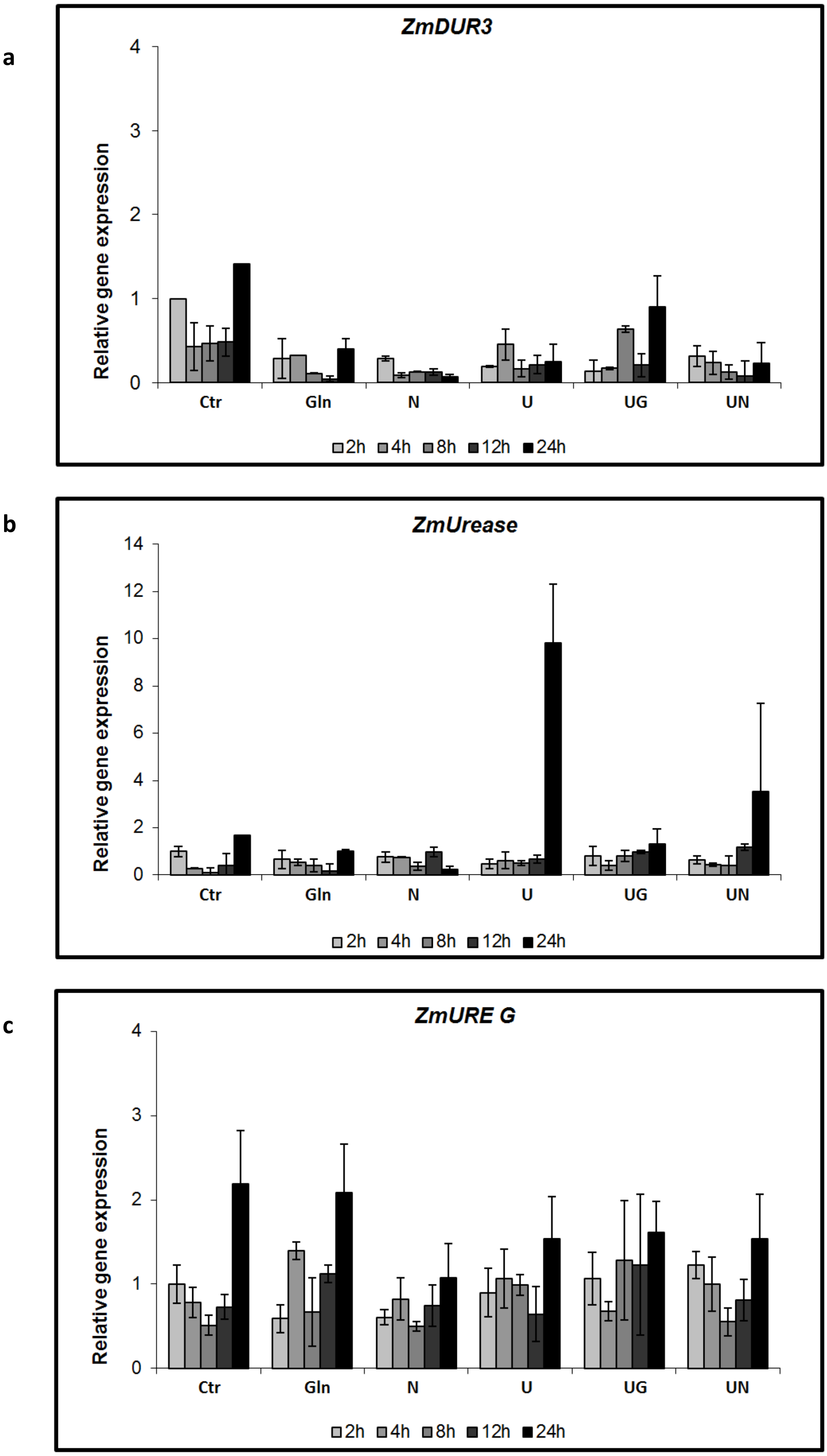


Figure 38. Real-time RT-PCR analyses of *ZmDUR3, ZmUrease, ZmUreG* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different

nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). Analysed genes encode: a, b and c, , high affinity urea transporter (ZmDUR3), urease (ZmUrease), urease accessory protein (ZmUreG).

3.2.2.1.6 ZmArginase gene expression

The enzyme arginase is a key enzyme in the urea cycle, catalysing the conversion of arginine to ornithine and *urea*. The expression analyses of the gene *ZmArginase* was performed to investigate if the root exposition to different nitrogen sources might be causing modifications in the endogenous metabolism, i.e. regulating the nitrogen remobilization from arginine.

During the time span of the experiment, the expression pattern registered for control and treated roots did not show significant modulations, since the values were closed to 1 of relative gene expression. Also among the treatments were not observed substantial differences (Figure 39).

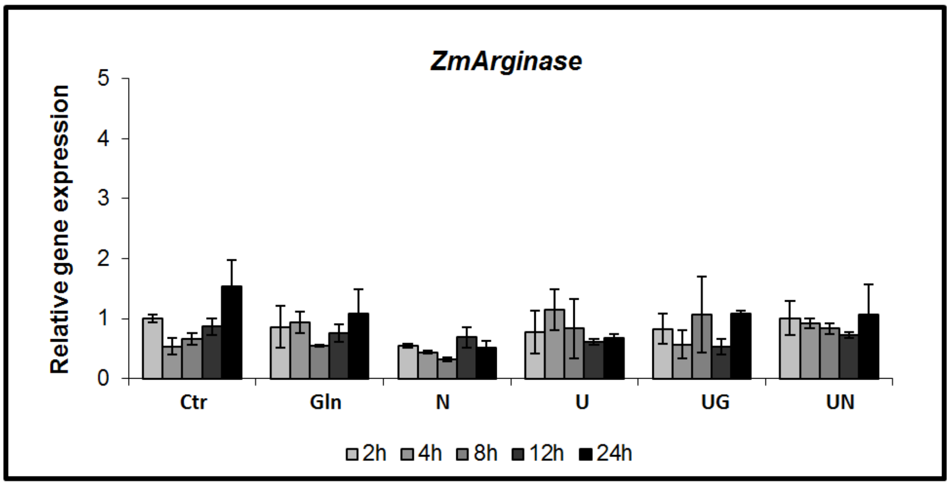


Figure 39. Real-time RT-PCR analyses of *ZmArginase* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmArginase* gene encodes for the enzyme arginase, which is involved in urea cycle.

3.2.2.1.7 *ZmPAL gene expression*

From microarray data the influence of nitrogen treatments on the expression of enzymes involved in the shikimic pathway was registered (Table 7). In particular the urea and nitrate treatment (UN) induced the transcriptional up-regulation of an down-stream enzyme for the synthesis of shikimic acid (shikimate kinase ,SK). Moreover the nitrogen sources influenced the transcription of genes encoding for enzymes that are involved in the transformation of phenylalanine derivatives into a phenilpropanoid, like synapate. For this reason the gene modulation of the enzyme phenylalanine ammonia-lyase (PAL), which catalyses the deamination of phenylalanine into cinnamic acid, was investigated. This reaction represents an essential regulatory step in the formation of many phenolic compounds.

During the time span of the experiment, stable expression levels of *ZmPAL* were found in control roots, and in presence of urea: under U, UG and UN treatments. Significant transcript modulation was observed under glutamine (Gln), in fact a strong increase in the expression levels was registered, reaching a peak after 4 hours of exposition to the amino acid. In the following hours, the expression levels declined already after 8 hours to values comparable to those detected in control roots.

Also the nitrate treatment induced the overexpression of *ZmPAL*, even if in this case the induction was less important than that registered under glutamine. After gradual increase in the first 8 hours, the transcript amount was stable from 8 to 24 hours being two times more abundant than in control roots (Figure 40).

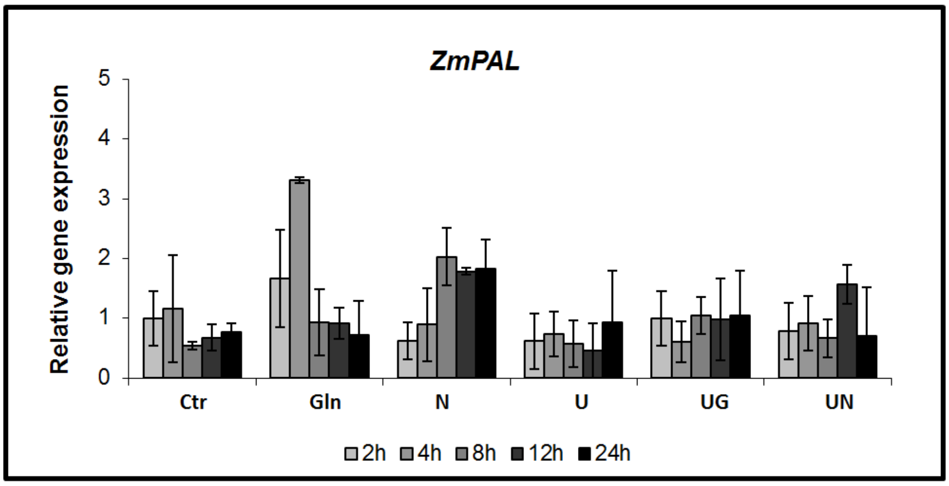


Figure 40. Real-time RT-PCR analyses of *ZmPAL* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmPAL* gene encodes for the enzyme phenylalanine ammonia-lyase (PAL), which, catalysing the deamination of phenylalanine, allowes the synthesis of many phenolic compounds.

3.2.2.1.8 ZmANR1 gene expression

Several studies have demonstrated that plants, including maize, respond to a localize nitrate treatment stimulating lateral root elongation (Granato and Raper, 1989; Zhang and Forde, 1998). One component of the nitrate signaling pathway has been identified in *Arabidopsis Nitrate Regulated-1 (ANR1)* gene, which is encoding for a member of the MADs box family transcription factors (Zhang and Forde, 1998). In Arabidopsis, *AtANR1* is exclusively expressed in roots (Zhang and Forde, 1998; Burgeff et al., 2002) and it is a positive regulator of a signal transduction pathway linking the external nitrate concentration in the soil with an increase in the rate of lateral root elongation (Zhang and Forde, 1998).

By real time RT-PCR, the expression profile of *ZmANR1* was examined in roots of maize exposed to the different nitrogen sources (Figure 41). The root exposition to 1 mM nitrate in the external medium (N treatment) induced the expression of *ZmANR1*. Under N treatment, the transcript amounts detected during the experimental time span were higher than those measured in control plants.

The highest up-regulation of *ZmANR1* was detected in root exposed to glutamine (Gln treatment) reaching peak after 4 hours, than the transcript amount declined suggesting the involvement of feedback regulation.

On the other hand, under U, UG and UN, the amounts of *ZmANR1* transcript did not show significant differences within 24 hours.

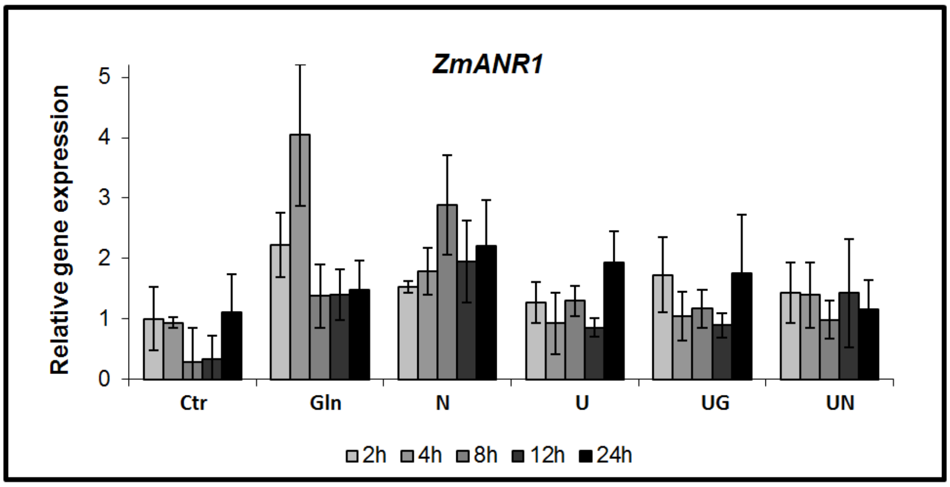


Figure 41. Real-time RT-PCR analyses of *ZmANR1* gene expression levels in maize roots. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Root samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmRPS4*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmRPS4* in roots of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmANR1* gene encodes for *Arabidopsis Nitrate Regulated-1* (ANR1) MADS-box transcription factor, a positive regulator of lateral root growth involved in nitrate signalling pathway.

3.2.2.2 Gene expression in maize leaves

3.2.2.2.1 ZmNR1, ZmNR2, ZmGS2 gene expression

In leaves, the expression levels of genes involved in nitrogen assimilation, such as nitrate reductase (NR), glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) was investigated. For most of them, the gene expression analysis was performed detecting the same gene isoforms that were measured in roots, in this way the effect of nitrogen treatments on modulation of genes, like *ZmNR2*, *ZmGS2,* and *ZmFd-GOGAT*, has been analyzed in time course within 24 hours in root and leaf tissues.

Two isoforms, *ZmNR1* and *ZmNR2,* of nitrate reductase were measured. Concerning the expression of *ZmNR1* (Figure 42, a) no great variations were observed in control and treated leaves, except under N and UN treatment. After 24 hours of root exposition to nitrate (N), leaves induced the expression of *ZmNR1* more than 4 times in comparison to the amount of transcript detected in control (2h Ctr). Interestingly, leaves of UN plants overexpressed *ZmNR1* already after 2 hours and, during all the experimental time span, the average of transcript amounts (mean value: 2.9 of relative gene expression) was higher than those detected in control leaves.

Despite the *ZmNR2* expression was strongly induced in roots by nitrate treatments (N and UN), in leaves this isoform was not induced by nitrogen sources (Figure 42, b). In leaves of control and treated plants, no significant up-regulation was observed within 24 hours. In particular N and UN showed expression levels comparable with control at 2 hours (around 1 of relative gene expression). However the profile pattern registered in control plants was not stable, indeed, after 8 hours, the amount of *ZmNR2* mRNA was 4 times downregulated (0.23 of relative gene expression). In urea-treated plants, the downregulation was delayed, occurring after 24 hours of root exposition to this organic nitrogen source. Intermediate and stable values were detected in plants treated with glutamine (Gln and UG treatments) ranging from 0,4 and 1 of relative gene expression.

As reported for *ZmNR2*, despite in roots *ZmGS2* was up-regulated by nitrate treatments, in leaves this gene was not induced under N and UN. Also other treatments did not induce significant gene modulation of *ZmGS2* and the transcript levels were comparable with those registered in control leaf tissue. Nevertheless, during the experimental timespan, a really weak increase in the amount of *ZmGS2* mRNA was observed in UG and UN treatments.

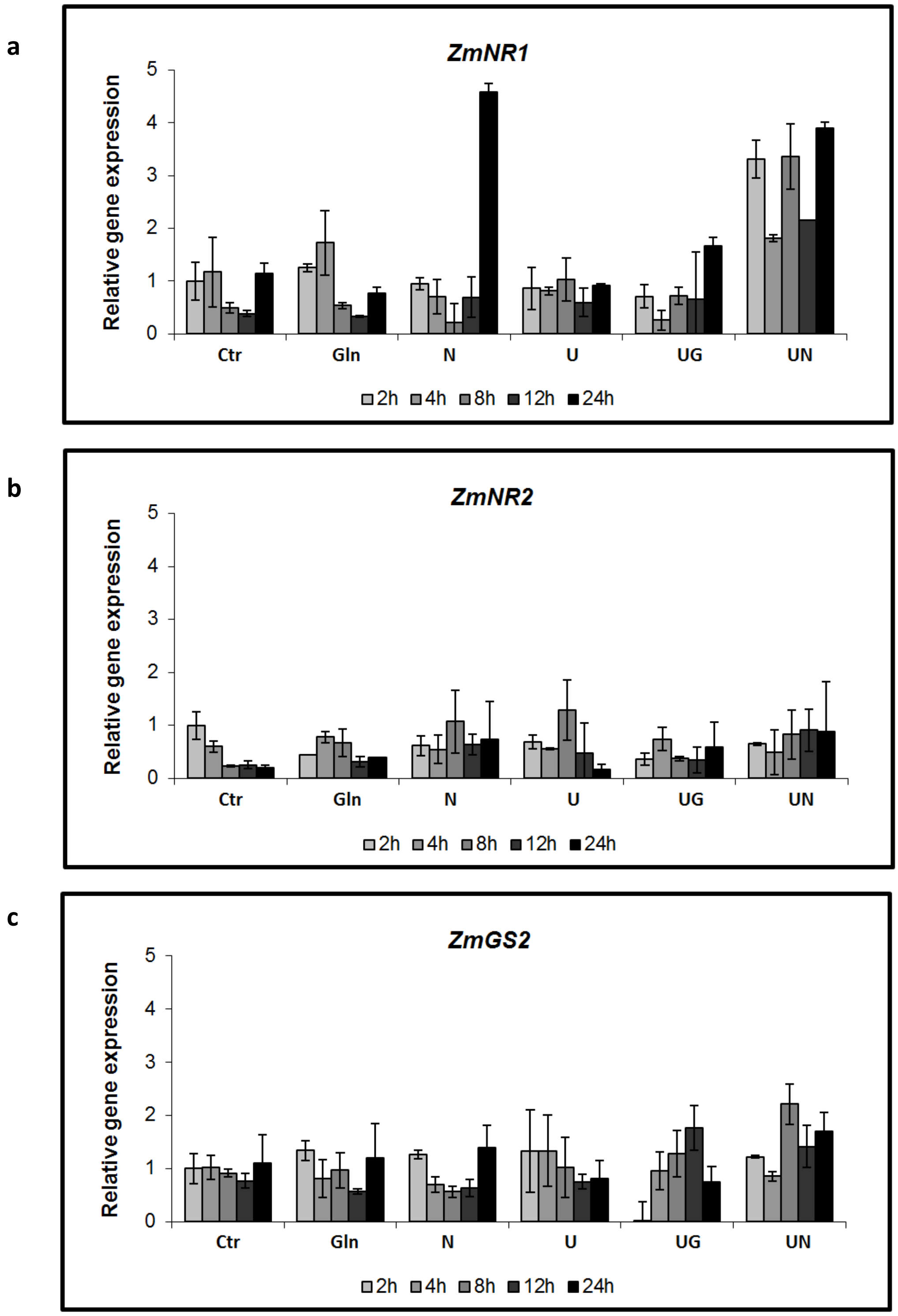


Figure 42. Real-time RT-PCR analyses of *ZmNR1, ZmNR2, ZmGS2* gene expression levels in maize leaves. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Leaf samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmPolyUBI*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmPolyUBI* in

leaves of control plants (Ctr) at 2 hours (Relative gene expression=1). Analysed genes encode: a, b and c, nitrate reductase (NR1 and NR2) and glutamine synthetase (GS2).

3.2.2.2.2 ZmFd-GOGAT gene expression

In leaves, the ferredoxin-dependent type of glutamate synthase (Fd-GOGAT) is found in chloroplasts and serves mainly in photorespiratory nitrogen metabolism for the (re)assimilation of the produced ammonia. The amount of Fd-GOGAT protein and its activity increase with light levels. Also the transcription of *ZmFd-GOGAT* gene was reported to be light regulated. In particular, Suzuki and coworkers (2001) examined the transcript amounts in 11-day-old second green leaves of maize plants grown under 16-h light/8-h dark cycle. The steady-state mRNA level varied between day and night: the higher mRNA level reached about 4 h after the onset of light, and then decreased by about 20% to the minimal level at the middle of the day. Interestingly, the *ZmFd-GOGAT* mRNA induction in the morning and repression at the middle of the day correlate with the lowest and highest levels of glutamate, respectively (Suzuki et al., 2001). The authors suggested that the reduction in *ZmFd-GOGAT* mRNA in the morning is reminiscent of a repression of the nitrogen assimilatory genes by high nitrogen metabolites or low carbon metabolites (Suzuki et al., 2001).

Our real time RT-PCR data (Figure 43) are in agreement with the previous observations reported by Suzuki et al. (2001), since in general a downregulation of *ZmFd-GOGAT* was measured in the middle of the light phase, correspondent to samples harvested after 4-8-12 hours of exposition to the treatments. On the other hand, higher values of expression were registered in 2- and 24-hour leaf samples, which were harvested after 3 and 1 hours, respectively, from the onset of light.

An interesting pattern was observed in UN leaves, where the minimal expression level of *ZmFd-GOGAT* was detected after 4 hours of root exposition to the sources and gradually increased reaching the maximum after 24 hours of treatment. After 13 hours from the onset of light (12h sample), the transcript amount was the equal to the one detected after 3 hours of light phase (2h sample). So, this data suggest that the expression of *ZmFd-GOGAT* is maintained higher when roots are exposed to urea and nitrate applied in combination than when are applied singularly.

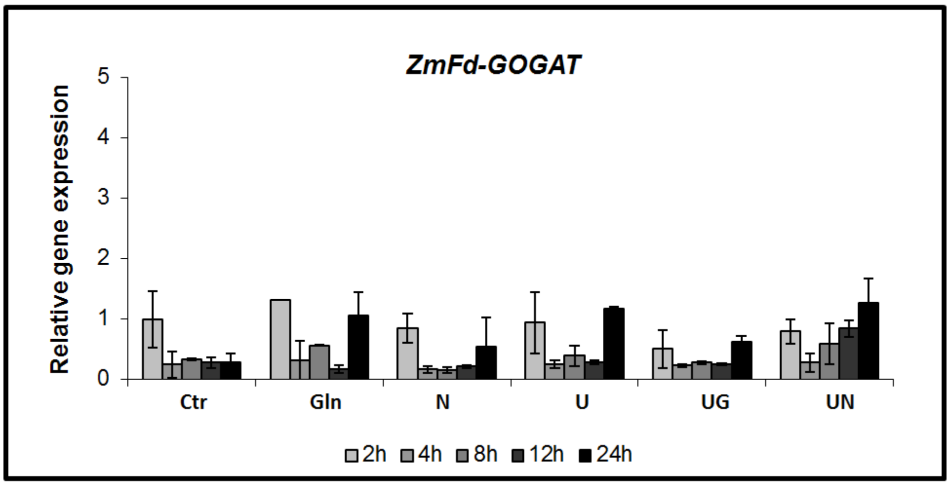


Figure 43. Real-time RT-PCR analyses of *ZmFd-GOGAT* gene expression levels in maize leaves. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Leaf samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmPolyUBI*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmPolyUBI* in leaves of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmFd-GOGAT* gene encodes for an enzyme involved in ammonium assimilation: glutamine oxoglutarate aminotransferase ferrodoxin dependent (Fd-GOGAT).

3.2.2.2.3 *ZmArginase gene expression*

The expression profile of *ZmArginase* was examined in roots (Figure 39) and in leaves (Figure 44). In both tissues *ZmArginase* did not show any significant variation in gene expression levels in control and treated plants and the values were very stable during the time span of the experiment.

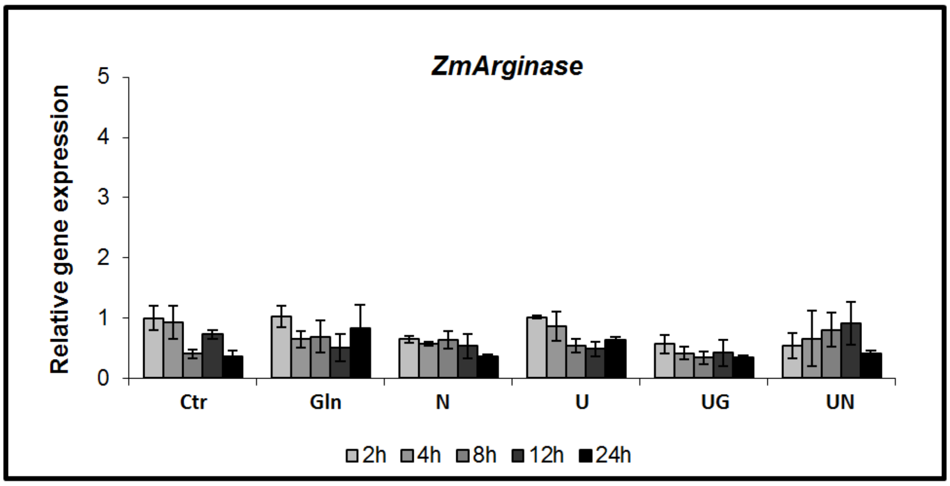


Figure 44. Real-time RT-PCR analyses of *ZmArginase* gene expression levels in maize leaves. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Leaf samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmPolyUBI*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmPolyUBI* in leaves of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmArginase* gene encodes for an enzyme, arginase, involved in the urea cycle for the remobilization of nitrogen stored in form of arginine.

3.2.2.2.4 ZmPIP4 gene expression

The maize gene *Plasma-membrane-Intrinsic-Protein 4 (ZmPIP4)* encodes for an aquaporin that belongs to the protein classes of water channel putatively localized at plasma membrane or PIPs. By heterologous expression in *Xenopus oocytes*, Marilia (2001) demonstrated the capacity of the maize protein PIP4 to transport urea.

By microarray analysis performed on root tissues, PIP4 did not show any variation at transcriptional level after 8 hours of exposition to N, U, UN or Ctr. So, a real time RT-PCR analysis was performed in maize leaves to investigate if, in leaves, *ZmPIP4* is modulated by the nitrogen sources.

Different expression patterns were observed for *ZmPIP4* in control and treated samples (Figure 45). Surprisingly, *ZmPIP4* was progressively accumulated in urea-treated plants (U) reaching a maximum after 8 h (5 times higher than 2h Ctr) and then decreased. Also under glutamine (Gln), *ZmPIP4* was up-regulated showing already after 2-4 hours a high amount of transcript. Differently the transcriptional profiles registered under N and UG were constantly below that detected in control leaves at 2 hours. Within 24 hours, Ctr and UN showed fluctuations in the transcript amounts and, in both case, the minimal value was reached after 12 hours of treatment.

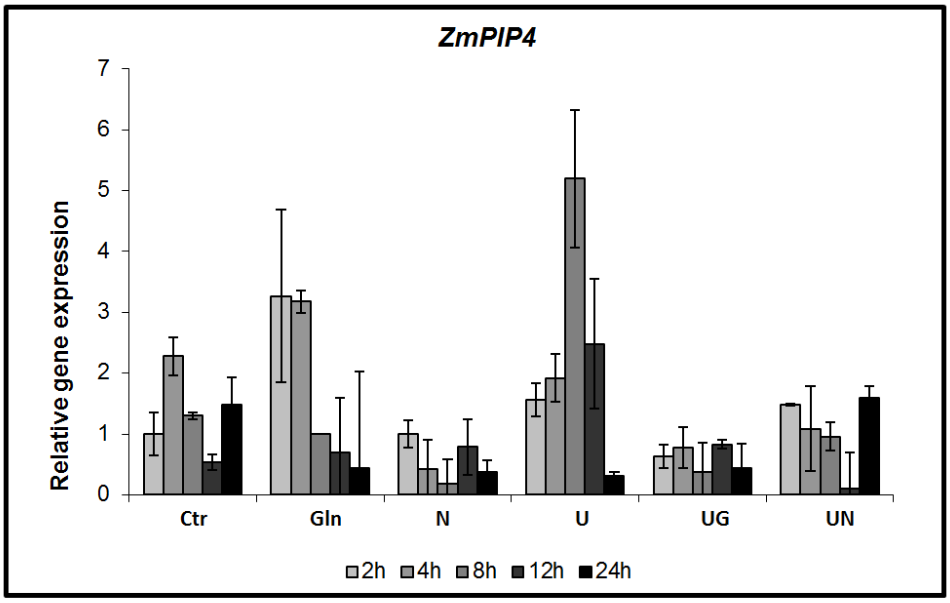


Figure 45. Real-time RT-PCR analyses of *ZmPIP4* gene expression levels in maize leaves. 5 day-old maize plants were exposed for a maximum of 24 hours to nutrient solution supplied with different nitrogen treatments: glutamine (Gln, 1 mM Ntot), nitrate (N, 1 mM Ntot), urea (U, 1 mM Ntot), urea and glutamine (UG, 2 mM Ntot), urea and nitrate (UN, 2 mM Ntot). As control few plants were maintain in nitrogen starvation, exposed to nutrient solution without addition of any nitrogen source (Ctr). Leaf samples were harvested after 2-4-8-12-24 hours of treatment. Data were confirmed by two independent biological replications, however the graphs are referred to representative experiments where data are mean ± SD (technical replications: n=3). Gene mRNA levels were normalized with respect to the transcript level of the housekeeping gene *ZmPolyUBI*; relative changes in gene transcript levels were calculated on the basis of the mean transcript level of housekeeping gene *ZmPolyUBI* in leaves of control plants (Ctr) at 2 hours (Relative gene expression=1). *ZmPIP4* gene encodes for an aquaporin: Plasmamembrane-Intrinsic-Protein 4 (ZmPIP4).

3.3 Functional and molecular characterization of ZmDUR3*,* the high affinity urea transporter in maize

In the last decades urea has become an extensively used N-fertilizer. However, despite its great agricultural importance the role of urea as a nitrogen source for plants is still not very well understood. In addition, besides the chemical input as fertilizer, urea is also a natural organic molecule synthetized by most organisms (Kojima et al., 2006; Wang et al., 2008). While in plants urea represents an important metabolite for nitrogen storage, in mammals the urea production is associated to the detoxification of nitrogen compounds (Sands, 2004). As a consequence urea is ubiquitously released in natural soils and water bodies at constant micromolar concentrations (less than 10 µM; Cho et al., 1996; Mitamura et al,. 2000a; Mitamura et al., 2000b). Despite the fact that fertilization events can contribute to increased urea concentration in soils, Gaudin et al. (1987) estimated that also in fertilized crop-planted soils the urea concentration is maintained in a micromolar range (up to 70 µM). In part this is due to the presence of microbial ureases in the soil solution, which rapidly hydrolyse urea into carbon dioxide and ammonia. However, as pointed out by Dalal (1985), the microbial urease activity shows an affinity constant in the millimolar range, so low concentrations of urea could remain in the soil also after microbial degradation. As a logical consequence plants might have developed strategies to use this nitrogen source, even if present in a micromolar concentration range.

The evolution of a high affinity urea transporter in plants can represent an important strategy to increase the nitrogen uptake capability especially when the external urea concentrations are very low (Kojima et al., 2006). However only few studies have investigated the molecular basis of the urea transporters in higher plants, the first research was published by Liu et al. in the 2003(a) reporting the cloning and characterization of a high affinity urea transporter of Arabidopsis, called *AtDUR3*. In particular the coding sequence of *AtDUR3* showed a weak homology with an ortholog of yeast (*ScDUR3*), a member of the sodium-solute symporter (SSS) gene family, which is widespread in microorganisms, animals, and humans (Reizer et al., 1994; Jung, 2002). Members of the SSS family have been described to transport a various range of solutes, such as sugars, amino acids, nucleosides, inositols, vitamins, anions, and urea (Reizer et al., 1994; Turk and Wright, 1997; Saier, 2000). Further investigations on AtDUR3 showed no significant homology to any other protein of Arabidopsis (Liu et al., 2003a); thus, it represents the only member of this gene family in plants. Similarly, in the rice genome, *OsDUR3* is the only gene that has significant homology with *AtDUR3*, suggesting that plant DUR3 proteins might represent a transporter subfamily consisting of only one member (Wang et al., 2012). To date, among higher plants only the Arabidopsis and rice orthologous urea transporters (AtDUR3 and OsDUR3) have been characterized at molecular and physiological level (Liu et al., 2003a; Kojima et al., 2007; Wang et al., 2012).

3.3.1 Isolation and cloning of *ZmDUR3* from maize roots

Only one putative full length coding sequence (ZmAC202439_FGT006) in the maize genome was identified by homology to *AtDUR3* (At5g45380), using a BLAST (Basic Local Alignment Search Tool) search algorithm performed using the Aramemnon plant membrane protein database (http://aramemnon.botanik.uni-koeln.de/index.ep, ARAMEMNON v. 7.0© Flügge Lab, Botanical Institute at the University of Cologne) (Figure 46).

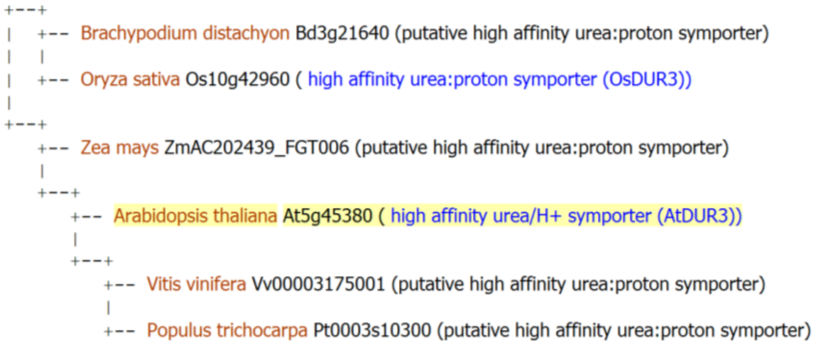


Figure 46. Orthologous cluster of related plant protein sequences to AtDUR3 (from Aramemnon DB http://aramemnon.botanik.uni-koeln.de)

Further *in silico* analysis on GenBank EST database revealed a group of maize ESTs (BQ164112, BQ164020, FL011289, FL448872, DV550376, AW400387, BQ163839, BQ163822, FL011290) proofing expression of this gene. Most of the ESTs covered the 3'- region of ZmAC202439_FGT006 with the exception of FL011289 and FL011290 which aligned at the 5'- region (Figure 47).

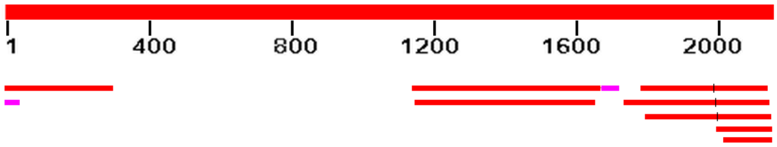


Figure 47. Distribution of ESTs on the putative transcript ZmAC202439_FGT006. ESTs were identified performing a BLASTN analysis on NCBI (*National Center for Biotechnology Information,* http://blast.ncbi.nlm.nih.gov).

In this part of the work, the coding sequence of the high affinity urea transporter (*ZmDUR3*) was isolated from maize root mRNAs. Using gene specific primers, a transcript from maize root was amplified by RT-Assembly-PCR and cloned into the yeast expression vector pDR197 (Doris Rentsch, unpublished). The sequencing results showed an open reading frame of 2196-bp coding for 731 amino acids. The alignment with the genomic sequence revealed four exon regions of 192, 108, 663 and 1233 bp, which differed in number and size from the predicted ones (Figure 48). In addition, in comparison to the predicted transcript sequence (ZmAC202439_FGT006), the isolated *ZmDUR3*-ORF contained three non-synonymous changes in the nucleotide sequence; one of this variation was also detected in a maize EST sequence (BQ164112). The same three nucleotides were also identified in the rice sequence, *OsDUR3* (Wang et al., 2012).

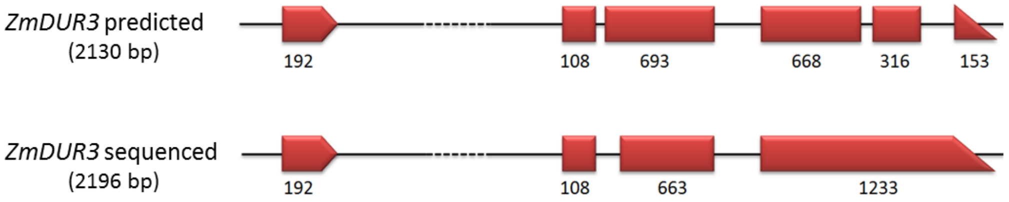
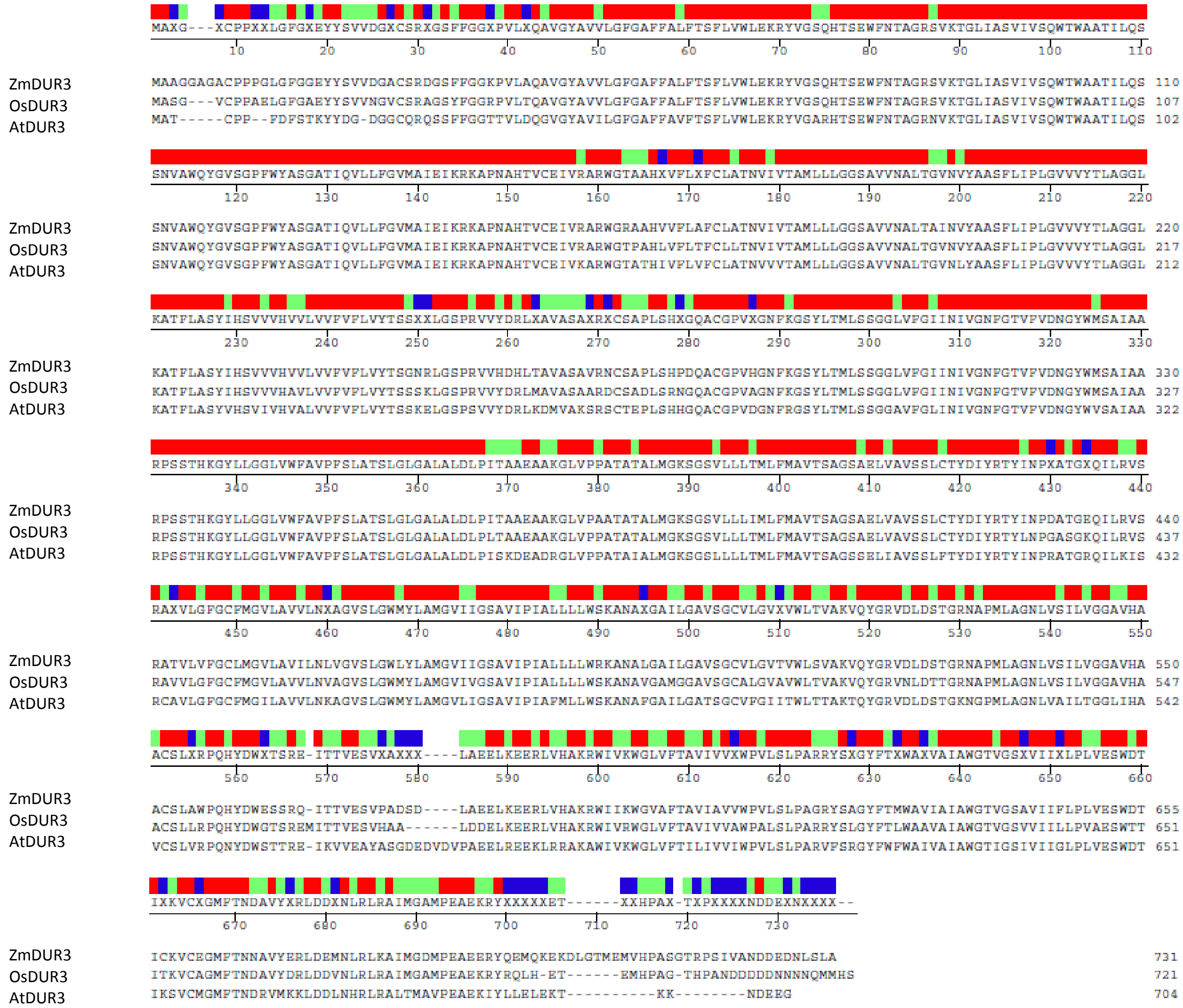


Figure 48. Schematic representation of the exonic regions of ZmAC202439_FGT006. Comparison between the predicted exons and the coding sequences found by cDNA sequencing (exons are represents as red boxes).

Blast analysis revealed that *ZmDUR3* cDNA had a high similarity with *OsDUR3* rice sequence (84% nucleotide sequence identity with a 94% of query coverage). Similar percentages were also observed at amino acid level with an identity of 83 and 75% to protein OsDUR3 and AtDUR3, respectively (Figure 49). ZmDUR3 contains 731 amino acids and is predicted as an integral membrane protein containing fifteen predicted transmembrane spanning domains with outside position of the N-terminus of the protein (TMSDs, prediction performed by TOPCONS, http://topcons.cbr.su.se/, and confirmed by TMHMM 2.0, http://www.cbs.dtu.dk/services/TMHMM/). In addition, since the expected number of amino acids in transmembrane helix in the first 60 amino acids of the protein was around 17 (larger than 10 amino acids), the presence of a "possible N-terminal signal sequence" was predicted (TMHMM 2.0, http://www.cbs.dtu.dk/services/TMHMM/).

The comparison between ZmDUR3 and the rice ortholog OsDUR3 (721 amino acids) revealed a similar predicted topology (Figure 50), especially with regard to number of TMSDs, and N- and C-terminal orientation.



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        MAXG---XCPPXXLGFGXEYYSVVDGXCSRXGSFFGGXPVLXQAVGYAVVLGFGAFFALFTSFLVWLEKRYVGSQHTSEWFNTAGRSVKTGLIASVIVSQWTWAATILQS
                10        20        30        40        50        60        70        80        90       100       110

ZmDUR3  MAAGGAGACPPPGLGFGGEYYSVVDGACSRDGSFFGGKPVLAQAVGYAVVLGFGAFFALFTSFLVWLEKRYVGSQHTSEWFNTAGRSVKTGLIASVIVSQWTWAATILQS 110
OsDUR3  MASG---VCPPAELGFGAEYYSVVNGVCSRAGSYFGGRPVLTQAVGYAVVLGFGAFFALFTSFLVWLEKRYVGSQHTSEWFNTAGRSVKTGLIASVIVSQWTWAATILQS 107
AtDUR3  MAT-----CPP--FDFSTKYYDG-DGGCQRQSSFFGGTTVLDQGVGYAVILGFGAFFAVFTSFLVWLEKRYVGARHTSEWFNTAGRNVKTGLIASVIVSQWTWAATILQS 102

        SNVAWQYGVSGPFWYASGATIQVLLFGVMAIEIKRKAPNAHTVCEIVRARWGTAAHXVFLXFCLATNVIVTAMLLLGGSAVVNALTGVNVYAASFLIPLGVVVYTLAGGL
               120       130       140       150       160       170       180       190       200       210       220

ZmDUR3  SNVAWQYGVSGPFWYASGATIQVLLFGVMAIEIKRKAPNAHTVCEIVRARWGRAAHVVFLAFCLATNVIVTAMLLLGGSAVVNALTAINVYAASFLIPLGVVVYTLAGGL 220
OsDUR3  SNVAWQYGVSGPFWYASGATIQVLLFGVMAIEIKRKAPNAHTVCEIVRARWGTPAHLVFLTFCLLTNVIVTAMLLLGGSAVVNALTGVNVYAASFLIPLGVVVYTLAGGL 217
AtDUR3  SNVAWQYGVSGPFWYASGATIQVLLFGVMAIEIKRKAPNAHTVCEIVKARWGTATHIVFLVFCLATNVVVTAMLLLGGSAVVNALTGVNLYAASFLIPLGVVVYTLAGGL 212

        KATFLASYIHSVVVHVVLVVFVFLVYTSSXXLGSPRVVYDRLXAVASAXRXCSAPLSHXGQACGPVXGNFKGSYLTMLSSGGLVFGIINIVGNFGTVFVDNGYWMSAIAA
               230       240       250       260       270       280       290       300       310       320       330

ZmDUR3  KATFLASYIHSVVVHVVLVVFVFLVYTSGNRLGSPRVVHDHLTAVASAVRNCSAPLSHPDQACGPVHGNFKGSYLTMLSSGGLVFGIINIVGNFGTVFVDNGYWMSAIAA 330
OsDUR3  KATFLASYIHSVVVHAVLVVFVFLVYTSSSKLGSPRVVYDRLMAVASAARDCSADLSRNGQACGPVAGNFKGSYLTMLSSGGLVFGIINIVGNFGTVFVDNGYWMSAIAA 327
AtDUR3  KATFLASYVHSVIVHVALVVFVFLVYTSSKELGSPSVVYDRLKDMVAKSRSCTEPLSHHGQACGPVDGNFRGSYLTMLSSGGAVFGLINIVGNFGTVFVDNGYWVSAIAA 322

        RPSSTHKGYLLGGLVWFAVPFSLATSLGLGALALDLPITAAEAAKGLVPPATATALMGKSGSVLLLTMLFMAVTSAGSAELVAVSSLCTYDIYRTYINPXATGXQILRVS
               340       350       360       370       380       390       400       410       420       430       440

ZmDUR3  RPSSTHKGYLLGGLVWFAVPFSLATSLGLGALALDLPITAAEAAKGLVPAATATALMGKSGSVLLLIMLFMAVTSAGSAELVAVSSLCTYDIYRTYINPDATGEQILRVS 440
OsDUR3  RPSSTHKGYLLGGLVWFAVPFSLATSLGLGALALDLPLTAAEAAKGLVPPATATALMGKSGSVLLLTMLFMAVTSAGSAELVAVSSLCTYDIYRTYLNPGASGKQILRVS 437
AtDUR3  RPSSTHKGYLLGGLVWFAVPFSLATSLGLGALALDLPISKDEADRGLVPPATAIALMGKSGSLLLLTMLFMAVTSAGSSELIAVSSLFTYDIYRTYINPRATGRQILKIS 432

        RAXVLGFGCFMGVLAVVLNXAGVSLGWMYLAMGVIIGSAVIPIALLLLWSKANAXGAILGAVSGCVLGVXVWLTVAKVQYGRVDLDSTGRNAPMLAGNLVSILVGGAVHA
               450       460       470       480       490       500       510       520       530       540       550

ZmDUR3  RATVLVFGCLMGVLAVILNLVGVSLGWLYLAMGVIIGSAVIPIALLLLWRKANALGAILGAVSGCVLGVTVWLSVAKVQYGRVDLDSTGRNAPMLAGNLVSILVGGAVHA 550
OsDUR3  RAVVLGFGCFMGVLAVVLNVAGVSLGWMYLAMGVIVGSAVIPIALLLLWSKANAVGAMGGAVSGCALGVAVWLTVAKVQYGRVNLDTTGRNAPMLAGNLVSILVGGAVHA 547
AtDUR3  RCAVLGFGCFMGILAVVLNKAGVSLGWMYLAMGVLIGSAVIPIAFMLLWSKANAFGAILGATSGCVFGIITWLTTAKTQYGRVDLDSTGKNGPMLAGNLVAILTGGLIHA 542

        ACSLXRPQHYDWXTSRE-ITTVESVXAXXX----LAEELKEERLVHAKRWIVKWGLVFTAVIVVXWPVLSLPARRYSXGYFTXWAXVAIAWGTVGSXVIIXLPLVESWDT
               560       570       580       590       600       610       620       630       640       650       660

ZmDUR3  ACSLAWPQHYDWESSRQ-ITTVESVPADSD----LAEELKEERLVHAKRWIIKWGVAFTAVIAVVWPVLSLPAGRYSAGYFTMWAVIAIAWGTVGSAVIIFLPLVESWDT 655
OsDUR3  ACSLLRPQHYDWGTSREMITTVESVHAA------LDDELKEERLVHAKRWIVRWGLVFTAVIVVAWPALSLPARRYSLGYFTLWAAVAIAWGTVGSVVIILLPVAESWTT 651
AtDUR3  VCSLVRPQNYDWSTTRE-IKVVEAYASGDEDVDVPAEELREEKLRRAKAWIVKWGLVFTILIVVIWPVLSLPARVFSRGYFWFWAIVAIAWGTIGSIVIIGLPLVESWDT 651

        IXKVCXGMFTNDAVYXRLDDXNLRLRAIMGAMPEAEKRYXXXXXET------XXHPAX-TXPXXXXNDDEXNXXXX--
               670       680       690       700       710       720       730

ZmDUR3  ICKVCEGMFTNNAVYERLDEMNLRLKAIMGDMPEAEERYQEMQKEKDLGTMEMVHPASGTRPSIVANDDEDNLSLA                                   731
OsDUR3  ITKVCAGMFTNDAVYDRLDDVNLRLRAIMGAMPEAEKRYRQLH-ET------EMHPAG-THPANDDDDNNNNQMMHS                                 721
AtDUR3  IKSVCMGMFTNDRVMKKLDDLNHRLRALTMAVPEAEKIYLLELEKT----------KK--------NDEEG                                        704
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Figure 49. Amino acid alignment between orthologous proteins (in order ZmDUR3, OsDUR3 and AtDUR3). Performed by "MegAlign" Lasergene software using Clustal-W method.

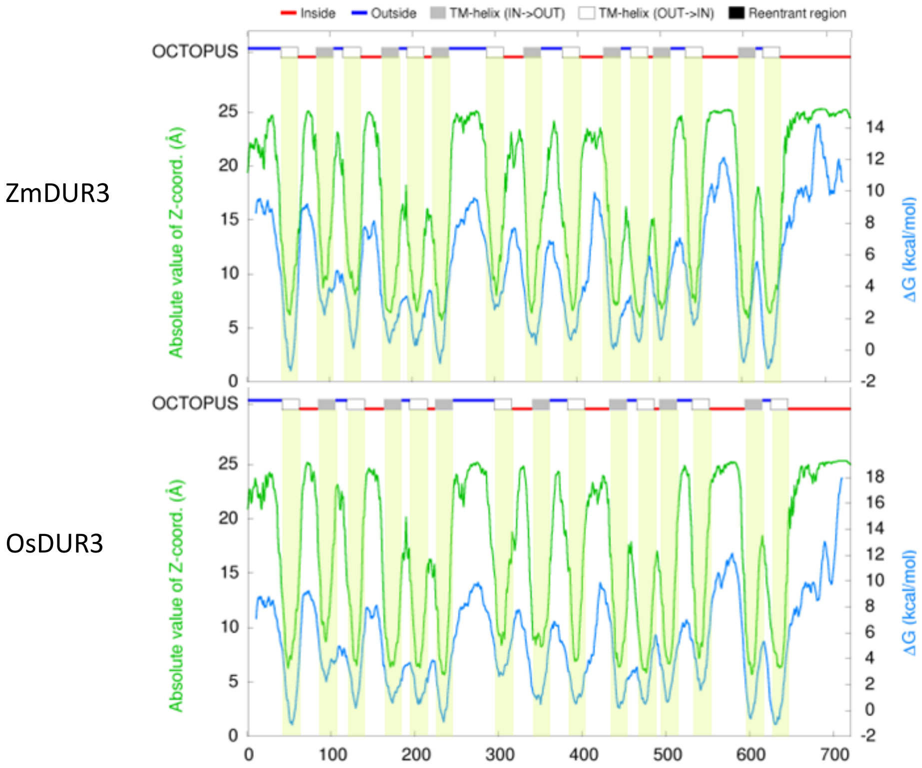


Figure 50. Comparison between predicting transmembrane protein topologies of ZmDUR3 and OsDUR3 (prediction performed by http://topcons.cbr.su.se/)

The functional characterization was performed by using two different approaches in heterologous systems: i) a functional complementation of a mutant strain of *S. cerevisiae*, ii) transport function assays by two electrode voltage clamp and radiolabeled uptake experiments in *Xenopus* oocytes. Since *ZmDUR3* has a high GC-content (around 80% GC content in the first 100 bp), its level of expression in other organisms may be limited. In order to reduce the GC content and favor the expression of *ZmDUR3*, we modified the first part of *ZmDUR3* at nucleotide level (less than. 200 nt). These modifications are all synonymous substitutions occurring only at the third base of the codons (the codon-usage preference in yeast is referred to http://www.kazusa.or.jp/codon/). In this way a second version of *ZmDUR3* was obtained (called *ZmDUR3"Modified"*) which differs from the *ZmDUR3"Native"* only at nucleotide level, while the amino acids remain unchanged (Figure 51).

ZmDUR3"Native": 5'- ATG GCC GCT GGC GGC GCC GGC GCG TGC CCT CCG CCG GGG CTG GGC TTC GGC GGG GAG TAC TAC TCG GTG GTG GAC GGC GCG TGC AGC CGC GAC GGC AGC TTC TTC GGC GGG AAG CCG GTG CTG GCG CAG GCC GTT GGG TAC GCC GTC GTC CTT GGC TTC GGC GCC TTC TTC GCG CTC TTC ACC TCA TTC CTG…-3'

MAAGGAGACPPPGLGFGGEYYSVVDGACSRDGSFFGGKPVLAQAVGYAVVLGFGAFFALFTSFL

ZmDUR3"Modified": 5'- ATG GC**T** GCT GG**T** GG**T** GC**T** GG**T** GC**T** TG**T** CCT CC**A** CC**A** GG**T** CT**A** GG**T** TT**T** GG**T** GG**T** GA**A** TA**T** TA**T** TC**T** GT**T** GT**T** GA**T** GG**T** GC**T** TG**T** AG**T** CG**T** GA**T** GG**T** AGC TT**T** TT**T** GGC GG**T** AA**A** CC**A** GT**T** CT**A** GC**T** CA**A** GC**T** GTT GG**T** TA**T** GC**T** GTC GT**T** CTT GG**T** TT**T** GG**T** GC**T** TTC TTC GCG CTC TTC ACC TCA TTC CTG…-3'

MAAGGAGACPPPGLGFGGEYYSVVDGACSRDGSFFGGKPVLAQAVGYAVVLGFGAFFALFTSFL

Figure 51. **Nucleotide differences between *ZmDUR3*"Native" and *ZmDUR3*"Modified" sequences.** The nucleotide sequence of the first exon was modified substituting only the third base of the codons (bold letters), originating *ZmDUR3*"Modified", with no difference occurring at the amino acid level.

3.3.2 Yeast complementation assay in *dur3* mutant yeast strain YNVW1

In order to verify the capacity of *ZmDUR3*-ORF to transport urea, a yeast complementation assay was performed using a *dur3* mutant strain of *Saccharomyces cerevisiae*, as described previously by Liu et al. (2003a). The mutant YNVWI (Δura3, Δdur3) is defective in urea uptake and can not grow on <5mM urea as the sole nitrogen source (Liu et al., 2003a). After complementation, both versions of *ZmDUR3*-ORF (*ZmDUR3"Native*" and *ZmDUR3"Modified*") were tested on a medium containing urea at different concentrations (1, 2 or 3 mM) as the sole nitrogen source.

Results showed that the *dur3* mutant strain transformed with the empty vector pDR197 was unable to grow on a medium containing only urea. On the other hand, the heterologous expression of both versions of *ZmDUR3*-ORFs enabled YNVWI to grow on urea medium (Figure 52). As positive control, the yeast strain 23346c (*Δura3*) containing the unmutated endogenous yeast urea transporter (ScDUR3) was transformed with the (empty) expression-vector pDR197 and grown on selective media. Growth of colonies of yeast strain 23346c were visible already after 3 days on 3 mM urea (data not shown).

In comparison to the positive control, the *ZmDUR3* transformants needed a longer incubation time since the colonies became visible only after 5 days.

Furthermore, the two *ZmDUR3*-ORF transformed lines (*ZmDUR3"Native"*- and *ZmDUR3"Modified"*-transformants) did not show any apparent growth difference on a media supplemented with ammonium (as ammonium sulfate, 0.5% $(NH_4)_2SO_4$) as the nitrogen source. When grown on selective plates supplemented with urea as a sole nitrogen source, however, growth differences between *ZmDUR3"Native"*- and *ZmDUR3"Modified"*-transformants became apparent. In particular the size of the colonies of *ZmDUR3"Modified"*-transformants was larger in comparison to colonies transformed with the native *ZmDUR3*-ORF version, and this different growth capacity was already visible on 1 mM urea plates. This result is clearly ascribable to the partial optimization of the codon usage in *ZmDUR3"Modified"*-ORF facilitating in yeast the translation of the maize sequence.

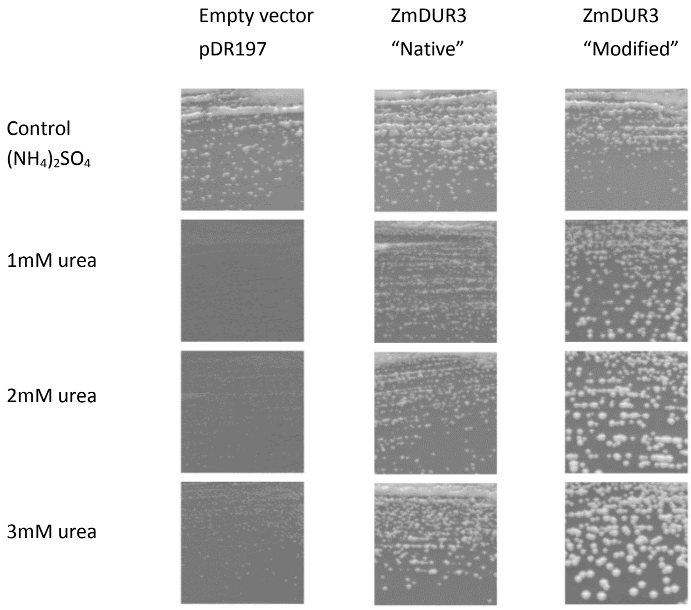


Figure 52. Yeast complementation assay. Growth of the urea uptake-defective strain YNVW1 by expressing *ZmDUR3*"Native" and *ZmDUR3*"Modified". The yeast mutant YNVW1 transformed with the empty vector pDR197(left column), and pDR197 carrying *ZmDUR3"Native"* (middle column) or *ZmDUR3"Modified"* (right column) ORFs. MM medium plates contained 0.5% of ammonium sulfate or urea at three different concentrations (1, 2 or 3 mM urea) as a sole nitrogen source. The pictures were taken after 5 days of incubation.

3.3.3 Kinetic studies in *X. laevis* oocytes (electrophysiology and ^{14}C-urea uptake)

To get information about kinetic parameters and transport features of ZmDUR3, both versions of *ZmDUR3*-cRNA (*ZmDUR3"Native*" and *ZmDUR3"Modified*") were injected into oocytes. Using this heterologous system, the ZmDUR3 transport activity was tested using two different approaches: two electrode voltage clamp experiments and influx assay of radiolabelled urea (^{14}C-urea).

The first approach is feasible when the transport process includes the net movement of charge across the membrane, such as with charged substrates or co-transport of substrate with ions such as protons. The application of this technique to oocytes allows the transporter-mediated currents to be assayed as a function of membrane potential, which is a component of the driving force in the transport system.

Until 1988 there was no significant experimental evidence for protein-mediated urea uptake by plant cell; in particular it was not clear if urea itself or its degradation products were moving across plant membranes. The first investigation in plants was performed by Wilson et al. (1988): short term ^{14}C -urea uptake in algal cells revealed a biphasic kinetics sensitive to the ATPase inhibitor DCCP or to the protonophore CCCP, indicating that urea uptake in plants was coupled to a proton gradient. More recently (Liu et al., 2003a) the functional characterization of AtDUR3 by electrophysiological assay could demonstrate that the high affinity urea transporter mediated the transmembrane movement of urea by a co-transport with protons. In that work the presence of urea 100 µM induced a small and reversible inward current (< 4 nA) in oocytes injected with *AtDUR3*-cRNA, corresponding to the net influx of positive charges across oocyte membranes (Liu et al., 2003a).

In order to characterize the transport of ZmDUR3, a two-electrode voltage clamp study in oocytes was performed (data not shown). Substrate induced currents were measured 4-6 days after injection of oocytes with *ZmDUR3"Native"* cRNA, *ZmDUR3"Modified"* cRNA or, as control, water. The analysis was performed exposing the oocytes to "Na-Ringer modified" buffer solution at pH 5.5 with or without the addition of 10 mM urea. However in our experiments no significant urea-induced currents could be measured in oocytes expressing ZmDUR3 indicating not, or only very low, urea uptake in ZmDUR3-expressing oocytes. Also the noise in all experiments was around 4 nA making it very difficult to measure currents ≤ 4 nA (as observed by Liu et al. (2003a) in AtDUR3-injected oocytes exposed to urea).

Therefore to verify the transport activity of ZmDUR3, a different approach was used i.e. measuring the influx of radiolabelled urea (^{14}C-urea). ZmDUR3-expressing oocytes were exposed for 5, 15, 30 or 60 minutes to Na-Ringer modified solution containing 200 µM urea (21 ± 1°C). Using this approach the capability of ZmDUR3 to transport urea and the linearity of ^{14}C-urea uptake could be shown in some experiments (Figure 53).

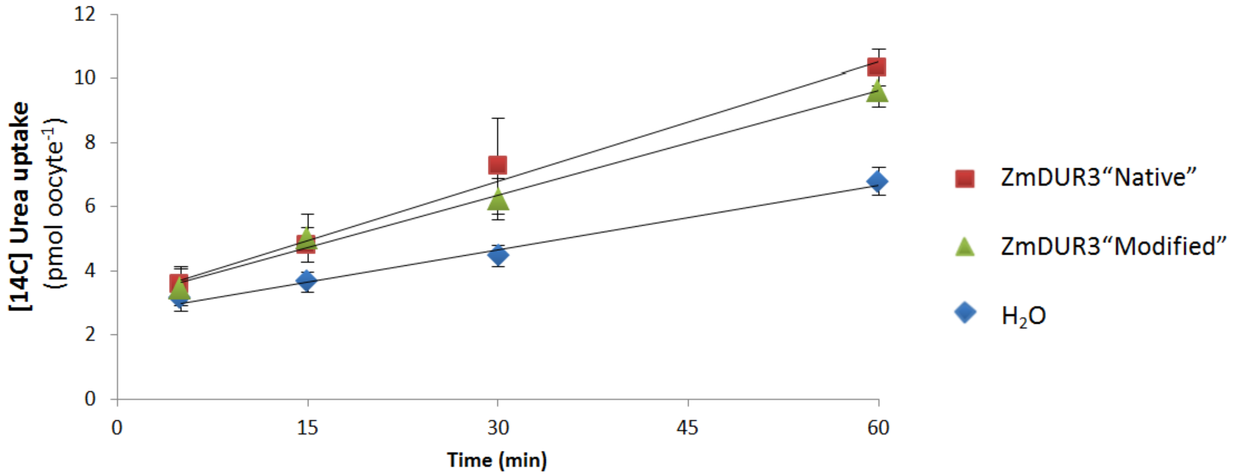


Figure 53. Influx assay of radiolabelled ^{14}C-urea in *Xenopus leavis* oocytes. To confirm the linearity of 200 µM urea uptake within 60 minutes, the ZmDUR3 uptake capacity was measured by ^{14}C-urea accumulation in water injected (H_2O) and in ZmDUR3 expressing oocytes (ZmDUR3"Native", ZmDUR3"Modified") exposed for 5, 15, 30 or 60 minutes (pH 5.5) to 200 µM urea. The values are means ± SD (n=5 oocytes).

In all experiments uptake of ^{14}C-urea was also observed in water injected oocytes. In batches of oocytes, where this background was low, transport function of ZmDUR3 could be shown (Figure 53) and measured; while in other batches (approximately 50%) of oocytes, the background was too high to allow significant measurements of ZmDUR3-mediated urea uptake. The background seems to be strongly different between different batches of oocytes.

Leung et al. (2000) published a study concerning the characterization of urea transport in *Xenopus* oocytes by a rabbit Na^+-glucose cotransporter (rbSGLT1). Their results showed that urea influx in non-injected oocytes was temperature sensitive; in particular the assay was performed by incubating oocytes at three different temperatures 14, 22 and 30°C. The component of unspecific urea influx across the plasma membrane was reduced by more than two times in oocytes incubated at 22°C as compared to 30°C.

For this reason, to discriminate the background from urea influx due to the active transport by ZmDUR3, the kinetic experiments were conducted at 21 ± 1°C exposing oocytes to 5, 10, 50, 100 or 200 µM of ^{14}C-urea. The accumulation of ^{14}C-urea by ZmDUR3 saturated around 50 µM with a K_m value of 20 µM (Figure 54) comparable to the Km value reported for OsDUR3 (*c.* 10 µM, Wang et al., 2012). The data shown here are derived from a single experiment, so further repetitions of kinetic assay are needed to confirm this result.

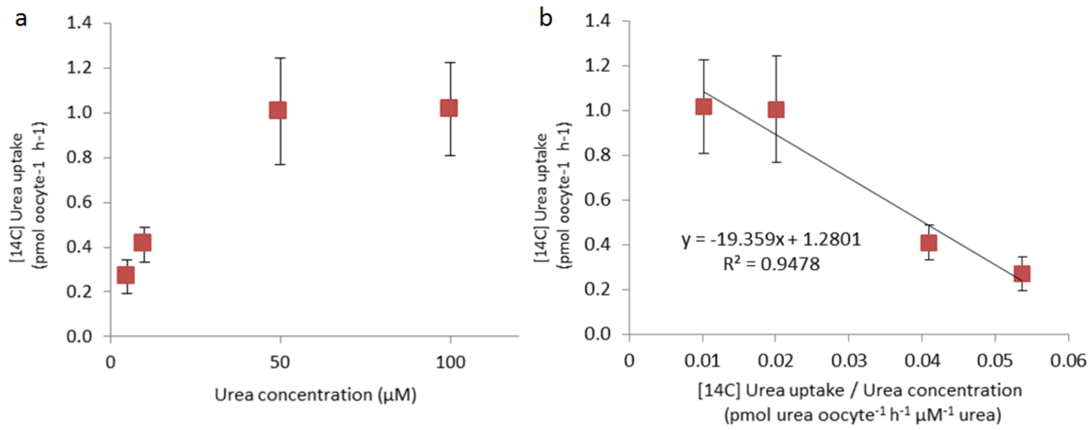
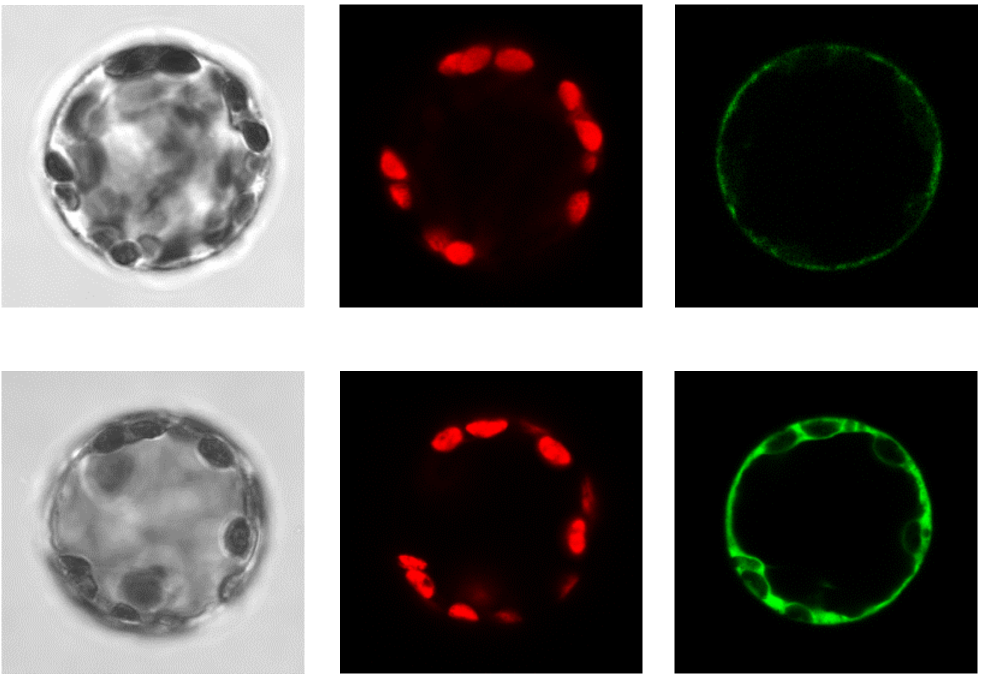


Figure 54. Biochemical characterization of urea transport by ZmDUR3"Modified" in *Xenopus leavis* oocytes. Concentration dependent ^{14}C-urea uptake was saturable displaying a Michaelis-Menten kinetic, in the graph the transport rate between 5 and 100 µM urea concentration are shown. Kinetic parameters were calculated after subtracting the linear component of the uptake rate detected in water injected oocytes. The data are depicted in two graphical representations, as: (a) Michaelis-Menten and (b) Headie Hofstee plot, the last one was used to calculate the affinity constant (K_m). The values are means ± SD of n=5 oocytes from one experiment, further repetitions have to be performed to corroborate these data.

3.3.4 GFP-localization in tobacco protoplasts

Functional complementation of yeast mutant YNVWI by ZmDUR3 and its functional expression in *Xenopus* oocytes indicated that in these heterologous systems the transporter is at least partially localized at the plasma membrane. To corroborate the subcellular localization, N- and C-terminal fusion proteins of ZmDUR3 and GFP (Green Fluorescent Protein) were transiently expressed in tobacco (*Nicotiana tabacum*) protoplasts. Tobacco protoplasts were also transformed with the vector encoding the free GFP (pUC18-Sp-GFP6; Komarova et al., 2012), which was used as negative control.

In Figure 55 the fluorescent signal derived from the ZmDUR3-GFP fusion protein and free GFP are shown. In the negative control (GFP) the fluorescent signal was in the cytoplasm, while in ZmDUR3-GFP expressing protoplasts the signal was mostly localized in internal membranes and maybe to the plasma membrane.



a) ZmDUR3-GFP

b) GFP

Figure 55. **Subcellular localization of ZmDUR3 protein fused with green fluorescent protein (GFP) in tobacco protoplasts.** a) ZmDUR3-GFP: protoplasts transformed with GFP fused to the C-terminal of ZmDUR3 (*ZmDUR3"Modified"-GFP* fusion construct); b) GFP, as control free GFP localized in the cytoplasm. Images were taken using a confocal laser-scanning microscope: bright-field images (left column) , chlorophyll fluorescent image (red, middle column), GFP-fluorescent image (green, right column) are shown.

To clarify the subcellular targeting of ZmDUR3, two colocalization experiments were performed using tobacco protoplasts which transiently expressed ZmDUR3-GFP or GFP-ZmDUR3 and AtPTR1-YFP. YFP-tagged AtPTR1 was used as reference, which was shown to be localized at the plasma membrane (Komarova et al., 2012). So, the localizations of ZmDUR3 and AtPTR1 was visualized as green and purple fluorescent signals, respectively (Figure 56).

Tobacco protoplasts expressed ZmDUR3-GFP and GFP-ZmDUR3 mainly showed green fluorescence at internal membranes, although a difference between the experiments was registered. When GFP was fused at the C-terminus of ZmDUR3 the green signal did not overlap with the signal of AtPTR1-YFP. On the other hand, the fusion of GFP at N-terminus of the protein showed a better co-localization with the AtPTR1-YFP.

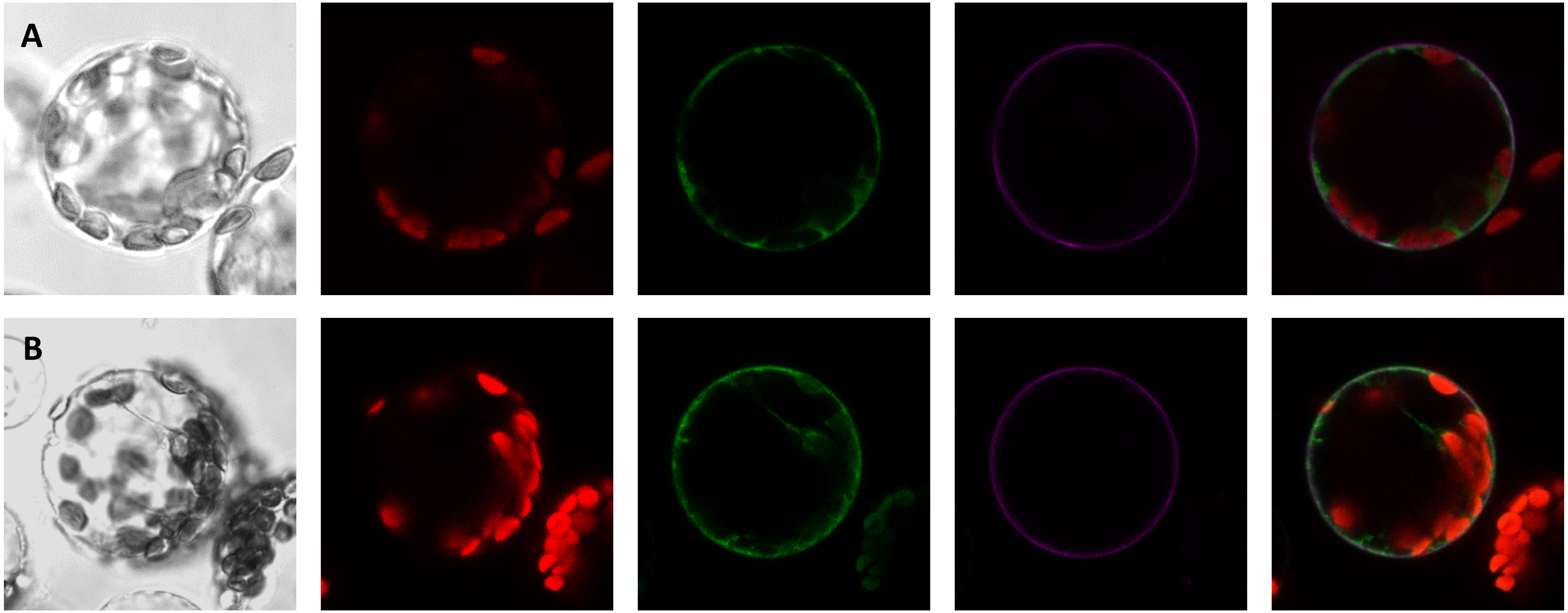


Figure 56. Colocalization in tobacco protoplast of (A) ZmDUR3-GFP and AtPTR1-YFP fusion proteins and (B) GFP-ZmDUR3 and AtPTR1-YFP fusion proteins. Images were detected using a confocal laser-scanning microscope: bright-field images (first column), chlorophyll fluorescent image (red signal, second column), GFP-fluorescent image (green signal, third column); YFP-fluorescent image (purple signal, fourth column) are shown. In the last column, merged images show chlorophyll fluorescence (red), GFP-fluorescence (green) and YFP-fluorescence (purple). Diameter of protoplasts was approximately 40 µm.

The results reported in this work look very similar to the GFP-localization images published for OsDUR3 and AtDUR3 (Wang et al., 2012; Figure 57). OsDUR3-GFP and AtDUR3-GFP fusion proteins were transiently expressed in Arabidopsis protoplasts. The authors reported that "*The green-fluorescence signal derived from* OsDUR3-GFP *expression was restricted to a thin ring comparable to the size of the protoplasts most similar to the signal resulted from* AtDUR3-GFP *fusion-protein…*" concluding that *"*OsDUR3 *was mostly targeted to the plasma membrane.*" (Wang et al., 2012).

In comparison to tobacco, the Arabidopsis protoplasts are characterized by a smaller size and a lower number of chloroplasts making it difficult to discriminate between the plasma membrane and the cytoplasm. Indeed in Arabidopsis, the cytoplasm may be also observed as thin ring located directly underneath the plasma membrane. In addition in the AtDUR3 and OsDUR3, the fluorescence signals were not uniformly distributed at the periplasm of protoplasts which may indicate that the protein may not be exclusively found at the plasma membrane, but may also localize in internal membranes.

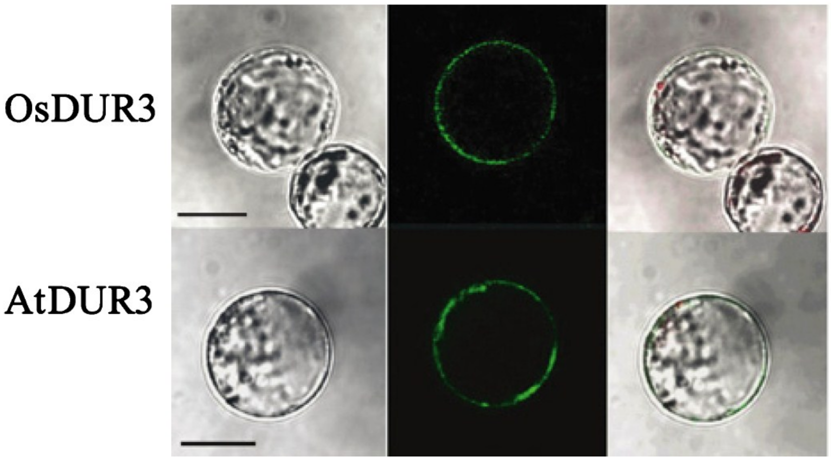


Figure 57. Subcellular localization of OsDUR3 and AtDUR3 protein fused with green fluorescent protein (GFP) (Wang et al., 2012). Transmission image (left column), fluorescence image (middle column) and merged image (right column) are shown. GFP fluorescence from Arabidopsis protoplasts transformed with OsDUR3-GFPand AtDUR3-GFP fusion constructs.

In conclusion, ZmDUR3 seemed to localize mainly in internal membranes although it can not be excluded that ZmDUR3 partially also reaches the plasma membrane. Moreover the subcellular localization of ZmDUR3 showed a comparable result to those reported for the rice and Arabidopsis orthologs, OsDUR3 and AtDUR3 (Wang et al., 2008).

4 Discussion

Plant nutrition is a research topic of great interest for scientists. It has been estimated that in the next years world population will increase, reaching 9.15 billion by 2050 (Alexandratos and Bruinsma, 2012). This, in turn, will determine an increase in food demand, requiring a higher cereal production (about +38% by 2025 according to Dobermann and Cassman, 2005).

It is well known that nitrogen nutrition is one of the key factors limiting plants growth, this is especially true for cereals, such as maize, mainly because of their low Nitrogen Use Efficiency (NUE) (Raun and Johnson, 1999). Actually a common agronomic practice to improve the cereal yield consists into apply nitrogen fertilizers based on nitrate, ammonium or urea. So in the future, especially if losses of cereal cropping area continue at the rate of the past 20 years (-0.33% per year) and the NUE of cereals will not be increased substantially, the cereal demand will lead to a 60% increase of the global nitrogen use (Dobermann and Cassman, 2005). The wide use of chemical inputs for food production will lead to negative impacts at ecological and also economical level. Core objective of modern agriculture and researchers has to be the improvement of cereal yields in a more cost-effective and eco-compatible way improving nitrogen use efficiency of cereals and minimizing losses from both inorganic and organic nutrient sources. This could be done by optimizing plant-soil relationships in order to improve the acquisition of native and applied nitrogen (Tomasi et al., 2009).

Despite the use of urea as the most worldwide nitrogen fertilizer, little information is available concerning the mechanisms of its acquisition in higher plants. The urea transporter gene was isolated and characterized for the first time in higher plants using Arabidopsis (*AtDUR3*, Liu et al., 2003a). The transformation of knock-out Arabidopsis mutants (*atdur3-1* and *atdur3-3*) with AtDUR3 restored the wild type phenotype growing on urea as sole nitrogen source. This experimental evidence, with other functional assays, supported the view that AtDUR3 codes for the major high affinity transporter for urea uptake in roots. To date only the orthologous gene of rice, *OsDUR3*, has been cloned and functionally characterized. Besides DUR3, the only other plant proteins to show urea permeability belong to the super-families of aquaporins, *Major facilitator Proteins* (MIPs): membrane channels that may facilitate the transport of water and small solutes, as urea. Particularly, in maize, five aquaporins have been characterized: ZmPIP1-5 (Gaspar et al., 2003); ZmNIP2-1, ZmNIP2-4, ZmTIP4-4 (Gu et al., 2012); and ZmPIP4 (Marilia, 2001). Several experimental evidence identified aquaporins as responsible for the low affinity urea transport system in higher plants (Siefritz et al., 2001; Liu et al., 2003b; Gaspar et al., 2003; Klebl et al., 2003). Even if their localization has not yet been clarified, these channels may play a crucial role in urea acquisition from soil, especially after a fertilization event when high urea concentration occurs in soil solution.

However, in agricultural soils urea is rapidly hydrolysed to ammonium by microbial urease action, thus its concentration is usually low and does not exceed the µM range (estimated to be up to 70 µM). For this reason the physiological role of DUR3 can be relevant to the uptake of urea from the soil.

This thesis focuses on the characterization of the mechanisms of urea acquisition in maize roots at physiological, transcriptional and molecular level. Moreover, with the aim to mimic more closely soil conditions, particular attention was paid to the influence of other nitrogen sources, such as nitrate, on the urea uptake system.

In the first part of the work, a physiological characterization of the high affinity transport system of urea was performed using intact maize roots. The effect of other,organic and inorganic nitrogen sources, on the uptake rate of urea was also evaluated by short-term experiments (within 24 hour). In particular, two sources were analysed in detail: i) nitrate, an inorganic source that occurs in soil solution as a product of nitrification process or derived from fertilization; ii) glutamine, an amino acid that in soil may be present as free molecule or as constituent of decomposing organic matter. Glutamine also represents the first metabolic product deriving from urea and nitrate assimilation pathway.

In order to assess the existence of a correlation among physiological and transcriptional responses, changes in the transcriptomic profile were evaluated by microarray analysis in roots exposed to urea and/or nitrate. In addition, to corroborate the microarray data and to analyse the transcriptional modulation of other genes during the experimental time span, real time RT-PCR analyses were performed within 24 hours of treatment with urea, nitrate or glutamine.

Finally, with the aim to identify at molecular level the urea transport system in maize, the *ZmDUR3* gene orthologous to rice and Arabidopsis, encoding for the high affinity urea transporter, was cloned from root samples and characterized in heterologous systems.

4.1 Physiological characterization of urea uptake

In agricultural soils, plant roots are exposed to a combination of different nitrogen sources, organic and inorganic ones, whose use efficiency is not yet completely clarified. Several studies reported the ability of plants to grow in presence of nitrate or ammonium as a sole nitrogen source, even if, depending on plant species, negative effects on plant development have been reported under ammonium nutrition (Britto and Kronzucker, 2002). On the other hand, concerning the use of organic sources by roots, very few information is available. Due to its great agronomical importance, urea is the organic nitrogen form that has been studied in more detail, especially in the last years. However, only few works have provided evidence that plants can use urea as sole nitrogen source, demonstrating the root absorption of the molecule (Coïc et al., 1961; Mérigout et al., 2008a). Furthermore, it was demonstrated that urea could sustain growth of crop species, like rice (Wang et al., 2012), wheat and maize (Mérigout et al., 2008b).

In order to confirm this result, in the present work, a first series of experiments was performed using maize plants grown in hydroponic conditions and exposed for 1 week to urea or to other nitrogen sources, such as nitrate, ammonium and glutamine; the different N-forms were applied singularly or in combination with urea (Figure 18). At the end of the experiment, strong differences were reported in the root architecture (Figure 18, B.2), while no great variation was observed in the shoot development. The morphological observation, together with the data on roots and leaves fresh weights, gave an indication that maize plants are able to grow in presence of urea as a sole nitrogen source. Particularly, plants fed with urea showed a very well developed root system with an extensive proliferation of secondary roots. The positive influence of urea on the root structure was previously described by Kirkby and Mengel (1967) in tomato plants. Biomass accumulation in plants treated with urea was similar to that, found in plants exposed to nitrate or to ammonium nitrate (Figure 18, A). Interestingly the highest yields in shoot fresh matter were registered in plants exposed to urea and nitrate (UN treatment). Cumulative effects of nitrate and urea have been reported also in wheat (Garnica et al., 2009) and oilseed rape (Arkoun et al., 2012b) with respect to nitrogen accumulation.

Starting from data on growth and morphological changes, time- and concentration-dependent uptake experiments were performed to characterize in detail the mechanisms of urea acquisition in maize roots. Among higher plants, the kinetic characterization of urea uptake was previously described only in Arabidopsis and rice (Kojima et al., 2007; Wang et al., 2012), using ^{15}N-urea short-term influx assay. A different approach was used in the present work: urea depletion from the root external media was measured, as described by Kyllingsbaek (1975), with a colorimetric assay where only intact urea molecules are the substrate for the colorimetric reaction. Results obtained with this approach were comparable to those reported in previous works, since the exposition of intact maize roots to urea up to 300 µM showed a saturation kinetic corresponding to the Michaelis-Menten model (Figure 19). This behavior is compatible with the presence in maize roots of a high-affinity transport system for urea uptake with kinetic features similar to those found in Arabidopsis and rice (Table 9).

Table 9 Comparison between Arabidopsis, rice and maize kinetic features of high-affinity urea transport system in roots. Arabidopsis and rice data are referred to ^{15}N-urea kinetic assay reported by Kojima et al. (2007) and Wang et al. (2012), respectively; whereas maize data are referred to Figure 19. *Not induced*: kinetic assay performed on nitrogen starved plants*; Induced with urea: kinetic* assay performed on plants exposed for 4 h to 1 mM urea. (*) data gathered from Figure 6 in Kojima et al. (2007).

Plants		V_{max}		K_m		References
Arabidopsis*	*Not induced*	~16	µmol urea g^{-1} DW h^{-1}	8.5	µM urea	Kojima et al. (2007)
Rice	*Not induced*	2.48	µmol urea g^{-1} DW h^{-1}	7.55	µM urea	Wang et al. (2012)
Maize	*Not induced*	8	µmol urea g^{-1} FW h^{-1}	4	µM urea	In the present work
Maize	*Induced with urea*	20	µmol urea g^{-1} FW h^{-1}	27	µM urea	In the present work

The kinetic assay in maize roots revealed an important aspect of urea uptake that was not previously described in detailed in higher plants: data (Figure 19) suggested that, in the high-affinity concentration range, urea induces its own uptake, causing a modification of the kinetic parameters. Moreover this response was found to be dependent on substrate concentration in the treatment solution (Figure 20). The root exposition to high urea concentration induced a higher and earlier up-regulation of influx rates than plants exposed to lower urea concentrations.

In a previous work (Mérigout et al., 2008a) up-regulation of urea uptake by itself was reported for Arabidopsis. This conclusion was inferred from influx assay performed by exposing plants to a solution containing 10 mM ^{15}N-urea (corresponding to 20mM Ntot). The influx capacity of urea-fed plants (U, over 300 µmol urea g^{-1} DW h^{-1}) was higher than N-starved plants or plants fed with ammonium nitrate (AN) or ammonium nitrate plus urea (ANU), which showed values around 200 µmol urea g^{-1} DW h^{-1}. Thus, under the experimental conditions employed in that work urea uptake was in main part due to the low affinity transport system. Results of the present work indicate that in maize urea can act on the induction of the high affinity transport system.

The induction by substrate is well noted feature of nitrate high-affinity transport system (iHATS) (e.g. Orsel et al., 2002; Monte, 2004) and was also confirmed here (Figure 24; Figure 25, B).

Time-course experiments also showed that a down regulation of uptake rates occurred prolonging exposition of the roots to urea; this would indicate the involvement of a feedback regulation by end-products or urea itself, similarly to what has been oserved for nitrate uptake (Glass et al., 2001). This physiological response might be connect to urea assimilation pathway. Indeed, previous experimental evidence (Mérigout et al., 2008a) suggest that urea taken up by Arabidopsis roots is assimilated directly in root cells releasing ammonium which is the substrate for glutamine synthesis by GS; this conclusion is supported by accumulation of glutamine in roots. So, glutamine, as well ammonium, might act as feedback reulators for the urea uptake. This idea is consistent with the observation that in maize (present work) the presence of glutamine with urea in the external medium (UG) exerted a negative effect on urea uptake rates limiting the induction of urea uptake system (see below).

Interactions among different N-sources was investigated in detail studying the reciprocal influence between urea and nitrate at the level of their influx rates in maize roots (Figure 23; Figure 24; Figure 25). Results showed that in the short time the simultaneous exposition of roots to nitrate and urea exerted negative effects on the induction of the uptake systems for each N-form. The up-regulation in the high-affinity uptake of urea was much reduced in plants treated with nitrate and urea in comparison to plants exposed to urea alone (Figure 23) and the same held true for nitrate uptake when urea was also present in the treatment solution.

This result is consistent with previous observations in Arabidopsis (Mérigout et al., 2008a) obtained analyzing the interactions, at the level of ^{15}N-influx, among different nitrogen sources. In this plant the presence of ammonium nitrate in the growth medium reduced the influx rate of urea. From its side urea exerted a repressive effect on nitrate influx while enhancing ammonium uptake.

Different results were obtained in wheat (Bradley et al., 1989; Garnica et al., 2009). In these works nitrate enhanced the uptake of urea when roots were simultaneously exposed to these N-sources (UN). As reported by Garnica et al. (2009) this effect was much more evident in UN fed plants between 24 and 96 hours. However, in both reports, uptake was evaluated as an accumulation during the time span of the exposition to the N-source (24 to 96 hours), while in the experiments reported in this thesis uptake rates were evaluated at each time of treatment (after 2, 4, 8, 12 and 24 hours) incubating intact maize roots for a very short period (10 minutes) in 200 µM urea assay solution. The results of the present work clearly indicate that rate of uptake of urea and nitrate can be modulated each other, especially when induction processes are considered. This would imply that reactivity of maize roots to the nitrogen forms could be influenced by the relative composition of the soil solution. So, when mixed to urea, nitrate did not affect plant growth in the long term (Figure 18) while limited the maximum capacity to uptake urea in the short term (Figure 23); *vice versa* the urea exerted similar effect on the rates of nitrate uptake (Figure 24).

In oilseed rape, Arkoun et al. (2012b) analysed the accumulation of ^{15}N on plants exposed for 15 days to solutions containing as sole nitrogen source ^{15}N-urea or ^{15}N-nitrate applied singularly or in conjunction. In contrast to the data reported in wheat (Garnica et al., 2009), in oilseed rape the simultaneous presence of nitrate along with urea led to preferencial nitrate uptake. The authors suggested that depending on the nitrophilic character of species, plants may prefer the uptake of a specific N-source among those available in soil solution. This hypothesis might explain the contradictory behavior observed among wheat, maize, Arabidopsis and oilseed rape.

Very few information is available about the use of organic nitrogen sources for plant nutrition. Single amino acids or peptide are present in the soil and might represent an accessible nitrogen source for crop growth. The Arabidopsis transporters, LHT1 and AAP5 are important components of the root amino acid uptake system, characterized by different amino acid specificities and function at amino acid concentrations seen in field conditions (Svennerstam et al., 2011). Several putative amino acid transporter have been described in a large variety of species (Lipson and Näsholm, 2001) suggesting a possible capacity of plants, beside Arabidopsis, to use these organic molecules as nitrogen sources.

Glutamine was shown to be the most effective amino acid in promoting Arabidopsis growth, although glutamine-fed plants were, on average, only about half the size of plants grown with nitrate (Svennerstam et al., 2007). In other species, glutamine was even the only amino acid that could support plant growth (Turnbull et al., 1995; Bonner and Jensen, 1996). Moreover, in plants glutamine is the first amino acid to be synthetized during the nitrate/ammonium assimilation pathway and its regulatory role in the gene transcription of high-affinity nitrate transporters, NRT2, has been clearly described (Glass et al., 2001).

Part of the work of this thesis focused on the interactions between root uptake of urea and of other N-sources, like nitrate and glutamine. Glutamine is an organic nitrogen source naturally occurring in soil, besides being the first metabolic product synthetized from urea and nitrate assimilation. Long term experiments (7 days) could confirm that this amino acid is able to sustain maize growth (Figure 18), even if, as reported in Arabidopsis (Svennerstam et al., 2007), glutamine-fed plants showed a reduced biomass accumulation as compared to plants grown with nitrate.

Thus, the effect of glutamine on urea uptake was studied in more detailed by short-term influx experiments using plants exposed for 24 hours to the different N-sources. Glutamine and nitrate exerted similar effect on urea uptake, since the combination of these sources with urea (UG and UN treatments), prevented the induction of the high-affinity urea uptake system (Figure 26). This result would suggest a possible role of glutamine as a negative (feedback) regulator of urea uptake.

In soil urea is rapidly hydrolyzed to ammonium and carbon dioxide by action of microbial ureases. It has been calculated that a significant portion of applied urea (more than 50%) is lost through ammonia volatilization (Terman, 1979), decreasing the amount of nitrogen available for plant nutrition. A common agronomic practice to increase the efficiency of urea-based fertilizers consists into applying urease inhibitors in conjunction with urea fertilizers. Actually the most used and commercially available urease inhibitor is the N-(n-butyl) thiophosphoric triamide (nBTPT) (Watson, 2005).

Very few studies have provided experimental evidences of the physiological effects of nBTPT on plants (Watson and Miller, 1996; Cruchaga et al., 2011). In particular, a main question is if this molecule can pass across the plasma membrane thus inhibiting also the plant ureases. In perennial grass, Watson and Miller (1996) reported a negative effect of nBTPT on the endogenous urease activity and transient formation of leaf-tip scorch. Similar effects were also reported in pea, where nBTPT was absorbed by plants causing clear inhibition of the leaf and root urease activity associated with the development of necrotic leaf margins (Cruchaga et al., 2011).

In this thesis the effect of the urease inhibitor nBTPT on the root capacity to absorb urea was investigated. Results showed that the exposition of plants to nBTPT limited the induction of the high-affinity uptake system (Figure 29). A 30% inhibition of the uptake rate of urea-induced plants by nBTPT was observed also adding nBTPT directly in the assay solution (10 minutes in 200µM urea, Figure 30). Previous works performed in our laboratory showed the capability of nBTPT to strongly inhibit also the induction of nitrate high-affinity transport system (Monte et al. unpublished). However, no direct effect of nBTPT on nitrate high-affinity transport was observed (Monte et al. unpublished).

These data represent an interesting starting point for improving knowledge about molecular mechanisms of nBTPT action on urea transporters. In addition, the capacity of nBTPT to enter into the roots limiting the enzymatic activity required for urea assimilation (*i.e.* acting on plant urease) should be considered.

4.2 Transcriptomic analyses

From the physiological data it is clearly evident that urea can induce its own uptake. Indeed the root exposition to a solution containing urea at millimolar range induced, after few hours (4-8 h), a peak value in the uptake rates and subsequently the influx decreased. Moreover, it was clear that the presence of urea along with nitrate reduced the induction of the high affinity uptake systems of each nitrogen source. With the aim to clarify if these physiological responses could be related to variations at transcriptomic level, genome-wide analyses were performed on maize roots treated for 8 hours with urea (U), nitrate (N) or urea and nitrate (UN) and compared with the transcriptomic profile of maize roots maintained without nitrogen supply (Ctr). Using a maize chip developed by NimbleGen Technology, the microarray analyses allowed the simultaneous monitoring of around 60,000 transcripts of the root transcriptomic profiles from the different treatements.

To date only one study concerning the transcriptomic modulation induced by urea treatment in the model plant Arabidopsis (Mérigout et al., 2008a) has been reported, while a large variety of microarray studies on the modulation of gene expression by nitrate in plants, including Arabidopsis (Wang et al., 2000; Wang et al., 2003; Wang et al., 2004) and maize (Liu et al., 2008), are available.

In the present work, the microarray analyses revealed that the transcriptional modulation induced by nitrogen treatments concerned only few genes, in particular comparing the different treatments 132 differentially expressed genes were found in UN *vs* Ctr, 89 in N *vs* Ctr, 62 in UN *vs* U, 57 in N *vs* U, 4 in U *vs* Ctr and 0 in UN *vs* N (Table 5). These data suggested that the transcriptomic variations were mostly induced by nitrate rather than urea. In fact, N and UN roots did not show significant differences in their transcriptomic profiles. On the other hand, the highest transcriptional variation was registered under nitrate, expecially when analyzing UN *vs* Ctr (132) and N *vs* Ctr (89). The modulated transcripts were manually annotated and grouped in main functional categories according to biological process terms of Gene Ontology (GO). The most representative classes were: "metabolic process", "localization" and "biological regulation".

The microarray data reported in this work were corroborated by experimental evidence documented in previous transcriptional works of Arabidopsis and maize. In particular the presence of nitrate induced the strong overexpression of genes involve in its assimilation, as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT); all these genes are well known to be nitrate-induced in plants, as also described by Wang et al. (2000). Interestingly microarray data showed that the treatment with urea and nitrate determine an increase in the up-regulation of genes induced by nitrate. This means that the transcriptional effect of urea involves changes in the amounts of transcripts more than the type of transcript, showing a difference at a quantitative scale more than at a qualitative one with respect to nitrate. Neverthless, as suggested by Mérigout et al. (2008a), the upregulation of those genes by urea might be correlated with the proposed pathway for N-urea assimilation (Figure 33).

Besides genes that belong to GS/GOGAT cycle, also transcripts encoding for protein involved in abiotic stress response, as well in scavenging of ROS, were identified. However most of them resulted to be modulated essentially by nitrate. The only exception was represented by *ZmSnRK2.4* gene which was found up-regulated only when nitrate was supplied along with urea. However its role in the signaling of abiotic stress depending on nitrogen nutrition state has still to be clarified.

The most interesting and novel founding microarray data concerns the modulation of transcripts involved in the shikimic pathway (*Shikimate kinase*, #78). In particular the presence of urea in conjunction with nitrate induced the up-regulation of genes (*Arogenate dehydrogenase isoform 2*, #77) for the synthesis of tyrosin, an aromatic amino acid precursor of several secondary metabolites. Moreover results indicate that under this nitrogen treatment (UN), urea may stimulate also the phenilpropanoid pathway (*Putative ferulate 5-hydroxylase*, #46) leading to the synthesis of compounds derived from phenylalanine.

In a previous study on tobacco plants, microarray data showed as the expression of genes involved in the central steps of the phenylpropanoid metabolism could be regulated by nitrate levels (Fritz et al., 2006). Results reported the stimulation of phenylpropanoid metabolism in nitrogen-deficient tobacco as a response to low nitrate *per se*, rather than low levels of amino acids. Thus, in maize (data in the present thesis), the effect of urea nutrition might lead to changes in nitrate levels of the roots, simulating a condition of nitrogen starvation.

Thus, these results suggest that urea, when supplied in combination with nitrate (UN), stimulates nitrogen assimilation by activation of GS/GOGAT cycle, amino acid biosynthesis and secondary metabolite (Tyr-derivate compounds). However, at the same time, possibly due to a change in C/N balance, urea determines up-regulation of genes involved in the synthesis of Phe-derivates leading to higher levels of carbon compounds.

With respect to the putative transcription factors belonging to "biological regulation" category, further analyses are nedded to identify in more detailed the encoded biological function of these transcripts and their involvement in nitrogen nutrition in maize roots.

Concerning the transcriptional modulation induced by urea itself, only four transcripts were identified in comparison to control roots (U *vs* Ctr, Table 8), which were found strongly up-regulated: a *MATE transporter* (8 FC), two *Asparagine synthetases* (both 5 FC) and a *Zinc finger protein* (4 FC). The over-expression of *Asparagine synthetase* (*AtASN1*), as induced by urea was previously described in Arabidopsis; this result was consistent with an increase in the Asp amounts in urea-fed plants (Mérigout et al., 2008a). Regarding the two other transcripts identified in the present work, further analysis is needed to clarify their involvement in the response mechanism of maize roots to urea.

In agreement with previous results obtained in Arabidopsis (Mérigout et al., 2008a), root exposition to urea did not induced modulation in the expression of genes coding for urease or enzymes involved in the urea cycle.

In order to confirm the microarray data and focusing on gene expression level of transcripts involved in nitrogen uptake and metabolism, real time RT-PCR analyses on root and leaf tissues were performed. Plants were treated with the same nitrogen sources applied for the microarray experiments, urea and nitrate, and moreover the effect of a nitrogen metabolite, *i.e.* glutamine, was evaluated. A time-course experiment run over 24 hours of treatment allowed to follow the dynamics of expression of the genes considered. As expected, results showed a strong up-regulation by nitrate of genes involved in the uptake of the anion and in its assimilatory pathway.

Concerning the uptake of nitrate, the expression of two putative high-affinity nitrate transporters were analysed (NRT2.1; 2.2, Figure 34 A, B) as well of an accessory protein needed for their functionality (NAR2.2, Figure 34 C). These genes were not found differentially modulated by the nitrogen treatments at the microarray analysis, while real time RT-PCR revealed that their expression was induced by the exposition to nitrate reaching a peak value after 2 hours for NAR and 4 hours for the two NRT2 transporters. These latter genes were found overexpressed also when nitrate was applied together with urea, showing, especially for NRT2.2, even higher values in presence of urea (UN) than in its absence (N). A higher up-regulation of *BnNRT2.1* was also reported after 24 h treatment of oilseed rape roots with urea plus nitrate as compared to nitrate alone, while changes in *BnNRT2.1* was not evaluated (Arkoun et al., 2012b). In the present work it appeared that while *ZmNRT2.2* expression was enhanced by the simultaneous provision of urea and nitrate, *ZmNRT2.1* expression was somehaw decreased in roots exposed to nitrate and urea; however this latter was maintained at higher level till the end of the experiment (24 h) as compared with the sole nitrate supply. This would suggest a kind of modulation of the two high-affinity transporters (Rizzardo et al., 2012). Interestingly, a role for ZmNRT2.2 in nitrate translocation has been also hypothesized (Trevisan et al., 2008). Negative modulation of *NAR2.2* gene is consistent with a decrease in nitrate uptake induction in UN roots.

Confirming microarray data, genes that are involved in nitrogen assimilation, such as nitrate reductase (*NR*), nitrite reductase (*NiR*) and glutamine synthetase (*GS2*), were found to be up-regulated only by nitrate (N) and by urea and nitrate (UN) treatments (Figure 36). Real time RT-PCR confirmed that when nitrate was applied in combination with urea, the expression levels of these genes was maintained higher for longer time and even at higher levels (*GS2*) than when nitrate alone was supplied.

A different situation was detected for the expression of the gene encoding Fd-GOGAT (Figure 37), that was found to be modulated also by treatments with glutamine and/or urea. However the differences observed after 8 hours of exposition are somehow in agreement with the microarray analysis, where the amounts of Fd-GOGAT were only two-fold those registered in control roots (UN *vs* Ctr FC 2.10).

Particularly with respect to the expression of *ZmGS* and *ZmFd-GOGAT*, several experimental evidence have previously demonstrated that nitrate could induce the expression of genes involved in its assimilation (Redinbaugh et al., 1993), while the positive influence of urea was not clearly reported in other works. Interestingly, the higher induction of nitrogen assimilatory genes in plants supplied with nitrate plus urea would suggest a positive effect of the two forms in accelerating nitrogen metabolism. This would possibly affect feedback control over the transporters gene expression (see above). Of course, this assumption needs to be supported by further analysis at the enzymatic and metabolomic level.

Besides genes involved in nitrate uptake and assimilation, also the transcriptional variations of genes related to urea transport and metabolism were investigated. Under the experimental conditions employed in the present work, no significant modulation in the gene expression of *ZmDUR3* was found (Figure 38, A). During the time span of the experiment (24 hours), the transcript amounts were comparable among the different nitrogen treatments.

In rice the relative expression of *OsDUR3* was found up-regulated 6 days after germination, while in Arabidopsis, the amount of DUR3 protein in roots was strongly induced after 3 days of nitrogen starvation (Kojima et al., 2007). An interesting study was performed by Arkoun et al. (2012a), who analysed the effect of urea and nickel (Ni) nutrition on the expression level of *BnDUR3.* This element is an important cofactor of the enzyme urease and is requested for its activity. These authors observed that depending on the presence of nickel in the external medium, rapeseed roots could differentially express *BnDUR3*. Plants were grown hydroponically for 1 week in nitrogen starvation and then were exposed for 7, 15 and 21 days to urea with or without nickel. After 7 days of treatment, the amount of transcript observed in urea-fed plants were higher than the expression level recorded in (urea+Ni)-fed plants.

This experimental evidence suggests that the expression of genes coding for DUR3 transporter may be up-regulated by nitrogen starvation and also by urea treatment under nickel deficiency. Thus, as future prospective to clarify the transcriptional regulation of *ZmDUR3,* further analysis will be done extending the time span of the experiment and changing the nickel concentration in the external medium.

In planta the fate of urea taken up by soil solution is not yet clarified, however reported in Arabidopsis it has been reported that the root exposition to urea determined an increase in urea contents also in shoots (Mérigout et al., 2008a). To data few information concerns the urea translocation in higher plants. Kojima et al. (2006) suggested the involvement of aquaporins for the allocation of urea in leaf tissues. Particularly in maize, aquaporins that belong to the protein classes of PIPs and TIPs, have been characterized as permeable to urea (Gaspar et al., 2003; Gu et al., 2012; Marilia, 2001). By heterologous expression in *Xenopus oocytes*, Marilia (2001) demonstrated the capacity of the maize protein PIP4 to transport also urea along with water. By real time RT-PCR analyses it was shown that the root exposition to urea treatment determined the overexpression of *ZmPIP4* in leaves, reaching a peak value after 8 hours of treatment (Figure 45).

The expression pattern of other genes involved in nitrogen assimilation in leaves were tested. Beside *ZmNR2, ZmGS2, ZmArginase* which showed no great variations during the experimental time span and under different nitrogen treatments, interesting changes in the transcriptional profiles were detected for the genes *ZmNR1* and *ZmFd-GOGAT*. In particular the presence of urea along with nitrate (UN) led to rapidly increase the leaf amounts of *ZmNR1* transcript (Figure 42) reaching values higher than those recorded when sources were applied singularly (U or N treatment). Concerning *ZmFd-GOGAT,* the transcriptional profiles confirmed the well known photoperiod regulation reported for this gene, showing a down regulation in the middle of the light phase (Suzuki et al., 2001). Neverthless the simultaneous exposition of roots to urea and nitrate (UN) positively reduced the repression of *ZmFd-GOGAT*, especially after 12 hours of treatment (Figure 43). The leaf gene expression analyses contribute to corroborate the hypothesis that urea in the external medium may act at transcriptional level to stimulate the expression of genes that are involved in nitrogen metabolism.

The microarray and real time RT-PCR experiments gave important information about the transcriptional modulation that may occur in roots when urea and/or nitrate are applied as nitrogen sources. Results are in agreement with the well-known modulation by nitrate of genes involved in the nitrogen uptake and assimilation. To our knowledge, in this work, the effect of urea on the gene expression was investigated for the first time in a crop species. Surprisingly the influence of this organic molecule at transcriptional level concerned only a limited number of genes, while its effect mainly concerns the quantitative level of transcripts. Indeed for genes involved in nitrate uptake and assimilation, the presence of urea in the external medium along with nitrate determined higher amounts of transcripts than those detected under nitrate treatment.

Interesting data were reported in leaves for the *ZmPIP4* transcript (Figure 45*),* which may be involved in the translocation and allocation within plants of urea taken up by roots. On the other, despite physiological results would suggest that urea may induce its own uptake, at transcriptional level this response did not seem to be supported by modulation in the gene expression profile of *ZmDUR3*. This result might be related to the experimental conditions employed, like e.g the short-term treatment as compared with previous work; indeed urease gene expression was clearly evident in urea-fed plants only after 24 hours of treatment (Figure 38, B). Alternatively it might indicate that for the transitory induction of uptake a new synthesis of the transporter is not needed and that a post-translational regulation might occur.

4.3 Functional and molecular characterization of ZmDUR3

In the last part of this work the open reading frame (ORF) of the *ZmDUR3* gene was isolated from maize roots and functionally characterized. The only ScDUR3 orthologs to be previously identified and characterized in higher plants are the urea transporters of Arabidopsis, AtDUR3 (Liu et al., 2003a), and rice, OsDUR3 (Wang et al., 2012). The functional characterization of ZmDUR3 was carried out by expressing the isolated ORF-sequence in different heterologous systems.

In the first approach, the urea transport activity of ZmDUR3 was verified by complementing the *dur3* yeast mutant strain (YNVWI, *Δura3 Δdur3*) which is defective in the endogenous urea transporter, ScDUR3. This mutant strain is unable to grow on a medium containing less than 5 mM urea as sole nitrogen source. The expression of *ZmDUR3* in YNVWI cells complemented the urea-uptake deficiency and restored the ability of the yeast strain to grow on a medium containing ≤3 mM urea when applied as unique source of nitrogen (Figure 52). This result provided a clear evidence about the physiological role of ZmDUR3 as transporter of urea across plasma membrane.

As positive control, the yeast strain 23346c (*Δura3*) that was transformed with the (empty) expression-vector showed colonies on 3 mM urea plates already after 3 days (data not shown). On the other hand, colonies of the yeast mutant transformed with *ZmDUR3* became visible only after a relative long incubation time of around 5 days. In agreement with this observation, also the complementation of the YNVWI phenotype by OsDUR3 required a long time of incubation of about 5 days (Wang et al., 2012).

Slow growth of *ZmDUR3*-transformant YNVWI colonies might be due to a low urea transport rates of ZmDUR3. Moreover, the expression of a maize protein in a heterologous system might lead to problems with the transcription and translation of the *ZmDUR3*-construct or to an incomplete targeting of ZmDUR3 to yeast plasma membrane. All these possible explanations might result in the delay of growth of the ZmDUR3-transformed *dur3* yeast mutants.

In order to facilitate the expression of *ZmDUR3*-ORF in yeast, an ORF variant was prepared with a yeast-preferred codon usage and lower GC content. So, in the first part (10%) of the ORF, G and C in the third codon position were replaced with A or T nucleotides making up codons which are more frequently used in yeast. The resulting sequence, *ZmDUR3"Modified",* showed a decreased in the GC content and therefore a partial optimization for the expression in yeast. Interestingly the *ZmDUR3"Modified"*-transformants grew slightly faster than yeast mutants transformed with the unmodified *ZmDUR3*-ORF (*ZmDUR3"Native"*). So, after 5 days of growth on selective plates, differences in colony size between *ZmDUR3"Native"*- and *ZmDUR3"Modified"*-transformants were detected. Since the two constructs differed only at nucleotide level, the lower translation of the maize transgene was partially responsible of the slow rate of yeast growth.

These results confirmed that especially for plant species with a high GC content, the ORF-optimization strategy may be a valid method to improve the expression of transgenes in yeast or oocytes allowing a better molecular characterization of plant proteins.

The yeast complementation assay gave a clear evidence that the functional activity of ZmDUR3 is to transport urea across the plasma membrane, so its characterization was deepened performing further experiments.

The measurement of the kinetic parameters of ZmDUR3-mediated urea transport could be performed also in yeast cells by uptake experiments with labeled urea (^{14}C-urea or ^{15}N-urea). Nevertheless, as previously pointed out by Liu et al. (2003a), the ^{14}C-urea uptake studies in yeast cells with AtDUR3 were difficult to perform due to the rapid degradation of urea by endogenous yeast ureases. In particular, the rapid hydrolysis of radiolabeled urea resulted in ^{14}C-CO$_2$ release with aftermathes for the safety and for the accuracy of the kinetic measurements. Although this problem could be solved using ^{15}N-labeled urea, ^{15}N measurements are less sensitive than using a radioactive tracer. Moreover the measurement of ^{15}N-content into cells did not allow a discrimination between the uptake of nitrogen as being derived from intact molecules of urea or from its hydrolysis products. In addition, the slow rate of growth of transformants may indicated a low capacity of these cells to take up urea, which could limited the analyses.

For these reasons, a different approach was chosen expressing ZmDUR3 in a different heterologous system, i.e. *X. laevis* oocytes, which are often used to investigate protein-facilitated urea transport processes (Liu et al., 2003a,b; Wang et al., 2012). A first series of experiments was conducted using two electrode voltage clamp (TEVC) technique. However no substrate-induced currents in response to 10 mM urea at pH 5.5 could be measured. In the electrophysiological experiments reported by Liu et al. (2003a), AtDUR3-expressing oocytes exhibited a very small (less than 4 nA) inward-directed currents when 100 µM urea was present in the medium. Under the experimental conditions used for ZmDUR3 analyses, the background noise did not allow to measure urea transport (< 4 nA). The problem to record significant current could be due to the heterologous expression of a maize protein in *Xenopus* oocytes, resulting in low amounts of transporters and, as a consequence, of urea uptake which resulted under the detection limit.

For this reason, more sensitive analyses were carried out using ^{14}C-labeled urea. During these uptake assays, it was observed that the measurement of ZmDUR3-mediated urea uptake into frog cells was possible to detect only in some batches of oocytes with low endogenous urea uptake ability. This background uptake of urea in water injected oocytes was very different depending on the batch of oocytes, since in few experiments it reached low values of ~4 pmol urea oocyte^{-1} h^{-1}; while in other experiments, very high values of around 27 pmol urea oocyte^{-1} h^{-1} were measured. Two possible factors could mainly contribute to this background: first, due to its neutral character and low molecular weight, urea can cross membranes by simple diffusion; and second, the presence of endogenous aquaporins on oocyte plasma membrane can facilitate a passive influx.

However, using one batch of oocytes with a low background of urea uptake, a kinetic assay of ZmDUR3 transport activity was carried out. A saturable kinetic of urea accumulation was observed and the concentration of urea permitting a half-maximal uptake (K_m) was around 20 µM (Figure 54). Even if further repetitions are necessary to confirm these data, the uptake rates of urea found for ZmDUR3 were comparable with those reported for OsDUR3, which showed a maximal ^{14}C-urea uptake around 13 pmol urea oocytes^{-1} h^{-1} (Wang et al., 2012). Moreover ZmDUR3 showed a similar K_m value to those reported for its orthologs from *Arabidopsis thaliana* (AtDUR3, K_m of 3 µM; Liu et al., 2003a, b), *Oryza sativa* (OsDUR3, K_m of 10 µM; Wang et al., 2012) and the fungus *Paxillus involutus* (PiDUR3, K_m of 31,8 µM; Morel et al., 2008).

Since the K_m value of ZmDUR3 for urea import into oocytes is consistent with the K_m value found for urea uptake by intact maize roots (K_m 27 µM in urea-treated plants) and no additional high-affinity urea permease are to date predicted from the fully-sequenced maize genome. Therefore ZmDUR3, might represent the most significant component of the high-affinity urea uptake system from soil in maize roots.

These further experimentsconfirmed that *ZmDUR3* codes for a transporter which is able to move urea as an intact molecule across either yeast or oocyte plasma membranes and that the kinetic parameters are similar to those of known urea transporters.

In conclusion the molecular characterization of ZmDUR3 in yeast and oocytes allowed to test its functional activity resulting in a preliminary indication of the kinetic parameters. In these heterologous systems, ZmDUR3 showed a comparable behavior to those observed for the Arabidopsis and rice orthologs, AtDUR3 and OsDUR3. Indeed in the yeast complementation assay ZmDUR3 transformants showed a relative slow growth on selective plates, as well as previously reported for OsDUR3 (Wang et al., 2012). Also urea uptake rates in ZmDUR3-injected oocytes were very low and in a comparable range to those detected for AtDUR3 and OsDUR3 (Liu et al., 2003a; Wang et al., 2012). It is possible that the difficulties to perform the analyses in yeast and oocytes might be related to the expression of plant proteins in heterologous systems. In particular, in comparison to plant cells, the fungal and animal cells display different capacity to perform post-translational modifications, which might be requested *in planta* before the DUR3 proteins becomes completely functional. Moreover, it remains possible that in these cells, even if efficiently incorporated into the organelle/membranes, the heterologous proteins were incorrectly or incompletely targeted to the plasma membrane. As consequence, different factors might have contributed to reduce the transport activity of ZmDUR3 making the molecular characterization of this transporter difficult.

For these reasons and to clarify *in planta* the cellular localization of ZmDUR3, tobacco protoplasts were transiently transformed with *ZmDUR3"Modified"*-ORF fused with *GFP*. The fluorescent signal was mostly detected in internal membranes although it is not excluded that ZmDUR3 partially also reaches the plasma membrane. Indeed in the colocalization experiments, a minor fraction of GFP-ZmDUR3 signal overlapped with the fluorescence detected for the reference protein of plasma membrane (AtPTR1-YFP) (Figure 56).

Transient expression of GFP fusion protein has limitations. In certain cases the wild-type protein and the GFP-fusion protein might differ in their subcellular locations, since the presence of GFP could hinder proper localization, ZmDUR3/GFP-fusion protein might result in incorrect or incomplete localization. Another disadvantage consists in improper folding or instability of the encoded fusion protein, so that little or not well targeted fluorescent signal was detected.

A further consideration about this technique concerns the plant species and the tissue which was used to express the transgene. In particular the analyses were conducted using tobacco protoplasts derived from leaf mesophyll cells, because these were easy to isolate and also the chloroplast autofluorescence allowed a good identification of the cytoplasm.

However it is possible that the expression of ZmDUR3 in root tissues might show a better targeting of the fusion protein. This hypothesis may be corroborated by the experimental evidences reported by Kojima et al. (2007), who showed by two immunological approaches that AtDUR3 protein resides predominantly in the plasma membrane of root cells. So, it is reasonable to assume that the ZmDUR3/GFP might showed a better localization at the plasma membrane when expressed in protoplasts isolated from maize root tissue.

Besides these considerations, in this work of thesis several experimental evidences indicated that the plasma membrane localization of ZmDUR3 is highly probable. First, the physiological experiments showed the capacity of maize roots to take up this organic molecule and since to date ZmDUR3 appears as the only predicted urea transporter in maize, it most likely represents the major component for the high affinity uptake of urea from the soil. Second, by characterization in heterologous system, the function of ZmDUR3 at the plasma membrane of yeast and oocytes was demonstrated. Third, the subcellular localization of ZmDUR3 showed comparable results to those reported for the rice and Arabidopsis orthologs, OsDUR3 and AtDUR3 (Wang et al., 2012). In particular for AtDUR3 the plasma membrane localization in Arabidopsis root cells was previously described by two immunological approaches. Kojima et al. (2007) used polyclonal antibodies against AtDUR3 in two independent analyses: a protein gel blotting analysis of membrane protein fraction from Arabidopsis roots and a immunohistochemical assay on whole-mount root samples. Both immunological techniques gave the same results: AtDUR3 localized mainly at the plasma membrane even if a minor fraction of the labeled protein appeared to be localized inside the cells. The authors suggested that a fraction of AtDUR3 might reside in endosomal compartments, reflecting proteins that were moving to or from the plasma membrane (Kojima et al., 2007). Another possibility is that for the membrane targeting of urea transporter, root auxiliary proteins or molecular signals are involved.

For example, concerning the subcellular-localization of a high affinity iron transporter of Arabidopsis (Iron-Regulated Transporter 1, IRT1), in Arabidopsis roots IRT1 was found mainly localized in the early endosomal compartments (Barberon et al., 2011) while at the plasma membrane the abundance of IRT1 was low and tightly regulated by ubiquitin-dependent trafficking. The turnover of IRT1 protein was investigated and the localization of IRT1 was explained by the authors as a result of a "rapid endocytosis and slower recycling to the plasma membrane, where it likely performs iron uptake from the soil, and is addressed to the lytic vacuole for turnover" (Barberon et al., 2011). So, the authors concluded that the specific contribution of these trafficking pathways controlled the amounts of IRT1 protein at the plasma membrane.

The consideration about IRT1 suggests that the presence of ZmDUR3 in internal membranes may reflect a similar situation where the abundance of the protein at the plasma membrane is controlled by a specific trafficking pathways in response to urea. It would be interesting to further analyze in root protoplasts if the presence of urea in the external medium might influence the membrane localization of this transporter.

As final approach to provide more detailed assessment of molecular and physiological role of this maize transporter *in planta*, the overexpression of ZmDUR3 in two *dur3* mutant lines of Arabidopsis is still ongoing. Kojima et al. (2007) identified two independent T-DNA insertion lines of Arabidopsis (ecotype Col-0), which were isolated from the insertion mutant collections of the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003). These mutants, called *atdur3-1* (SALK_042649) and *atdur3-3* (SALK_036318), are defective in the endogenous urea transporter AtDUR3 and therefore they showed impaired growth on a medium with urea (<5 mM) as sole nitrogen source. In particular both mutant lines are unable to grow on a medium containing 1 mM urea. Under these conditions *atdur3* lines became chlorotic and accumulated more anthocyanins than the wild type plants, indicating a condition of nitrogen deficiency. The transformation of both *dur3* mutants with *35sCaMV:ZmDUR3* by *Agrobacterium tumefaciens* was performed in order to obtain *ZmDUR3*-overexpressing transgenic plants of Arabidopsis. The possible complementation of the mutant phenotype by ZmDUR3 will confirm the physiological role of this protein as high affinity urea transporter for the uptake from soil and of its localization at the plasma membrane of root cells.

5 Conclusion

My thesis work reports, for the first time, the physiological characterization of urea uptake in roots of intact maize plants. Results indicate that at micromolar urea concentrations (up to 300 µM urea), maize roots are able to take up this nitrogen source, using a high affinity transport system characterized by saturation kinetic. These data are consistent with those reported in Arabidopsis and rice.

This transport system appeared to be inducible by urea itself, retro-regulated and dependent on the external urea concentration as well as on the time of root exposure to the molecule. Furthermore it was affected by the presence of nitrate or glutamine in the external medium, which limited the induction of the urea transport system. These data support the idea of a cross-interaction among different N-sources, inorganic and organic, present in the soil solution.

At least under the experimental conditions employed in this work, the modulation of the urea uptake rate due to the presence of urea did not appeared to be regulated at transcriptional level, since no parallel variation in the expression levels of the high-affinity urea transporter *ZmDUR3* was found. Indeed, analysis of the transcriptomic profile revealed that the presence of urea in the external medium as the sole N-source, determined the up-regulation of only four genes. On the other hand, the effect of urea was more appreciable when this nitrogen organic source was applied to roots in combination with nitrate. Under this treatment a higher increase in the expression levels of genes known to be induced by nitrate was registered.

So, when applied simultaneously to roots, urea and nitrate at physiological level limited reciprocally the inducible component of each other high-affinity transport system, while at transcriptional level they increased the amounts of transcripts involved in the mechanism of nitrogen acquisition and assimilation. These data might be considered in a context of a more efficient use of the nitrogen sources available for maize plants, where acquisition and utilization mechanisms are efficiently modulated.

The microarray analysis has been also an important tool to detect transcripts of genes, whose involvement in response to urea and/or nitrate has not been yet elucidated. So, further molecular analyses will be performed to characterize these transcripts and clarify their biological role in nitrogen nutrition.

Besides the physiological characterization of high affinity urea transport in maize roots, and of the related molecular aspects, an important result of the present work was the identification and characterization of ZmDUR3 by heterologous expression in yeast mutant, *X.laevis* oocytes and tobacco protoplasts. With the aim to provide convincing evidence that ZmDUR3 does fulfill a function in urea acquisition from soil and use within the plant, the transformation of two Arabidopsis mutant lines (*atdur3-1* and *atdur3-3*, Kojima et al., 2007), which are defective for the endogenous transporter AtDUR3 and are not able to grow on a medium containing urea (<5mM) as sole nitrogen source will be carried out.

This work sheds light on the regulation of urea acquisition mecchanisms in maize roots and on the role of *ZmDUR3* in the high affinity urea transport system, improving the knowledge on the overall nitrogen acquisition and on the use efficiency of nitrogen fertilizers in crop plants.

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