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Transgenerational responses to nitrogen deprivation in *Arabidopsis thaliana*

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SUMMARY

Living organisms are constantly exposed to environmental stresses, which may have negative impacts on their growth, development, and reproduction. Such conditions are often so crucial to determine the environmental and geographical distribution of species, and provide a selective evolutionary pressure on a given population.

Different strategies can be adopted by living organisms to minimize stress influence. Because of their sessile nature, plants are expected to have evolved sophisticated mechanisms to cope with unavoidable adverse environmental conditions.

As perturbations may occur repeatedly, it would be advantageous to plants to retain the "memory" of past events and to use it to adapt to new environmental challenges (i.e. pathogens attack, drought stress). According to this, in *Arabidopsis thaliana* has been demonstrated that stress signals are usually transduced into effects on gene expression. Furthermore, it has been frequently reported that changes in gene expression induced by stresses in plants could be transmitted to the progenies stabilizing stress-dependent modifications; however, the explanation of this phenomenon remains still controversial.

One of the major stress types to which plants are frequently exposed is nutritional stress. The most critical example of this kind of stress is the deprivation of nitrogen, the essential mineral element required in greatest amount for plant growth due to its role as a constituent of many primary metabolites, such amino acids, nucleic acids, pigments as well as secondary metabolites, such as amines, phytohormones, alkaloids. Symptoms of nitrogen deficiency are slow growth, chlorosis of leaves and their fall off, as well as woodiness of stems and accumulation of anthocyanin pigments.

Nitrogen concentration can rapidly fluctuate in the soil because of leaching, volatilization of ammonia, soil denitrification and especially human activities. Thus, this leads plants to efficiently adapt by adjusting their acquisition mechanisms to ensure an adequate element supply, a proper plant development as well as an appropriate yield in crops. Moreover, it is known that nitrate, the main source of nitrogen for plants growing in aerobic soils, is a signal

capable of eliciting the rapid expression of transporters and assimilatory enzymes as well as changes in root morphology.

The aim of this PhD thesis was to evaluate the possible establishment of a "*transgenerational stress memory"*, at physiological and transcriptomic level, using an experimental setup based on three successive generations of the model plant *Arabidopsis thaliana* (Col-0), subjected to nitrate deprivation. Using nitrate uptake measurements and genome-wide gene expression analyses (RNA-sequencing), it was observed an increase in the capability to take up the anion between the first and the second generation, involving a high-affinity and saturable transport system, paralleled by a considerable modulation of gene expression at the level of nitrate transporters (i.e. *NRT2.4* and *NRT2.5*). In order to assess the occurrence of a "*transgenerational memory"*, transcriptional analysis on the third generation showed an enduring down-regulation of genes involved in the response to light, like for example *LHCB2* and *LHCA3*, which are members of photosystem light harvesting complexes.

In the second part of the project, in order to reinforce the observed transcriptional patterns, we explored the possibility that the observed changes in the expression of nitrogen-related genetic pathways might be accompanied by epigenetic changes. Indeed, the epigenetic analysis, focused on the high-throughput detection of genome-wide DNA methylation, a potentially inheritable DNA modification, revealed changes, also termed differentially methylated regions (DMRs), between treated (N-deprived) and control plants which also involved genes pinpointed by the transcriptome analysis. However, the mechanism underlying the maintenance of expression changes in the progeny of stressed plants remains elusive and compels further investigation.

The results of this study highlight the molecular changes associated with the plant response to an important nutritional stress in a transgenerational perspective. Thus, this exploratory work paves the way for new lines of research that may help breeding for high crop production by reinforcing the natural resilience and adaptive attitude of plants in the context of nitrogen requirement and consequently reducing the use of N-fertilizers, that represents an expensive and environmentally costly strategy to achieve desirable crop yields.

1. INTRODUCTION

1.1 Nitrogen nutrition

1.1.1 Nitrogen cycle

Nitrogen is present in many forms in the biosphere. The atmosphere contains high amounts (about 78% by volume) of molecular nitrogen (N_2), which is a form unavailable to most living organisms because of the strength of the triple bond that holds the two nitrogen atoms together. Only a limited number of species of microorganisms have evolved the capability to convert N_2 to reactive nitrogen (Galloway et al., 2004).

Nitrogen fixation is the process where molecular nitrogen is transformed to ammonia. For many nitrogen-fixing systems, the overall reactions are thermodynamically favorable:

$$N_2 + 3H_2 \rightarrow 2NH_3$$

and microorganisms that are involved in this reaction can be classified by their energy source:

- Photosynthetic bacteria called *cyanobacteria*, which use light energy and electrons provided by water and other compounds;
- soil bacteria (sometimes in association with root system and their exudates), which use the reducing power and ATP from degradation of carbohydrates and organic matter;
- symbiotic microorganism, genus *Rhizobium* (gram-negative). These bacteria colonize plant cells within root nodules where they convert atmospheric nitrogen into ammonia and then provide organic nitrogenous compounds such as glutamine or ureides to the plant. The plant in turn provides the bacteria with organic compounds made by photosynthesis.

In the soil, nitrogen is generally present in organic forms mostly unavailable to plants, apart from small molecules that can be absorbed by roots in limited amounts. However, organic nitrogen can undergo microbial mineralization, which includes protein hydrolysis into peptides and amino acids and their following degradation to ammonia. Ammonia in turn can

be converted to nitrate through nitrification process, otherwise stay in the soil as ammonium (figure 1.1).

The ammonium (NH_4^+) and nitrate (NO_3^-) ions that are generated through fixation or released through decomposition of soil organic matter become the object of intense competition among plants and microorganisms. To remain competitive, plants have developed mechanisms to efficiently acquire these ions from the soil (Taiz and Zeiger, 2002).

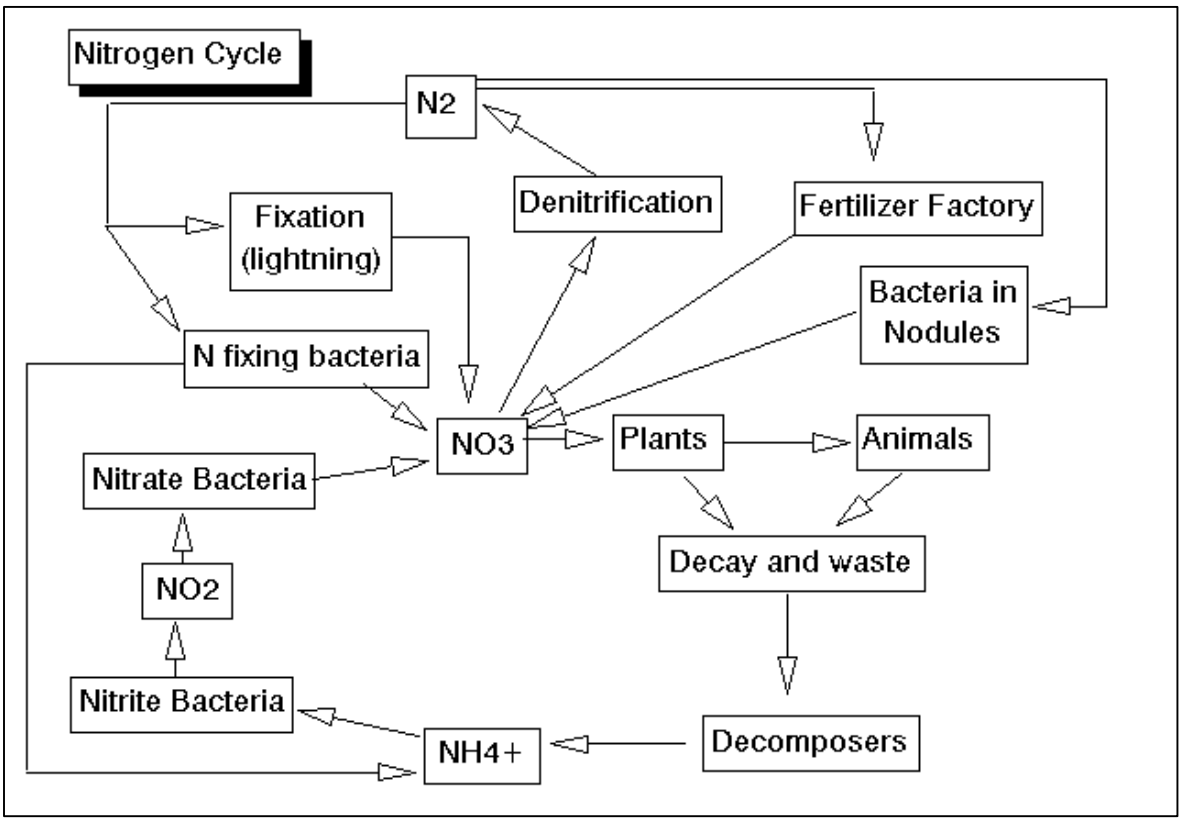


Figure 1.1. Schematic representation of N cycle.

1.1.2 The relevance of nitrogen in plants

Higher plants are autotrophic organisms that can synthesize their organic molecular components out of inorganic nutrients obtained from their natural environment. For many mineral nutrients, this process involves absorption from the soil by the roots and incorporation (assimilation) into the organic compounds, which are needed for growth. The distribution of mineral nutrients between different types of cells within a given tissue (e.g.,

epidermis cells, guard cells, mesophyll cells of a leaf) also provides important information about the functions of mineral nutrients (Marschner, 1995). Assimilation of some nutrients requires a complex series of biochemical reactions that are among the most energy-requiring reactions in living organisms (Taiz and Zeiger, 2002).

Nitrogen is the most required mineral element by plants. It is an essential constituent of many plant cell components, such amino acids, nucleic acids, pigments and secondary metabolites. Nitrogen is also an integral constituent of protein structure and apoenzymes, with a relevant role in the catalytic reactions of enzymes.

Thus, nitrogen deficiency slows down plant growth. If such deficiency continues, most plants show *chlorosis*, which is the yellowing of the leaves, especially in the older leaves (figure 1.2). If this deprivation becomes severe, leaves turn completely yellow and fall off the plant.

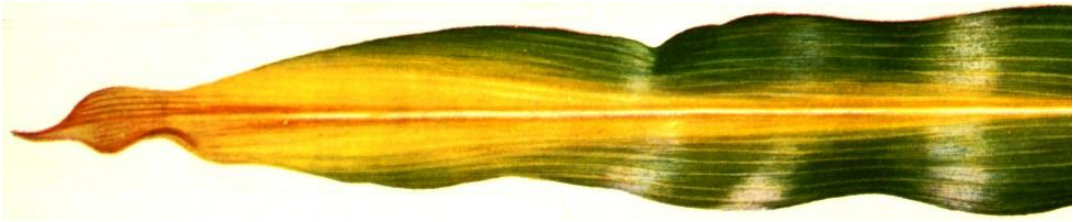


Figure 1.2. Leave of *Zea mays* with typical symptoms of nitrogen deficiency (http://nue.okstate.edu/).

At the beginning, younger leaves may not show these symptoms since nitrogen can be mobilized from older leaves. Another symptom of nitrogen deprivation in plants is woodiness of stems, caused by an excess of carbohydrates that cannot be used in the synthesis of amino acids or other compounds. These unused carbohydrates may also be used in anthocyanin synthesis, following an accumulation of that pigment (Taiz and Zeiger, 2002) (figure 1.3).

Nitrate and ammonium are the major sources of inorganic nitrogen taken up by the roots of higher plants. Most of the ammonium has to be incorporated into organic compounds in the roots, whereas nitrate is readily mobile in the xylem and can also be stored in the vacuoles of roots, shoots, and storage organs. Nitrate accumulation in vacuoles can be of considerable importance for cation-anion balance, for osmoregulation (Smirnoff and Stewart, 1985) and

for the quality of vegetable and forage plants. However, in order to be incorporated into organic structures, nitrate has to be reduced to ammonia.

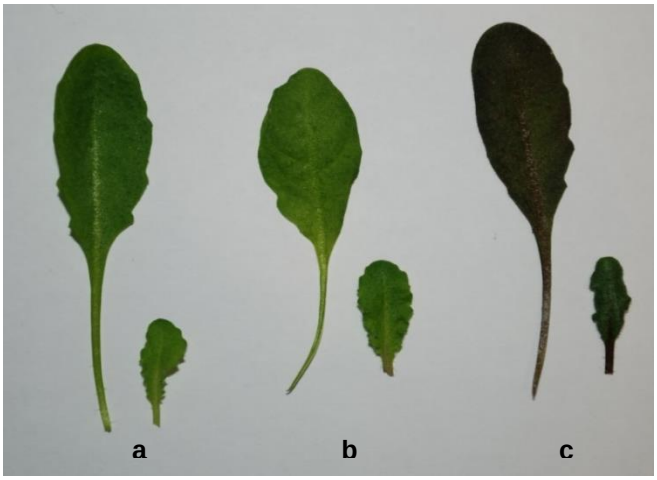


Figure 1.3. Effects of nitrogen deficiency on leaves of *Arabidopsis thaliana* subjected to different light condition and time of deprivation. a) plants grown at 8 h of light and with 0.5 mM NH_4NO_3. **b)** Plants grown with a photoperiod of 8 h of light and without nitrogen for 4 days. **c)** Plants with a photoperiod of 16 h of light and grown without nitrogen for 20 days (Massaro et al., unpublished work).

The importance of the reduction and assimilation of nitrate for plant life is similar to that of the reduction and assimilation of CO_2 in photosynthesis (Marschner, 1995). The preferred form in which nitrogen is taken up depends on soil conditions and plant species (Miller and Cramer, 2004, Marschner, 1995). For example, optimal growth of tomato roots occurs in soils with a ratio of nitrate to ammonium of 3:1 and it is inhibited if the concentration of ammonium is too high (Bloom et al., 1993). Generally, plants adapted to low pH and reducing soil conditions tend to take up ammonium. At higher pH and in aerobic soils, nitrate is the predominant form. Organic nitrogen compounds, such as amino acids, can also provide important nitrogen sources, especially in such environments where mineralization is slow (Miller and Cramer, 2004).

1.1.3 Nitrate uptake in plants

1.1.3.1 Fluctuations of nitrate concentration in soil

Nitrate is the main source of inorganic nitrogen for plants in aerobic soil conditions. According to Wolt (1994) the mean soil concentrations of nitrate were found to be 6.0 mM compared with 0.77 mM for ammonium. Because of its mobility, partially due to the negative charge of soil, the nitrate ion concentration can fluctuate in the solution by four orders of magnitude (Crawford and Glass, 1998). This rapid depletion of nitrate by both biotic (acquisition from root systems and microbial activities) and abiotic factors (rains and human activities) causes extensive seasonal and regional variations in nitrate concentrations in soils (Haynes, 1986). Temperature and other environmental factors, seasonally changing, affect the consumption of nitrate by plants and the generation of nitrate by soil microbes. For example, during the spring, soil nitrate levels initially rise and then decrease as increasing plant demand and uptake remove anion from the soil solution. Heavy seasonal rains may produce a "waterlogging" effect, producing anaerobic conditions that inhibit the generation of nitrate by soil microbes (Crawford and Glass, 1998).

1.1.3.2 Nitrate uptake

Because of their sessile nature, plants have evolved at least three adaptation processes to cope with the limited abundance of nitrate in soil. Firstly, plants can adapt their transport and assimilatory activities to nitrate availability (Gojon et al., 2009). Another plant strategy is to store nitrate in vacuoles to subsequently remobilize it. The rapid accumulation of nitrate in these organelles can prevent dispersion and uptake by neighboring plants (Martinoia et al., 1981). Furthermore, this process can play a relevant role in osmoregulation of cells, especially in plants in which nitrate reaches concentrations up to 300 mM (Hewitt et al., 1979). Finally, root architecture can change in response to nitrogen availability in the soil, a classic example of a plant developmental adaptation to its environment. In *Arabidopsis*, a localized nitrate treatment stimulates lateral root elongation (Zhang and Forde, 1998; Linkohr et al., 2002). It has been shown that nitrate itself, rather than a downstream metabolite elicits lateral root elongation by increasing a meristematic activity in the root tip

directly exposed to the signal (Zhang and Forde, 1998) (figure 1.4). Such morphological changes generally required longer time as compared to the regulation of transport activity and storage in vacuoles.

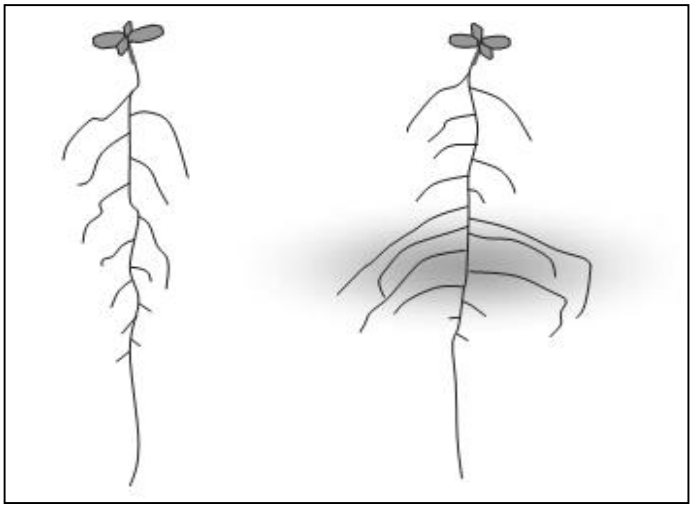


Figure 1.4. Root system of *Arabidopsis thaliana* grown with a uniform availability of nitrate (left) and localized (right). Lateral root branching is evident where nitrate is provided locally (right) (Forde and Walch-Liu, 2009).

As reported above, regulation of amount and activity of proteins involved in nitrate transport is crucial in order to sustain an efficient acquisition of the anion in plants. Furthermore, nitrate transport inside the root cells is an active process that requires energy to overcome the disadvantageous electrochemical gradient for the anion. The proton motive force is provided by the activity of the plasma membrane (PM) H^+-ATPase; protons extruded by the pump are then used to sustain the $NO_3^-/2H^+$ symport (Glass and Siddiqi, 1995) (figure 1.5).

1.1.3.3 Nitrate transport mechanisms in roots

Given the physiological role on nitrate, plants have developed different kinds of specific transporters, with peculiar features (Taiz and Zeiger, 2002).

It is generally acknowledged that roots have three nitrate$^-$ transport systems, with distinct kinetic characteristics:

- A constitutive high affinity transport systems (cHATS), with K_m values in the range 6–20 µM and V_{max} from 0.3 to 0.82 mmol g fw h^{-1};
- An inducible high affinity transport system (iHATS), with K_m values around 20–100 µM and V_{max} values in the range 3–8 mmol g fw h^{-1}. This system is induced within hours to days of exposure to NO_3^-;
- A constitutive low affinity transport system (LATS), which can significantly contribute to nitrate uptake at external concentrations above 250 µM, showing no saturation at NO_3^- concentrations as high as 50 mM.

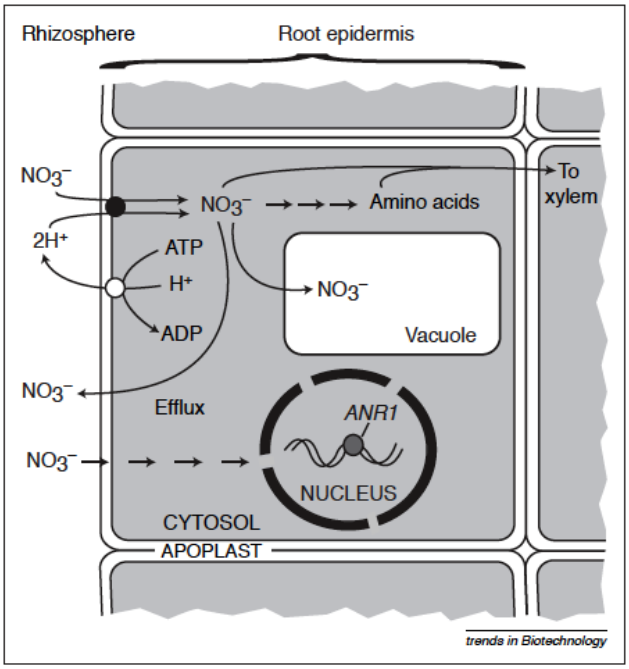


Figure 1.5. Nitrate uptake in root cells. The anion is transported inside the cell by plasma-membrane (PM)-located proteins using a symport with two protons. The difference in proton concentration depends on the activity of proton pump (PM H^+-ATPase) (Hirsch and Sussman, 1999).

The cHATS is expressed in the absence of nitrate, while iHATS expression is induced several fold by nitrate treatment (Kronzucker et al., 1995). Furthermore, iHATS is feedback-regulated by the products of nitrate assimilation (King et al., 1993; Forde, 2000). The distribution of these transporters is different along the radical axis. It has been suggested that LATS are

preferentially located in the root tip while HATS in the older part of the roots (Pinton et al., 2007).

1.1.3.3.1 Nitrate transporters in Arabidopsis

Several molecular evidences have described the existence of two principal families of genes encoding nitrate transporters among eukaryotes, namely the *NRT1* and *NRT2* families. These families correspond to the low- (LATS) and high-affinity (HATS) nitrate transporters, respectively (Crawford and Glass, 1998; Forde, 2000). In *Arabidopsis thaliana*, twelve members of the *NRT1* family (Tsay et al., 2007; Dechorgnat et al., 2011) and seven members of *NRT2* family (Orsel et al., 2002; Dechorgnat et al., 2011) have been identified. In addition in *Arabidopsis* two transporters, *NAXT1* (Segonzac et al., 2007) and *NRT1.5* (Lin et al., 2008), mediate nitrate efflux in cortex and xylem loading, respectively. The NRT1 family was also named NRT1/PTR because NRT1/PTR members in animals, fungi, and bacteria transport dipeptides. To be more precise the members of the NRT1 (PTR) family were categorized as two distinct subtypes, namely nitrate transporters and peptide transporters. Up to now no nitrate transporters have been found to have peptide transport activity and no peptide transporters have been found to transport nitrate (Tsay et al., 2007). In *Arabidopsis*, there are 53 NRT1/PTR transporters. It is possible that the nitrate transport activity of this family evolved from an ancient dipeptide transporter. Most nitrate and peptide transporters characterized in the NRT1/PTR family are proton-coupled transporters. In contrast to NRT1/PTR transporters, which have different substrate specificity, all NRT2 transporters isolated from *Aspergillus*, *Chlamydomonas*, and higher plants transport only nitrate (Wang et al., 2012).

A member of the NRT1 family, the *NRT1.1* gene, is expressed in the apical part of the *Arabidopsis* root; this gene codes for a protein that, at the same time, has the double role of nitrate transporter (Tsay et al., 1993) and nitrate sensor to activate the expression of nitrate-related genes. The *NRT1.1* gene is also called *CHL1* (Chlorate resistant mutant 1). Chlorate, a nitrate analog, can be taken up by plants using nitrate uptake systems and converted by nitrate reductase (NR) into chlorite, which is very toxic for plants (Tsay et al., 2007). Firstly,

NRT1.1 was described as a low-affinity nitrate transporter characterized by a K_m of 5 mM, involved in the LATS. The expression of the *NRT1.1* gene is inducible by nitrate, suggesting a role of the protein in the inducible component of the LATS (figure 1.6). The same protein was also shown to be involved in HATS (Liu et al., 1999). *NRT1.1* is now considered to be a dual affinity nitrate transporter, regulated by a phosphorilation/dephosphorilation mechanism (see next paragraph).

On the other hand *NRT2.1*, is absent from the *Arabidopsis* root tip, but expressed in the mature part of the root. The *NRT2.1* protein is localized on the plasma membrane (Chopin et al., 2007) and is the master protein of the HATS system. Another important gene is *NRT2.2*, located near the *NRT2.1* on chromosome I. Experimental evidences by Li (Li et al., 2007) have demonstrated that *NRT2.1* is responsible for 72% of HATS activity. These results suggest that *NRT2.2* gives only a small contribution to the uptake system, providing a compensation mechanism when *NRT2.1* function is lost (Li et al., 2007). Together with *NRT2.1* and *NRT2.2*, *NRT2.4* transporter contributes to nitrate uptake at very low external nitrate concentration (figure 1.7). The localization in the plasma membrane of roots, makes *NRT2.4* an important player for scavenging even very low amounts of nitrate from the external medium. Furthermore, analysis of multiple mutants suggested that the interplay between *NRT2.1*, *NRT2.2*, and *NRT2.4* is important for optimal adaptation to nitrogen starvation (Kiba et al., 2012). *NRT2.5* is also a plasma membrane-localized high-affinity nitrate transporter playing an essential role in adult plants under severe nitrogen starvation. The expression of such transporter is induced under long-term starvation and after 10 days of deficiency becomes the most abundant NRT2 transcript in adult plants (Lezhneva et al., 2014).

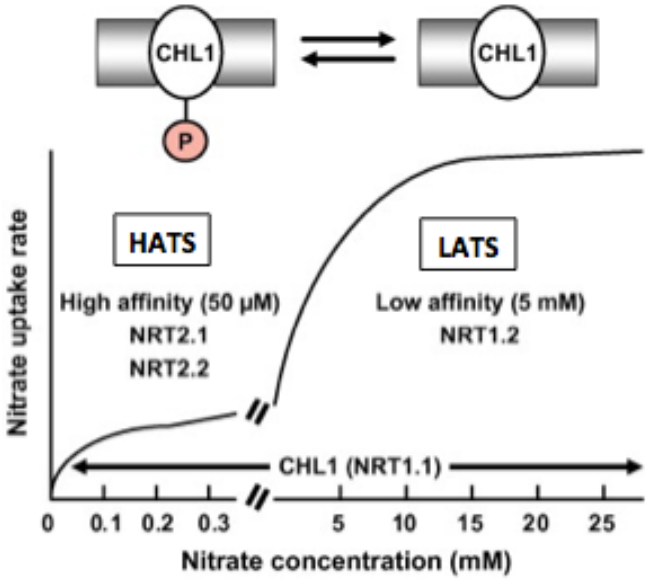


Figure 1.6. Examples of different transporters for nitrate uptake in *Arabidopsis*. *NRT1.1* (*CHL1*) is a dual affinity nitrate transporter involved in both HATS and LATS transport. *NRT2.1* and *NRT2.2* are high-affinity nitrate transporters involved mainly in iHATS. The mode of action of *CHL1* is switched by phosphorylation and dephosphorylation (Tsay et al., 2007).

Moreover *NRT2.4*, expressed in the major vein of source leaves, is involved in carrying nitrate into the phloem for remobilization under N limited condition (Kiba et al., 2012). The same function can be attributed to *NRT2.5*, expressed in the minor veins of leaves (Lezhneva et al., 2014). The transporter *NRT2.6* is weakly expressed in most plant organs, its expression being higher in vegetative organs than in reproductive ones. This transporter has an unusual behavior: conversely to other NRT2 members, *NRT2.6* expression was not induced by limiting but rather by high nitrogen levels. Interestingly, plants with a decreased *NRT2.6* expression showed a lower tolerance to pathogen attack. Finally, probably there is a link between *NRT2.6* activity and the production of ROS in response to biotic and abiotic stress (Dechorgnat et al., 2012).

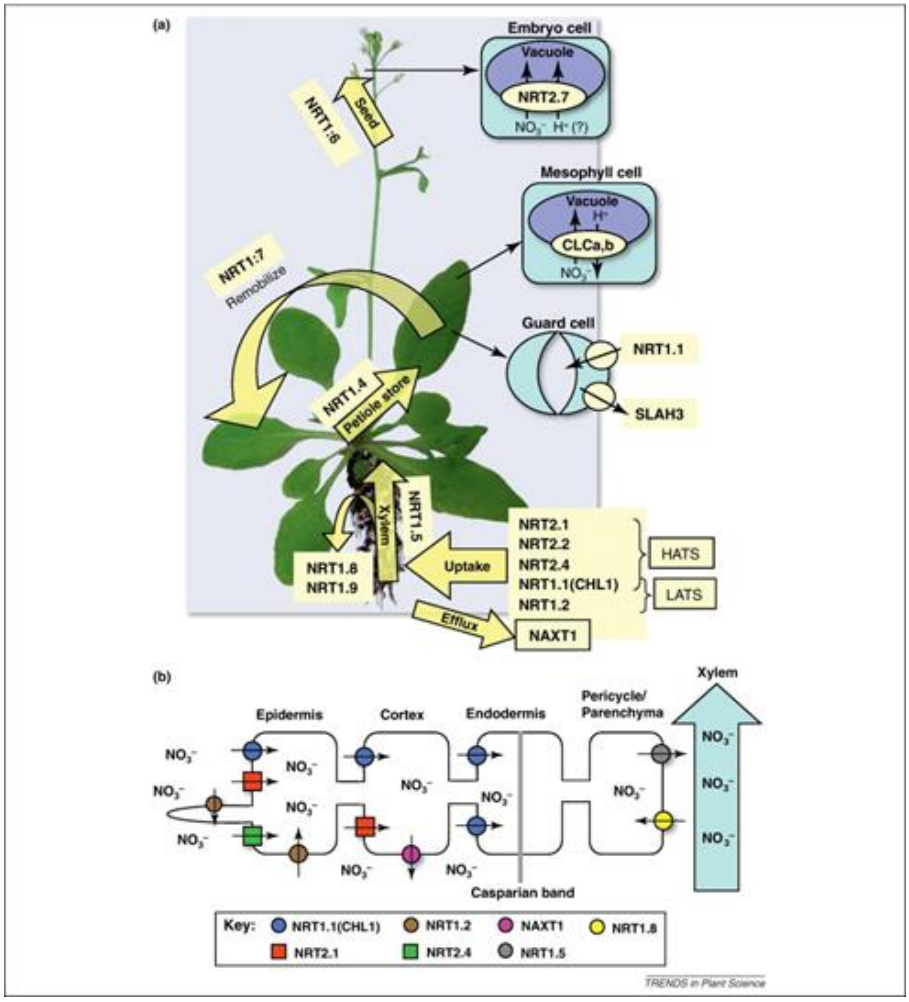


Figure1.7. Physiological functions of *Arabidopsis* nitrate transporters. **a**) Contribution of nitrate transporter 1 (NRT1), NRT2, chloride channel (CLC) a/b, and slow anion channel-associated 1 homolog 3 (SLAH3) to different steps of nitrate uptake and allocation. Nitrate is taken up by roots from soils and transported to shoots and seeds for storage and/or further assimilation. Abbreviations: HATS, high-affinity transport system; LATS, low-affinity transport system. **b**) Detailed illustration of nitrate uptake and movement in roots. NRT1.1 (CHL1), NRT1.2, NRT2.1, NRT2.2, and NRT2.4 are involved in nitrate uptake from soils. Nitrate excretion transporter 1 (NAXT1), a transporter in the NRT1 family, mediates nitrate efflux under acid load. NRT1.5, NRT1.8, and NRT1.9 play a role in regulating root-to-shoot xylem transport of nitrate. The illustration does not mean that these transporters have a polarized distribution in the root cells (Wang et al., 2012).

1.1.3.3.2 Primary response to nitrate: regulation of transporters involved in nitrate assimilation in Arabidopsis.

In the last years a lot of evidences have suggested that, in higher plants, nitrate serves not only as a nutritional source, but also as a signaling molecule. In fact, nitrate induces the expression of several genes involved in its transport and subsequent assimilation (Stitt, 1999), induces leaf expansion (Walch-Liu et al., 2000), stimulates lateral root branching (Zhang and Forde, 1998), regulates abscisic acid (ABA)-independent stomatal opening (Guo et al., 2003) and other important events in plants development. Although these nitrate-induced responses could be regulated by downstream assimilation products, various studies have indicated that nitrate itself regulates gene expression. The rapid transcriptional induction in response to nitrate is termed "*the primary nitrate response*" (Redinbaugh and Campbell, 1991).

Nitrate elicits rapid changes in gene expression for about 10% of the detectable transcriptome, with specific genes responding within 5–10 min of root exposure to sub-micromolar concentrations of nitrate or nitrite (Krouk et al., 2010). Nitrogen deprivation also elicits a strong transcriptional response (Krouk et al., 2010).

Genes regulated by nitrate include nitrate transporters. Generally, roots have a basal uptake in absence of nitrate, that is sustained by constitutive transporters. The exposure to the anion induces the expression of transporter genes in order to raise the amount of proteins involved in the uptake process. Time and entity of the response depends on the plant species and external nitrate concentration. By considering the energy required for nitrate uptake, the expression of the plasma-membrane proton pump is also induced (Santi et al., 2003); increased transcription of a series of metabolic genes ensures a proper nitrogen assimilation (Girin et al., 2007). In some promoters of genes of these transporters have been identified sequences binding transcriptional factors induced by nitrate (Girin et al., 2007). Intermediate metabolites such as nitrite and ammonium and various nitrate assimilation products like glutamine, asparagine and arginine repress nitrate uptake (Loqué et al., 2003; Cai et al., 2007).

One of the well-known player in this phenomenon is *NRT2.1.* His expression profile follows nitrate influx, increasing rapidly upon supply of nitrate to nitrogen-starved roots and decreasing when the nitrate concentration becomes stable.

Expression of *NRT2.1* is induced by low nitrate concentration (Okamoto et al., 2003; Wirth et al, 2007), feedback repressed by NH_4^+ and amino acids, in particular glutamine (Vidmar et al., 2000), and stimulated by light and sugars (Leyay et al., 2003). These mechanisms may modulate root anion uptake, coordinating nitrogen and carbon metabolism of the plant. Recently, *NRT2.1* has been shown to be down-regulated by nitrate itself, through a mechanism independent of the feedback repression exerted by reducing forms of nitrogen, but specifically triggered by the *NRT1.1* transporter (Krouk et al., 2006) (figure 1.8). However, it has been suggested that NRT2 transporters also possess a post-transcriptional regulation, participating to the modulation of root nitrate uptake in response to environmental changes.

NRT2.1 does not seem to be able to mediate nitrate transport on its own. It needs to be co-expressed with a *NAR2* protein, to allow efficient nitrate transport. This has suggested that the actual transport system corresponds in fact to a dual component (NRT2/NAR2) transporter (Orsel et al., 2006). A crucial role of the *NRT2.1* putative partner *NAR2.1*, also termed *NRT3.1*, is confirmed by the observation that mutants disrupted in the *NAR2.1* gene show an even stronger defect in HATS activity than the *NRT2.1* mutants in *Arabidopsis*. It has also been hypothesized that *NRT2.1* might play a role as a nitrate sensor, or a signal transducer (Zhou et al., 2000). In addition the inducible transport of *NRT2.1* is almost put off if *NRT3.1* is not correctly expressed (Okamoto et al., 2006; Orsel et al., 2006).

It has been demonstrated that for *NRT2.1* there are also post-translational regulation mechanisms; the C-terminal domain can be removed, causing inactivation of transporter (Wirth et al, 2007).

In the last years experimental evidences suggested that also *NRT1.1* plays an important role in "*the primary nitrate response"*. Through genetic and genomic approaches, it has been demonstrated that *NRT1.1* can sense a wide range of nitrate concentrations in the soil and can use a phosphorylation switch to change the transport activity and signaling output. Indeed, when threonine 101 (T101) is phosphorylated, *NRT1.1* functions as a high-affinity

nitrate transporter and dephosphorylated transporter acts as a low-affinity transporter (Krouk et al., 2010) (figure 1.6).

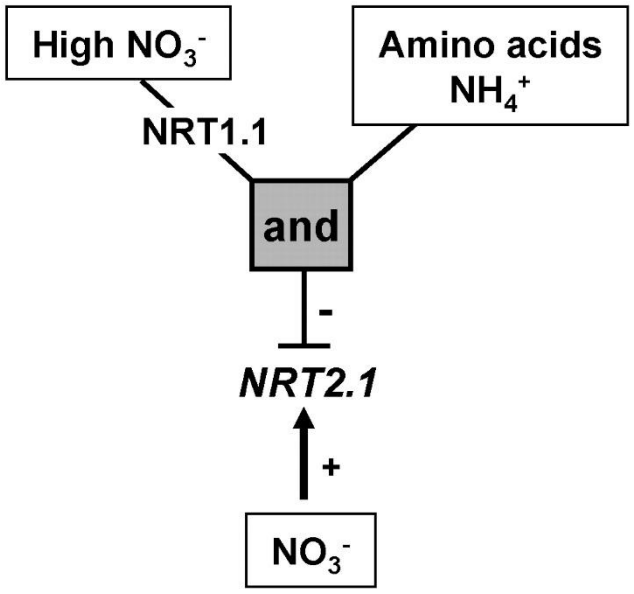


Figure1.8. Model for N regulation of *NRT2.1* expression in *Arabidopsis* roots (Krouk et al., 2006).

Two nitrate-inducible protein kinases CIPK8 and CIPK23 (calcineurin B-like interaction protein kinase) seem to be involved in the regulation of the *NRT1.1* dual affinity. In response to low concentrations of nitrate, CIPK23 is responsible for phosphorylating *NRT1.1* at T101. The target site of CIPK8 remains to be determined (Krouk et al., 2010).

These mechanisms suggest that the nitrate sensing system can sense not only the presence or absence of nitrate but also the concentration of nitrate.

The sensor activity of NRT1.1 is independent from its transport activity (Muños et al., 2004) and it is required to induce *NRT2.1* transcription and activity (Ho et al., 2009).

1.1.4 Ammonium uptake in plants

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, especially 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 (Ullrich et 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).

1.1.4.1 Nitrate and ammonium assimilation

Once NO_3^- is taken up into the cell it has four possible fates: firstly, reduction to NO_2^- by the cytoplasmic enzyme nitrate reductase; secondly, efflux back across the plasma membrane to the apoplasm; additionally, influx and storage in the vacuole; or whereas transport to the xylem for long-distance translocation to the leaves (figure 1.9). Indeed after the uptake of nitrate into the cell, the next step in the nitrogen assimilation pathway is reduction of nitrate to nitrite. This step competes with both efflux of nitrate from the cell and transport of nitrate into the vacuole. However, different steps are modulated at both transcriptional and post-translational levels. The enzyme that catalyzes the reduction reaction, nitrate reductase (in *Arabidopsis* called *NIA1*), is located primarily in the cytosols of root epidermal and cortical cells and it responds to many signals (Crawford and Forde, 2002; Crawford, 1995).

Nitrate reductase transfers two electrons from NAD(P)H to nitrate via three redox centers composed of two prosthetic groups (flavin adenine dinucleotide, FAD, and heme) and a MoCo cofactor, which is a complex of molybdate and pterin. The complexity of nitrate reductase is reflected in its size: it is a homodimer or homotetramer of 110-kD subunits (Crawford, 1995). Each redox center is associated with a functional domain of the enzyme that has activity independent of the other domains. For example, just, two domains of nitrate reductase, the heme and FAD domains, are needed to catalyze a reaction in which cytochrome c is used instead of nitrate as an alternate electron acceptor. Cytochrome c reductase activity involves transfer of electrons from NAD(P)H to the FAD domain, which reduces the heme domain, which in turn reduces cytochrome c. The MoCo domain need not be intact for this partial reaction to work (figure 1.10).

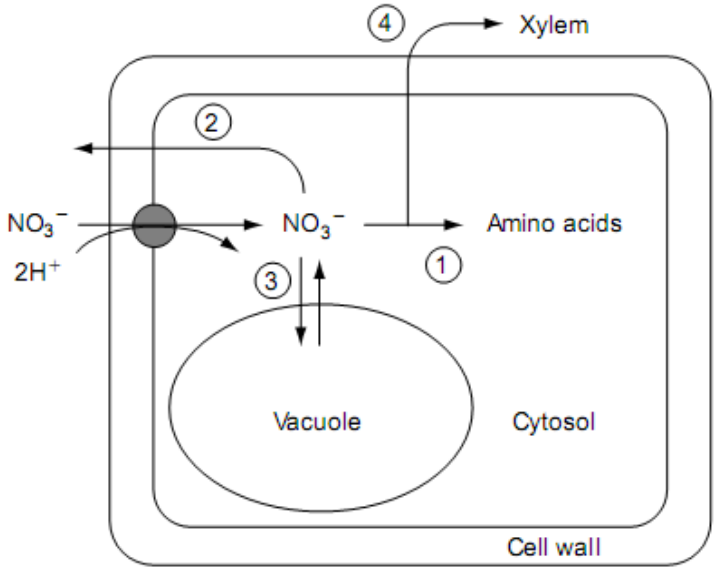


Figure 1.9. Different fates of nitrate once taken up into cell: (1) reduction to NO_2^- by the cytoplasmic enzyme nitrate reductase; (2) efflux back across the plasma membrane to the apoplasm; (3) influx and storage in the vacuole; or (4) transport to the xylem for long-distance translocation to the leaves (Crawford and Glass, 1998).

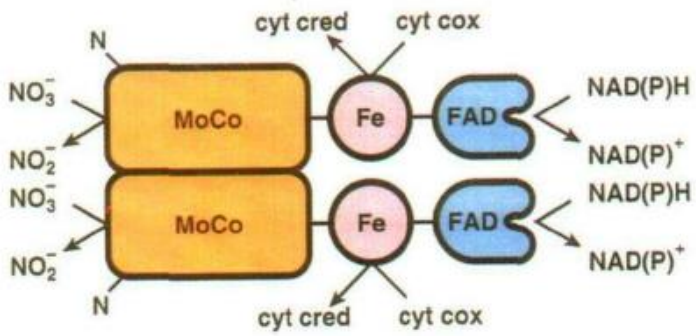


Figure 1.10. Schematic representation of the nitrate reductase homodimer with the functional domains indicated. FAD, flavin domain; Fe, heme domain; MoCo, molybdenum cofactor domain; N, N terminus of NR; cyt c, cytochrome c, which can be used as an alternate electron acceptor in vitro; cyt cred and cyt cox, the reduced and oxidized forms of cytochrome c, respectively (Crawford, 1995).

As for transporters, nitrate rapidly activates transcription of the *NIA* genes. Sucrose, light and cytokinin enhance *NIA* induction while ammonium and amino acids repress expression. *NIA* gene also responds to circadian rhythms. Another important aspect to consider is the regulation at protein level. Nitrate reductase is phosphorylated by one of several kinases on a

serine residue in the first hinge region, which separates the Mo-cofactor and heme-binding regions of NR. As reported above the reductase has three redox centers and the Mo-cofactor transfers electrons to nitrate while the heme carries across electrons from a FAD prosthetic group to the Mo-cofactor. If nitrate reductase is phosphorylated in the hinge 1 region, it can bind a 14-3-3 dimer, which in turn inactivates the enzyme. Removal of the 14-3-3 dimer or dephosphorylation of enzyme by a phosphatase results in reactivation of the enzyme (Crawford and Forde, 2002).

Nitrite produced by *NIA* is potentially toxic to plants, thus it is transported into the chloroplasts in green tissues, where it is reduced to ammonium by nitrite reductase (NiR) using reduced ferredoxin. The general reaction is:

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

NiR is a nuclear-encoded enzyme that is transported into the chloroplast, with a 30-amino acid transit sequence (Gupta and Beevers, 1987). The holoenzyme is a monomer (60 to 70 kD) with two redox centers: a heme-Fe center and an iron-sulfur center. In addition it has been suggested that the C-terminal half of *NiR* contain the redox centers; the N-terminal half is thought to bind the reducing agent ferredoxin. Ferredoxin reduced by the chloroplast noncyclic electron transport system provides the electrons for reducing nitrite. Several promoter elements have been identified that are needed for NO_3^- induction of *NiR* gene (Rastogi et al., 1993).

Enzymes required for ammonium assimilation are glutamine synthetase (GS) and glutamate synthase (GOGAT). The glutamine synthetase catalyzes the condensation of glutamate and ammonia to form glutamine, using ATP and bivalent ion as cofactors (Mg^{2+}, Mn^{2+} o Co^{2+}). The catalyzed reaction is:

$$\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i$$

Glutamine synthetase is composed by 8 identical subunits (350 kDa) and there are two isoforms. One of these is in the cytosol and the other one in plastids of roots and chloroplast of shoots. The cytosolic forms are expressed in germinating seeds or in the vascular bundles

of roots and shoots and produce glutamine for intracellular nitrogen transport. The GS in root plastids generates amide nitrogen for local consumption; the GS in shoot chloroplasts reassimilates photorespiratory NH_4^+ (Lam et al., 1996). Light and carbohydrate levels alter the expression of the plastid forms of the enzyme, but they have little effect on the cytosolic forms. Elevated plastid levels of glutamine stimulate the activity of glutamate synthase. This enzyme transfers the amide group of glutamine to 2-oxoglutarate, producing two molecules of glutamate. Plants contain two types of GOGAT: The first accepts electrons from NADH:

$$\text{Glutamine} + \text{2-oxoglutarate} + \text{NADH} + \text{H}^+ \rightarrow \text{2-glutamate} + \text{NAD}^+$$

the second accepts electrons from ferredoxin (Fd):

$$\text{Glutamine} + \text{2-oxoglutarate} + \text{Fd}_{\text{red}} \rightarrow \text{2-glutamate} + \text{Fd}_{\text{ox}}$$

The NADH type of the enzyme (NADH-GOGAT) is located in plastids of non-photosynthetic tissues such as roots or vascular bundles of developing leaves. In roots, NADH-GOGAT is involved in the assimilation of ammonium absorbed from the rhizosphere; in vascular bundles of developing leaves, NADH-GOGAT assimilates glutamine translocated from roots or senescing leaves. The ferredoxin-dependent type of glutamate synthase (Fd-GOGAT) is found in chloroplasts and serves in photorespiratory nitrogen metabolism. Both the amount of protein and its activity increase with light levels. Roots, particularly those under nitrate nutrition, have Fd-GOGAT in plastids. Fd- GOGAT in the roots presumably functions to incorporate the glutamine generated during nitrate assimilation. Once assimilated into glutamine and glutamate, nitrogen is incorporated into other amino acids via transamination reactions (Taiz and Zeiger, 2002) (figure 1.11).

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*.

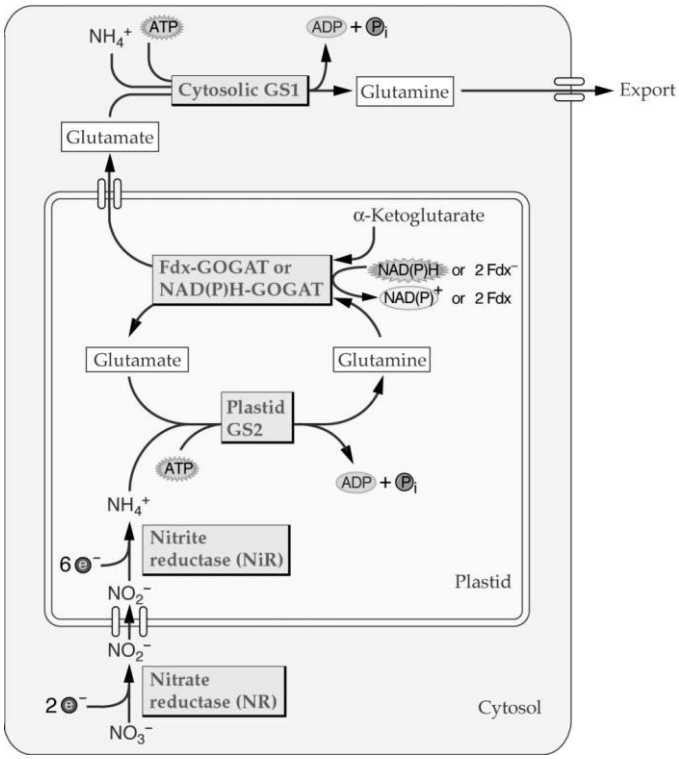


Figure 1.11. Assimilation of nitrate. Many enzymes (NR, Nir, GS, GOGAT) and reactions are involved to convert nitrate in reduced forms of nitrogen and other organic forms as amino acids (Buchanan et al., 2002).

1.2 Nitrogen fertilizers use efficiency

World population is expected to increase to be 50% higher by 2050. Such increase in population growth will intensify pressure on the world's natural resource base (land, water, and air) to achieve higher food production. An increase in the food production could be carried out by expanding the crops area and by increasing yields per unit area. Most of the land that could be brought under cropping has been utilized with exception of some land in Sub-Saharan Africa and South America (Borlaug and Doswell, 1993). For this reason, all over the world many agricultural soils are deficient in one or more of the essential nutrients to support healthy and productive plant growth. Especially human activity and farming practices have contributed to soil degradation and lowering of fertility across different agroecosystems.

Chemical fertilizers are one of the expensive strategies used by farmers to achieve desired crop production (figure 1.12). Nevertheless, recovery of applied inorganic fertilizers by plants is low in many soils (Cassman et al., 1998). Estimates of global efficiency of these applied fertilizers have been about 50% or lower for nitrogen, less than 10% for phosphorus, and near to 40% for potassium (Baligar and Bennett, 1986). These low efficiencies are due to significant losses of nutrients by leaching, volatilization of NH_3 and soil denitrification. These losses can potentially contribute to degradation of soil and water quality and finally of the overall environment. During the last 50 years, global nitrogen fertilizer applications have increased steadily, rising almost twenty fold to the present rate. These are important reasons of the need to increase the Nitrogen Use Efficiency (NUE). NUE is generally used to indicate the ratio between the amount of N-fertilizer removed from the field by the crop and the amount of N-fertilizer applied. However, focusing on the importance of the crop plant itself as an integral component of this system, and particularly with respect to nitrogen fluxes between plant and soil, there are several physiological pressures upon nitrogen uptake by roots of crop plants that facilitate the documented losses and contribute to inefficient nitrogen utilization (Glass, 2003).

The higher use of N-fertilizer leads to improve crop yields but decreases NUE (figure 1.13). The average NUE of cereal crops is close to 30-35% (Eickhout et al., 2006).

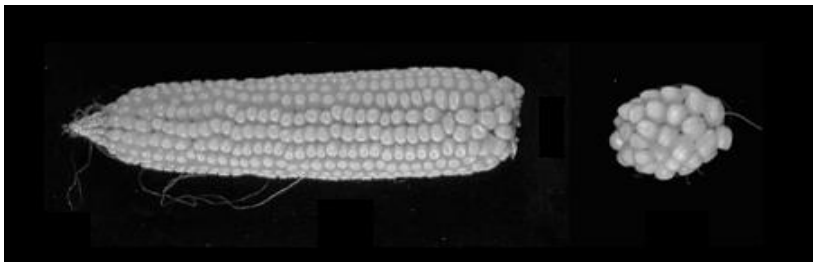


Figure 1.12. Ears of corn of plants grown in N sufficiency (left) and deficiency (right) conditions (Hirel et al., 2007).

Thus, the environmental consequences of N-fertilizer use in food production are strongly related to the NUE. Each year commercial value of dispersed fertilizer reaches around 10

billion dollars, to which needs to add costs of environmental damages. Now days, innovative fertilizer use efficiency technologies enable increased crop production, for example improving agricultural production systems and the exploitation of alternative sources (Roy et al., 2002).

In the next future, possible strategies to increment NUE could be for increasing the cultivar selection, based on the ability to uptake nitrate and finally plant breeding and genetic modifications (Eickhout B. et al., 2006).

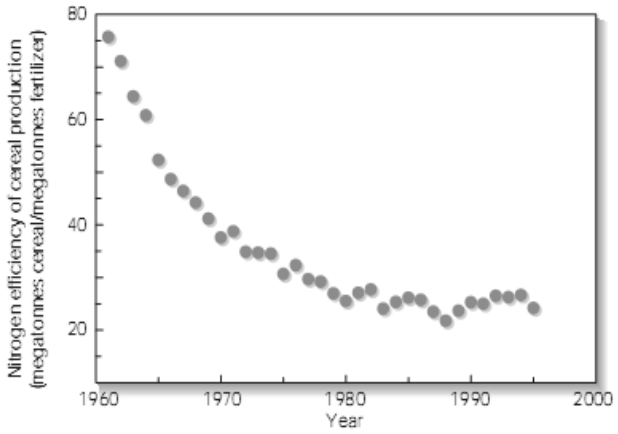


Figure 1.13. Trends in the nitrogen-fertilization efficiency of crop production (annual global cereal production divided by annual global application of nitrogen fertilizer), during the period 1960-2000 (Tilman et al., 2002).

1.3 Nutritional stress and epigenetics

1.3.1. Plant responses to stress

Organisms are constantly exposed to environmental stimuli named stresses, and they have the ability to establish mechanisms of protection and adaptation. Stress has the most significant and mainly negative effect on organism growth, development, and reproduction. It often determines the distribution of species, and more importantly provides a selective evolutionary pressure on a given population (Doroszuk et al., 2006; Boyko and Kovalchuk, 2008). Two types of stresses exist: biotic such as pathogens, and abiotic stress, like temperature, salt as well as cold. For these reasons three different strategies can be applied to minimize stress influence. They are tolerance, resistance, and avoidance or ultimately escape. Obviously, because of their sessile nature, plants are restricted to the first three mechanisms only. Hence, it can be expected that they have evolved sophisticated mechanisms to cope with adverse environmental conditions. Generation of new traits followed by selection of the adaptive qualities represents a long-term surviving strategy. In contrast to animals that can compensate a slow evolution rate by escape or limited exposure to stress, plants require efficient short-term strategies based on the manipulation of the existing genetic information (Boyko and Kovalchuk, 2008). These strategies include an alteration of plant homeostasis during the somatic growth (Shinozaki et al., 2003) and heritable or transgenerational modifications of gene expression. (Whitelaw and Whitelaw, 2006).

Many genes respond to drought, salt and/or cold stress at the transcriptional level and their products are involved in the stress response and tolerance (Bray et al, 2000; Shinozaki et al., 2003). Transcriptome analyses using microarray technology, for example, (Seki et al., 2001) have identified several genes that are induced by abiotic stresses. Such genes have been classified into two important groups (Bray et al, 2000). The first one encodes products that directly protect plant cells against stresses, whereas the products of the second group regulate gene expression and signal transduction in abiotic stress responses. Various different sets of cis- and trans-acting elements have been identified as involved in stress-responsive transcription. For example it has been noticed that many genes are modulated by

both drought and cold stress, suggesting the existence of crosstalk between the drought and cold-stress signaling pathways as well as other networks (Shinozaki et al., 2003).

The second type of modifications can occur without changing the original DNA sequence and are known as epigenetic. They can be established on several interdependent levels including methylation of DNA sequence, various histone modifications, and chromatin remodeling (Wagner, 2003; Boyko and Kovalchuk, 2008) (figure 1.14).

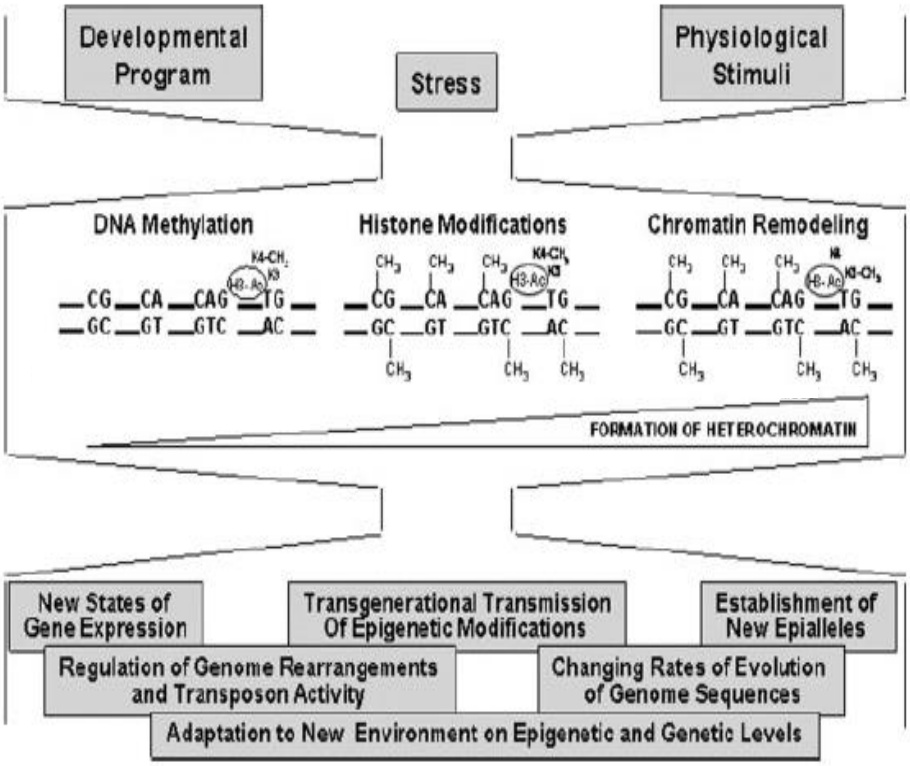


Figure 1.14. Possible mechanisms of response to stress via different chromatin modifications. Epigenetic modifications allow the reversible generation of new, potentially heritable, states of gene expression without modifying coding and regulatory DNA sequences, and can be introduced on three distinct levels that include DNA methylation, histone modifications, and chromatin remodeling. Transition of chromatin from eu- to heterochromatin can be initiated by a number of external and internal stimuli (modified from Boyko and Kovalchuk, 2008).

1.3.2 Epigenetics

Conrand Waddington defined epigenetics as "the branch of biology which studies the casual interaction between genes and their products, which bring the phenotype into being" (Waddington, 1942). In this original formulation, the science of epigenetics was already considered as a sort of conceptual bridge between genotype and phenotype, which could be tackled from different perspectives and at many different levels. For example, even though the vast majority of cells in a multicellular organism share an identical genotype, during development a diversity of cell types is generated with disparate profiles of genes expression and distinct cell functions. Consequently, cell differentiation may be regarded as an epigenetic phenomenon in Waddington's view. Although the definition of epigenetics has significantly changed ever since, we due to Waddington the intuition that cell differentiation is largely governed by changes in what Waddington described as the "epigenetic landscape" rather than in the primary sequence of DNA (Waddington, 1957) (figure 1.15).

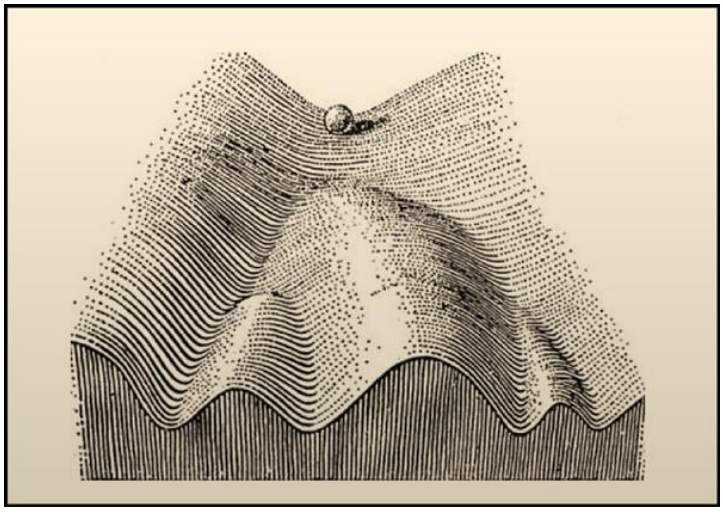


Figure 1.15. Imagine of Waddington's concept of an epigenetic landscape to represent the process of cellular decision-making during development. At various points in this dynamic visual metaphor, the cell (represented by a ball) can take specific permitted trajectories, leading to different outcomes or cell fates (Waddington, 1957).

Indeed, in a modern key epigenetics is defined as the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA (Goldberg et al., 2007). DNA methylation, histone modifications and nucleosome positioning are the three main epigenetic mechanisms underlying these changes, though non-coding RNAs, in particular small RNAs, are also involved. This work is focused on DNA methylation.

1.3.2.1 DNA methylation

DNA methylation is among the best characterized chemical modifications of chromatin. This important mechanism plays a crucial role in the regulation of gene expression, in the activity of transposable elements, in the defense against foreign DNA, and even in the inheritance of specific gene expression patterns. It provides the addition of a methyl group to a cytosine base, producing a 5-methyl cytosine. As reported in literature, DNA methylation appears to be evolutionarily ancient and primarily associated with gene silencing in eukaryotes, including plants, animal and fungi. However, this epigenetic mark has been curiously lost in some well-studied model organisms, such as *Caernorabditis elegans* and *Drosophila melanogaster,* baker's yeast and fission yeast (Zemach and Zilberman, 2010). To the best of our knowledge, *Arabidopsis,* one of the most important model organisms, has the most widely characterized DNA methylome of any plant organism.

In *Arabidopsis* and other plants investigated thus far, DNA methylation commonly occurs in all sequence contexts: the symmetric sites CG and CHG (where H= A, T or C) and the asymmetric site CHH. In *Arabidopsis*, DNA methylation levels of approximately 24%, 6.7% and 1.7% are observed for CG, CHG, and CHH contexts, respectively (Cokus et al., 2008). The most prominent difference in the DNA methylation patterns between plants and animals is that in animal genomes the fraction of modified cytosines is substantially higher, and asymmetrical cytosine methylation is restricted to specific cell lines and developmental stages (Lister et al., 2008).

1.3.2.2. Maintenance of methylation in CG context

DNA methylation can be established *de novo* or maintained through cell division. The maintenance of already established DNA methylation patterns occurs in CG context and in the others sites. Regarding CG methylation in plants, genetic evidences have demonstrated that homologs of the mammalian proteins Dnmt1, the MET1 DNA methyltransferase (Vongs et al., 1993), the VARIANT IN METHYLATION (VIM) (Woo et al., 2008; Law and Jacobsen, 2010), and the DDM1 chromatin remodeling factor (Vongs et al., 1993; Hirochika et al., 2000) are required to maintain CG methylation, suggesting that plants and mammals maintain CG methylation in a similar manner. MET1 is associated with DNA replication sites and serves to restore the expected methylated state in hemimethylated DNA generated during DNA replication. Early studies have clarified that methyltransferase is recruited to replication machinery via an interaction with the proliferating cell nuclear antigen (PCNA), essential for the replication **(**Law and Jacobsen, 2010). Recently, it was shown that MET1 also interacts with another chromatin associated protein, VIM, which is required for the association of MET1 with chromatin. The SRA domain of VIM specifically binds to hemimethylated CG dinucleotides, bringing to the idea that VIM recruits MET1 to hemimethylated DNA **(**Law and Jacobsen, 2010) (figure 1.16 a). It is well known that in *Arabidopsis* around 1/3 of genes have CG methylation in their coding region, which is maintained by MET1 (Lister et al., 2008; Zilberman et al., 2007). Differently from methylation at transposons, CG methylation within gene bodies does not seem to cause gene silencing and methylated genes tend to be quite expressed in many tissues anyway (Zilberman et al., 2007). Nonetheless, as reported by Zilberman, the expression of some body methylated genes is up-regulated in *met1* mutants (Zilberman et al., 2007), and highly or lowly expressed genes, in contrast with moderately expressed genes, tend to not have body methylation, suggesting in any case a link among transcription and body methylation, albeit counterintuitive. The presence of body CG methylation at some genes has also been reported in other invertebrate organisms, suggesting it may be a common trait of eukaryotic genomes (Cokus et al., 2008). However, the function of body methylation still remains not well understood **(**Law and Jacobsen 2010).

1.3.2.3. Maintenance of methylation in non-CG contexts

CHG methylation is thought to be maintained through a mechanism involving histone and DNA methylation (Johnson et al., 2007) (figure 1.16 b). Genome wide analyses showed that histone H3K9 dimethylation and DNA methylation are highly correlated (Bernatavichute et al., 2008). CMT3 is the DNA methyltransferase largely responsible for maintaining CHG methylation (Lindroth et al., 2001; Bartee et al., 2001) and is functionally related to SU(VAR)3–9 HOMOLOG 4 (SUVH4)/ KRYPTONITE (KYP) the histone methyltransferase involved in H3K9 dimethylation (Jackson et al., 2002). A loss of these two components results in a dramatic decrease in DNA methylation (Jackson et al., 2002). Two other H3K9 histone methyltransferases, SUVH5 and SUVH6, also contribute to global levels of CHG methylation (Ebbs et al., 2005). The observed interdependence of DNA and histone modifications could arise from the multidomain structure of CMT3 and KYP. KYP has also an SRA domain that specifically binds CHG methylation (Johnson et al., 2007), suggesting that CHG methylation recruits KYP. Furthermore, CMT3 possesses a chromodomain that binds methylated histone H3 tails (Lindroth et al., 2004) indicating that histone methylation by KYP maybe need to recruit CMT3. Such interplay between DNA and histone methylation is also observed in mammals and, in many cases, the connection between these modifications appears to involve protein-protein interactions between the histone and DNA methyltransferases themselves. Whereas direct protein interactions between CMT3 and KYP occur and help to maintain CHG methylation, but in plants this is to be verified.

Methylation in the CHH asymmetric context is maintained by DRM2 and RNA-directed DNA methylation (RdDM), which is involved in *de novo* methylation. However, at some loci this kind of methylation is provided by CMT3 and DRM2 (Cao et al., 2003). RdDM also requires proteins with SRA domains, as CG and CHG methylation. SUVH9 and SUVH2 own SRA domains that preferentially bind CHH and CG methylation, respectively, and these proteins are thought to act late in the RdDM pathway (figure 1.17), possibly functioning to recruit or retain DRM2 at loci targeted for methylation (Johnson et al., 2008).

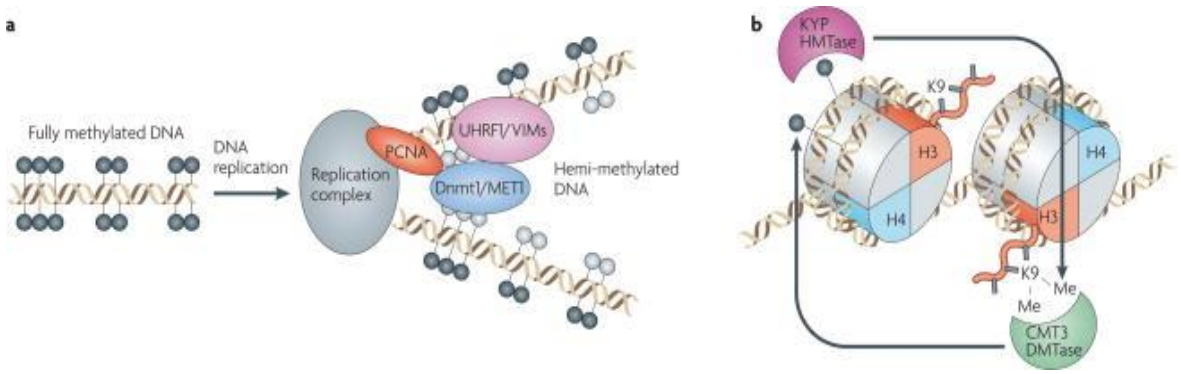


Figure 1.16. a) Model of maintenance of CG methylation during replication. In plants, MET1, and the VIM family of SRA domain proteins, homologs of Dnmt1 and UHRF1, respectively. MET1 is proposed to be recruited to replication machinery through interactions with VIM, an SRA domain protein that specifically interacts with hemi-methylated DNA, and with PCNA. Black and Gray circles represent methylated and unmethylated cytosines respectively. **b) Mechanism of CHG methylation maintenance.** The CMT3 DNA methyltransferase maintains methylation in the CHG context, which is recognized by the SRA domain of the KYP/SUVH4 histone methyltransferase. KYP catalyzes H3K9 dimethylation (H3K9me2), a modification that is required for the maintenance of CHG methylation, and the chromodomain of CMT3 binds methylated histone 3 (H3) tails **(**Law and Jacobsen, 2010).

1.3.2.4. De novo methylation in plants

De novo methylation in plants occurs through a phenomenon initially observed by Wassenegger (Wassenegger et al., 1994) termed RNA-directed DNA methylation (RdDM) (Matzke et al., 2009). This kind of mechanism, in addition to the canonical RNA interference (RNAi) machinery (that is, members of the Dicer and Argonaute families) and DRM2, also requires two plant specific RNA polymerases, Pol IV and Pol V, with important and various functions (Huettel et al., 2007), two putative chromatin remodeling factors, and several other recently identified proteins (Matzke M. et al., 2009). Biogenesis of the 24 nt small interfering RNAs (siRNAs) required to target DNA methylation depends on Pol IV, RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and DICER-LIKE 3 (DCL3). Other RdDM components including DRM2, ARGONAUTE 4 (AGO4), and Pol V are needed for siRNA accumulation for a subset of loci (figure 1.17). However, these proteins do not appear to be involved in the initial production of siRNAs and are proposed to reinforce siRNA biogenesis by an unknown mechanism (Henderson and Jacobsen, 2007) Additional subunits or interacting partners of Pol IV and Pol

V have recently been identified (Lahmy et al., 2009; Ream et al., 2009). While some subunits are shared with Pol II, others are unique to Pol IV, Pol V, or both (Ream et al., 2009). Pol IV is hypothesized to initiate siRNA biogenesis by producing long single-stranded RNA transcripts. These transcripts are then thought to be acted upon by RDR2, generating double-stranded RNAs that are processed into 24nt siRNAs by DCL3 and loaded into AGO4 (Matzke et al., 2009). AGO4 interacts with the Pol V subunit, NUCLEAR RNA POLYMERASE E1 (NRPE1) (El-Shami et al., 2007) and this interaction is required for RdDM, leading to the hypothesis that this complex functions as a downstream effector of DNA methylation. *In vivo*, AGO4 co-localizes either with Cajal bodies or with NRPE1, NRPE2 and DRM2 at a separate discrete nuclear body termed the AGO4/NRPDE1 (previously termed NRPD1b) (AB) body (Li et al., 2008). The AB body is located adjacent to *45S* ribosomal DNA and may be a site of active RdDM (Li et al., 2008; Law and Jacobsen, 2010).

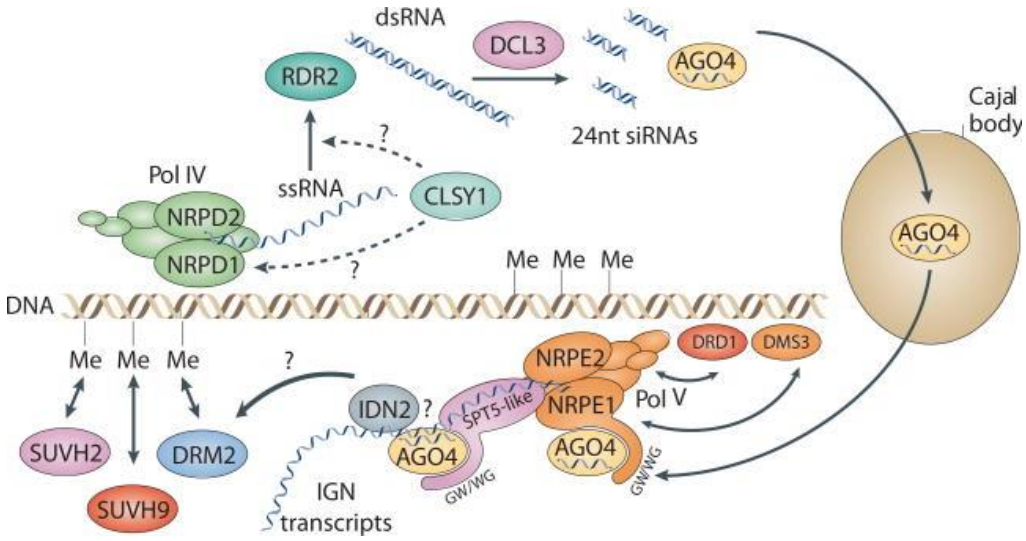


Figure 1.17. Model for RNA directed DNA methylation (RdDM) (Law and Jacobsen, 2010).

1.3.3. Histone modifications

Histone modifications represent one of the most important levels in epigenetic regulation. Chromatin is composed of nucleosomal subunits which consist of about 146 base pair (bp) of

DNA wrapped around an octamer of core histones. The histone octamer contains two copies of each histone 2A (H2A), histone 2B (H2B), histone 3 (H3), and histone 4 (H4) proteins (Marks et al., 2001). Each core histone has an N-terminal tail which protrudes from the nucleosome and is rich in lysine (Marks et al., 2001). Amino acids on H3 and H4 tails are easier to modify chemically than other histone amino acids (Chen et al., 2010b). Various modifications, such as acetylation, methylation, phosphorylation, ubiquitination, biotinylation, and ribosylation, requiring different enzymes, modify the nucleosome distribution and change chromatin structure allowing activators or inhibitors to bind to DNA sequences (Chen et al., 2010b). Histone modification enzymes, such as histone methyltransferase (HMT), histone de-methylase (HDM), histone acetyltransferase (HAT), and histone de-acetylase (HDAC) are fundamental factors of chromatin remodeling (Kim et al., 2010).

Histone tail modification is a key control point of chromatin structure and gene regulation (Zhang et al., 2007; Zhu et al., 2008; Chen et al., 2010b). In general, acetylation, phosphorylation, and ubiquitination of histone tails are associated with active transcription, while de-acetylation and biotinylation result in gene repression (Chen et al., 2010b). In total, 28 histone modification sites have been recognized in *Arabidopsis*; some of them are conserved in mammalian and yeast cells, while some are unique to plants (Zhang et al., 2007; Kim et al., 2008). Each histone modification according to its position can be used to determine the status of target genes. For example, acetylation of H3 Lys9 (H3K9ac) and mono/di/tri-methylation of H3 Lys4 (H3K4me3/2/1) are often positive marks associated with transcriptionally active genes. On the other hand, H3K9 de-acetylation, H3K9me2/3, H3K27me2/me3 are generally negative marks associated with transcriptionally repressed genes (Zhou, 2010).

Furthermore, the euchromatin state is correlated with hyper-acetylation of H3 and H4 along with methylation of H3K4, whereas the heterochromatin structure is dependent on hypo-acetylation of H3 and H4, de-methylation of H3K4, and methylation of H3K9 residues (Bender, 2004). It is also suggested that the deposition of two independent H3 variants differing in sequence and post-translational modifications, particularly in the enrichment of

methylated K9 and K27, result in different methylation levels of H3 and transcriptional activity of their corresponding genes (Zilberman et al., 2007). Studies of different histone modifications on a global scale have also revealed that, in plants, one histone modification can interact with another histone modification or DNA methylation and these diverse modifications function together in gene regulation (Zhang et al., 2009).

Roudier and coworkers showed that the distribution of the twelve marks (H3K4me2 and 3, H3K27me1 and 2, H3K36me3, H3K56ac, H4K20me1, H2Bub, H3K9me2, H3K9me3, H3K27me3 and DNA methylation) along the genomic sequence defines four main chromatin states, which preferentially index active genes, repressed genes, silent repeat elements and intergenic regions (Roudier et al., 2011). The four prevalent associations of marks are H3K27me1 + 5mC + H3K9me2 + H4K20me1 + H3K27me2, H3K56Ac + H2Bub + H3K4me3 + H3K4me2 + H3K9me3 + H3K36me3, H3K27me3 + H3K27me2 + H3K4me2 and H3K27me3 + H3K27me2. Indeed the first combination is almost exclusively associated with TE sequences, the other three are mainly present over genes (Roudier et al., 2011).

1.3.3.1 Histone modifications in responses to abiotic stress

Histone modifications play a critical role in both plant development and plant responses to stress (Chen et al., 2010a). There are many reports on dynamic alterations of histone tail modifications in response to abiotic stresses in plants. For example, exposure of tobacco and *Arabidopsis* cells to salinity, cold and Abscisic Acid (ABA) treatment resulted in a rapid and transient increase in the global enrichment level of H3 Ser10 phosphorylation (H3S10ph), H3 Ser10 phospho-acetylation (H3S10ph-ac), and H4 Lys14 acetylation (H4K14ac). The dynamic changes in these histone modifications were followed by the up-regulation of stress specific genes (Sokol et al., 2007). Using chromatin immune precipitation (ChIP) assays, Kim et al (2008) showed an increase in the enrichment of H3K4me3 and H3K9ac marks on the coding regions of *Arabidopsis* drought stress responsive genes (*RD*29A, *RD*29B, *RD*20, and *RAP*2.4), as well as an increase in the enrichment level of H3K23ac and H3K27ac on the promoters and coding regions of *RD29B* and *RAP*2.4 during dehydration stress. These changes were associated with activation of the genes (Kim et al., 2008). Exposure to UV-B triggered an

increase in the enrichment of H3K9/14ac on the promoter of *ELIP*1 in *Arabidopsis* and wheat (Cloix and Jenkins, 2008). Chen et al. (2010a) also showed that gene expression induced by ABA and salt stress is associated with the induction of gene activation marks, such as H3K9/14ac and H3K4me3, and the reduction of gene repression marks, such as H3K9me2, at ABA and abiotic stress-responsive genes. They also showed that HDA6, a histone de-acetylase, is involved in ABA and abiotic stress responses, and in ABA- and salt stress-induced gene expression in *Arabidopsis*. Further, they suggested that histone acetylation and methylation may mutually interact and affect each other in order to regulate gene activity in response to stress conditions (Chen et al., 2010a). Taken together, these studies prove that histone acetylation/de-acetylation and methylation/de-methylation play important roles in gene regulation, plant stress response, and tolerance to abiotic stresses (Cloix and Jenkins, 2008; Kim et al., 2008; Chen et al., 2010a).

1.3.4. The "priming effect"

As mentioned above, plants are constantly exposed to stressful situations. "The priming effect" has been defined as an induced state whereby a plant reacts more rapidly and more efficiently to a stress (Conrath et al., 2002). Priming events can occur as a result of interindividual or interspecies communication, such as induced resistance mediated by rhizobacteria, mycorrhizal fungi, or virulent or avirulent pathogens or by natural compounds (Conrath et al., 2006; Balmer et al., 2015). Plants 'remember' such events and, depending on the type of primary stimulus (initial trigger for priming) and the system involved (target of priming), primed plants can set up various groups of defense mechanisms. Recently, it has become apparent that the specific defense responses also depend strongly on the priming state and priming has been divided into three phases: a '*priming phase*', a '*post challenge primed state'*, and a '*transgenerational primed state*' (Gamir et al., 2014).

In nature, a plant's capacity for priming is a crucial survival parameter, especially when it is confronted by changing environmental conditions. The availability of rapidly growing transcriptomic, proteomic, and metabolomic datasets allows such plants to have an increased capacity to cope with stress and therefore ultimately lead to a lower input of

protective/curative chemicals into the environment. These traits are very important for crop plants (Balmer et al., 2015).

From the perspective of our work, '*transgenerational primed state*' appears very interesting. Although events in this state remain largely unknown, some recent studies indicate an epigenetic basis regulating this state of defense priming (Balmer et al., 2015). Jaskiewicz described long-lasting priming following acibenzolar S-methyl treatment or bacterial infection that was regulated by chromatin remodeling and histone modifications (Jaskiewicz et al., 2011). In addition, Slaughter (Slaughter et al., 2012) described the reactions of *Arabidopsis* plants that had been either primed with b-aminobutyric acid (BABA) or with an avirulent isolate of the bacteria *Pseudomonas syringae pv tomato* (PstavrRpt2). The descendants of primed plants showed up a faster and higher accumulation of transcripts of defense-related genes in the salicylic acid signaling pathway and enhanced disease resistance upon challenge inoculation with a virulent isolate of *P. syringae*. In addition, the progeny of primed plants was also more resistant against the pathogen *Hyaloperonospora arabidopsidis*. Furthermore Sani and coworkers demonstrated that primed plants, in hyperosmotic priming, display reduced salt uptake and enhanced drought tolerance after a second stress exposure, depending on alteration in the epigenomic landscape. These kind of changes, due to the priming treatment, were small, but they were specific to the treated tissue, varied in number and direction depending on the modification, and preferentially targeted transcription factors (Sani et al., 2013).

1.3.5. Epigenetic memory for stress response

Regarding the "priming state", recently it has been shown that plants can remember past environmental events and can use these memories to aid responses when these events recur. As environmental perturbations may occur repeatedly, it is advantageous to plants to be able to remember past incidents and to use this stored knowledge to adapt to new challenges (Kinoshita and Seki, 2014).

Drought stress is one of the most diffused type of stress all over the world. The importance of available water has been demonstrated in *Arabidopsis*, in which drought signals are

transduced into effects on gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). Considering that changes in gene expression patterns are generally accompanied by changes in the chromatin state (Campos and Reinberg, 2009), the modification of histone tails in response to dehydration has been analyzed in the chromatin surrounding drought stress-responsive genes (Kim et al., 2008). Furthermore, Ding et al. (2012) reported that multiple exposures to drought stress conditions enable plants to respond to a new stress by more rapid adaptive changes to gene expression patterns compared with plants not previously exposed to a drought stress (figure 1.18). Nevertheless, to date no correlation has been identified between gene expression patterns and modifications in DNA methylation levels in *Arabidopsis*, suggesting the lack of a stress memory (Colaneri and Jones, 2013).

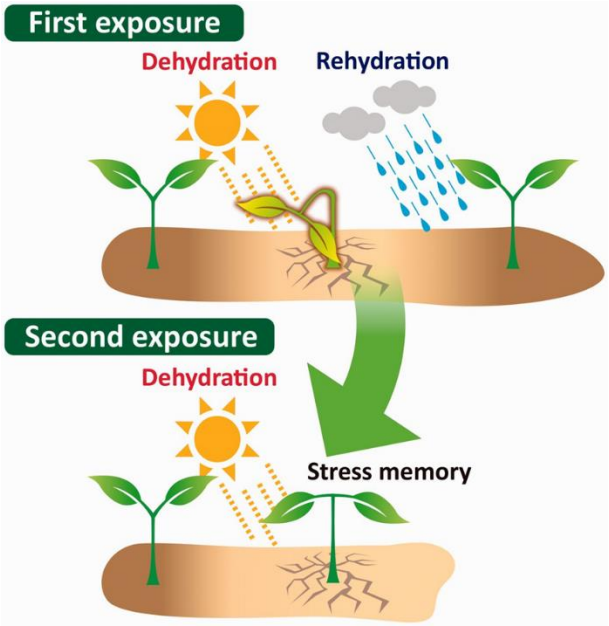


Figure 1.18. An example of stress memory in plants. For example, a plant that experiences a period of drought wilts under the drought stress and then recovers after rehydration; during a second drought stress, the plant 'remembers' the past stress experience, allowing it to raise better resistance to dehydration and improve its survival prospects (Ding et al., 2012).

On the other hand, low humidity conditions provide a stress to plants. In fact, water loss through the stomata is a crucial aspect of the processes for obtaining and absorbing

nutrients from the soil, and for respiration and photosynthesis (Kinoshita and Seki, 2014). Under low humidity conditions, wild-type plants and plants with the chromomethyltransferase 3 (*CMT3*) mutation show reduced dry weight; in contrast, plants with double mutation of domain rearranged methyltransferase 1 and 2 (*DRM1/2*) or with the methyltransferase1 (*MET1*) mutation are resistant to the effects of low humidity. Hence, in this case modification in methylation levels are involved as well as an interesting transgenerational effect of low humidity stress has been identified for the stomatal index phenotype in *Arabidopsis*, where the ratio of stomata to other epidermal cells is developmentally controlled by the levels of relative humidity (Tricker et al, 2012).

Moreover, the mechanisms of stress response to elevated temperature levels have gained much attention (Hedhly et al., 2009). Recently, transposition of the retrotransposon ONSEN in *Arabidopsis* has been reported to involve an epigenetic mechanism (Ito et al., 2011; Matsunaga et al., 2012). Experimental evidences have demonstrated that transposition of ONSEN occurs more frequently in the progeny of the RdDM machinery mutants that have been exposed to heat stress, confirming that the RdDM machinery has a role in the prevention of transgenerational propagation of retrotransposons in this organism (Ito et al., 2011). Another important stress still poorly considered for plants in literature is nutritional stress, in particular deficiency of nutrients.

Players often involved in these mechanisms are accumulated levels of key signaling metabolites, plant hormones and proteins involved in their synthesis, or transcription factors and the kinases/phosphatases regulating their activity, that have been considered potential 'memory factors' (Bruce 2014; Conrath, 2011; Santos et al., 2011; Kinoshita and Seki, 2014; Vriet et al., 2015; Avramova, 2015). For example the transcription factor *MYC2* was identified as the critical component that determines the memory behavior of a specific subset of *MYC2*-dependent genes (Liu et al., 2014). On the other hand, the capability of chromatin to be subjected to both dynamic and stable changes in its structure in response to stress stimuli has been recognized as a mechanism for stress memory propagation (Van Oosten et al., 2014). Recent studies propose that proteins involved in signaling pathways, like mitogen-activated protein kinases, may transfer signals to chromatin/nucleosome structure through

chromatin-modifying enzymes (Avramova, 2015). Consequently, chromatin may act as memory 'storage' where 'signal transduction pathways converge upon sequence-specific DNA binding factors to reprogram gene expression' (Badeaux and Shi, 2013; Johnson and Dent, 2013; Avramova, 2015). For example trithotax group (TrxG) methyltransferases as ATX1, SDG8, ASHH2 and ASHR are responsible for methylation of H3K4me3, involved in both developmental and biotic/abiotic stress responses (Ding et al., 2009; Ding et al., 2011; Berr et al., 2010; Wang et al., 2014) and less known in stress memory responses. As reported above, ATX1 has been implicated in the memory responses of dehydration stress-response genes (Ding et al., 2012). In addition, the Polycomb group methyltransferases, involved in methylation of H3K27me3, like the CURLY LEAF (CLF) also functions in a gene-specific manner in the dehydration stress responding pathway (Liu et al., 2014). This particular group of proteins is also involved in the phenomenon of vernalization (Angel et al., 2011), a growth and development stage modulated by environmental signals. In *Arabidopsis*, involves downregulation and epigenetic silencing of the gene encoding the floral repressor FLOWERING LOCUS C (FLC), which encodes a MADS domain protein that acts as a repressor of flowering (Michaels and Amasino, 1999). This epigenetic silencing is quantitative and increases with the duration of exposure to cold (Song et al., 2012; Sheldon et al., 2000). This mechanism localized nucleation of silencing during periods of cold (Kilian et al., 2007; Swiezewski et al., 2009), and spreading of the silencing complex over the whole gene after the exposure to cold (Angel et al., 2011; De Lucia et al., 2008; Finnegan and Dennis, 2007).

Furthermore, recent studies have observed into the roles of H3K4me3, H3K36me3, histone H3 acetylation (H3Kac) and ubiquitination of H2B (H2Bub) in the transcriptional process. However, which transcription phases are affected by the silencing modifications H3K27me3, H3K9me3/me2 and methylated cytosines is less clear (Avramova, 2015).

Moreover, active/ inactive transcriptional states induced by both developmental and stress-generated signals have been associated with altered nucleosome occupancies and H2A.Z histone variant patterns (Berr et al., 2010; Han et al., 2012).

It is very important to underline the importance of distinguishing between environmental adaptation (considered stable and heritable) and acclimation (considered plastic and

reversible) (Avramova, 2015). Mitotic/meiotic inheritance of stress-acquired traits is related to short-/long-term memory responses and, consequently, to the plant's acclimation/adaptation ability (Avramova, 2015).

Between many stress triggered epigenetic modifications, the most intensely studied is the trans-generational propagation of changed DNA methylation patterns, often associated with reactivation of transcriptionally silent loci (Boyko et al., 2010; Boyko and Kovalchuk, 2011; Bilichak et al., 2012; Saze et al., 2012; Migicovsky et al., 2014). Since changes in DNA methylation, occurring sporadically or triggered by environmental stresses, may be inherited by successive generations, they are a potential factor in adaptive and evolutionary mechanisms in plants (Avramova, 2015). Additionally, it is also important that mechanisms for epigenetic reprogramming, involving chromatin remodeling factors and small non-coding RNAs, function during gametogenesis and in early embryo development to prevent and reduce the transmission of acquired chromatin states (Hsieh et al., 2009; Mosher et al., 2009; Slotkin et al., 2009; Lang-Mladek et al., 2010; Iwasaki and Paszkowski, 2014), although mitotic and meiotic transmission of histone modifications is less well-understood (Avramova, 2015).

1.3.6 Nutritional stress and transgenerational memory

Although nutritional stress is poorly studied in plants, in literature it has been already reported that heritable epigenetic modifications can be induced by different nutritional stresses in mammals. In spite of these promising observations, the role of epigenetics in "*transgenerational stress memory"* is controversial in plants although stable changes of gene expression induced by stress have been frequently reported as above.

It is known that both maternal and paternal unbalanced diet can have long-lasting effects on the health of the offspring. Studies implicate that macronutrients play an important role in fetal programming, although the importance of micronutrients is also becoming increasingly apparent. Folic acid and vitamins B2, B6 and B12 are essential for one-carbon metabolism and are involved in DNA methylation. They can therefore influence the programming of the offspring's epigenome. Also, other micronutrients such as vitamins A and C, iron, chromium,

zinc and flavonoids play a role in fetal programming (Vanhees et al., 2013). Both animal and human studies have shown that low caloric diet of the mother during pregnancy are linked to an increased risk for adult offspring developing type 2 diabetes (Dumortier et al., 2007). Several studies on fetal programming showed that fetal nutritional deprivation (maternal caloric or macronutrient deficiency during pregnancy) is a strong programming stimulus (McMillen et al., 2008; Vanhees et al., 2013). The father's diet may also play a role in fetal programming. Male rats exposed to a high-fat diet, which bred with females on a control diet, produced female offspring with an early onset of impaired insulin secretion and glucose tolerance, as well as altered gene expression in pancreatic islets, increasing their risk for diabetes later in life (Ng et al., 2010). Methyl-donors (betaine, folate, choline), methionine, and related cofactors (vitamins B12 and B6) were described to induce epigenetic modifications at different exposure times and ages. Mice fed a folate-deficient diet for 32 weeks exhibited global DNA hypomethylation (Linhart et al., 2009). Choline and betaine were reported to alter both global and gene specific DNA methylation, with possible roles in fetal brain development (Mehedint et al., 2010; Niculescu, 2012). Although it has been suggested that some epigenetic variations can be conserved over generations, most studies, both in humans and animals, address the effect of in utero nutrition on fetal programming in first and second generation offspring. However, to examine true transgenerational inheritance in response to diet, the third generation, being the first 'unexposed' generation, should be studied. The first filial generation is directly exposed to the maternal diet, while the second generation results from gametes that were exposed in utero (Vanhees et al., 2013).

1.4 Aim of work

Nitrogen is the most prominent mineral nutrient required by plants and nitrate, being a primary source of it, is a prominent signal eliciting the rapid gene expression of transporters and metabolism enzymes as well as changes in root morphology and physiology. As a result, nitrogen deficiency represents one of the most severe nutrient-related stresses challenging plant growth.

The preliminary goal of this project was the characterization of the morphological, physiological and molecular responses to nitrate availability using an experimental setup based on the model plant *Arabidopsis thaliana*. Building on these observations, we also set out to evaluate the potential establishment of a "transgenerational memory" in response to an extended nitrogen starvation in *Arabidopsis* plants in three successive generations. This potential "memory" was studied both at physiological and transcriptional level, using a specific assay for the measurement of nitrogen uptake in roots and next generation sequencing (NGS) technologies, respectively. Furthermore, we investigated if changes in nitrogen efficiency, in particular nitrate acquisition, could be paralleled by the accumulation of epigenetic changes at the methylation level and if these modifications were prone to be transmitted to the progeny of N-stressed plants.

By this approach we aimed at characterizing priming effects and a potential transgenerational response to nitrogen deprivation that could be revealed by reinforced resistance to stress, enhanced uptake capabilities, morphological adaptation, gene pathway dysregulation and methylation changes in different contexts.

The overall scenario obtained with these data aimed at providing a comprehensive view of the plastic capability of plants to adapt to different disadvantageous conditions and lay the foundation for new lines of research to improve breeding.

2. MATERIALS AND METHODS

2.1 Plants growth

Arabidopsis thaliana (ecotype Columbia) seeds were surface sterilized by immersing them in 95% ethanol for 1 minute and in a solution with 25% NaClO and 0.1% Triton X -100 for 7 minutes, followed by seven rinses in sterile water. Afterwards they were germinated on agar medium (0.7% Phyto agar, Duchefa Biochemie, containing nutrients: 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.125 mM K_2SO_4, 0.125 mM $CaCl_2$, 0.5 mM NH_4NO_3, 5 µM H_3BO_3, 0.25 µM $MnSO_4$, 0.25 µM $ZnSO_4$, 0.1 µM $CuSO_4$, 0.005 µM Na_2MoO_4, pH adjusted to 5.8 with 1 M KOH) and placed in a growth chamber under controlled climatic conditions (day/night photoperiod, 8/16 h; light intensity, 220 µmol m−2 s−1; temperature day/night, 25/20°C; relative humidity, 70 to 80%).

After 15 days, the seedlings were transferred in hydroponic conditions for 5 weeks in an aerated nutrient solution containing: 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.5 mM NH_4NO_3, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 $CuSO_4$, 0.01 µM Na_2MoO_4 (pH adjusted to 5.8 with 1 M KOH).

After 5 weeks, half of plants were subjected to a total nitrogen deprivation for seven days (T0), while the other plants were maintained under the same nutrient condition as reported above (0.5 mM NH_4NO_3; C0). At the 6th week, after the control or N-deprivation treatment (C0, T0; first generation), physiological measurements and root sampling for molecular analyses were performed.

Some C0 and T0 plants were grown to maturity under long day condition (light day/night photoperiod, 16/8 h). Then, seeds were collected from three biological replicates for each treatment (C0, T0). These batches of second-generation seeds were vernalized for 21 days at 4°C. At this point, six batches (3 independent sets from both C0 and T0 seeds) of seeds were germinated and plants were grown in the same conditions (Control or N-deprivation) as reported above. Hence, four different treatments were obtained in second generation (CC, TC, TT, CT – see figure 2.1; second generation).

After 6 week of cultivation at the end of the N-deprivation treatment, physiological measurements and root sampling for molecular analyses were performed. Another set of plants for each treatment were transferred to a 16-h light period to induce the production of seeds. Seeds were collected, vernalized and germinated as described above. Plants of the third generation were grown for six weeks only in control conditions (CCC, TCC, TTC, CTC; third generation) and roots were sampled in order to perform molecular analyses.

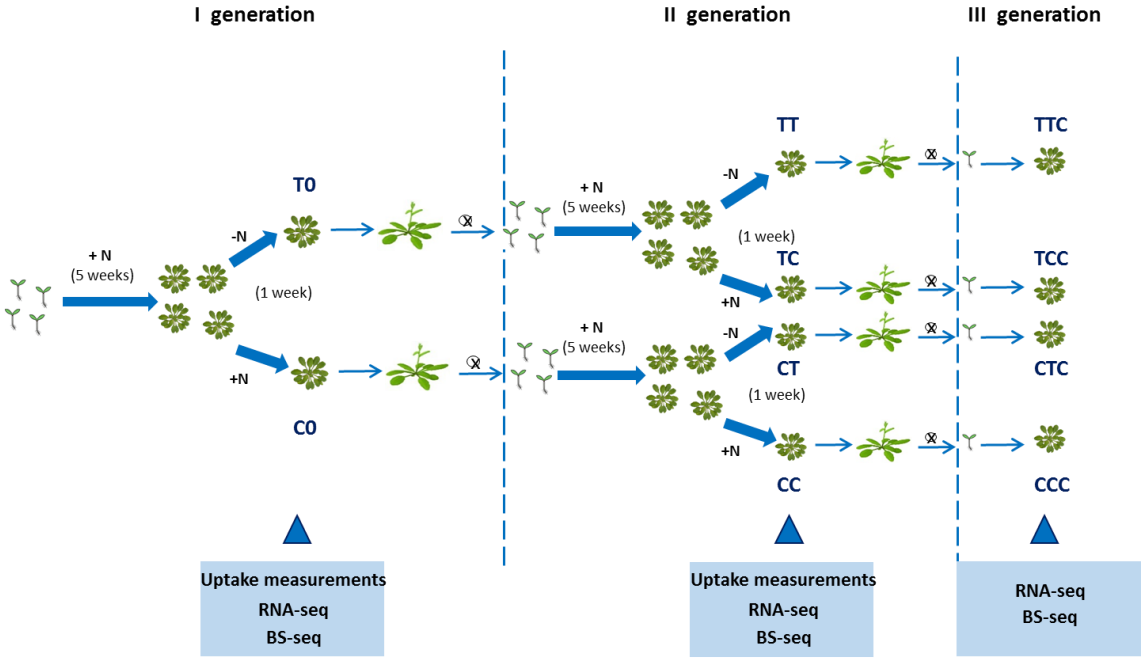


Figure 2.1. Schematic representation of the experimental setup. It consists of three successive generations of *Arabidopsis* plants exposed to two different treatments, *i.e*. absence (T0, TT, CT) or presence (C0, CC, TC) of nitrogen in the last week of growth (sixth). On the first and second generations were performed NO_3^- uptake measurements on roots with a specific assay, RNA-seq to study transcriptional differences and BS-seq to analyze methylation. Finally, in the third generation were performed only BS-seq and RNA-seq analyses, to try to evaluate a possible establishment of a memory. Letter T stands for treatment, which indicates a nitrogen deprivation for 1 week in the sixth week of growth, whereas C stands for control, which indicates a growth in a standard nutrient solution for six weeks.

2.2 Net high-affinity nitrate uptake in *Arabidopsis* plants

The day of experiment, nutrient solutions were renewed and supplied with 0.5 mM $Ca(NO_3)_2$ (induction); as control, no supply of nitrate was done (not-induction). In time course (after 4, 6, 8, 10, 24 h and at the beginning of induction period), roots of two intact seedlings were rinsed in 0.5 mM $CaSO_4$ and then immersed in 12 ml of a constantly agitated solution containing 0.5 mM $CaSO_4$ and 0.1 mM KNO_3. Net uptake was measured as NO_3^- depletion from the solution per unit of time (Cataldo et al., 1975), removing samples (0.05 ml) for NO_3^- determination every 2 min for 10 min, span time during which uptake had a linear trend. Aliquots of 0.05 ml were mixed thoroughly with 0.2 ml of 5% (w/v) salicylic acid in concentrated H_2SO_4. After 20 min incubation at room temperature, 4.750 ml of 2 M NaOH was added. Samples were cooled to room temperature and NO_3^- amounts were determined spectrophotometrically by measuring the absorbance at 410 nm. The uptake rate of nitrate was determined on *Arabidopsis* plants of first and second generation.

2.3 RNA extraction

Total RNA was isolated from roots of *Arabidopsis* plants. A pool of three roots was used for each sample. The RNA extractions were performed using the Invitrap Spin Plants RNA mini kit (Stratec Molecular, Berlin, Germany) as reported in the manufacturer's instructions (http://www.stratec.com/). Seventy mg of maize tissue were homogenized in liquid nitrogen and the powder was mixed with 900 µl of DCT solution and dithiothreitol (DTT). In order to verify the absence of genomic contamination, 1 µg of total RNA was analyzed electrophoretically running on 1% agarose gel. The concentration and integrity of RNA were checked on the Qubit® 2.0 Fluorometer (Life Technologies) and on the Agilent 2100 Bioanalyzer system following manufacturer's protocol (Agilent Technologies, Santa Clara, CA, United States) (figure 2.2).

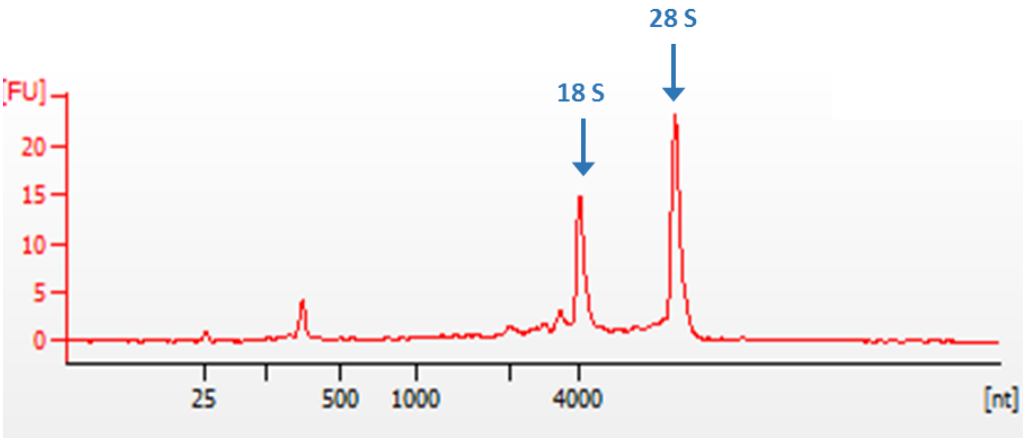


Figure 2.2. Typical output of RNA quality control on Agilent 2100 Bionalyzer. The electropherogram shows two distinct ribosomal peaks corresponding to either 18S and 28S of ribosomal RNA. The baseline until the 18S rRNA peak is relatively flat and free of small rounded peaks corresponding to smaller RNA molecules that are degradation or 5S, 5.8S and tRNAs. The RNA integrity number (RIN) that indicates the quality of RNA is 8.3 for this sample. Only RNA with a RIN greater than 8 were used for RNA-seq analysis.

2.4 RNA-sequencing

2.4.1 Preparations of cDNA libraries

Barcoded cDNA libraries were prepared for multiplex sequencing on the Illumina HiSeq2500 platform using the TruSeq™ Stranded mRNA sample preparation kit according to manufacturer's instructions. Thirty libraries were prepared from each of three biological replicates for each treatment. To obtain sufficient material for sequencing, the libraries were amplified by PCR for 15 cycles following the recommendations of the TruSeq™ Stranded mRNA sample preparation protocol. Final elution of each library was in 30 µl of total volume. Library concentrations and quality were assessed using the Qubit 2.0 Fluorometer (Life Technologies) and the Caliper - LabChip GX/GXII (Amv, United Kingdom) (figure 2.3). Sequencing was performed on the HiSeq2500 instrument, at the Applied Genomic Institute (IGA) at Udine, Italy.

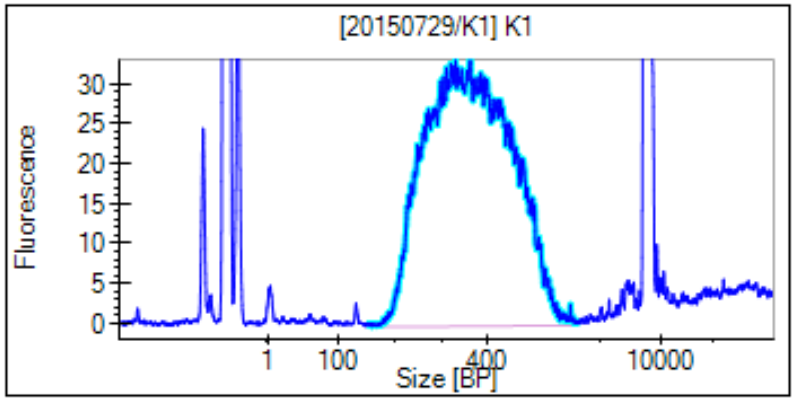


Figure 2.3. Example output of Caliper-LabChip GX/GXII for a cDNA library prepared with TruSeq™ Stranded mRNA sample preparation kit (Illumina).

2.4.2 Sequence Processing

Sequence data in FastQ format were checked for their quality using FastQC software (version 0.11.3, www.bioinformatics.babraham.ac.uk/projects/fastqc/); all samples had high overall quality. Sequences were aligned onto the latest *Arabidopsis* Col-0 genome assembly (released November 2010) using TopHat (version 2.0.6 www.ccb.jhu.edu/software/tophat/index.shtml, Trapnell et al., 2012) and BowTie (version 2.0.2 www.bowtie-bio.sourceforge.net/index.shtml) with the following parameters: -p8 -G TAIR10 annotation.gff --library-type fr-firststrand -o. The accepted_hits.bam file produced by TopHat was used in subsequent analysis steps. This binary alignment file (Li et al., 2009) contained both spliced and unspliced read alignments. SAMtools version 0.1.19 (Li et al., 2009) and the "NH" (number of hits) Sequence Alignment Map flag were used to separate alignments according to the number of times a read mapped onto the reference genome. Reads mapping to a unique location in the genome were used for differential expression. Differential expression analysis of RNA-seq read alignment counts was performed using Cuffdiff, with Cufflinks version 2.2.0.

2.4.3 MapMan Display

The data were visualized and figures produced using the MapMan software (Thimm et al., 2004). A downloadable version for local application and a servlet version are available at http://mapman.gabipd.org/web/guest/mapman-download, as well the mapping file of *Arabidopsis* transcriptome (mapping release AGI_TAIR9_Jan2010) and a selection of schematic maps of metabolism and cellular processes. The overview and metabolism figures in this work are prepared using version 3.6.0RC1.

2.5 Real Time PCR

Total RNA (0.5 µg) was retrotranscribed in cDNA using 100 pmol of Oligo-d(T)23 (Sigma Aldrich, Milano, Italy), 20 U Prime RNase Inhibitor (Eppendorf, Hamburg, Germany), 200 U of RNase H derivative of moloney murine leukemia virus (EuroClone, Pero, Italy), according to the manufacturer's protocol, keeping 1 h at 42°C. 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 real-time RT-PCR complete mix, FluoCycle™ sybr green (20 µl final volume; Euroclone, Pero, Italy), in a DNA Engine Opticon Real Time PCR Detection (Biorad, Hercules, USA). Specific primers (58°C melting temperature, Tm) were designed to generate PCR products (range 100-120 bp) with Primer3 software version 0.4.0 (Howard Hughes Medical Institute, the National Institutes of Health and National Human Genome Research Institute, www.bioinfo.ut.ee/primer3-0.4.0/) and they were synthesized by Sigma Aldrich (Milano, Italy, Table 2.1). The analyses of real-time result were performed using Opticon Monitor 2 software (Biorad) and R (version 2.7.2; http://www.r-project.org/) with the qPCR package (version 1.1-8). Data were normalized with respect to the transcript level of the average of three housekeeping (HK) genes: *Actin2,* forward primer GCCATCCAAGCTGTTCTCTC and reverse primer ACCCTCGTAGATTGGCACAG (80.5% efficiency, NM_112764); *Apt1,* forward primer GACAGTGAAATGGCGACTGA and reverse primer AAAGGCCTCAGTGTCGAGAA (80.8% efficiency, NM_102509.3); *CBP20,* forward primer TACTGGCTCATTGGGAGCTT and reverse primer CTCTTCCATGGCGATTTTGT (81.15% efficiency,

NM_123787.3)**.** The expression data were analyzed using $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = (C_{T,Target} - C_{T,HK})_{Time\ x} - (C_{T,Target} - C_{T,HK})_{Time\ 0}$ (Livak and Schmittgen, 2001).

Table 2.1. List of specific primers used to perform Real Time-PCR analyses.

GENE	AGI CODE	TRANSCRIPT CODE	Primer FOR (5'- -3')	Primer REV (5'- -3')	EFFICIENCY
NRT2.1	AT1G08090	NM_100684	GGAGTTGCCTCTGTCTCTGG	CACGAAGCTCATGGAGAACA	76.9%
NRT2.2	AT1G08100	NM_100685	AGTACAATCTGCCGGTGGAC	CGTCGAAACAAAACATGTGG	68.7%
NRT2.4	AT5G60770	NM_125470	GTTGGGCCTCAGATATTGCG	CATAGGCAGAAGAAACCGCC	76.5%
NRT2.5	AT1G12940	NM_101165	AGCAGGGATTATAGCAGCGA	CCTCTCATCCCAAACCGTCT	63.2%
NRT1.1	AT1G12110	NM_101083	GCCGTACTTGTTGCCTTGAA	TCCAACTCAATCCCCACCTC	84.2%
NRT1.3	AT3G21670	NM_113062	CGAAGAGAAGGATCCGTGGA	TTCGTTGCCCAAATCGGTAC	74.0%
AHA4	AT3G47950	NM_114664	TTTCCGAAAGCTTGCCTCAG	ACGTTCCACAAAAGACCAGC	80.9%
AHA7	AT3G60330	NM_115897	CACAGCACGGTTTAGAGACG	TCTGCACGCCTTTTAGCTTC	77.3%
NIA1	AT1G77760	NM_106425	GTGGTCGGATGGTTAAATGG	CAGCTCAGCATCAACGAGAG	76.1%
NIR1	AT2G15620	NM_127123	GGCTTAGTTGCTTGTACCGG	GCATCCTTATCGGTCTTGGC	76.5%
GLN1;4	AT5G16570	NM_121663	CTTATGGGGTGTGGCAAACC	CATGTTCGAAGCTGGCCTAC	74.4%
NADH-GOGAT(GLT1)	AT5G53460	NM_124725	AAAATTTGCTGGGGAGCCTG	CGCACGACCAATCATCTCAG	85.5%
	AT5G38200	NM_123181	ACTTGAAGTTGGAGCCGAGT	ATGATCCTCCGTTCCTCACC	78.9%
ADC2	AT4G34710	NM_119637	TCGTTGCTCTCTGATGGTGT	GTCAATCCCCAAACCACCAC	85.5%
PAL3	AT5G04230	NM_120505	AGTGGAACGAGTCAGGACAG	GTCTCGAGTTCATTCCGCAC	89.5%
TIP2;3	AT5G47450	NM_124117	TGCGGCTAACATTTCTGGTG	GCCAAGACACTGAGCAATCC	90.0%
	AT3G09340	NM_111768	TGATGGAGTCGGGTTTCACA	AAACTGCGTGACCACTGAAC	80.0%
MLS	AT5G03860	NM_120467	GCATTCACTGGTCACATGGG	CTCCTCGCGGTATTTGAAGC	57.5%
ALMT1	AT1G08430	NM_100716	GTTCGCGCTGATATCGTTGT	AGGATGCAACTGACTCCTCC	78.5%
NAS1	AT5G04950	NM_120577	CCAAGTTTCACCTCCCCAAC	AGAGACGAGGTTTGAAGCGA	85.4%
SEL1	AT1G78000	NM_106449	ATATCTCCGCCTTGCCTTCA	GCGTGGGAAAGGAAATCGAT	76.8%

2.6 Statistical analyses

Statistical significance was determined by one-way analysis of variances (ANOVA) using Holm-Sidak method for net high-affinity nitrate uptake essays and Student-Newman-Keuls method for Real-Time PCR analyses, taking $P < 0.05$ as significant. Statistical analyses were performed using SigmaPlot Version 12.0 software.

2.7 DNA extraction

Total DNA was isolated from the same pool of *Arabidopsis* roots used from isolation of total RNA. The DNA extractions was performed using hexadecyl trimethyl-ammonium bromide (CTAB, Murray and Thompson, 1980) from 50-70 mg of grinded roots with the addition of 700 µl of 2X CTAB. Samples were incubated at 68°C for 30 min with frequent shaking. After a centrifuge at 13000 rpm for 5 minutes at room temperature, the supernatant was transferred into new tube, adding 3 µl of RNAse (10mg/ml). Then, it was incubated at 37°C for 30 min. One volume of chloroform: isoamyl alcohol (24:1) was added and it was vortexed and centrifuged at 13000 rpm for 5 minutes. Afterwards, aqueous supernatant was transferred into new tube. Ice-cold absolute ethanol (2.5 volumes) was added and DNA was precipitated at -20°C for 14 hours. Thereafter samples were centrifuged at 13000 rpm for 20-30 min at 4°C and the pellet was washed with 80% ethanol. Finally pellet was dried and resuspended in 50 µl of H_2O. The concentration of DNA was checked with Qubit 2.0 Fluorometer (Life Technologies).

2X CTAB buffer

100 mM	Tris HCl (pH = 8.0)
1.4 M	NaCl
20mM	EDTA (pH = 8.0)
2% (w/v)	CTAB

2.8 Bisulfite-sequencing

2.8.1 Preparation of Bisulfite libraries

Bisulfite libraries were prepared for multiplex sequencing on the Illumina HiSeq2500 platform following bisulfite conversion (figure 2.4) with the EZ DNA methylation-Gold ™ Kit (Zymo Research Corporation 17062, Murphy Ave. Irvine, CA 92614, U.S.A.) according to manufacturer's instructions. To estimate bisulfite conversion efficiency 0.5 ng of λ DNA were used as a spike-in control for each sample. Eight libraries were constructed following the TruSeq DNA Methylation sample preparation kit (Illumina, San Diego, CA 92122 USA). In order to obtain sufficient material for sequencing, the libraries were amplified by PCR for 10 cycles following protocol recommendations. Final elution of each library was in 20 µl of total volume. Library concentration and quality were assessed using the Qubit 2.0 Fluorometer (Life Technologies) and the Caliper - LabChip GX/GXII (Amv, United Kingdom). Sequencing was performed at the Applied Genomic Institute (IGA) in Udine, Italy

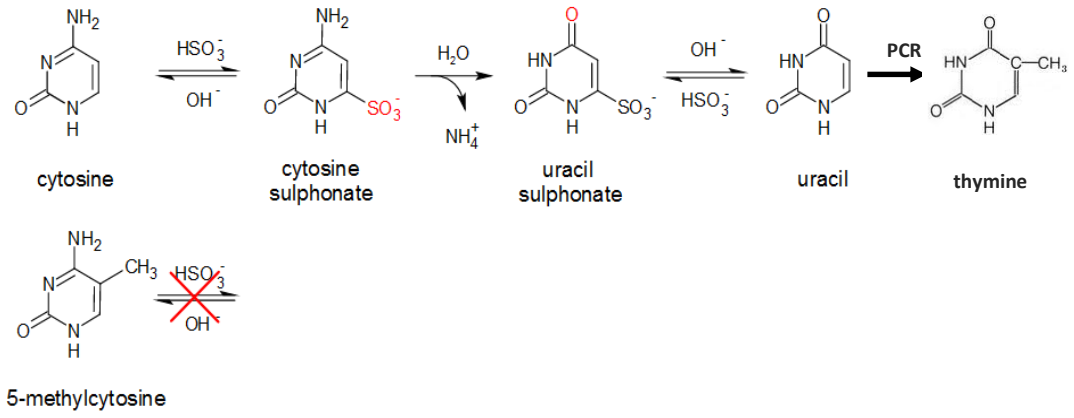


Figure 2.4. Schematic representation of the chemical transformation of cytosine caused by bisulfite. Treatment of DNA with bisulfite converts cytosine residues into uracil, but leaves 5-methylcytosine residues unaffected.

2.8.2 Bisulfite Sequence Processing

Bisulfite sequencing (BS-seq) libraries were sequenced in paired-end mode on Illumina Hiseq 2500 sequencer. Following quality check of raw sequences by the FASTQC software (version

0.11.3, www.bioinformatics.babraham.ac.uk/projects/fastqc/), quality trimming and adapter sequence removal was performed using Trim Galore wrapper script (version 0.4.0, www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with the following settings: --phred33 --illumina –paired --trim1 --clip_R1 20 --clip_R2 6 --three_prime_clip_R1 4 --three_prime_clip_R2 4. Quality of trimmed reads was controlled again after the trimming process using FastQC. Accepted reads were aligned to the *Arabidopsis* reference genome (TAIR10 release) in paired-end mode using Bismark (version 0.14.3, www.bioinformatics.babraham.ac.uk/projects/bismark/) with the following settings: --bowtie2 --phred33 -N 1 -p 2 --non_bs_mm. Read pairs discarded by Bismark in paired-end mode that could be mapped in single-end mode in proper orientation were included in the alignment and reconstituted as pairs. Duplicate reads generated during the PCR amplification were recognized as alignments of reads mapping to the same position in the genome and removed using the deduplicate_bismark_alignment_output.pl script. Finally, DNA methylation calls were extracted from the deduplicated Bismark output using the methylation_extractor script included in the Bismark distribution.

2.8.3 MethylKit analyses

A clustering analysis of the samples, based on the similarity of their methylation, was performed with the MethylKit package of v. 3.2.3 R software (Akalin et al., 2012). This function allows clustering the samples and drawing a dendrogram. With the same package a PCA analysis (*principal component analysis)* was also performed. This statistical procedure uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables. Differentially methylated regions (DMRs) were screened by pairwise comparisons of samples, using Fisher's Exact Test to evaluate methylation differences in the three contexts (CG, CHG and CHH) within 500 bp genomic windows as implemented in the MethylKit package. Custom scripts and a bioinformatic suite (http://bedtools.readthedocs.org/en/latest/) were utilized to intersect DMR coordinates with gene and TE annotation.

2.8.4 Circos

Using the software package *Circos* (www.circos.ca, Krzywinski et al., 2009), the relationships among density of genes, density of transposable elements (TE), and DNA methylation in each sample was performed and visualized with circular layout.

3. RESULTS

Most studies on nitrogen nutrition in higher plants have focused on the physiological and molecular characterization of nitrate uptake systems in response to deprivation and/or supply of the nutrient. However no research has investigated the potential establishment of a "*transgenerational stress memory"* at physiological and transcriptomic level. Indeed, according to "priming state" theory (see § 1.3.4), it has been shown that plants can retain memory of past environmental events and use it to induce responses when these events occur again (Kinoshita and Seki, 2014).

The aim of this thesis is to assess this potential "*transgenerational memory"* at least at physiological and transcriptional stage and consequently try to verify if one of epigenetic modifications (methylation) are involved in this mechanism.

In order to adopt standard controlled conditions of growth and to limit environmental perturbations during the development of the three different generations, *Arabidopsis* plants were grown in hydroponic solutions.

The choice to analyze only root tissue for RNA-seq and BS-seq analyses was driven by the need to assess the presence of this "*memory"* and not to understand the mechanism of propagation of this "*memory"* signal between generations of plants.

3.1 Morphological variations in plants exposed to nitrogen deprivation

3.1.1 Morphological differences in the first generation

Preliminary experiments were conducted in order to verify the growth response of *Arabidopsis* plants in absence or presence of nitrogen: plants were fed with a nutrient solution containing 0.5 mM NH_4NO_3. After 5 weeks half of plants were subjected to a total nitrogen deprivation for 1 more week. At the end of the treatment, marked differences between the two treatments were observed (figure 3.1). In particular, the effect was dependent on the photoperiod and the duration of nitrogen deficiency. After only 4 days of deficiency and with a photoperiod of 8 hours, leaves showed an initial chlorosis (figure 3.1, A–B, b). If the starvation continued until 20 days and photoperiod increased up to 16 h of light, negative effects became more evident. Plants blossomed (figure 3.1, A, c) as well as there was an increase in purple coloration of leaves, probably due to an accumulation of anthocyanin and linked to an excess of carbohydrates that could not be used in the synthesis of amino acids or other compounds or a chlorophyll decomposition in older leaves (figure 3.1, A-B, c). Younger leaves did not show these kind of symptom because of remobilized nitrogen from older leaves (figure 3.1, B, c) (see § 1.1.2).

Especially aerials parts of control plants, during flowering (16 h of light), produced more siliques and leaves and showed an increased number of flowers (figure 3.2, A, a). On the other hand, treated plants showed an increase in purple coloration in leaves, sign of a prolonged deficiency (figure 3.2, A, b).

Roots of plants subjected to a prolonged deficiency appeared to be more developed and longer than those of control plants (figure 3.2, B, a-b).

Figure 3.1. Effects of nitrate deprivation on plants subjected to different light condition and time of deprivation. A) Plants were grown at 8/16 h light/dark and with 0.5 mM NH_4NO_3 (a) or at 8/16 h light/dark and without nitrogen for 4 days (b). Plants were grown at 16/8 h light/dark and grown without nitrogen for 20 days (c). B) Details of single leaves from plants in A (a, b, c).

Figure 3.2. Morphological differences in aerials parts (A) and in roots (B) between two plants of the first generation during flowering time grown in presence (a) or in absence (b) of nitrogen starting from the sixth week of growth.

3.1.2 Morphological variations in the second generation

Results obtained in the first generation were in general confirmed in the second generation (figure 3.2-3.3). Indeed, shoots of stressed plants grew less than control ones. On the other hand, even more in this generation, root system of plants grown without nitrate showed a greater development.

The supply of nitrogen allowed a good development of *Arabidopsis* plants, although variations in root system morphology could be highlighted depending on the nitrogen treatment received during the first generation. Four different treatments were tested in the second generation:

- CC treated plants: at first and second generation, plants were grown under complete nutrient solution;
- TC treated plants: at first generation, plants were grown for five weeks with 1 mM total nitrogen and then grown for one additional week under N-starvation. At second generation plants were grown under complete nutrient solution (1 mM total nitrogen);
- CT treated plants: at first generation, plants were grown under complete nutrient solution (1 mM total nitrogen). At second generation, plants were grown for five weeks with 1 mM total nitrogen and then were grown for one additional week under N-starvation;
- TT treated plants: at first and second generation, plants were grown for five weeks with 1 mM total nitrogen and then were grown for one additional week under N-starvation.

Under these experimental conditions, CT plants appeared to be smaller than the other plants and leaves showed a partial chlorosis. Also roots appeared to be less developed than in TT plants, although being greater than in CC plants (figure 3.3, A-B, a, b, c, d). In comparison to CC and TC plants, the root system of CT plants showed a good development and was more extended. In shoots and roots, no great differences were observed between TC and CC plants.

Most interesting morphological modifications occurred in TT plants. Shoots of TT plants showed green leaves and no chlorosis or anthocyanin accumulation were visible, as conversely observed in CT plants. A positive effect of a repeated nutritional stress (TT) could be also verified in roots, which appeared more developed and with a higher density of lateral roots than the other treatments as reported in the literature (Remans et al., 2006).

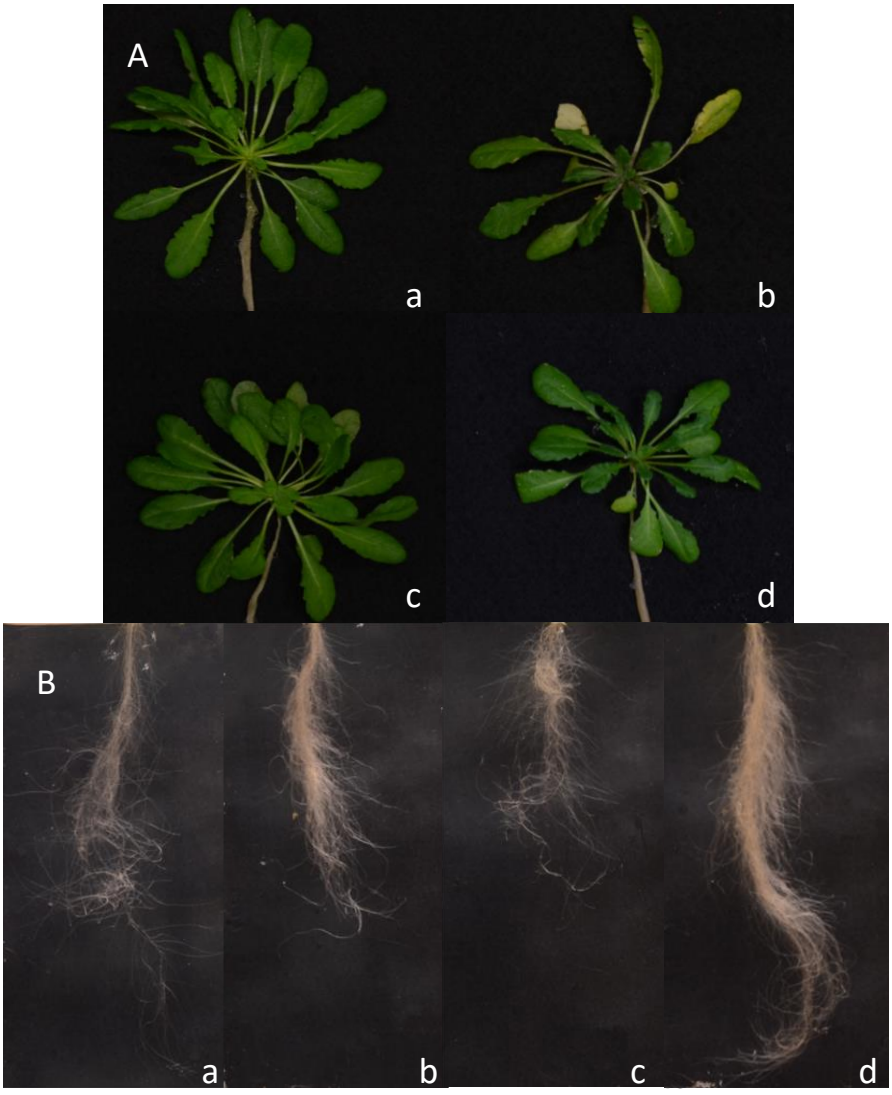


Figure 3.3. Morphological differences in shoots (A) and in roots (B) among plants of the second generation at the end of the sixth week of growth. Small letters refer to: **a**, CC plants (grown in control condition in first and second generation); **b**, CT plants (treated with a nitrogen deficiency only in second generation); **c**, TC plants (treated only in first generation); **d**, TT plants (double deficiency of one week in both generations).

As shown in figure 3.2 and 3.3 there were morphological differences in shoots and root system of plants in the two generations and under different treatments. To understand if these observations involve changes in plant biomass, fresh weight of leaves and roots of *Arabidopsis* were measured (figure 3.4).

In leaves of first generation plants, no significant variation was detected between C0 and T0 plants, although slight differences were recorded (figure 3.4, a). Also leaf biomass of second generation plants (CC, CT, TC and TT) did not displayed significant differences but only slight changes. These little variations in leaf weights reflected morphological differences found and described above.

In plants of second generation differences in root weights were observed (figure 3.4, b). Indeed roots of CT and TT plants showed higher biomass in comparison to CC and TC plants, reflecting morphological differences observed in figure 3.3.

In roots of third generation plants, no significant changes were observed among treatments, although TTC plants showed a slight increase in root weight than those recorded in CTC and TCC plants. The double treatment of nitrogen deprivation in TTC plants, may represents an encouraging signal of a probably adaptation to stress.

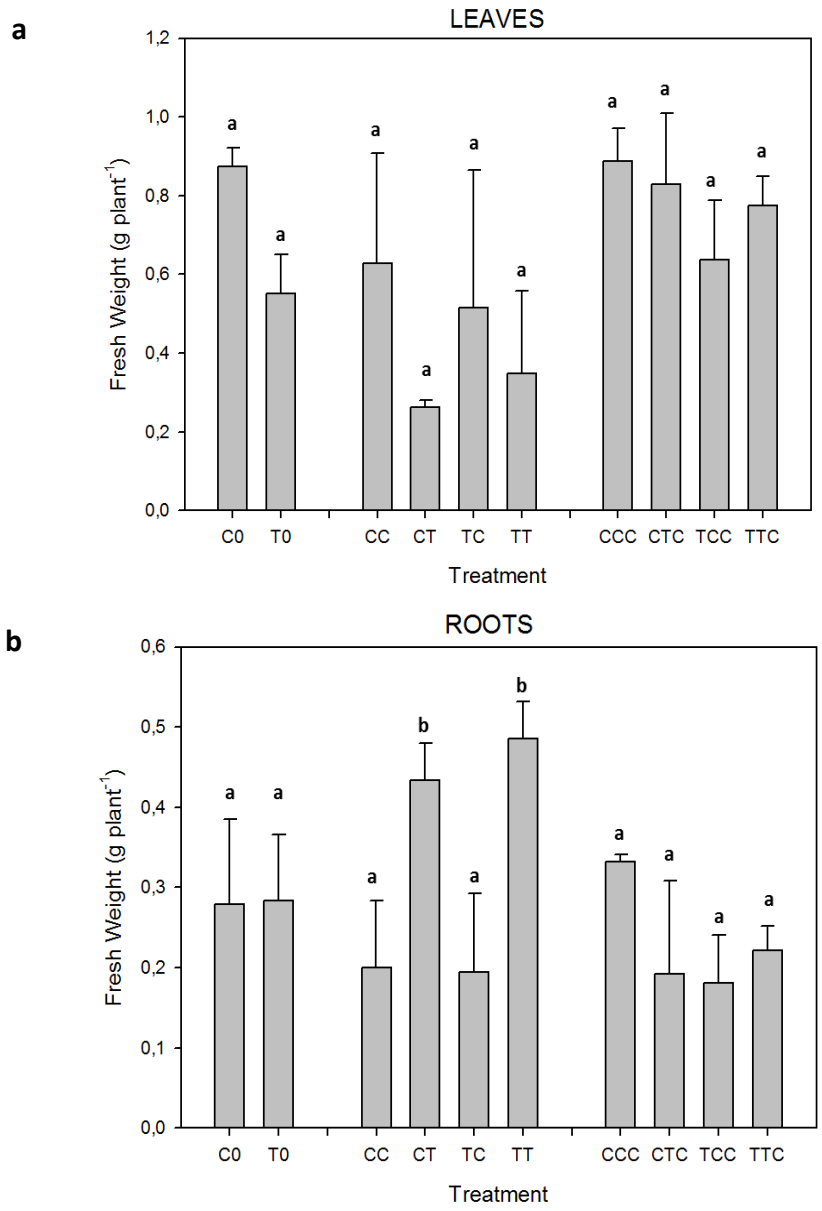


Figure 3.4. Differences in biomass of leaves (a) and roots (b) among plants of the three generations at the end of the sixth week of growth. *First generation plants*: C0, grown in control condition; T0, treated with a nitrogen deficiency. *Second generation plants*: CC, grown in control condition in first and second generation; CT, treated with a nitrogen deficiency only in second generation; TC, treated only in first generation; TT, double deficiency of one week in both generations. *Third generation plants*: CCC, CTC, TCC, TTC plants grown in control condition and derived from treatments in second generation. Small letters refer to statistical significance in the same generation. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.2 Physiological study of nitrate acquisition in *Arabidopsis* plants

It is well known that nitrate serves as a signaling molecule and that the exposure of roots to the anion induces its own uptake (see § 1.1.3.3.2). This process is due to a higher rate of nitrate uptake in root cells. The maximum increase is followed by a feedback regulation caused by the accumulation of downstream metabolites of nitrate assimilation and of the anion itself.

To evaluate the capacity of *Arabidopsis* roots to take up nitrate and to verify the occurrence of changes in this behavior during generations exposed to nutritional stress, a net influx analysis was performed using six-week-old plants. Plants were hydroponically grown in a standard nutrient solution (0.5 mM NH_4NO_3) for 5 weeks and then transferred to an N-free nutrient solution or to a standard nutrient solution for 1 week. During the uptake experiment, control plants and stressed plants, were exposed for 24 hours to a nutrient solution containing 1 mM nitrate as N source (induced plants). Net uptake rates were determined measuring nitrate depletion from assay solutions, containing 0.1 mM of nitrate, representative of the high-affinity NO_3^- transport system (HATS).

3.2.1 Net high-affinity uptake in the first generation

In the first generation four different treatments have been considered:

- plants grown in presence of 0.5 mM NH_4NO_3 for six weeks and not induced with nitrate during the time course of the experiment (C0c);
- plants exposed to N-deprived conditions in the last week of growth (sixth week) and not induced with nitrate during the time course of the experiment (T0c);
- plants supplied with 0.5 mM of NH_4NO_3 for six weeks and then induced with 1 mM nitrate during the time course of the experiment (C0i);
- plants grown in N-deprived condition in the last week of growth (sixth week) and then induced with 1 mM nitrate during the time course of the experiment (T0i).

Results confirmed previous observations (Okamoto et al., 2003): plants previously subjected to nitrogen deprivation for 7 days and then exposed to nitrate (T0i), increased their capability to acquire the anion (figure 3.5). These plants steadily increased their uptake rate, which reached a peak after 8 hours of exposure (25 µmol NO_3^- g^{-1} root FW h^{-1}): afterwards, the rate rapidly decreased showing, after 24 hours, values similar to those registered for not induced plants. On the other hand, during the 24 hours, T0c plants showed a decrease in the net uptake rate (6 µmol NO_3^- g^{-1} root FW h^{-1} after 8 h of exposure). These data confirmed that high affinity nitrate transport in *Arabidopsis* roots is a substrate-inducible process, involving feedback-regulation mechanisms.

Plants not subjected to nitrogen deficiency (C0i and C0c) displayed a net efflux of nitrate (figure 3.5), possibly due to the high concentration of the anion in roots.

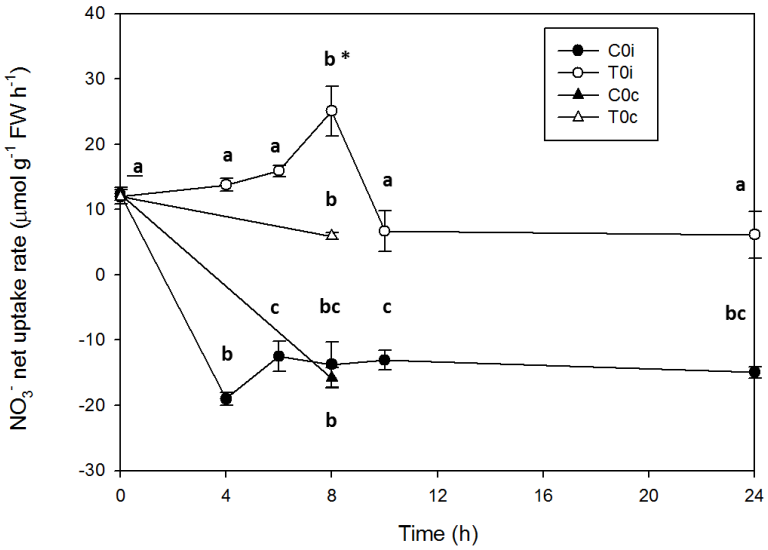


Figure 3.5. Time-course of nitrate uptake in *Arabidopsis* roots. Six-week old plants were treated in the last week of growth with standard solution with 1 mM of total N concentration (C0) or an N-free solution (T0). During the 24 h of the experiment plants were supplied with 1mM nitrate (induced: C0i, T0i) or with no nitrate (not induced: C0c, T0c). After 0, 4, 6, 8, 10, 24 h groups of six plants from each treatment were transferred into the assay solution containing 100 µM nitrate. The values are means ± SD of three experiments (n=3). Small letters refer to statistical significance in the same treatment. Letter "a" refers to the same starting point. Asterisk refers to statistical significance at 8 h between T0i and T0c. Statistical analyses were performed with Holm-Sidak method ($P < 0.05$).FW, fresh weight.

3.2.2 Net high-affinity uptake in the second generation

To analyze the capability of plants to take up nitrate also in the second generation, the same time-course net nitrate influx assay was performed on plants of the second generation. In this case only four treatments were considered, depending on 1 mM total nitrate supply during the time course (24 hours) of the experiment (*induction,* TTi, CTi, CCi, TCi).

Specifically, plants exposed to nitrogen deficiency in the second generation (TTi and CTi) showed the same behaviour as plants of first generation treated in same conditions (figure 3.6, a). Roots of plants subjected to a double deprivation (TTi) displayed a significant increase in the capacity to absorb nitrate, reaching a peak after 8 hours of exposure to the anion (43.5 µmol NO_3^- g^{-1} root FW h^{-1}). Plants subjected to N-deficiency stress only in the second generation (CTi) also showed an induction of uptake although to a lesser extent (35 µmol NO_3^- g^{-1} root FW h^{-1}).

In contrast, plants maintained in standard conditions during the second generation (CCi and TCi), displayed again a net efflux of nitrate (figure 3.6, b).

These results show that the maximal induction of nitrate uptake, recorded after 8 hours of exposure to the anion, is higher in plants subjected to a double N-deprivation treatment in comparison with other plants in both generations (figure 3.7).

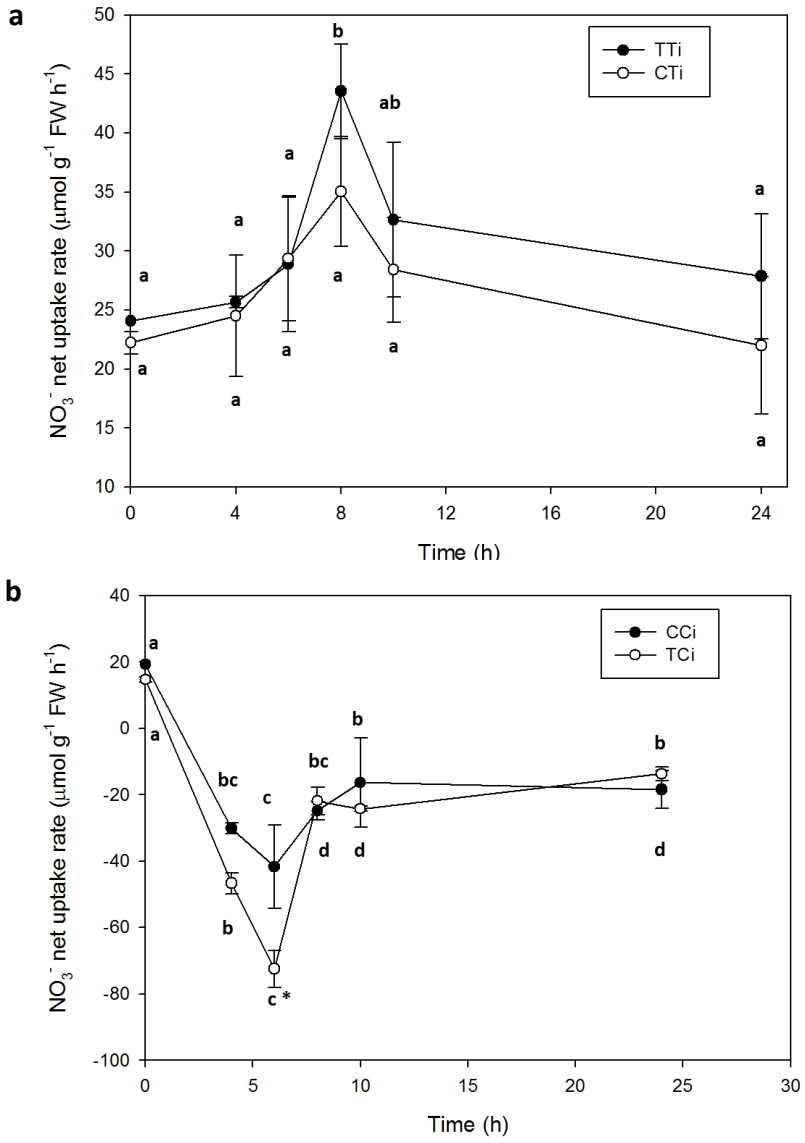


Figure 3.6. Time-course of nitrate uptake in *Arabidopsis* roots. a) Six-week old plants were supplied during the 24-h experiment with 1mM nitrate (*induced*). Plants were grown in N-deprived condition in the last week of growth in both generations (TTi) or only in the second one (CTi). **b)** Plants were fed with 1 mM of total nitrogen for six weeks in both generations (CCi) or plants grown in N-deprived condition in the last week of growth only during the first generations (TCi). After 0, 4, 6, 8, 10, 24 h groups of six plants from each treatment were transferred into the assay solution containing 100 µM nitrate. The values are means ± SD (n=3). Small refer to statistical significance in the same treatment. Asterisk refers to statistical significance at 8h between CCi and TCi. Statistical analyses were performed with Holm-Sidak method ($P < 0.05$). FW, fresh weight.

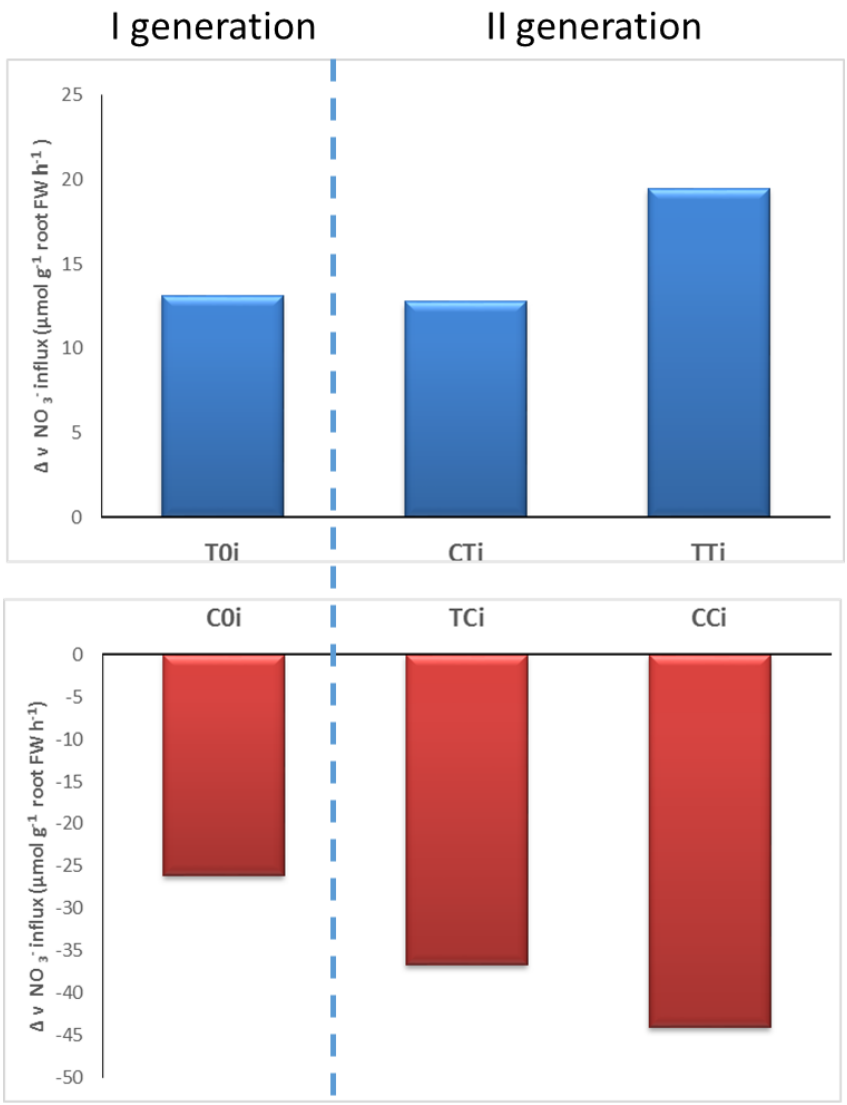


Figure 3.7. Changes in nitrate net-uptake rate (influx) after 8 hours of induction (Δ $v = v_{0h} - v_{8h}$) with nitrate in *Arabidopsis* roots of first and second generation subjected to different treatments. In *blue*: positive changes, in *red*: negative changes. Treatements (T0i, CTi, TTi C0i, TCi, CCi) as indicated in figures 3.5 and 3.6.

3.3 Transcriptional variations and trangenerational effects of nitrogen deprivation

3.3.1 RNA-sequencing in first and second generations

In order to investigate the transcriptomic changes involved in the physiological responses, a genome-wide gene expression analysis was performed using the same experimental conditions reported in the previous chapters. Plants of first and second generation were grown for five weeks with 1 mM of total nitrogen concentration (0.5 mM NH_4NO_3) and in the last week of growth subjected to different N nutritional conditions (C0, T0, CC, CT, TT, TC). At the end of sixth week of growth three roots for each treatment were collected and processed to extract RNA as a single sample in order to perform RNA-sequencing analysis. From 18 total samples was obtained an average of 56 M total reads, but only 55 M (98%) were accepted, which of 53.8 (97.8%) mapped on *Arabidopsis* genome (TAIR10 release Nov 2010), which contains 27,416 protein coding genes, 4827 pseudogenes or transposable element genes and 1359 ncRNAs (non-coding RNAs). Quality of sequencing was very high, as resulted in FASTQC files (figure 3.8).

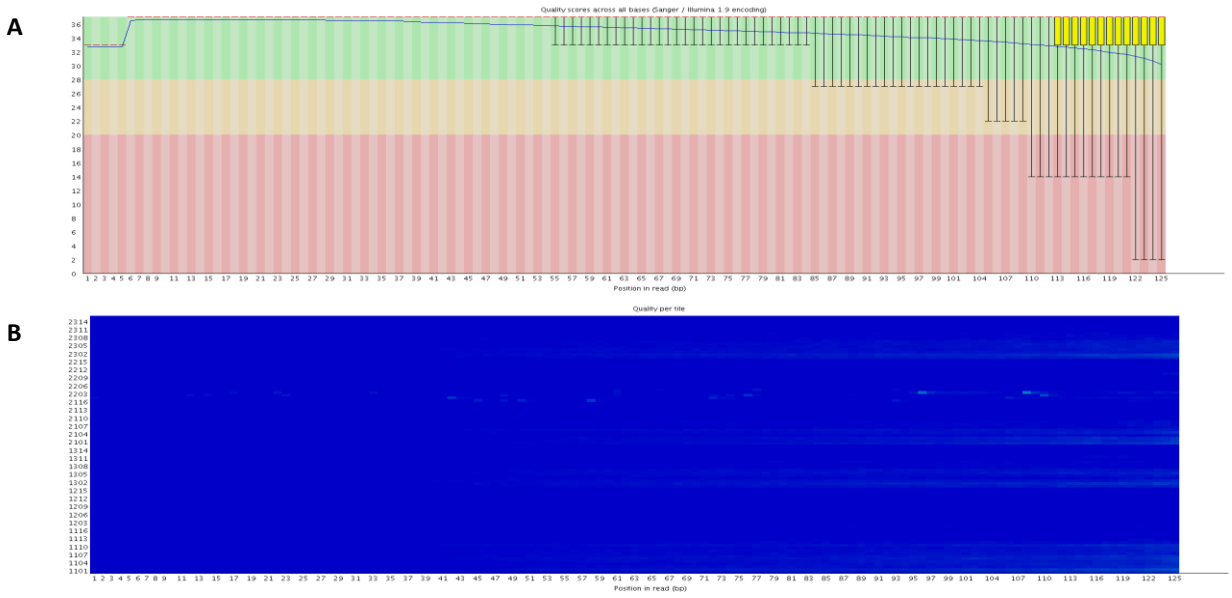


Figure 3.8. Examples of FASTQC output files. **A**) The first graph represents per base sequence quality and shows the average and range of the sequence quality values across the read. **B**) The graph per tile sequence quality allows looking at the quality scores from each tile across all of your bases to see if there was a loss in quality associated with only one part of the flow cell.

Three comparisons of transcriptomic profiles (T0 vs C0, TT vs CC, CT vs CC) were carried out to identify genes potentially involved in the response to nitrate deprivation across two different generations. As reported in Table 1 the number of genes differentially expressed was higher in the second generation and surprisingly the number of genes differentially expressed was the same in the comparisons TT vs CC and CT vs CC, although regulation level of up-regulated and down-regulated genes was different. A lower number of differentially expressed genes was observed in the comparison T0 vs C0. However, even if the number of modulated genes was very high, the intensity of this modulation was quite moderate (n-fold change was lower than |2|).

It is evident from Table 1 that in first and second generation the number of up-regulated genes was greater than those down-regulated, this behavior being particularly evident in comparison the TT vs CC.

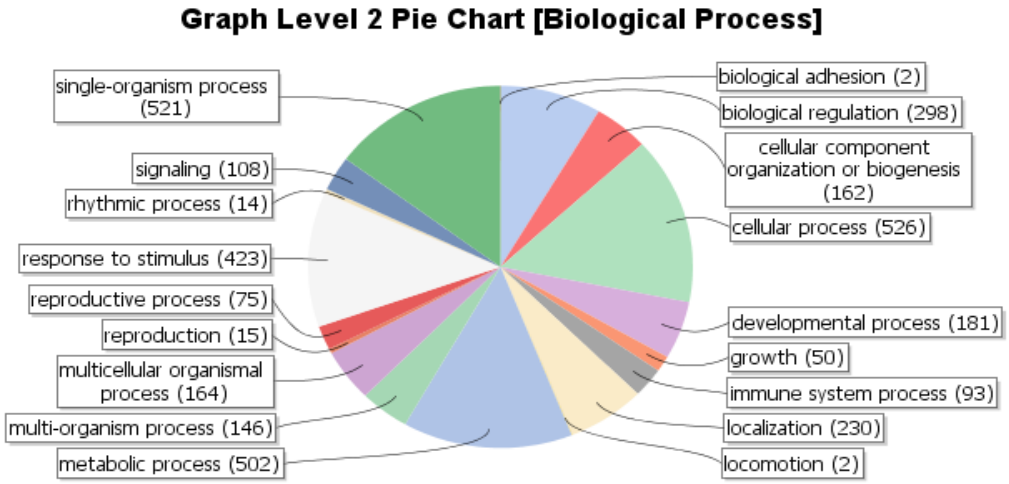
Table 1. Number of gene differentially expressed by nitrogen treatments in the three comparisons: T0 vs C0, TT vs CC and CT vs CC. Data provided by RNA-seq analyses performed on mRNA extracted from roots of *Arabidopsis* grown under different nitrogen treatment in two generations. For each comparison, 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; respectively, in second and third rows are reported the number of up- and down- regulated transcripts. This experiment was performed using three independent biological replicates and the data were statistical analyzed using Cufflinks tool.

	T0 vs C0	**TT vs CC**	**CT vs CC**
total transcripts	1893	2599	2599
up-regulated	1048	1737	1653
down-regulated	845	862	946

Using Blast2GO software, a BlastX analyses was performed to annotate differentially expressed transcripts at biological process level, in order to retrieve a functional profile of gene set obtained from high throughput sequencing of transcriptomes. The most

representative functional categories were “metabolic process”, “cellular process”, “response to stimulus”, “single organism process”, while less abounded were “localization”, “biological regulation”, “developmental process”, “cellular component organization or biogenesis”, “signaling”. Figure 3.9 shows that distribution of ontology classes of differentially expressed genes in the comparisons T0 vs C0 and TT vs CC were quite homogeneous. On the other hand, as previously shown, the number of modulated genes broadly changed in the two generations. The same result was obtained for comparison CT vs CC (data not shown).

a



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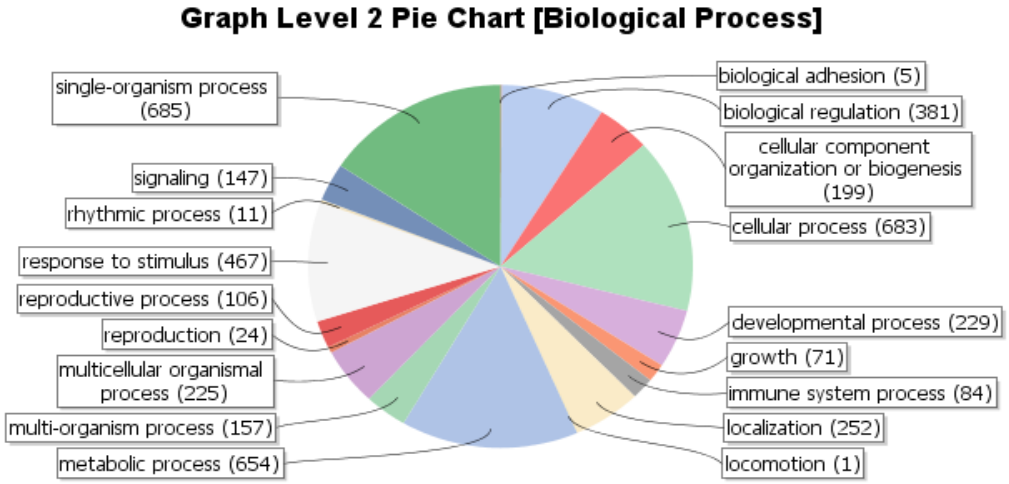
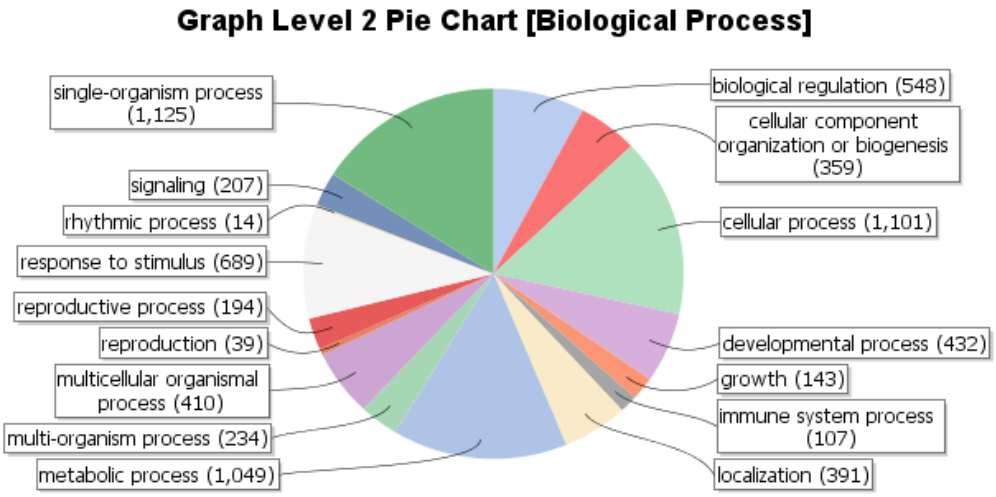


c



d

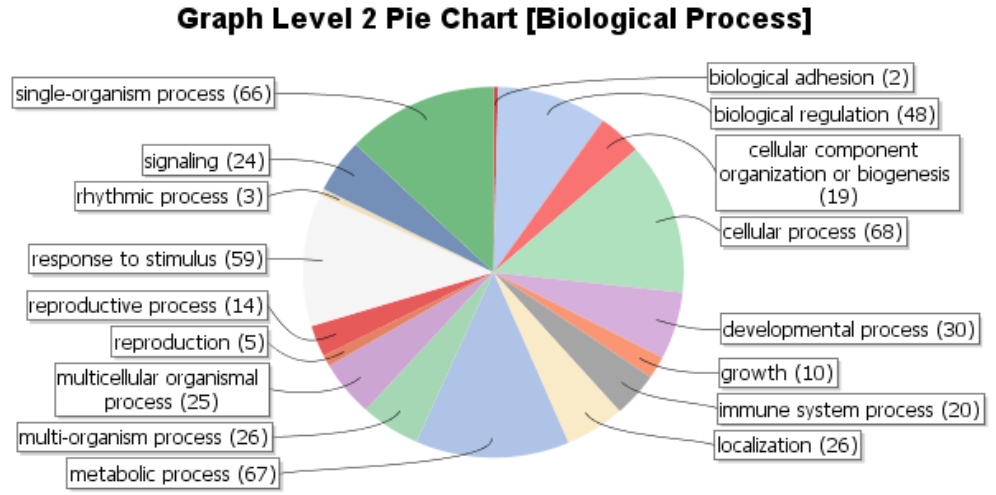
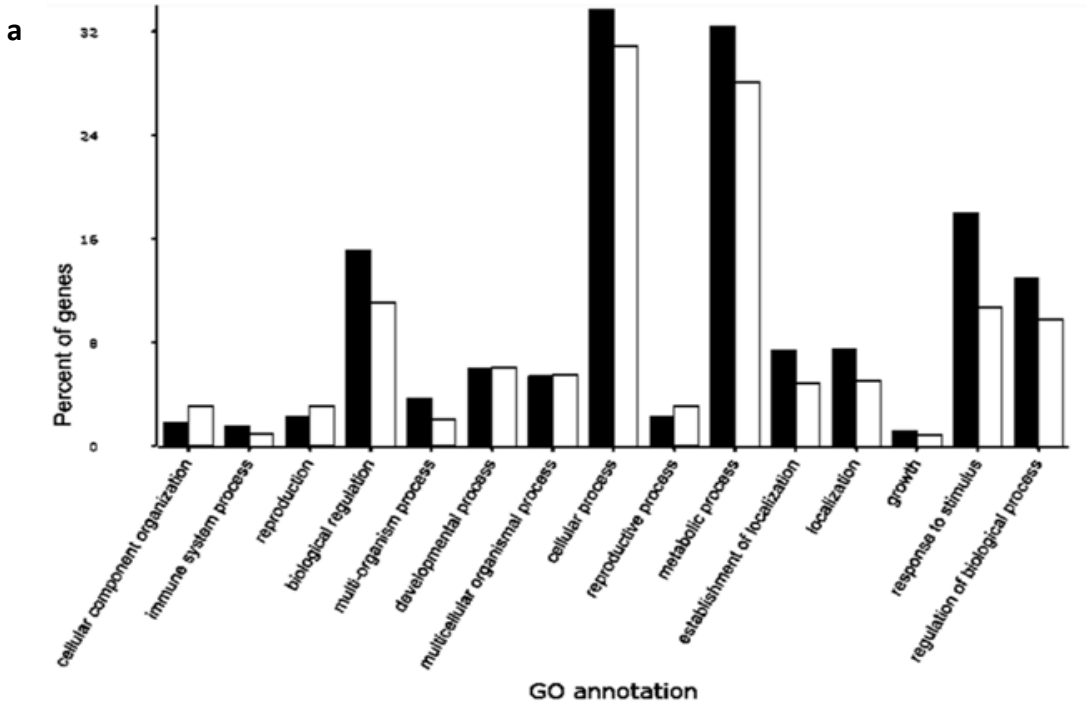


Figure 3.9. Distribution of gene ontology classes of differentially expressed genes in comparison T0 vs C0 and TT vs CC at biological process level. Ontological distribution of regulated genes: **a**, up-regulated genes in comparison T0 vs C0; **b**, down-regulated in T0 vs C0; **c**, up-regulated in TT vs CC; **d**, down-regulated in TT vs CC. The number of genes not corresponds to the numbers showed in Table 1 because same genes can belong to more ontological classes.

Another way for interpreting genome-wide expression profiles is to perform enrichment analysis, which allows to compare gene ontology classes over-expressed or down-regulated in samples with *Arabidopsis*'s transcriptome reference. Graphs in figure 3.10 shows the rates of each class in the comparisons T0 vs C0, CT vs CC and TT vs CC against the same reference. Firstly, response to stimulus and biological regulation, as well as localization classes increased in all three comparisons. In addition, in the comparison T0 vs C0 and CT vs CC also cellular and metabolic process classes raised. The only relevant difference was in cellular and metabolic process of the comparison TT vs CC in the second generation, where the differences between samples and reference decreased, probably as a consequence of a prolonged stress.



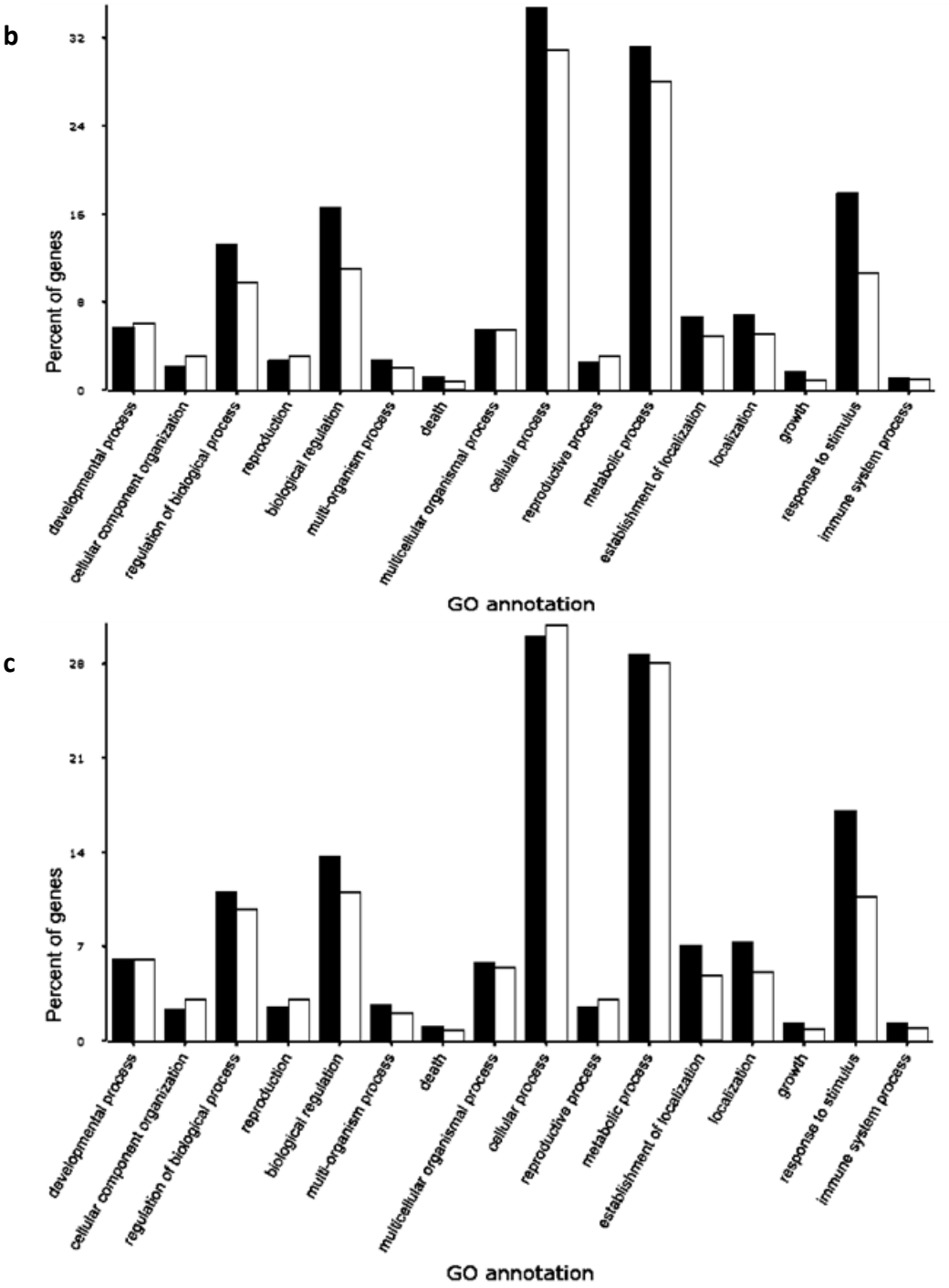


Figure 3.10. Enrichment analyses and distribution in main functional categories according to the GO "biological process" for three different comparisons T0 vs C0 (**a**), CT vs CC (**b**) and TT vs CC (**c**). Singular Enrichment Analysis (SEA) was performed with AgriGO toolkit (Du et al., 2010) using as reference the *Arabidopsis* transcriptome (TAIR10). Black bars refer to reference (TAIR10), while white bars refer to sample data.

3.3.1.1 Nitrate transport and assimilation

In Table 2 are reported the differentially expressed transcripts belonging to the pathway of nitrate uptake and assimilation. The same transcripts are presented using the data visualization tool MapMan (Thimm et al., 2004) (figure 3.11 – 3.12). The results were firstly presented for the response to N-deprivation in the three considered comparisons. There were a considerable number of genes that changed directly involved in nitrate transport and assimilation among first and second generation.

In the comparison T0 vs C0, as well as in TT vs CC and CT vs CC *NRT1.1* was strongly down-regulated and this result confirmed its double role as nitrate low-affinity transporter (Tsay et al., 1993) and as nitrate sensor (Ho et al., 2009). On the contrary, results in the three comparisons corroborated the idea that *NPT3*, also called *NRT1.3***,** encoding a dual-affinity nitrate transporter similar to the *NRT1.1*, was directly up-regulated by the absence of nitrate and down-regulated by the supply of the anion to the roots, as demonstrated in the model legume *Medicago truncatula* (Morère-Le Paven et al., 2011). Other nitrate transporters, steadily induced by N-deficiency in all comparisons, were *NRT2.5* and *NRT2.4*; *NRT2.2* was highly up-regulated in CT vs CC and even more in TT vs CC. According to literature these transporters are important for adaptation to long-term nitrogen starvation (Kiba et al., 2012, Lezhneva et al., 2014). Surprisingly, *NRT2.6* was up-regulated only in TT vs CC under severe nitrate deprivation condition, in contrast with what reported by Dechorgnat and coworkers (Dechorgnat et al, 2012).

NIA1 and *NIR1* were down-regulated only in the second generation, curiously much more in CT vs CC. Conversely, *NIR1* was over-expressed in the first generation. Interestingly *UPM1*, which encodes an enzyme producing a cofactor for *NIR1*, was also repressed in this generation according to results of Wang (Wang et al., 2003).

In addition *NiRT*, encoding a chloroplastic nitrite transporter (Sugiura et al., 2007), was similarly induced in all the comparisons. Once nitrite is converted into ammonium by NIR1, the latter needs to be incorporated into amino acids and other organic molecules. Glutamine synthetase catalyzes the incorporation of ammonium into glutamate thereby producing

glutamine. *GLN1.1, GLN1.3, GLN1*.4 are *Arabidopsis* genes coding for isoforms of glutamine synthetase (GS). These isoforms were over-expressed in all treated (N-deprived) samples.

In particular *GLN1.1*, the cytosolic form, is important under N-limited conditions (Ishiyama et al., 2004). Moreover *GLN1.3* is the low affinity GS1 isoenzyme expressed in *Arabidops*is roots and its expression was inhibited by high concentration of glutamate (Ishiyama et al., 2004). Finally, *GLN1.4* is the high affinity isoenzyme expressed under N-limited conditions, but its expression was two times higher than *GLN1.1*, contrary to what has been reported by Ishiyama and coworkers (Ishiyama et al., 2004). Subsequently, glutamate synthase transfers the amide amino group of glutamine to 2-oxoglutarate, yielding two molecules of glutamate.

In *Arabidopsis, GLT1* or *NADH-GOGAT* uses NADH as electron donor; this was down-regulated in all the three comparisons, whereas *GLU2* or *Fd-GOGAT*, using ferredoxin as electron donor, was up-regulated.

Glutamate dehydrogenase produces ammonium from glutamate; a specific isoform of glutamate dehydrogenase, *GDH3*, was highly expressed in T0 vs C0, TT vs CC, CT vs CC indicating a possible role in the recycling of ammonium during amino acid catabolism; probably this isoform is normally expressed at very low levels of nitrate. *GDH1* and *GDH2* were repressed, respectively, in all three comparisons and only in T0 vs C0.

As reported above (see § 1.1.4) the ammonium transporter (*AMT*) family comprises six isoforms in *Arabidopsis*. In the reported experiments four isoforms were expressed (Table 2) *AMT1.1* that might give a major contribution to ammonium influx in nitrogen-deficient *Arabidopsis* roots (Loqué et al., 2006), was over-expressed in all the three starved samples (T0 vs C0, TT vs CC, CT vs CC). In addition, *AMT1.2*, which mediates the uptake of ammonium in roots, was expressed only in T0 vs C0. Interestingly, *AMT1.5* was switched only in T0 vs C0, according to results reported by Yaun and coworkers (Yuan et al., 2007), which showed that transcripts accumulated considerably in roots of wild-type plants grown under nitrogen-insufficient conditions. Usually, *AMT2.1* showed a higher level of gene expression in shoots compared with roots, but in this case it was similarly expressed in the second generation.

These results suggest that the reduction pathway of nitrate is almost turned off in N starvation condition of first generation possibly due to a prevailing recycling pathway of

nitrogen. In the second generation this general behavior is more evident as the reduction pathway is fully turned off.

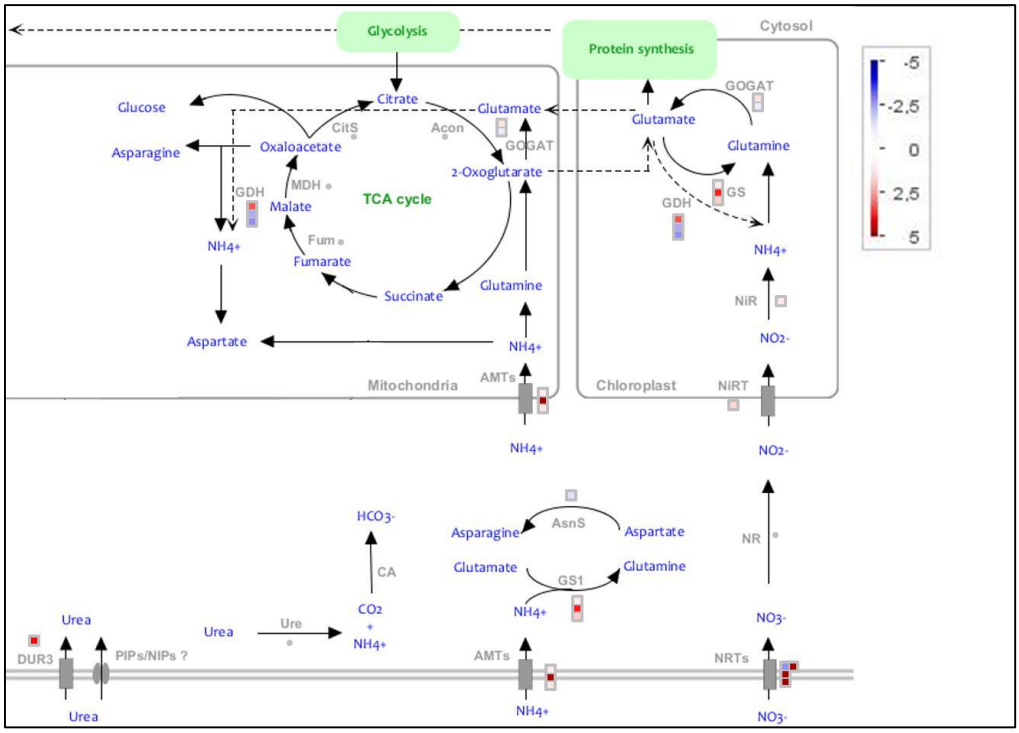


Figure 3.11. Expression of genes involved in the acquisition and metabolism of nitrate and ammonium in T0 vs C0 in the first generation. Schematic representantion of genes involved in nitrate and ammonium uptake and their assimilation. Not differentially regulated transcripts in T0 vs C0 are shown in gray, up-regulated genes are shown in red while down-regulated ones are visible in blue. The coulor scale refers to the $\log_2$ fold change of differentially regulated transcripts in the data set, a 2-fold change (=1 on a log2 scale) is required to produce a visible coloration and the scale saturates at a 32-fold change (=5 on a log2 scale). The results are the mean of three biological replicates. The output was created with MapMan software (Thimm et al., 2004). AMTs, ammonium transporters; NRTs, nitrate transporters; DUR3, high affinity urea transporter; Ure, urease; NR, nitrate reductase; NiR, nitrite reductase; NiRT, nitrite transporter; GS, glutamine synthetase; GDH, glutamate deydrogenase; GOGAT, glutamate synthase; Acon, Aconitase; CitS, citrate synthase; MDH, malate deydrogenase.

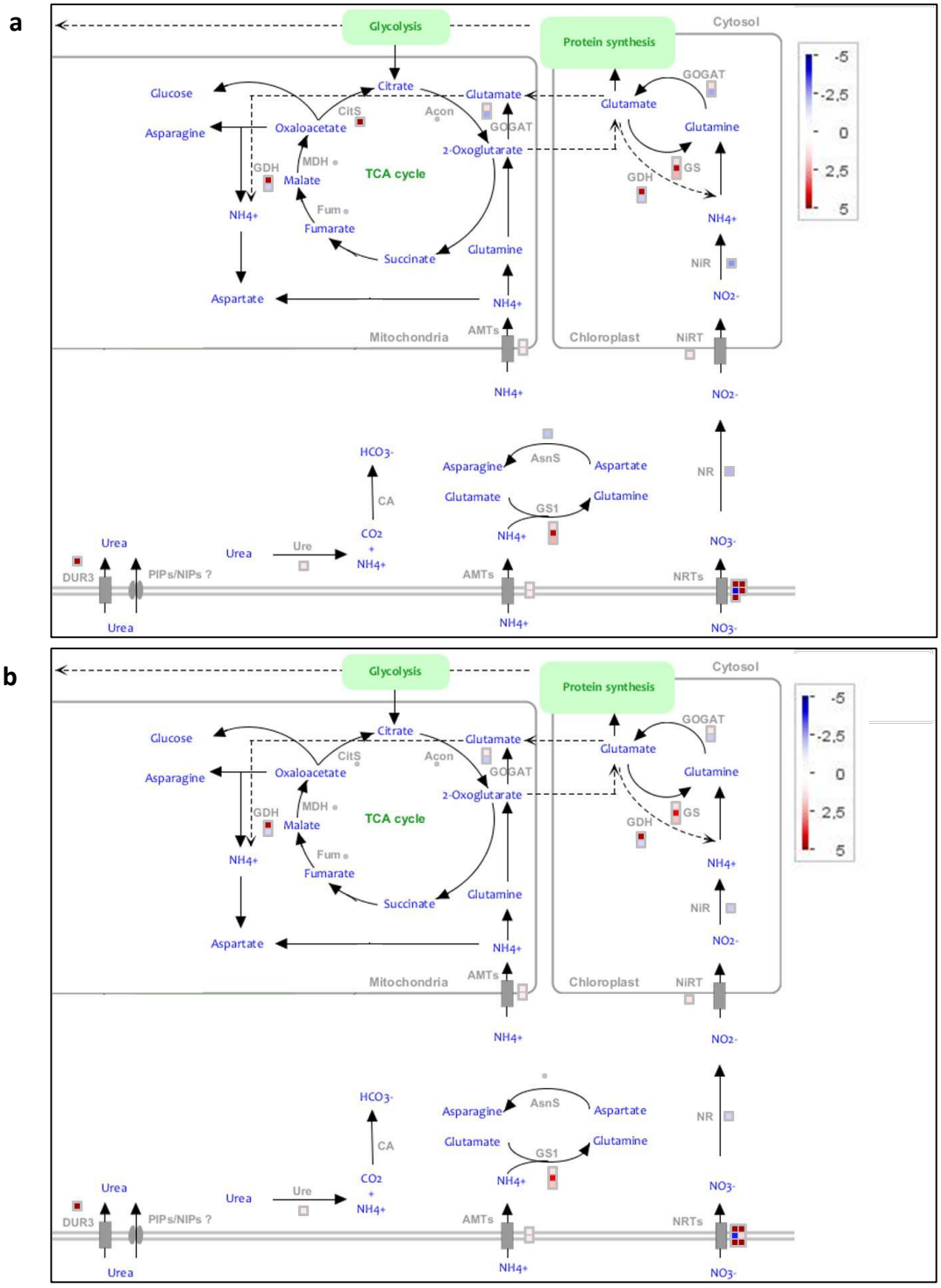


Figure 3.12. Transcripts levels of genes involved in nitrate and ammonium uptake and assimilation pathways. **a**) Expression levels in the comparison CT vs CC and **b**) in the comparison TT vs CC in roots of second generation plants. For more details see figure 3.11.

3.3.1.2 Coordinated regulation of asparagine and urea metabolism

An interesting transcriptional modulation is provided for asparagine synthase (*ASN*), which converts aspartate into asparagine by deamination of glutamine. It appeared to be down-regulated by a single treatment of N-deprivation (T0 vs C0, CT vs CC), but not modulated in a double treatment (TT vs CC). Another important modulated transcript is DUR3, a putative high-affinity urea transporter (Liu et al., 2003.), which was strongly up-regulated, as expected in condition of N-limitation (Kojima et al., 2007). *DUR3* in *Arabidopsis* showed a weak homology with an orthologous 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, inositol, vitamins, anions, and urea (Reizer et al., 1994; Turk and Wright, 1997; Saier, 2000).

Moreover, urease is a nickel-containing urea hydrolase involved in nitrogen recycling from ureide, purine, and arginine catabolism in plants. The process of urease activation by incorporation of nickel into the active site is a first example of chaperone-mediated metal transfer to an enzyme (Witte et al., 2005). In plants different urease accessory proteins are required for activation and results this work confirmed that *UREG*, coding for the urease accessory protein G, was induced in the second generation by nitrogen deficiency.

Table 2. List of transcripts involved in nitrate and ammonium uptake and in their metabolism, that were modulated in *Arabidopsis* roots in response to different N-deprivation treatments. In the table are reported: the *Arabidopsis* gene identifier (Gene_ID), the common name of the gene (Symbol), for each comparison, Log2 FC (Fold change) and relative p-value; the description of the function. Each transcript is discussed in detail in the text. With asterisks, infinite expression values have been replaced with|12.00| as theoretical maximal.

Gene_ID	Symbol	log2-ratio T0 vs C0	p-value	log2-ratio TT vs CC	p-value	log2-ratio CT vs CC	p-value	Description
AT1G12940	NRT2.5	11.345	0.0003	11.546	0.00005	11.905	0.00005	nitrate transporter 2.5
AT5G60770	NRT2.4	6.575	0.00005	6.286	0.00005	5.629	0.00005	nitrate transporter 2.4
AT1G08100	NRT2.2			9.163	0.0011	6.391	0.0011	nitrate transporter 2.2
AT3G45060	NRT2.6			1.588	0.00005			nitrate transporter 2.6
AT3G21670	NTP3	6.266	0.00005	5.929	0.00005	6.231	0.00005	nitrate transporter -like
AT1G12110	NRT1.1	-2.639	0.00005	-3.796	0.00005	-4.168	0.00005	nitrate transporter 1.1
AT1G77760	NIA1			-1.651	0.00005	-2.160	0.00005	nitrate reductase 1
AT2G15620	NIR1	1.151	0.00005	-1.806	0.00005	-2.620	0.00005	nitrite reductase 1
AT5G37600	GLN1.1	1.827	0.00005	1.934	0.00005	2.244	0.00005	glutamine synthase clone R1
AT3G17820	GLN1.3	1.082	0.00005	1.408	0.00005	1.502	0.00005	glutamine synthetase 1.3
AT5G16570	GLN1.4	3.827	0.00005	4.174	0.00005	4.696	0.00005	glutamine synthetase 1;4
AT2G41220	GLU2	1.306	0.00005	1.191	0.00005	1.415	0.00005	glutamate synthase 2
AT5G53460	GLT1	-1.066	0.00005	-1.798	0.00005	-2.373	0.00005	NADH-dependent glutamate synthase 1
AT3G03910	GDH3	3.329	0.00505	4.807	0.00005	4.789	0.00005	glutamate dehydrogenase 3
AT5G18170	GDH1	-2.668	0.00005	-1.632	0.00005	-1.758	0.00005	glutamate dehydrogenase 1
AT5G07440	GDH2	-2.317	0.00005					glutamate dehydrogenase 2
AT4G13510	AMT1.1	1.292	0.00005	1.183	0.00005	1.163	0.00005	ammonium transporter 1;1
AT1G64780	AMT1.2	1.073	0.00005					ammonium transporter 1;2
AT3G24290	AMT1.5	12*	0.00005					ammonium transporter 1;5
AT2G38290	AMT2.1			1.090	0.00005	1.055	0.00005	ammonium transporter 2
AT1G68570	NiRT	1.601	0.00005	1.317	0.00005	1.200	0.00005	proton-dependent oligopeptide transport (POT) family protein
AT3G47340	ASN1	-1.334	0.00005		0.00005	-1.912	0.00005	glutamine-dependent asparagine synthase 1
AT5G45380	DUR3	4.092	0.00005	5.122	0.00005	5.513	0.00005	probable urea active transporter 1-like
AT2G34470	UREG			1.147	0.00005	1.274	0.00005	urease accessory protein G
AT2G11270	citrate synthase 4					12*	0.0006	citrate synthase 4

3.3.1.3 Coordinated modulation of various metabolisms in the N-deprivation

As already reported (see § 1.1.3.3.2) nitrate elicits a wide range of genes involved in various metabolic processes, like the oxidative pentose pathway to produce reducing equivalents for nitrate assimilation (Wang et al, 2003), the production of organic acids (Wang et al. 2000), the root architecture (Zangh and Forde, 1998) and the production of secondary metabolites (figure 3.13 -3.14).

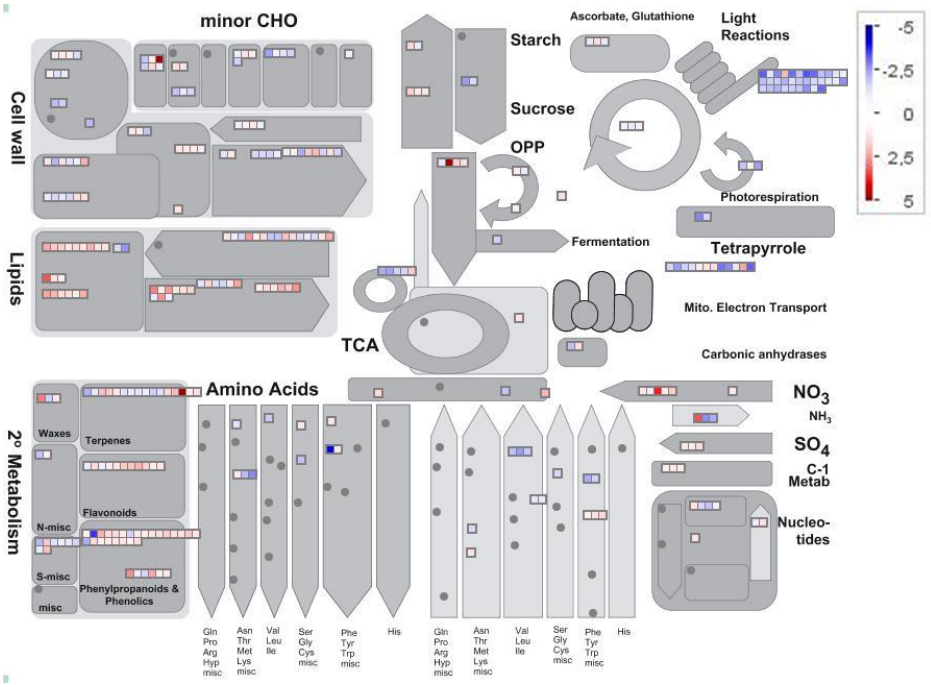


Figure 3.13. General overview of changes at transcriptional level in T0 vs C0 in the first generation.. Schematic representantion of genes involved in various metabolisms. Not differentially regulated transcripts in T0 vs C0 are shown in gray, up-regulated genes are shown in red while down-regulated ones are visible in blue. The coulor scale refers to the $\log_2$ fold change of differentially regulated transcripts in the data set, a 2-fold change (=1 on a log2 scale) is required to produce a visible coloration and the scale saturates at a 32-fold change (=5 on a log2 scale). The results are the mean of three biological replicates. The output was created with MapMan software (Thimm et al., 2004). For more details see Table S1 and text.

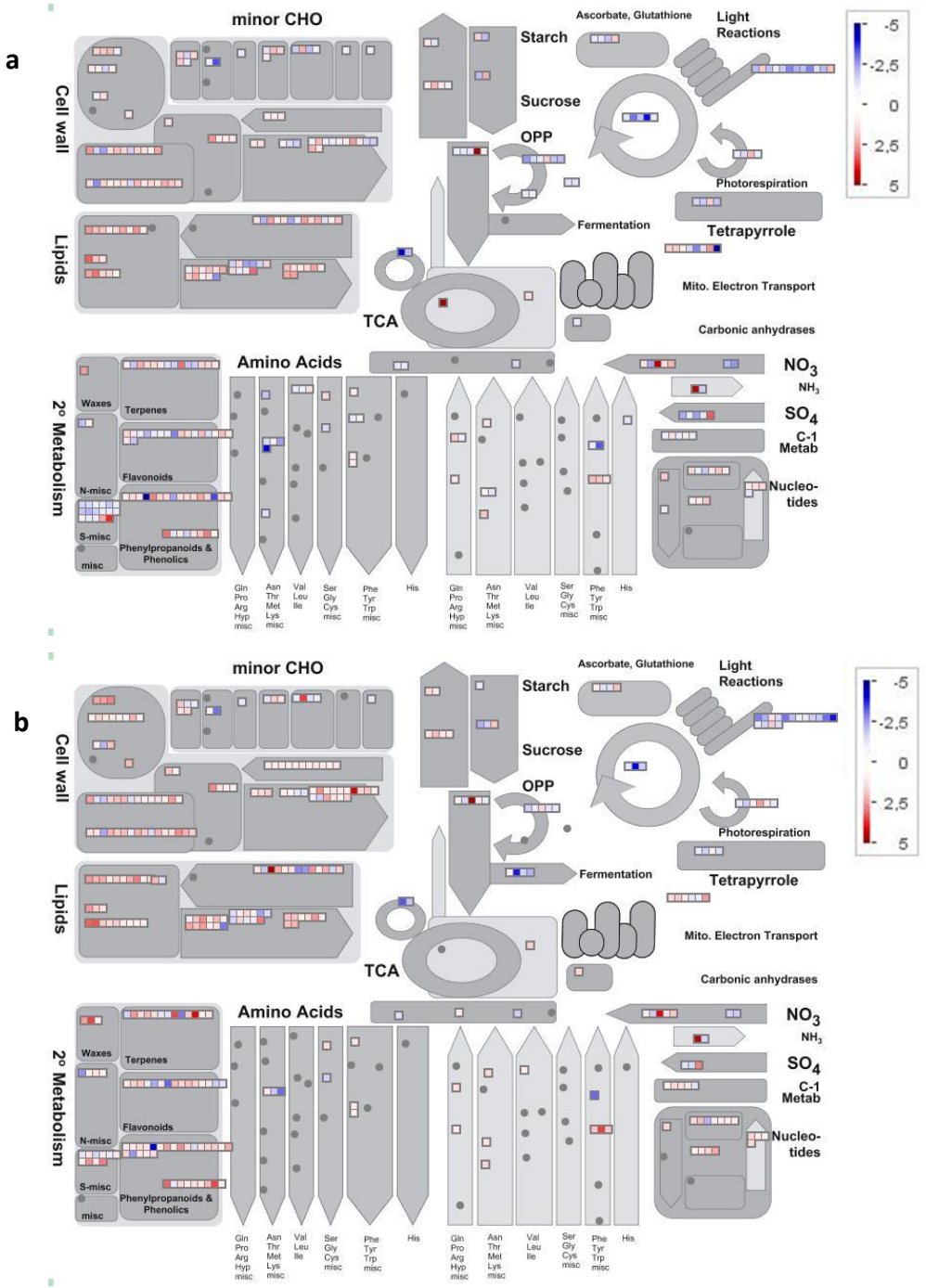


Figure 3.14. General overview of changes at transcriptional level in the second generation under N-deprivation condition. Expression levels in the comparison CT vs CC (**a**) and in the comparison TT vs CC (**b**) in the second generation showed in the output of MapMan software (Thimm et al., 2004). For more details see Table S1 and text and figure 3.13.

3.3.1.4 TCA cycle and electron transport chain

Five genes involved in TCA cycle were modulated in the two generations in response to nitrogen starvation. Three were carbonic anhydrase (CA) isoforms, that catalyze the reversible hydration of CO_2^- ; two of them (see Table S1, #370 and #371) were down-regulated respectively in CT vs CC and T0 vs C0, whereas alpha carbonic anhydrase 2 (*ACA2* - #372) was up-regulated in T0 vs C0 and TT vs CC; its role at ambient or high CO_2^- concentration or low CO_2^- concentration remains still controversial according to Fabre and coworkers (Fabre et al., 2007). Except for the turning on of a citrate synthase (CS)-like gene (#368) in the comparison CT vs CC, the expression levels of genes in the TCA cycle were unchanged.

Interestingly, the NADP-malic enzyme 3 (*NADP-ME3* - #369), a cytosolic isoform of the four present in *Arabidopsis*, was highly expressed in all the three comparisons. *NADP-ME3* has the highest catalytic efficiency for NADP and it might be involved in providing NADPH for downstream reactions (Wheeler et al, 2005).

NADH and $FADH_2$ produced by TCA cycle must be used to produce ATP via the mitochondrial electron transport chain. According to this the subunits of complex I (NADH-dehydrogenase) were mostly down-regulated in the first generation (from #373 to #376), but alternative pathway of electron transport chain seemed to be activated. Indeed, two external NAD(P)H dehydrogenase (#377 and #378), that introduce electrons at the level of ubiquinone pool, were respectively up-regulated in two generations and only in the first one. In agreement with these results, the alternative oxidase (AOX1A-#379) was induced by N-starvation (Moore et al., 2003). As reported by Møller (2001), these alternative enzymes have the potential to catalyze wasteful respiration but can also decrease the production of reactive oxygen species in the respiratory chain. Furthermore cytochrome c oxidase and ATPase subunits (from #380 to #383) were repressed by N-deficiency.

3.3.1.5 Pentose phosphate pathway and glycolysis regulation

Oxidative pentose phosphate (OPP) pathway, which generates NADPH is also modulated by nitrate. Nevertheless, in the present work three isoforms of glucose-6-phosphate

dehydrogenase and two of 6-phosphogluconate dehydrogenase were repressed in the two generation in comparisons T0 vs C0, TT vs CC, CT vs CC (#362, #363, #364, #366, #367, Table S1), as already reported by Bussell (Bussel et al., 2013). Although *G6PD3* (#364), preferentially expressed in roots, remained down-regulated in the second generation, it was up-regulated in T0 vs C0, possibly due to a lower sensitivity to feedback inhibition (NADPH); however its role in oxidative or other kind of stresses remains still unknown (Wakao et al., 2005).

On the other hand, 6-phosphogluconolactonase 1 (*PGL1* #365) was likewise over-expressed in the second generation; Bussel and coauthors (Bussel et al., 2013) have demonstrated that another isoform, 6-phosphogluconolactonase 3 (*PGL3*), is highly over-expressed under N-deficiency conditions.

OPP pathway is strictly related to glycolysis. In this light a phosphoglycerate mutase family protein (#359), producing 2-phosphoglycerate, was very highly expressed in the three comparisons, even more in the second generation. Moreover, pyruvate kinase was weakly activated by N-starvation in CT vs CC (#360) and phosphoenolpyruvate carboxylase 2 (#361) producing oxaloacetate, was over-expressed in TT vs CC. Other important genes involved in glycolysis not were modulated.

3.3.1.6 Amino acids transport and secondary metabolism

Results from a range of studies suggest that amino acids are potential N sources for plants (Forsum et al., 2008); thus in N-deprived plants used in this experiment several transmembrane amino acids transporters as well as amino acids permeases and specific amino acid transporters like for lysine (lysine histidine transporter 1, *LHT1* - #345) and other amino acids (tyrosine and histidine) were strongly over-expressed (from #341 to #358).

In addition to amino acids transport, the internal biosynthesis of some specific amino acids resulted to be modulated. As previously observed for glutamate, glutamine and asparagine (see § 3.3.1) aromatic amino acids biogenesis showed changes in gene expression under nitrate deficiency. Concerning this aspect, phenylalanine and tyrosine are provided by the shikimic acid pathway, which converts simple carbohydrate precursors to these aromatic

amino acids. Indeed, chorismate mutase 1 (*CM1* - #116) was up-regulated in T0 vs C0 and CT vs CC and two arogenate dehydratases (*ADT5*, *ADT1* - #114-#115) were similarly induced in CT vs CC and TT vs CC. Cho and coworkers (2007) demonstrated that *ADT1* is able to catalyze the decarboxylation/dehydration of prephenate directly, whereas the *ADT5* had an apparent strict requirement for arogenate (figure 3.15).

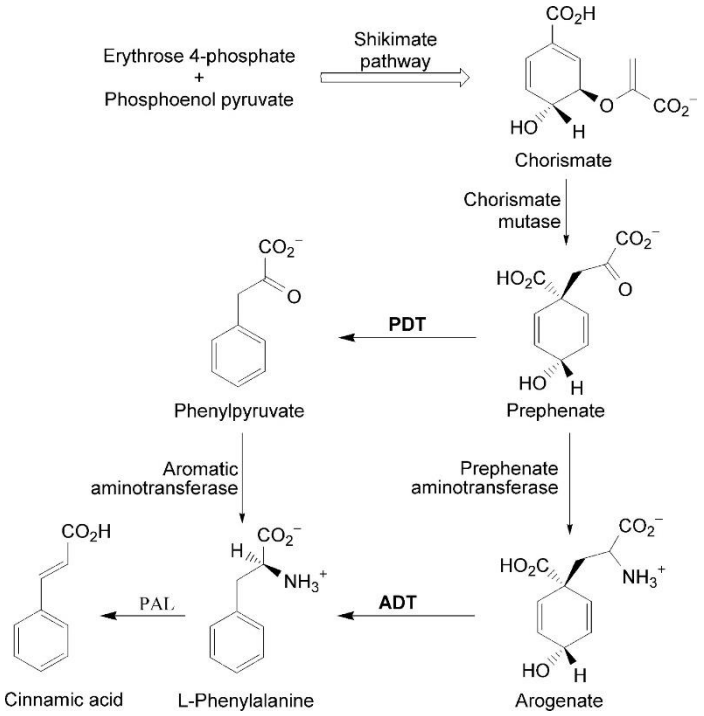


Figure 3.15. Proposed biosynthetic pathway from prephenate and arogenate to Phe in plants (Cho et al., 2007).

Phenylalanine is involved in the production of secondary metabolites like phenylpropanoids, via the elimination of an ammonia molecule to form cinnamic acid. This reaction is catalyzed by the phenylalanine ammonia lyase (*PAL*). In the present work two isoform of PAL were oppositely expressed. *PAL1* (#120) was over-expressed in all the three treatments, according to the observations that nitrate starvation up-regulates this gene (Olsen et al., 2009). In contrast, *PAL3* (#126) was down-regulated, highly in CT vs CC, maybe because *PAL3* shows a

small increase in response to nitrogen re-supply, as reported by Olsen and coauthors (Olsen et al., 2009). Two other isoforms, *PAL2* and *PAL4* (#135-136), were up-regulated in T0 vs C0. Interestingly, three caffeoyl- o-methyltransferases were strongly up-regulated in N-deficient plants, especially in the second generation (from #117 to #119), whereas caffeoyl-CoA 3-O-methyltransferase(#127) was steadily repressed, in particular in CT vs CC. Additional genes involved in phenylpropanoids metabolism were generally modulated (from #121 to #125 and from #128 to #149).

An important class of secondary metabolites is polyamines, including spermine, putrescine and cadaverine. They are implicated in many physiological processes and in particular their metabolism can be modulated in response to a variety of abiotic stresses (Alcazar et al., 2006). Such idea is supported by results shown here. Indeed, arginine decarboxylase 2 (*ADC2* - #175), which synthesizes putrescine from arginine, was down-regulated in all the three comparisons (T0 vs C0, TT vs CC, CT vs CC). After putrescine synthesis, next biosynthetic steps require the activity of S-adenosylmethionine decarboxylases to produce spermidine (figure 3.16). According to this, two of four SAM decarboxylases present in the *Arabidopsis* genomes (Urano et al., 2003), were also repressed, especially in the second generation (#172 - #173). Conversely, agmatine deiminase (#175), the second enzyme required in the biogenesis of putrescine releasing a NH_3, was up-regulated in the second generation.

Finally, the polyamine oxidase 1 (#176), which is involved in the catabolism of polyamines, was over-expressed by nitrogen deficiency in the second generation. This enzyme induces the back-conversion of spermine to spermidine, producing 4-aminobutanal, 1-3 diaminopropano and H_2O_2 (Cona et al., 2006, Alcazar et al., 2010). All these findings suggest that plants subjected to a prolonged nitrogen starvation try to retrieve nitrogen by different pathways, such as blocking the biogenesis of polyamine and releasing nitrogen from various N-containing compounds.

3.3.1.7 Oxidative stress responses

To cope with the accumulation of reactive oxygen species induced by different stresses, several defensive mechanisms are activated by plants. Results reported in Table S1 show that several genes encoding thioredoxin (from #150 to #154), glutaredoxin (from #156 to #169), dismutases and catalases (#170 -#171) as well as peroxidases (from #177 to #209) were induced and others repressed by nitrogen deficiency. To this category of genes belongs also *AHB1* (#155), one of two non-symbiotic hemoglobin genes of *Arabidopsis*. This is induced by nitrate (Wang et al., 2000) and by low oxygen content in roots (Trevaskis et al., 1997). Nitrate-starved plants showed a strong repression of this gene.

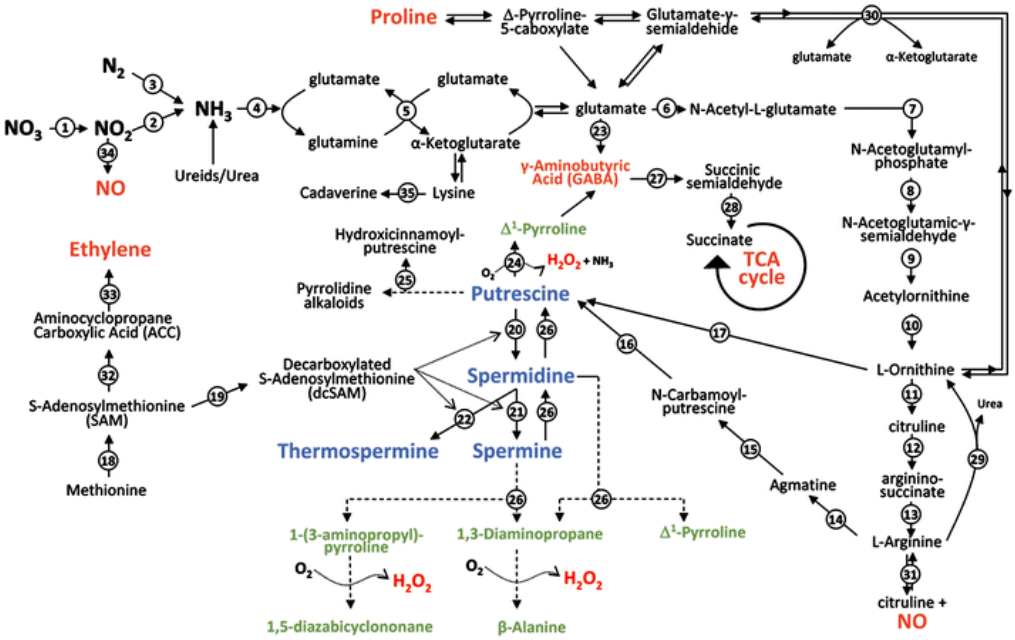


Figure 3.16. Schematic representation of polyamine metabolism and its interaction with other metabolic pathways. 1 nitrate reductase, 2 nitrite reductase, 3 nitrogenase, 4 glutamine synthetase, 5 glutamate synthase, 6 glutamate N-acetyltransferase, 7 acetylglutamate kinase, 8 N-acetyl-phosphate reductase, 9 acetylornithine transaminase, 10 acetylornithine deacetylase, 11 ornithine-carbamoyl transferase, 12 arginosuccinate synthase, 13 arginosuccinate lyase, 14 arginine decarboxylase, 15 agmatine iminohydrolase, 16 N-carbamoylputrescine amidohydrolase, 17 ornithine decarboxylase, 18 SAM synthetase, 19 SAM decarboxylase, 20 spermidine synthase, 21 spermine synthase, 22 thermospermine synthase, 23 glutamate decarboxylase, 24 diamine oxidase, 25 putrescine hydroxycinnamoyl transferase, 26 polyamine oxidase, 27 _-aminobutyrate aminotransferase, 28 succinic semialdehyde dehydrogenase, 29 arginase, 30 ornithine

aminotransferase, 31 nitric oxide synthase, 32 ACC synthase, 33 ACC oxidase, 34 nitrate reductase, 35 lysine decarboxylase. (Alcazar et al., 2010).

3.3.1.8 Transcriptional factors

The *Arabidopsis* genome encodes about 2,000 transcription factors or transcriptional regulators (Riechmann, 2002). Fifty-six genes, belonging to MYB family presented changes in expression under nitrogen deficiency treatment (from #210 to # 266). MYB proteins are a superfamily of transcription factors that play key roles in regulation of developmental processes and defense responses in plants (Yanhui et al., 2006). The WRKY proteins are a superfamily of transcription factors with up to 100 representatives in *Arabidopsis*. Twenty-three (from #307 to #330) WRKY DNA-binding proteins showed a regulation in all three comparisons (T0 vs C0, TT vs CC, CT vs CC).

In *Arabidopsis*, at least three genetically distinguishable pathways for defense signaling have been characterized: those mediated by salicylic acid, jasmonic acid, or ethylene. These signaling molecules increase during senescence and can modulate the expression of specific downstream genes (Buchanan-Wollaston et al., 2005). In particular, 39 genes (from #267 to #306) encoding for transcriptional factors and belonging to ethylene-responsive element binding protein family, were largely modulated in the two generations. Especially *EDF3* (#272), also called *RAV1*, plays an important role in regulating biotic and abiotic stresses (Sohn et al., 2006) and it was over-expressed in all three comparisons.

3.3.1.9 Histone proteins and cell wall modifications

Organogenesis in plants requires the continuous production of cells in the organ primordia, their expansion and a coordinated differentiation. Genome replication is one of the most important processes that occur during the cell cycle, as well as the maintenance of genomic integrity is relevant for development. As long as chromatin must be duplicated, a strong coordination occurs among DNA replication, the deposition of new histones and the introduction of histone modifications and variants (Sequeira-Mendes and Gutierrez, 2015).

Data of the present work revealed a strong regulation of genes involved in cell wall modification by nitrogen deprivation. One hundred and twelve (from #1 to #113) of these genes were modulated; 6 genes belonging to expansin family were up-regulated, especially in second generation, corroborating morphological modification of roots previously observed (see § 3.1.1 and 3.1.2).

In this context, changes in 10 principal histone proteins (from #331 to #340) seem to play a crucial role in cell division. However Ascenzi and Gantt (Ascenzi and Gantt, 1997) have hypothesized that *HIS1-3*, that is up-regulated in T0 vs C0 (#340) and encodes a structurally divergent linker histone in *Arabidopsis*, is induced by water stress and it may play a role in the structure and function of plant chromatin.

3.3.2 Clustering analysis of differential gene expression

A clustering analysis with k-means method was performed for whole genes differentially expressed in each treatment. K-means algorithm, also known as Lloyd's algorithm, aims to group n observations into k clusters in which each observation belongs to the cluster with the nearest mean. According to this, 7 more interesting genes of a cluster of 13 were always highly expressed in all control samples (C0, CC, TC) (figure 3.17, a). Plasma membrane intrinsic protein (PIP), as aquaporins, and tonoplast intrinsic proteins were classes mainly represented in the data. As demonstrated by di Pietro and coworkers (di Pietro et al., 2013) the regulation of PIP and tonoplast intrinsic protein abundance is involved in the response to a few treatments like NaCl, NO, and nitrogen starvation in *Arabidopsis* roots.

Furthermore, as suggested by Loqué and coauthors (Loqué et al., 2005), two tonoplast intrinsic proteins TIP2;1 (AT3G16240) and TIP2;3 (AT5G47450) confer tolerance to the toxic ammonium, allowing its transport into the vacuole. The high repression of such genes in N-starved plants supports the occurrence of N recycling pathways.

On the contrary, 5 more interesting genes of a cluster of 17 were always up-regulated in treated samples (T0, TT, CT) (figure 3.17, b); among these a peroxidase and two metallothioneins (cysteine-rich proteins that can bind metals and oxidant radicals). These findings corroborate the idea that such genes might affect responses to oxidative stress

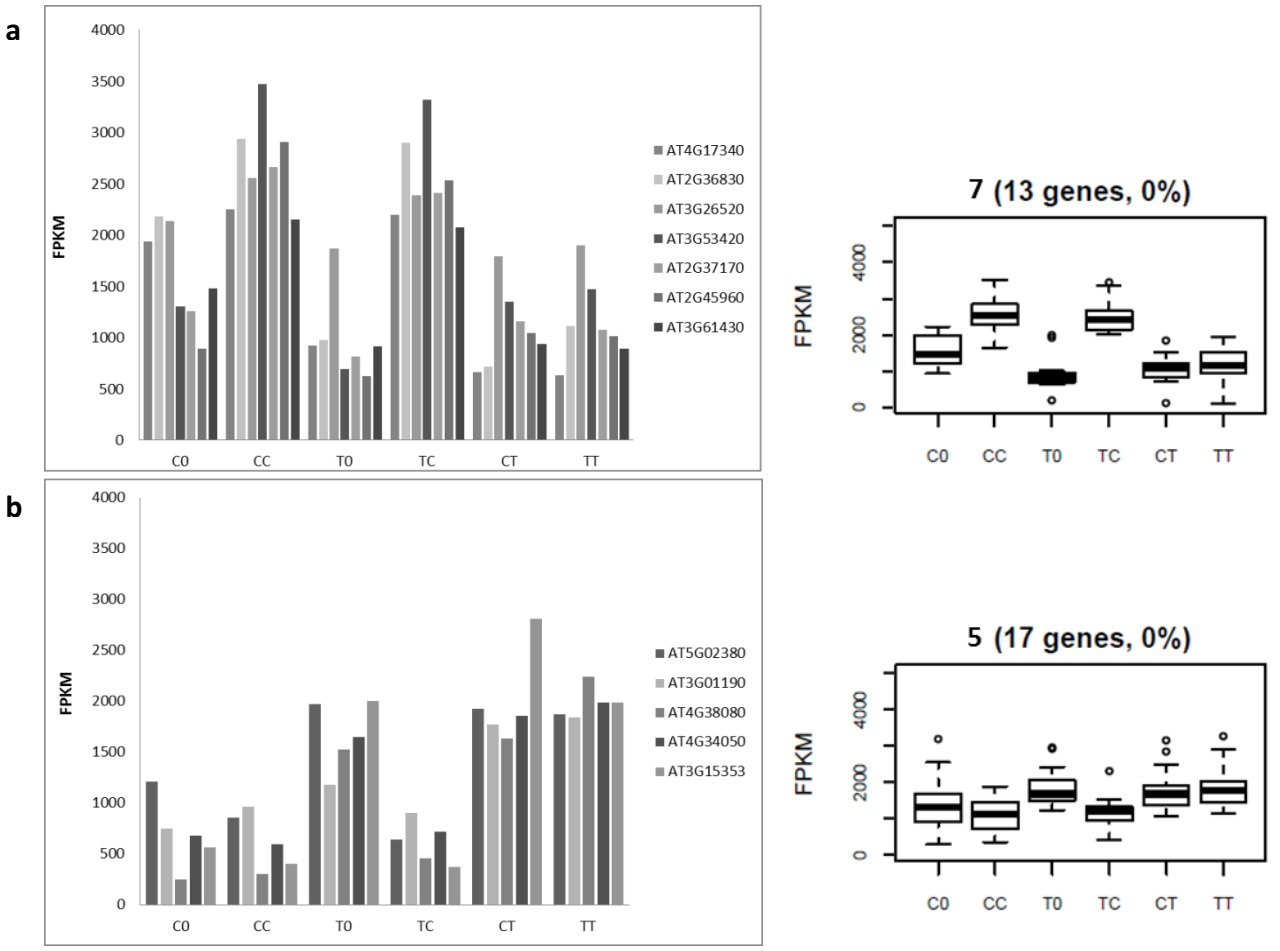


Figure 3.17. Clustering analysis of differentially expressed genes. a) AT4G17340 (tonoplast intrinsic protein), AT2G36830 (gamma tonoplast intrinsic protein), AT3G26520 (tonoplast intrinsic protein), AT3G53420 (plasma membrane intrinsic protein), AT2G37170 (plasma membrane intrinsic protein), AT2G45960 (plasma membrane intrinsic protein), AT3G61430 (plasma membrane intrinsic protein 1) are highly expressed in non N-deprived plants (C0, CC, TC). **b**) Conversely, AT5G02380 (metallothionein 2B), AT3G01190 (peroxidase superfamily), AT4G38080 (hydroxyproline–rich glycoprotein family protein), AT4G34050 (caffeoyl coenzyme A O-methyltransferase 1), AT3G15353 (metallothionein 3 (MT3)) are remarkably expressed in nitrogen starved plants (T0, TT, CT). The analysis was performed with k-means method using software R ($P < 0.05$; n=3).

3.4 Modulation of gene expression in time-course experiment

To corroborate results of time-course physiological experiments (see § 3.2) a quantitative analysis on specific genes expressed in roots were performed using Real Time PCR. In addition, to assess the effect of direct modulation of nitrate on specific genes, four distinct treatments of the second generation were considered:

- plants grown in control condition in the first generation and subjected to N-deprivation for 1 week in the second one (CTc);
- plants subjected to a double deficiency treatment for 1 week in both generations (TTc);
- plants fed with 1 mM of total nitrogen (control condition) in the first generation, subjected to N-deprivation for 1 week in the second generation and supplied with 1 mM nitrate ("*induction"*) during the 24h time-course experiment (CTi);
- plants subjected to N-deprivation treatment (1 week) in both generations and then supplied (TTi).

For the first two treatments only 0h and 8h of root exposure were considered, whereas for the last two 0h, 4h, 8h, 24h were examined. Real time PCR data were confirmed in two independent biological replicates, however the graphs are referred to only one representative experiment. The unit value (Relative gene expression = 1) reported in the graphs is referred to the 0 h of TTc. Genes discussed below, were chosen also on the basis of RNA-sequencing results (see Table 2 and Table S1).

3.4.1 NRT2.1, NRT2.2, NRT2.4 and NRT2.5 gene expression in Arabidopsis thaliana

The transcript amounts of *NRT2.1* and *NRT2.2*, which encode two high-affinity nitrate transporters, were measured. In not induced samples (CTc and TTc) there was no significant increase between 0h and 8h (figure 3.18, a - b) for the transcripts of the two transporters. *NRT2.1* was strongly induced at 8h for CTi treatment, whereas for TTi induction was higher at 4h of exposure. At 24h the expression sharply decreased.

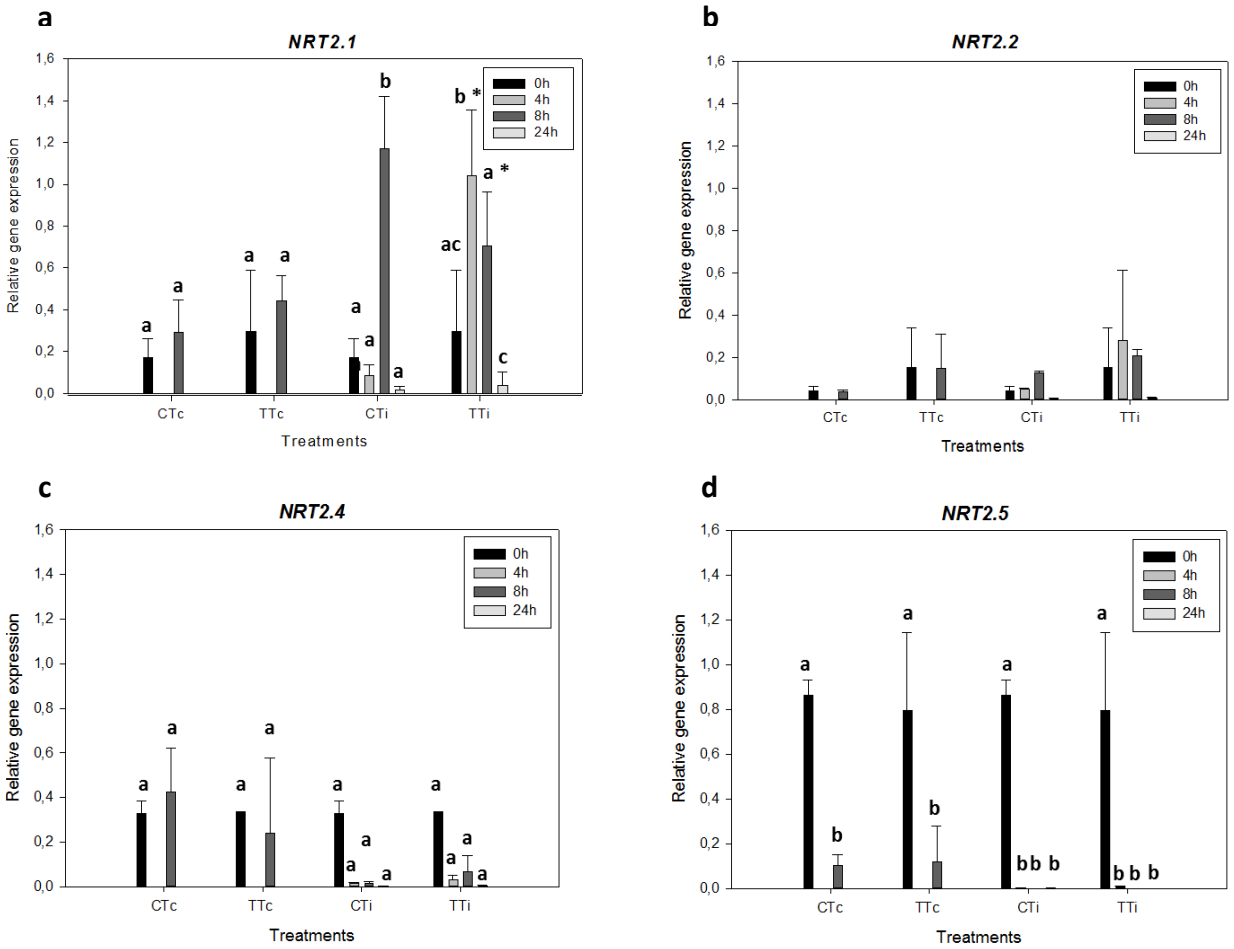


Figure 3.18. Real-time RT-PCR analyses of *NRT2.1* (a), *NRT2.2* (b), *NRT2.4* (c) and *NRT2.5* (d) gene expression levels in *Arabidopsis* roots. Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported; data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h and 8h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

Generally, the induction of *NRT2.2* was lower than that of *NRT2.1* and this latter was statistical evident at 4h for TTi and 8h for CTi. In fact for *NRT2.2* any statistical analyses were possible because the difference in the mean values among the treatment groups was not

great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference (P = 0,240). Moreover, for *NRT2.4* and *NRT2.5*, other high affinity nitrate transporters, stimulated by nitrogen starvation, were not induced by nitrate supply in CTi and TTi at 4h and 8h (figure 3.18, c-d).

3.4.2 NRT1.1, NRT1.3, AHA4 and AHA7-gene expression

Transcription of *NRT1.1* and *NRT1.3*, encoding for two low affinity nitrate transporters, were also investigated by Real-time PCR. In particular *NRT1.1* plays a dual affinity transporter role (high- and low-affinity), thanks to a threonine phosphorylation (T101). As reported by Liu and coworkers (Liu et al., 1999) there is a close temporal correlation between the increase of high-affinity nitrate uptake activity and the *NRT1.1* mRNA level in wild-type plants in presence of nitrate ("*induction"*) (Liu et al., 1999). Data of the present work agreed with these observations (figure 3.19, a). *NRT1.3***,** encoding a dual-affinity nitrate transporter similar to *NRT1.1*, is over-expressed by nitrate deprivation and repressed by the supply of the anion to the roots (Morère-Le Paven et al., 2011). In real time RT-PCR experiment these results were confirmed. In fact at 0h, with plants coming from a period N deprivation (CTc, TTc, CTi, TTi), the expression was higher than later during the nitrate supply.

Interestingly, considering the general trend of expression, plants with a single treatment of nitrogen deficiency and the subsequent supply of nitrate (CTi) showed a stronger down-regulation of genes in comparison with TTi (figure 3.19, b), but this difference was not statistical significant.

As previously reported (see § 1.1.3.2) the proton motive force to take up nitrate through the $NO_3^-/2H^+$ symport is provided by the activity of the plasma membrane (PM) H^+-ATPase (Glass and Siddiqi, 1995). Thus it is reasonable to expect a modulation of gene(s) coding for this enzyme. Indeed, *AHA4* and *AHA7*, two genes encoding for different proton pump isoforms, at 4h of the TTi treatment in comparison with CTi showed a strong induction of expression and this induction is statistical significant in comparison with 0h for *AHA7*. In the CTi treatments the up-regulation was bigger at 8h for AHA7, while no significant changes were found for *AHA4* in the CTi treatment. This shift of modulation from 8h in CTi to 4h in TTi may indicate

an adaptation mechanism in response to the duration and intensity of N-deprivation (figure 3.19, c-d).

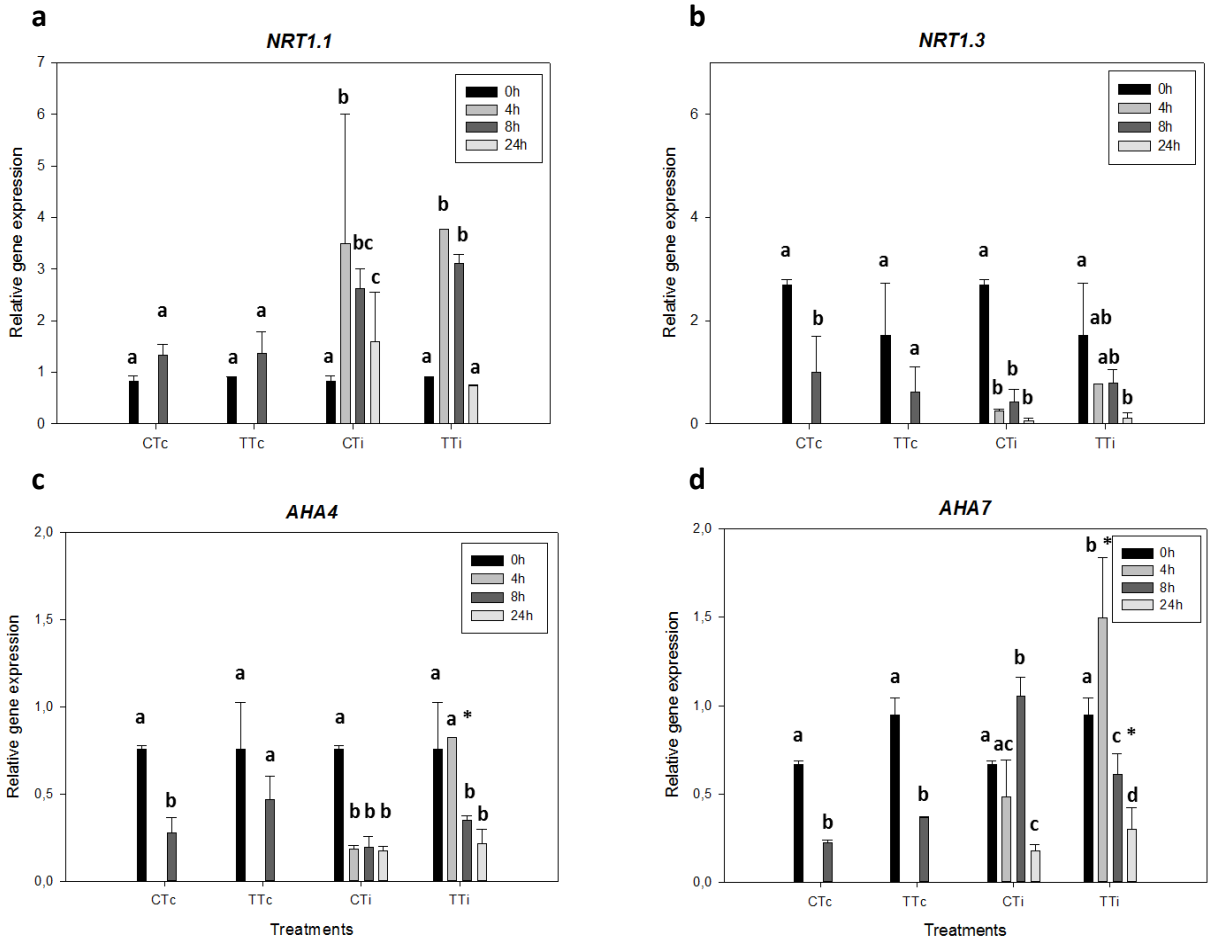


Figure 3.19. Real-time RT-PCR analyses of *NRT1.1* (a), *NRT1.3* (b), *AHA4* (c) and *AHA7* (d) gene expression levels in *Arabidopsis* roots. Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h and 8h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.4.3 N-metabolism involved genes modulation

One of these genes induced by nitrate treatment is *NIA1*, the cytosolic nitrate reductase. As shown in figure 3.20 (a) nitrate reductase was induced especially at 8h of the time-course experiment in CTi and even more in TTi with a statistical difference between two treatments. Another relevant gene in nitrate metabolism is *NiR1*, encoding for the nitrite reductase. Also *NiR1* was over-expressed in CTi and TTi at 8h (figure 3.20, b), but surprisingly in CTi the induction was higher at 4h of treatment with 1mM nitrate producing a statistical difference between the two treatments. As reported by Konishi and Yanagisawa *NIR1* promoter is induced by nitrate via a NRE (nitrate responsive element) sequence located on its promoter (Konishi and Yanagisawa, 2010).

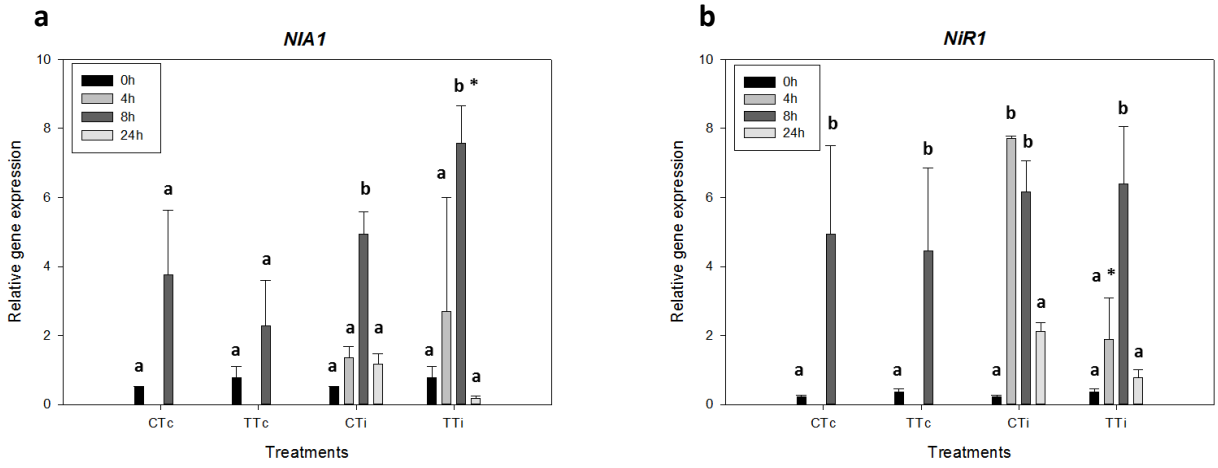


Figure 3.20. Real-time RT-PCR analyses of *NIA1*(a) and *NiR1* (b) transcript levels in *Arabidopsis* roots. Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h and 8h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

As mentioned above ammonium produced by nitrite reduction needs to be incorporated into organic compounds as amino acids. This step involves two genes: glutamine synthetase (GS) and glutamate synthase (GOGAT), also regulated by nitrogen and carbon metabolites. In particular, the cytosolic isoform of GS, *GLN1.4*, that is induced by nitrogen starvation did not display a significant induction of transcript level during the time-course experiment (figure 3.21, a). In addition, *NADH-GOGAT*, also called *GLT1* and expressed in roots, was weakly up-regulated for example at 4 h in TTi treatment (figure 3.21, b), but this is not statistical significant. In fact for *NADH-GOGAT* any statistical analyses were possible because the difference in the mean values among the treatment groups were not great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference (P = 0,662). In literature it was reported that *NADH-GOGAT* expression is stimulated in roots by low levels of ammonium (Hirose et al., 1997) and by nitrate in roots and leaves after nitrogen starvation in tobacco (Lancien et al., 1999).

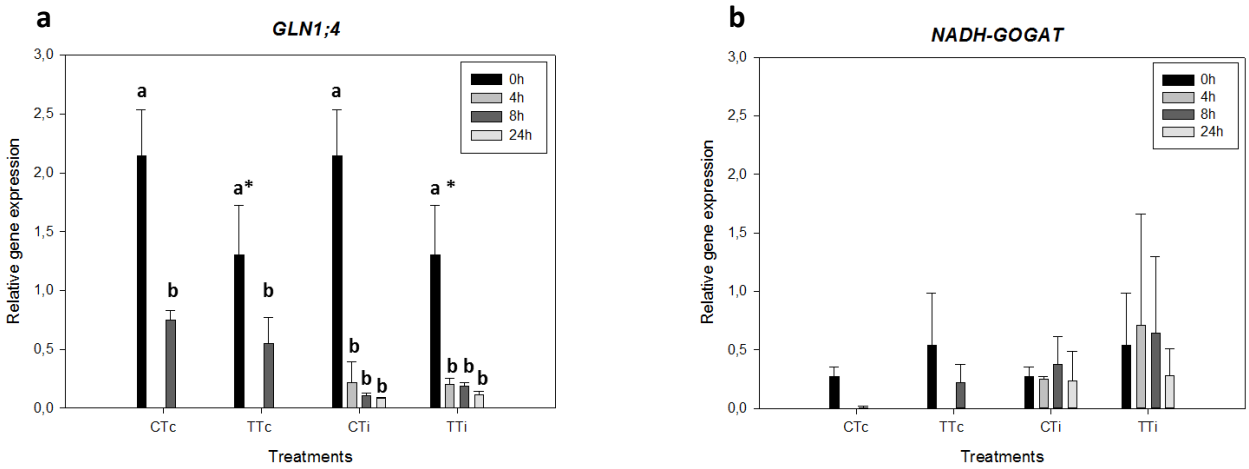


Figure 3.21. Real-time RT-PCR analyses of *GLN1;4* (a) and *NADH-GOGAT* (b) in *Arabidopsis* roots. Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 0h between TTi and CTi and CTc and TTc. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

Interestingly, the gene *AT5G38200*, belonging to a glutamine amidotransferase-like superfamily protein 1 (GAT1), showed a very high statistical significant induction in TTi especially at 4h, with a relative gene expression of 300 in comparison with TTc ,that slowly decreased in successive hours (figure 3.22). This particular class of proteins provides the amidation of known acceptor molecules (e.g. CTP synthesis). Zhu and Kranz demostrated that a homologuos protein *GAT1_2.1* (AT1G15040), is repressed over 50-fold by nitrogen stress, and it is also involved in the control of root branching (Zhu and Kranz, 2012)**.** The expression of latter gene represses shoot branching in *Arabidopsis*. Together with two close neighbors, AT5G38200 and AT1G66860, *GAT1_2.1* shows the conserved catalytic triad residues (Cys-His-Glu), characteristic of the amidotransferase active site, which removes the side chain ammonia from Gln (Zhu and Kranz, 2012). *Arabidopsis* annotation and various BLASTp analyses yielded 30 potential class I Gln amidotransferase proteins in *Arabidopsis* and this cluster is solely present in the plant kingdom.

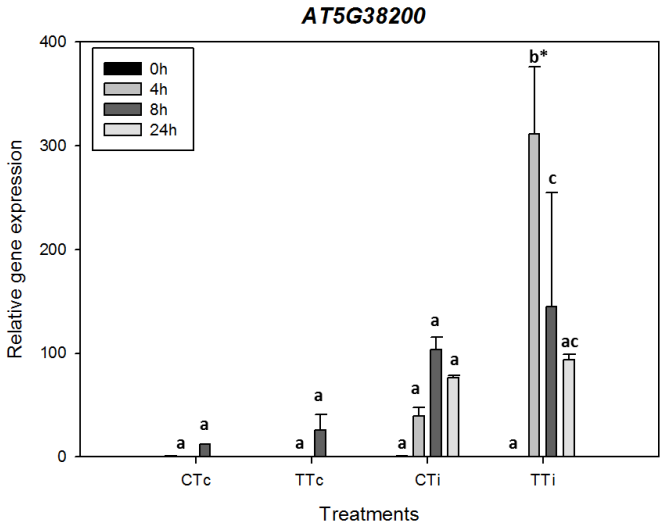


Figure 3.22. Real-time RT-PCR analyses of *AT5G38200*. Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.4.4 ADC2 and PAL3 gene expression

Real time RT-PCR analyses indicated two other genes involved in secondary metabolism that were modulated by different treatments, as already discussed in RNA-sequencing data (see § 3.3.1.6). The first was *ADC2*, isoform 2 of the arginine decarboxylase, which synthesizes putrescine, a polyamine, from arginine. Its expression was significantly induced in CTi at 8h in comparison with TTi, whereas in TTi at 4h the induction is higher (figure 3.23, a). At 24h the expression decreased similarly in CTi and TTi without significant increases in comparison with CTc and TTc.

Furthermore the other gene considered was *PAL3*, an isoform of phenylalanine ammonia lyase, already considered (see § 3.3.1.6) that was down-regulated by nitrogen deficiency. This gene showed a different induction of *ADC2* in terms of temporal modulation in CTi and TTi. Thus it significantly rose at 4h in CTi, but this increase was more than two times higher in TTi, as shown in figure 3.23 (b).

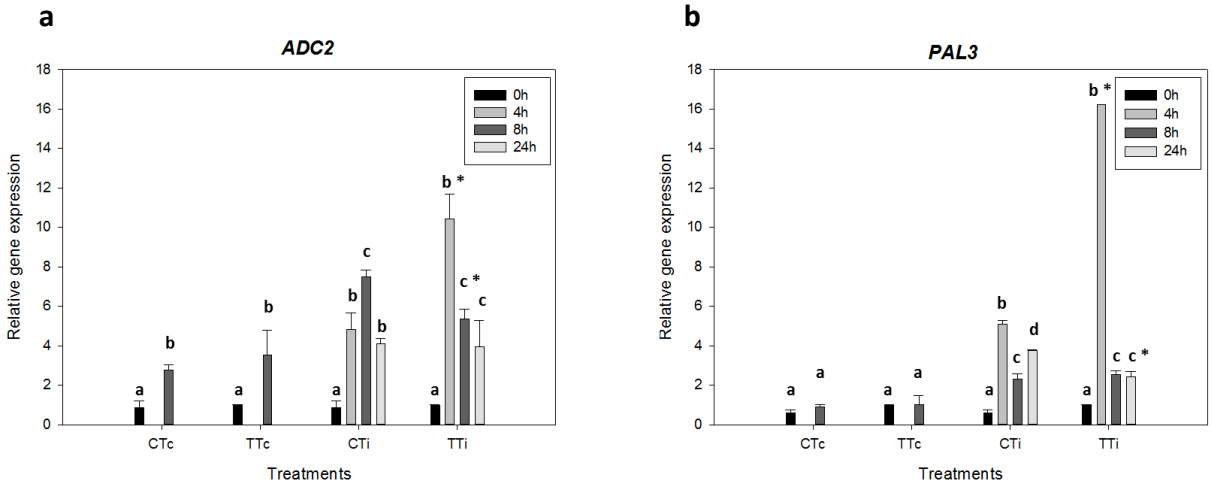


Figure 3.23. Real-time RT-PCR analyses of *ADC2* (a) *and PAL3* (b). Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h, 8h, 24h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.4.5 TIP2;3 and AT3G09340 modulation

By considering previous findings with K-means analyses (see § 3.3.2) the gene *TIP2;3* (AT5G47450), a tonoplast intrinsic proteins, was found to be highly repressed in N-starved samples. With the supply of 1mM nitrate for 24h there was a statistical greater modulation in CTi and TTi sample at 8h, whereas at 4h and 24h there was a decrease (figure 3.24, a). It is noteworthy the significant increment in up-regulation in CTi in comparison with TTi.

Genome-wide expression analysis of this experiment showed that several transmembrane amino acids transporters as well as amino acids permeases were up-regulated in N-deprivation conditions (see § 3.3.1.6). However with the supply of nitrate to deficient plants there was a significant increase in the expression of amino acids transporter (AT3G09340) at 4h of root exposure (figure 3.24, b) in TTi in comparison with CTi.

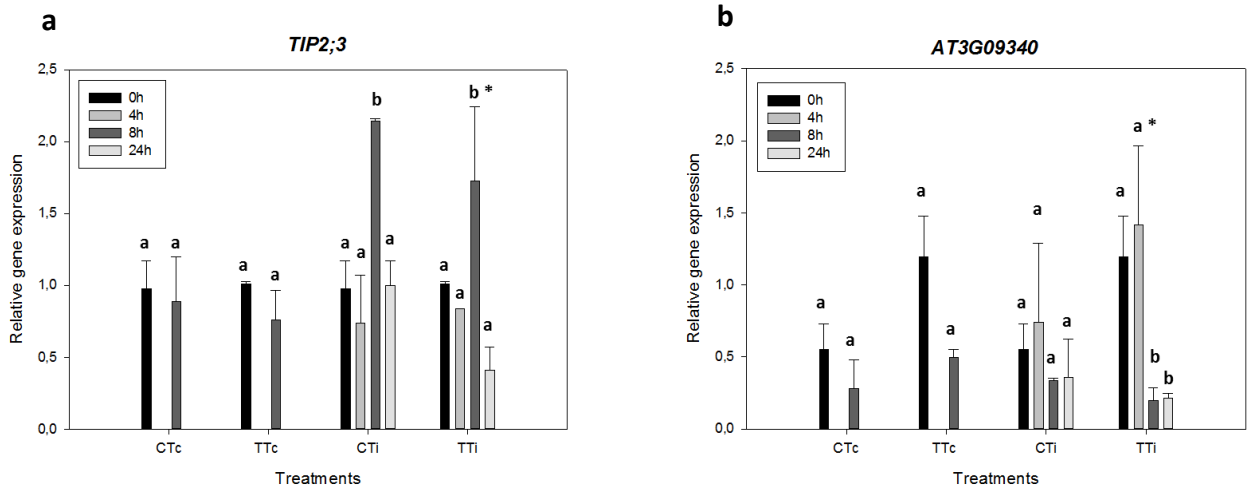


Figure 3.24. Real-time RT-PCR analyses of *TIP2;3* (a) and *AT3G09340* (b). Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h and 8h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.4.6 ALMT1 and MLS gene expression

Two other genes are strongly regulated by nitrate deprivation. The first is *ALMT1*, an aluminum-activated malate transporter; the highest up-regulation of this gene was observed in CTi at 4h, with a relative gene expression of about 70 (figure 3.25, a), significant different in comparison with TTi. As reported by Hoekenga and coworkers this is the best candidate, from the 14-member *AtALMT* family, for Al tolerance (Hoekenga et al., 2006). One of the best-documented physiological mechanism for Al tolerance in *Arabidopsis* and wheat involves the Al-activated release of organic acids from the roots (Kochian et al., 2004). These organic acids are deprotonated anions at the pH found in the cytosol; once transported out of the root, they can effectively form no toxic Al-complexes in the rhizosphere (Kochian, 1995; Kochian et al., 2004). A malate synthase gene (*MLS*), involved in glyoxylate cycle, was over-expressed in CTi at 4h and it was even more significantly up-regulated at 4h in TTi treatment, reaching a relative gene expression of about 180 (figure 3.25, b).

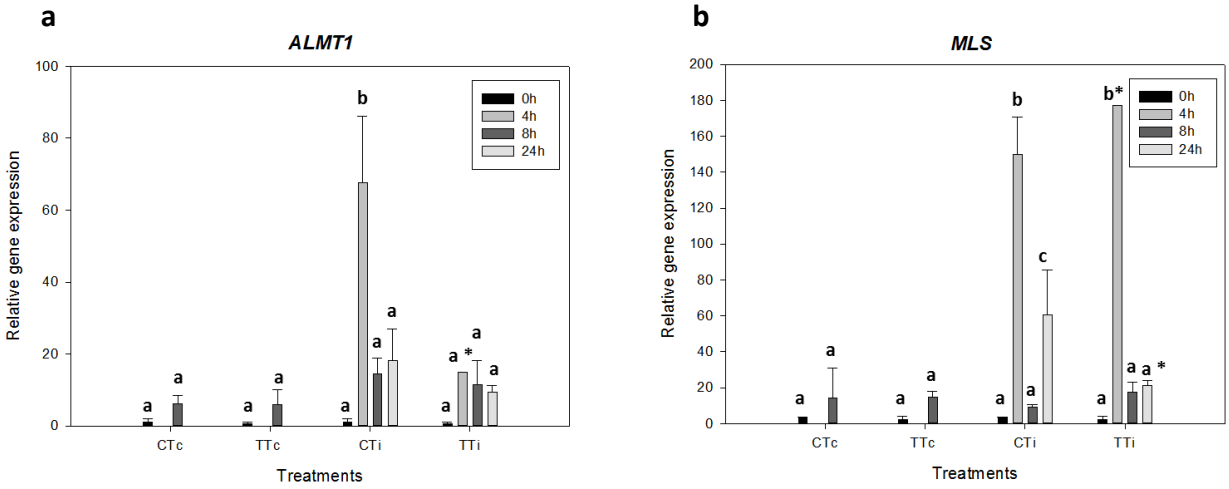


Figure 3.25. Real-time RT-PCR analyses of *ALMT1* (a) and *MLS* (b). Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h and 24h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.4.7 NAS1 and SEL1 gene modulation

In order to maintain the concentration of essential metals within physiological limits in plants, a sophisticated network of homeostatic mechanisms has evolved that serves to control their uptake, distribution, and accumulation within the plant tissues. The main components of metal homeostasis are chelators and transporters. For example metallothioneins as already reported (see § 3.3.2), are over-expressed in treated plants (T0, TT, CT). A higher nicotianamine synthase activity is a key factor for metal hyperaccumulation. Weber and coworkers demonstrated that nicotianamine synthase is implicated in Zn tolerance and hyperaccumulation in *A. halleri* (Weber et al., 2004). In the present work the isoform 1 of nicotianamine synthase (*NAS1*) was mainly induced in CTi and this induction rose until reaching a peak at 24h. Conversely, in TTi the expression increased at 4h and afterwards decreased (figure 3.26, a). The difference between the two treatments is statistical significant at 8h.

A sulfate transporter gene, *SEL1* also termed *SULTR 1;2* (AT1G78000), showed an evident induction of expression in TTi at 4h, also in comparison with CTi. Furthermore, in CTi the up-regulation appeared only at 8h of time course (figure 3.26, b). *SEL1* is primarily involved in importing sulfate from the environment into the root and mainly expressed in the root cortex, the root tip and lateral roots (Shibagaki et al., 2002).

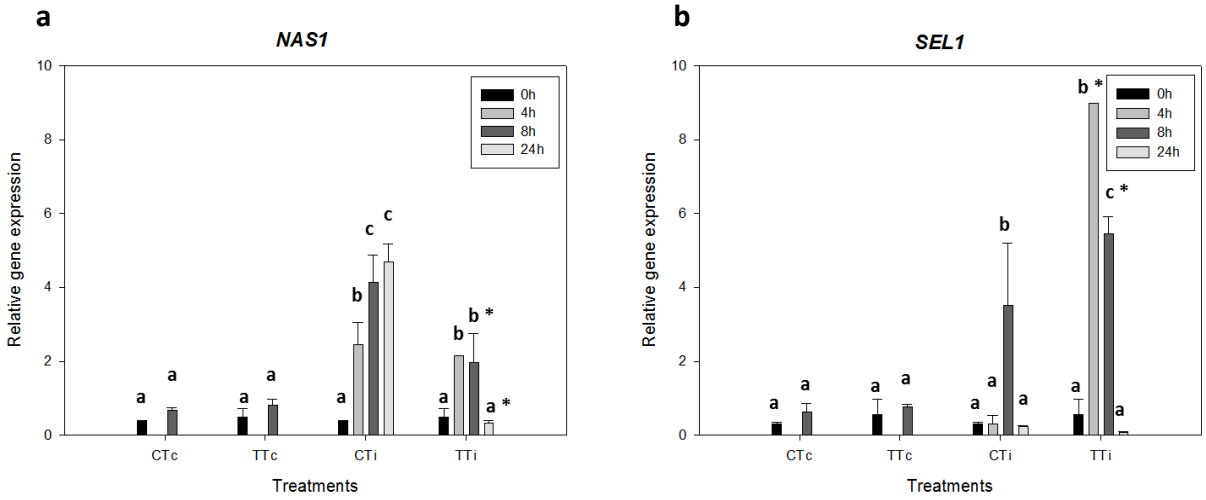


Figure 3.26. Real-time RT-PCR analyses of *NAS1*(a) and *SEL1* (b). Six week-old plants, previously subjected to different nitrogen treatments, were supplied for up to 24 hours with 1 mM nitrate (CTi and TTi). As control some plants were maintained in standard nutrient solution without the addition of nitrogen (CTc and TTc). Root samples were harvested after 0-4-8-24 hours of treatment. A representative experiment for each treatment is reported, data are mean + SD. Gene mRNA levels were normalized with respect to the transcript level of the mean of three housekeeping genes (*Actin2*, *Apt1* and *CBP20*). Small letters refer to statistical significance in the same treatment. Asterisks refer to statistical significance at 4h, 8h and 24h between TTi and CTi. Statistical analyses were performed with Student-Newman-Keuls method ($P < 0.05$; n=3).

3.5 Bisulfite sequencing

In order to assess the establishment of putative epigenetic changes in the *Arabidopsis* genome as a result of stress conditions, a genome-wide analysis of the DNA methylome was carried out on first and second generation. Plants of first and second generation were grown for five weeks with 1 mM of total nitrogen concentration (0.5 mM NH_4NO_3) and in the last week of growth they were exposed to deficiency conditions or maintained in control conditions, following the experimental scheme, in order to obtain 4 different combinations of treatments across two generations (C0, T0, CC, TT) with 2 biological replicates for each combination. At the end of sixth week three roots for each condition were collected and processed to extract DNA as a single pooled sample in order to perform bisulfite-sequencing analysis (BS-seq). From 8 total samples an average of 63 M total reads was obtained. Quality of sequencing was high, as shown by FASTQC analyses and the conversion rate of C (cytosine) to T (thymine) resulted to be over 98% for all samples. Despite this, only 27 M reads (47%) could be mapped on the TAIR10 genome release (Nov 2010) resulting into low coverage. In addition, the bioinformatic analysis revealed an excess of short fragments having been sequenced, with a negative impact on the actual sequencing information available (Emanuele De Paoli, personal communication). Unfortunately, the current Illumina method used for library preparation in this work had been recently developed and, considering the recent introduction of the protocol into the market, no sufficient literature exists about method and troubleshooting. Nevertheless, the sequencing data were deemed sufficient to perform the planned analyses. Indeed, as shown in the figure 3.27, the representation of the DNA methylome recapitulated the features described by the seminal work by Lister et al. (2008): the level of methylation in all the three different contexts (CG, CHG, CHH) was larger in pericentromeric regions, where gene density is lower and the frequency of transposable elements higher, than in the euchromatic chromosome arms (Lister et al., 2008). This methylation pattern was similar in each replication of each treatment in both generations (see figure S1, S2, S3).

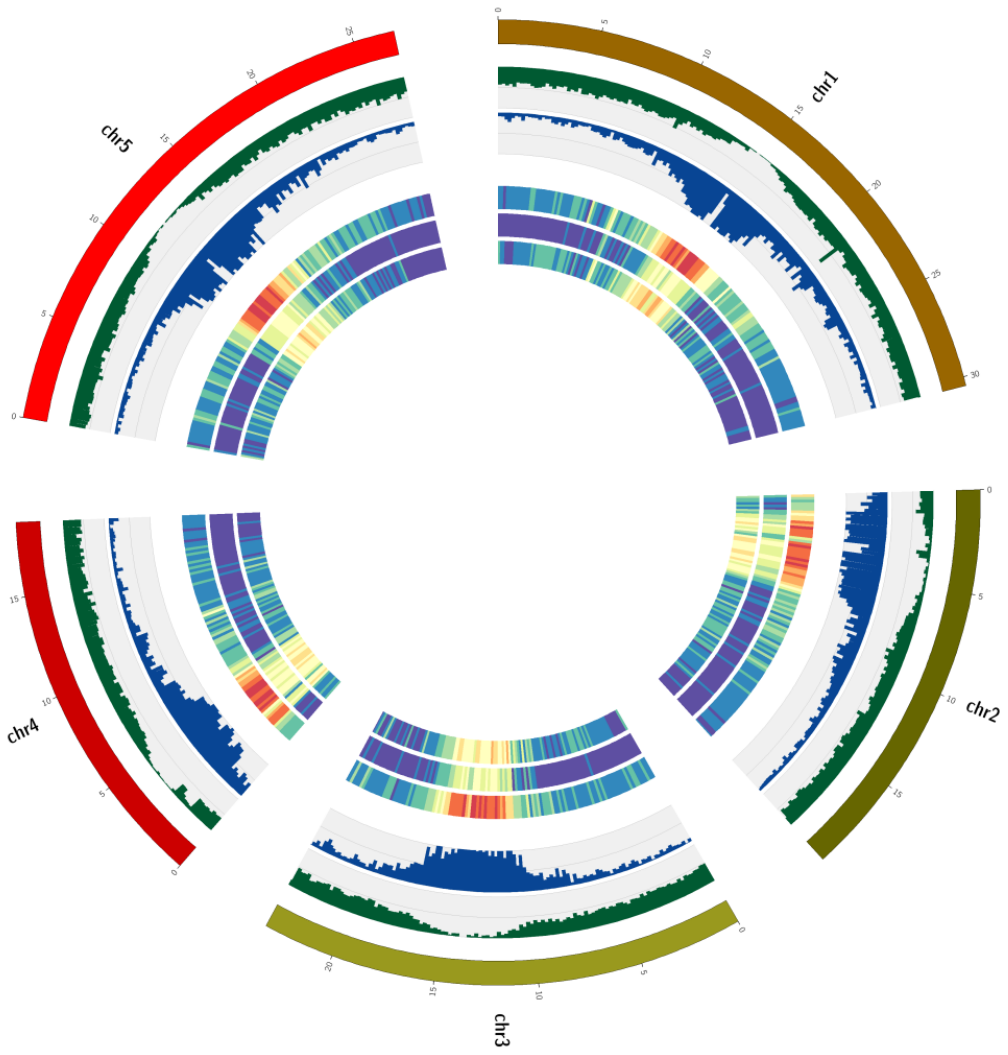


Figure 3.27. Schematic representation of the *Arabidopsis* DNA methylome in N-deprived samples of the first generation (T0). The graph shows, from the outer to the inner circle, chromosome ideograms of the *Arabidopsis* genome, gene density, frequency of transposable elements (TE), methylation in the CG context, methylation in the CHG context and methylation in the CHH context. Methylation in the CG, CHG, CHH contexts are shown with a color scale from blue (-100%) to red (+100%). The graph was produced with the Circos software (www.circos.ca - Krzywinski et al., 2009) and represented the average of two biological replicates.

3.5.1 Analysis of differentially methylated regions (DRMs)

A general analysis with the MethylKit Package (Akalin et al., 2012) of the R software was performed in order to collect information about the homogeneity of replicates, evaluate differentiation between replicates of samples treated with N-deficiency (T0-1, T0-2, TT-1, TT-2) and replicates of control samples (C0-1, C0-2, CC-1, CC-2) in first and second generation and finally identify differentially methylated regions (DRMs). All the analyses were performed by evaluating DNA methylation levels across 500 bp genomic windows, which assured a sufficient number of Cs being screened per windows. As shown in figure 3.28, performing a principal component analysis (PCA) of CG methylation all replicates of the treated samples appeared relatively similar. Conversely, all replicates of the control samples resulted very different and distant from each other.

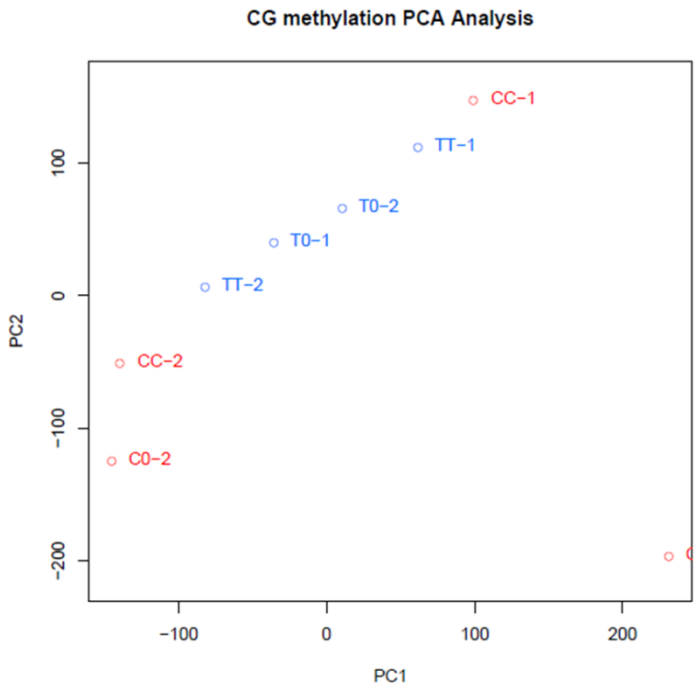


Figure 3.28. Principal component analysis (PCA) of methylation in CG context. A cluster analysis based on PCA method was performed in all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and in all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012).

These analyses were also performed on the CHG and CHH context, obtaining a different scenario where treated samples did not differentiate from controls in a consistent way as they did with the CG context (see figure S4 and S5). A hierarchical clustering analysis performed with the same package and using the Ward method, was carried out to correlate samples based on similarity of DNA methylation and confirmed the same sample structure when applied to the CG context. Indeed, all replicates of the treated samples (T0-1, T0-2, TT-1, TT-2) appeared strongly related to each other and isolated from the control samples. In contrast, the replicates of the control samples (C0-1, C0-2, CC-1, CC-2) did not cluster in a unique clade, indicating that the divergence within the set of control samples exceeded the distance between control and treated samples (figure 3.29). In light of these results, the N-deprivation treatment seemed to elicit an epigenetic response common to all the treated samples.

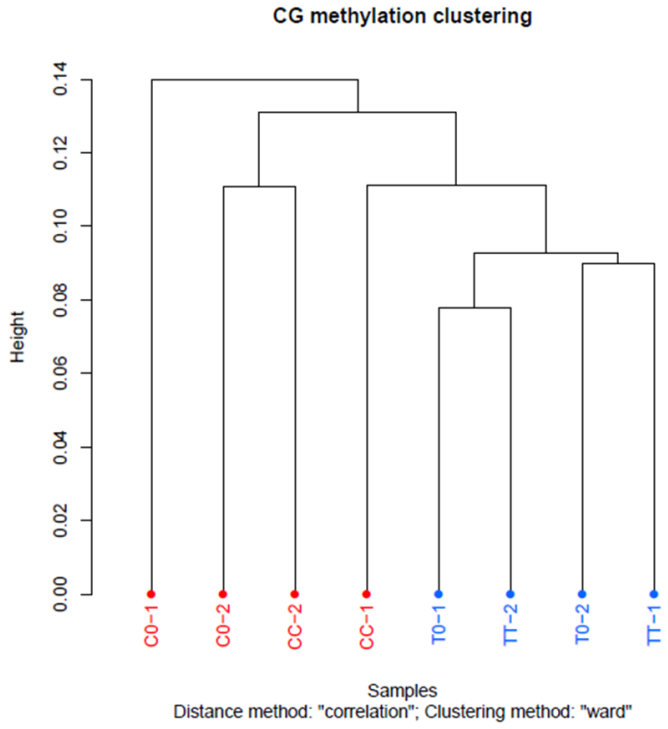


Figure 3.29. Clustering analysis of methylation in CG context. A clustering analysis based on CG methylation was performed on all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and on all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012).

These clustering analyses were repeated also for CHG and CHH context, but the separation between treated and control samples seemed to be less clear. In these dendrograms the effect of generations appears more prevalent than the effect of treatment itself, as shown in figure 3.30 and 3.31.

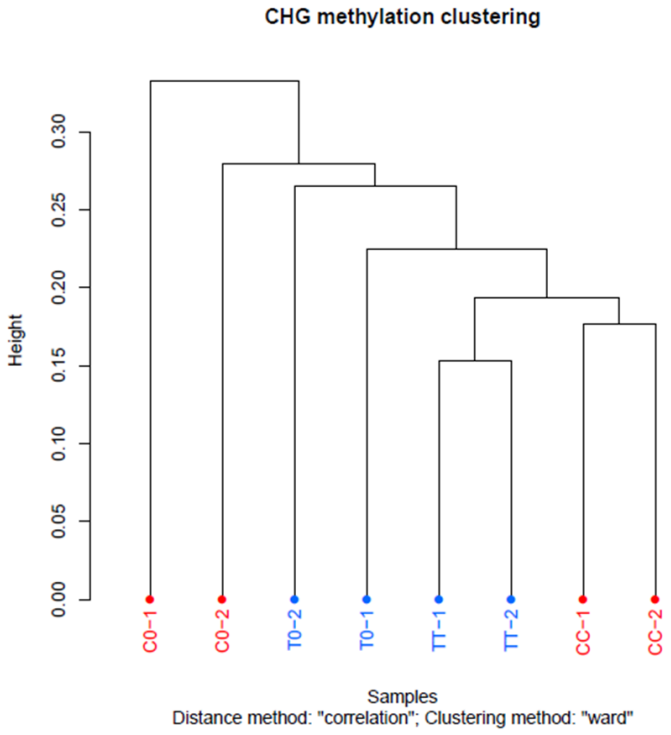


Figure 3.30. Clustering analysis of methylation in CHG context. A clustering analysis based on CHG methylation was performed on all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and on all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012).

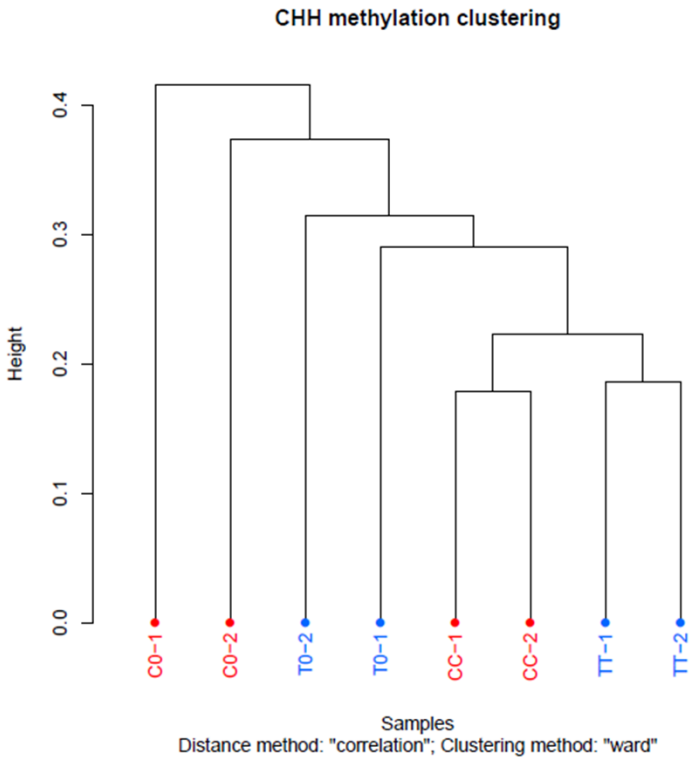


Figure 3.31. Clustering analysis of methylation in CHH context. A clustering analysis based on CHH methylation was performed on all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and on all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012).

In light of the macroscopic effect of the treatment on DNA methylation revealed by the clustering analysis, we asked whether a specific subset of genomic windows could lead the genome-wide correlation between methylation signal and treatment. Whereas K-means clustering of genomic windows based on their methylation level across the eight samples fell short in identifying regions leading this correlation (data not shown), we verified that the treated samples showed a quite consistent reduction of DNA methylation relative to controls in the CG and CHG sequence contexts genome-wide. In particular, hypomethylated 500 bp regions in the treated samples outnumbered the hypermethylated regions by 5% and 17% for the CG and CHG context respectively. The difference was significant in six out of eight possible treatment vs control comparisons for the CG context and in seven out of eight comparisons for the CHG context (Fisher's Exact Test, $P < 0.05$). In contrast, the CHH showed

an opposite trend with a 5% (Fisher's Exact Test, P < 0.05) excess of hypermethylated regions in the treated samples. Moreover, a meta-analysis of gene body DNA methylation levels across the total number of *Arabidopsis* annotated genes, including 2500 bp upstream and downstream regions, displayed a clear stratification with the treated samples being on average less methylated than controls across the entire genic sequence analyzed (figure 3.32).

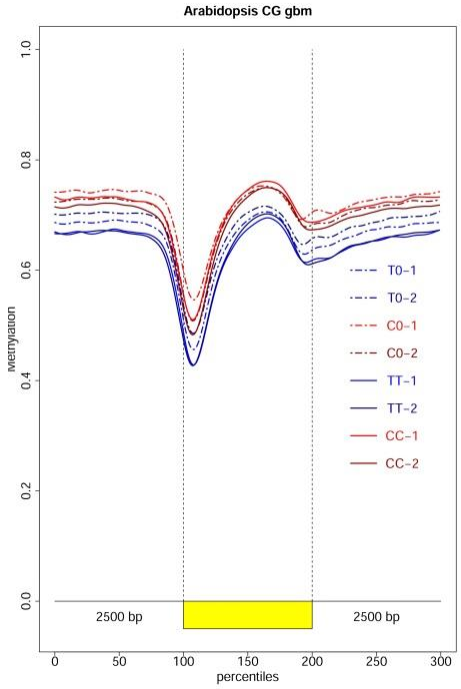
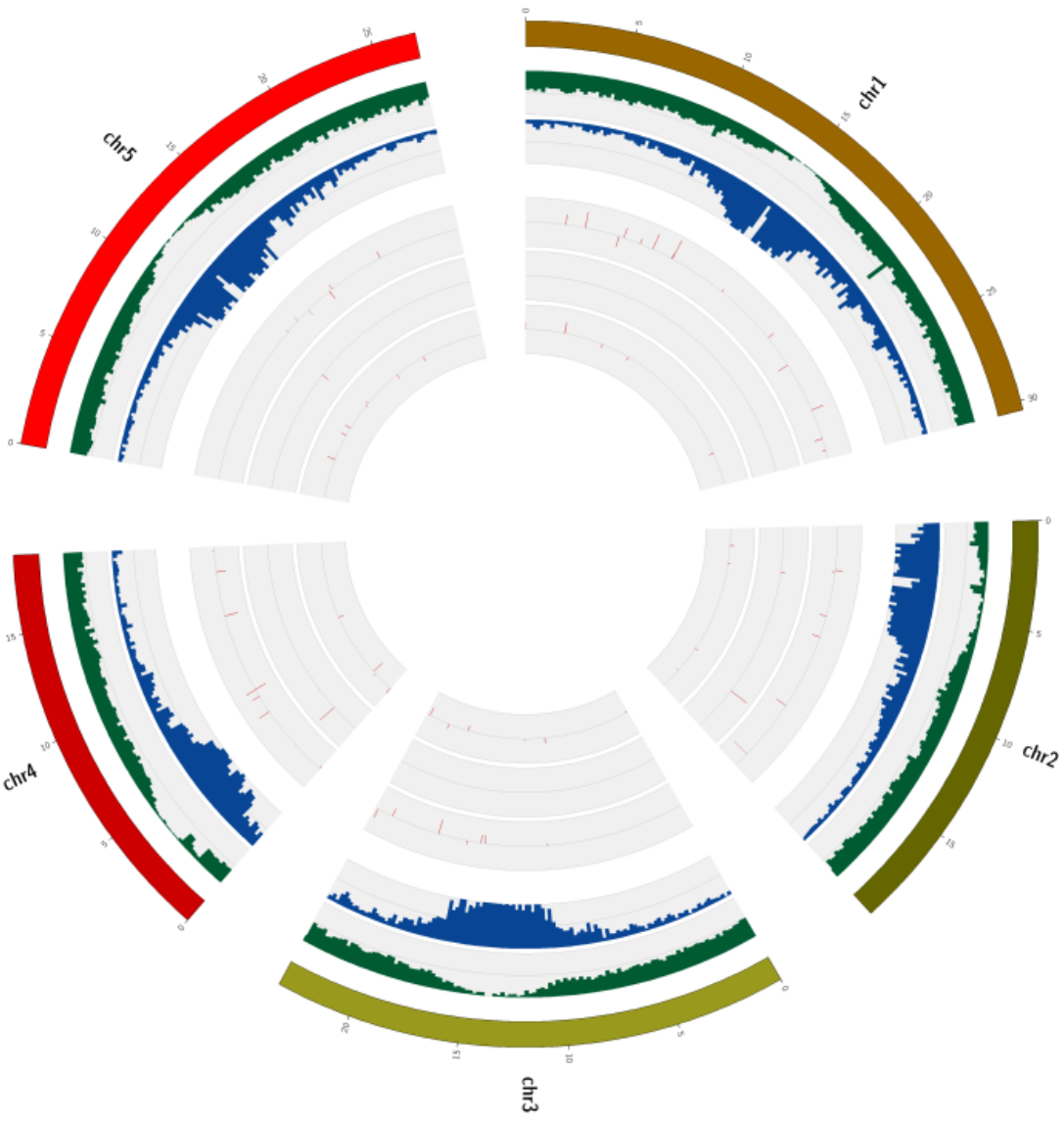


Figure 3.32. Meta-analysis of gene body (CG context) DNA methylation levels of *Arabidopsis* annotated genes. The analysis included 2500 bp upstream and 2500 bp downstream regions of the transcribed sequence. In blue treated samples of first and second generation (T0-1, T0-2, TT-1, TT-2); in red control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed using the R software.

The DNA methylation analyses above described were based on an unfiltered set of genomic windows distributed across the entire genome. In order to identify differentially methylated regions (DRMs) on a local scale and at high confidence, a differential analysis of methylation in all the three contexts (CG, CHG, CHH) was separately performed in first and second generation comparing each treated sample (T0-1, T0-2, TT-1; TT-2) with both of their

respective controls in the same generation (C0-1 and C0-2 for first generation and CC-1 and CC-2 for second generation). DMRs were taken into account for follow-up analyses if they were identified as significant in each treated vs control comparison within the same generation, in order to discard background noise. As clearly shown in figure 3.33, significant DMRs (Fisher's Exact Test, q-value <= 0.05) are more frequent in second generation (b), in particular in the CG context, whereas the frequency of DMRs in first generation is very low.

a



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b

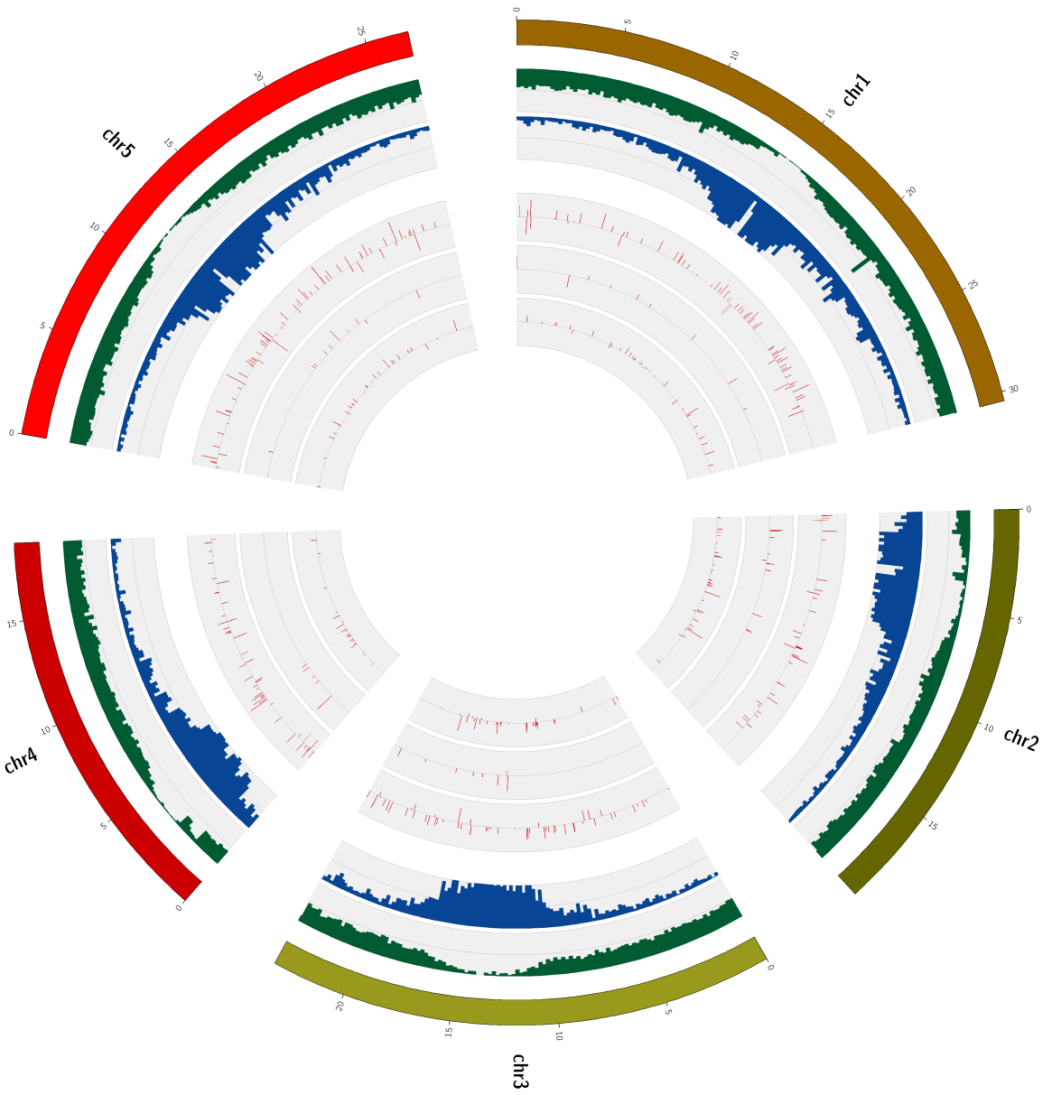

Figure 3.33. Schematic representation of common DRMs in the CG, CHG and CHH contexts of the *Arabidopsis* methylome identified in all N-deprived samples of first generation (a) and second generation (b) in comparison with their respective controls. The graph shows from the outer to the inner circle chromosome ideograms of the *Arabidopsis* genome, gene density, frequency of transposable elements (TE), DRMs in CG context, DRMs in CHG context, DRMs in CHH context. The graph was produced with the Circos software (www.circos.ca-Krzywinski et al., 2009).

3.5.1.1 Analysis of genes in DMRs

In order to verify a correlation between the variation in gene expression previously found and changes in the methylation status, a deeper analysis of DMRs was carried out. This analysis was separately performed in the two generations and in all three methylation contexts to try to identify genes involved in DMRs. Considering the possible effects of epigenetic changes in regulatory regions, genes were deemed in association with a DMR provided that the DMR were located within the transcribed sequence, in the 2 kb promoter or in the 2 kb trailer (region downstream to 3'-UTR end). Some of the identified genes had also appeared differentially expressed between treated and control samples in either or both generations (T0 vs C0, TT vs CC, CT vs CC comparisons). DMRs involving genes were more abundant for the CG and CHH contexts in the second generation but differentially expressed genes were mainly associated with DMRs in the CHG and CHH contexts. Results are summarized in Table 4.

Table 4. Number of genes found in association with DMRs and differentially expressed as a result of nitrogen treatments. Data provided by BS-seq analyses performed on DNA extracted from root of *Arabidopsis* grown under different nitrogen treatment in two generations. For each sequence context and generation numbers in the first row indicate the total number of DMRs, numbers in the second row are referred to the total number of genes found in association with DMRs (see main text for details); in the third row is reported the number of differentially expressed genes identified by RNA-seq in the following comparisons, T0 vs C0, TT vs CC and CT vs CC. . This experiment was performed using three independent biological replicates for the transcriptional analysis and two for the DNA methylation analysis.

	CG		**CHG**		**CHH**	
	1st generation	**2nd generation**	**1st generation**	**2nd generation**	**1st generation**	**2nd generation**
N° of treated vs control DMRs	43	284	5	45	27	162
N° of genes in association with DMRs	73	481	4	46	42	179
N° of genes with changes in expression value	0	0	0	5	6	26

An enrichment analysis of the ontological classes to which these genes belonged (Fisher's exact test, $P < 0.05$) identified a significant number of genes involved in transport of nitrate, nitrogen and ammonia metabolism, secondary metabolism of phenylpropanoids, abiotic stress and cell wall modification as discussed above in the RNA-seq results (see § 3.3.1 and Table 5). However, after Bonferroni Correction of p-values, only one class (misc. gluco-, galacto and mannosidases) remained statistically significant, with a q-value lower than 0.05.

Among the various differentially expressed genes associated with DMRs (see transcriptomic section, § 3.3.1 and Table S1), there were genes belonging to photosystem I, *LHCA2* (#1, Table S2) and *PSAL* (#12, Table S2), one gene of the transmembrane amino acid transporter family (#24, Table S2) discussed above (see § 3.3.1.6) as well as two genes of cytochrome P45 (#3 - #13, Table S2). All these genes were maintained down-regulated in the treated samples of the two generations. *LHCA2* and the two genes of cytochrome P45 showed a decrease in methylation, whereas PSAL and the genes encoding for the transmembrane amino acid transporter increased their methylation. The gene encoding the MATE efflux family protein (#5 and #26, Table S2) is over-expressed as associated with both CHG and CHH DRMs (decreased methylation). The gene *PSAL* (#12) down-regulated in first and second generation and found in association with a CHH DMR was maintained down-regulated also in third generation, where there was no N-deprivation treatment (see below § 3.6 - #4, Table 9).

DMRs can arise in different regions of a gene or near a gene. However, as shown in table S3, no obvious DMR enrichment was observed for any of the compartments taken into account.

Table 5. List of ontological category to which genes found in DMRs in the two generations and all three contexts (CG, CHG, CHH) belong. In the table are shown the list of enriched ontology classes (category), with a p-value < 0.05 (Fisher's exact test) and relative q-value (Bonferroni correction). The only class with q-value < 0.05 is highlighted in green.

Category	CG 1st generation		CG 2nd generation		CHG 1st generation		CHG 2nd generation		CHH 1st generation		CHH 2nd generation	
	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
tetrapyrrole synthesis uroporphyrin-III C-methyltransferase	0,006367	1,789023044										
minor CHO metabolism galactose	0,01898	5,333433714										
cell division	0,022294	6,264537643										
signalling receptor kinases	0,029308	8,235475343										
transport nitrate	0,031436	8,833458913										
protein post-translational modification			0,001022	0,287231								
gluconeogenesis / glyoxylate cycle citrate synthase			0,001862	0,523088								
misc plastocyanin-like			0,002237	0,628706								
lipid metabolism FA synthesis and FA elongation			0,004172	1,172466								
transport sugars			0,006288	1,766914								
cell wall pectin synthesis			0,00646	1,815158								
protein degradation			0,008251	2,318646								
misc dynamin			0,020199	5,67593								
N-metabolism ammonia metabolism			0,020199	5,67593								
RNA processing			0,020649	5,802505								
transport Major Intrinsic Proteins			0,024374	6,849111								
protein glycosylation			0,030399	8,542188								
tetrapyrrole synthesis uroporphyrinogen decarboxylase			0,040973	11,51336								
transport calcium			0,043214	12,143								
cell wall precursor synthesis			0,048535	13,63831								
secondary metabolism phenylpropanoids					0,014169964	3,98176						
cell wall hemicellulose synthesis							0,000651	0,182796				
transport misc							0,013621	3,827391				
not assigned ontology							0,023704	6,66082				
misc cytochrome P450							0,043763	12,29734				
cell cycle									0,000573	0,161094		
stress abiotic									0,017545	4,93021	0,009528	2,677427
amino acid metabolism synthesis									0,028334	7,961847		
not assigned unknown									0,045512	12,78875		
misc gluco-, galacto- and mannosidases											3,94E-05	0,011058
signalling calcium											0,007967	2,238652
gluconeogenesis / glyoxylate cycle Malate DH											0,01543	4,335741
lipid metabolism glyceral metabolism											0,035635	10,01342
lipid metabolism exotics (steroids, squalene)											0,041051	11,53538

3.5.1.2 Analysis of transposable element (TE) associated with DMRs

Transposable elements (TEs) are the major component of several plant genomes and significantly contribute to interspecific genome size variation (Zhang and Wessler, 2004). *Arabidopsis* holds all of the TE types found in larger plant genomes; however, their presence is generally limited, with all TEs estimated to account for only 10% of the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000). Furthermore, transposable elements can regulate genes epigenetically, but only when inserted within or very close to them (Lippman et al., 2004).

In this study, 48 TE families had members significantly (Fisher's exact test, $P < 0.05$) affected by DNA methylation changes (DMRs) identified in at least one generation and one cytosine sequence context (Table 7). However, only three TE families, namely ATDNA12T3, ATGP1 and ATGP3, remained significantly enriched after Bonferroni correction (q-value < 0.05).

Some TEs presenting DMRs in their sequence were located at limited distance (≤ 2000 bp) from the ends of annotated genes or even within the transcribed sequence (Table 6). However, only 14 distinct genes were differentially expressed in the T0 vs C0, TT vs CC, and CT vs CC comparisons already considered in RNA-seq analyses (see Table S4, Table S1 and § 3.3.1). Two TEs of the COPIA68 and SINE4 family respectively showed a reduced methylation (DMRs) in multiple sequence contexts and were associated with differentially expressed genes. The first gene was *LHCA2* (#4- #8, Table S4, §3.5.1.1), belonged to photosystem I light harvesting complex and was down-regulated in the T0 vs C0 comparison. The second gene encoded the MATE efflux protein (#9-#13, Table S4) and was up-regulated in T0 vs C0 and TT vs CC comparisons.

Table 6. Number of genes found near and within differentially methylated TEs. Data provided by BS-seq analyses performed on DNA extracted from root of *Arabidopsis* grown under different nitrogen treatment sin two generations. For each context and generation, in the first row the number of genes near and within differentially methylated TEs is reported in the table; in second row the number of these genes differentially expressed in comparison T0 vs C0, TT vs CC, CT vs CC. This experiment was performed using three independent biological replicates.

	CG		**CHG**		**CHH**	
	1st generation	**2nd generation**	**1st generation**	**2nd generation**	**1st generation**	**2nd generation**
Genes associated with differentially methylated TEs	8	35	3	11	15	55
N° of genes with changes in expression value	3	4		2	1	6

Table 7. Enrichment of TE families affected by DMRs. In the table is shown the list of TE families, with a p-value < 0.05 obtained with a Fisher test and relative q-value (Bonferroni correction, not always < 0.05, in green the family with q-value < 0.05).

TE family	CG 1st generation		CG 2nd generation		CHG 1st generation		CHG 2nd generation		CHH 1st generation		CHH 2nd generation	
	p-value	q-value	p- value	q-value	p-value	q-value	p-value	q-value	p-value	q-value	p-value	q-value
ARNOLD3							0,047514489	15,20463661				
ATCOPIA25											0,048068282	15,38185028
ATCOPIA3											0,043366631	13,87732189
ATCOPIA51											0,002750963	0,880308236
ATCOPIA68			0,009867611	3,157635672			0,045494956	14,55838602				
ATCOPIA82											0,038641909	12,36541103
ATCOPIA89			0,042361401	13,55564826								
ATCOPIA9							0,01052085	3,366671921				
ATDNA12T3_2											0,000128749	0,041199712
ATDNAI2T9B											0,020682025	6,618247975
ATGP1											1,02921E-05	0,003293482
ATGP2			0,012079318	3,865381605								
ATGP2N	0,000243659	0,077970855										
ATGP3							4,09093E-05	0,013090965				
ATHATN10			0,012079318	3,865381605								
ATHATN5	0,017771516	5,686884988										
ATHILA2	0,012493375	3,997879908										
ATHILA3							0,00221388	0,708441707			0,007760324	2,483303763
ATHILA4C							0,020364919	6,516774066				
ATHILA6A			0,001565957	0,501106168								
ATHILA8A					0,012508422	4,002694998						
ATHILA8B	0,032425295	10,37609431										
ATLANTYS3			0,012621144	4,038766168								
ATLINE1_4	0,042082081	13,46626606										
ATLINE1_5									0,046153934	14,76925879		
ATLINE1A					0,036674176	11,73573628						
ATN9_1									0,033317064	10,66146063		
ATREP12					0,002946261	0,942803434						
ATREP16											0,024256977	7,762232798
ATSINE4									0,049582608	15,8664347	0,013648277	4,367448603
BRODYAGA1			0,001677741	0,536877249								
META1									0,002301319	0,736422072	0,032530765	10,40984485
RathE1_cons	0,003506418	1,12205375										
RathE2_cons									0,037778813	12,08922023		
RathE3_cons											0,000207791	0,066493097
RP1_AT											0,001074993	0,343997836
SIMPLEHAT2	0,030402188	9,728700004										
TA11							0,012269883	3,926362597				
Unassigned											0,019686319	6,299622046
VANDAL1	0,033233446	10,63470272										
VANDAL14	0,010780707	3,449826312										
VANDAL15							0,007375917	2,360293467				
VANDAL16			0,009449369	3,023798113								
VANDAL18							0,015740945	5,037102366				
VANDAL2									0,022328778	7,145208975		
VANDAL2N1											0,028064609	8,980674837
VANDAL3							0,015359087	4,914907827				
VANDAL6			0,00361393	1,156457506							0,010618032	3,39777027

3.5.2 Methylation changes in specific genes

After a general analysis of differential methylation in all samples starting from the identification and characterization of anonymous DMRs (see § 3.5.1), a specific analysis of methylation variation was performed on single genes previously pinpointed by the transcriptomic analyses of three generations. About 150 genes were selected by analyzing their transcriptomic profiles.

For these analyses two replicates of four treatments were considered:

- control plants (C0) without N-deprivation in first generation;
- N-deprived plants for 1 week (T0) in first generation;
- plants grown without N-deficiency treatment in both generations (CC);
- plants subjected to a double deficiency treatment in both generations for 1 week (TT).

The methylation status of the 150 genes was examined by manual inspection, in order to check for possible changes in the shape of the methylation patterns that the DMR analysis previously performed by the MethylKit package might have overlooked. DNA methylation changes in all the three sequence contexts (CG, CHG and CHH) were analyzed between treated and control samples in first and second generation (T0 vs C0 and TT vs CC) taking into account promoter, transcribed region and trailer of each gene. Considering the technical issues encountered in bisulfite sequencing and in order to be less stringent in this approach, we considered a relaxed coverage threshold of 4X to accept Cs for methylation estimates. From this analysis only one gene, escaped from the previous DMR detection, was identified as changing in its methylation pattern.

This gene is AT4G21990, commonly named APR3, and encodes a protein disulfide isomerase-like (PDIL) protein, member of a multigene family within the thioredoxin superfamily. As shown in figure 3.34 the most significant change occurred in the gene body with a strongly increased level of methylation. Specifically, this increment was particularly observed in the CHH context and in samples treated in both generations (TT). The thioredoxin family was previously described in transcriptomic analyses as a large family modulated from N-starvation (see § 3.3.1.7) and involved in oxidative stress response. From transcriptomic data such gene resulted down-regulated in the TT vs CC and CT vs CC comparisons and when

considering the third generation, maintained in control condition, this gene remained down-regulated also in the TTC vs CCC and TCC vs CCC comparisons (Table 8), possibly in association with this evident methylation change.

Table 8. Expression changes in the APR3 gene. Different treatments in second and third generation were considered to evaluate expression variations. The gene was down-regulated in all the four comparisons considered.

Gene_ID	Symbol	log2-ratio TT vs CC	p-value	log2-ratio CT vs CC	p-value	log2-ratio TCC vs CCC	p-value	log2-ratio TTC vs CCC	p-value
AT4G21990	APR3	-1.52733	0.0001	-2.44327	0.00005	-1.47569	0.00005	-1.19084	0.00005

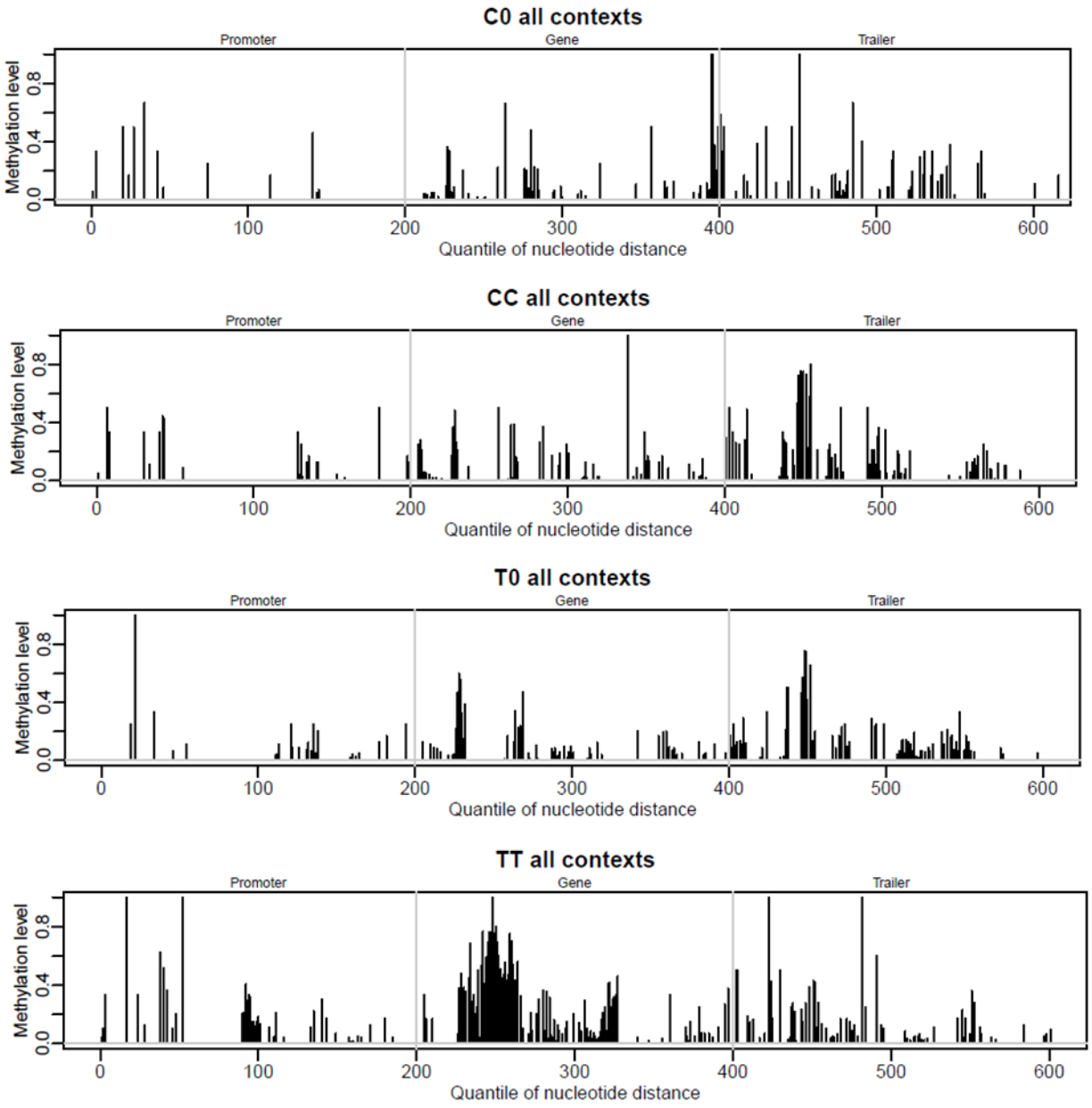

Figure 3.34. Representation of methylation variations in the APR3 gene. The graph shows the methylation level across the APR3 gene, including 2 kbp of promoter and downstream region, as a mean of methylation values in all the methylation contexts (CG, CHG, CHH). The value is also an average of 2 replicates for each of 4 combinations of treatments across first and second generation: C0, CC, T0, TT. The graph was produced with the R software.

3.6 Transgenerational transcriptomic "memory"

In order to evaluate the possible occurrence of a transgenerational memory, a genome-wide expression analysis was also performed in the third generation. Plants derived from the second generation were fed for six weeks with 1 mM of total nitrogen (0.5 mM NH_4NO_3). At the end of the sixth week three roots for each growth condition (CCC, CTC, TTC, TCC) were collected and processed to extract RNA, as reported above for the first and the second generation. By considering all the 12 samples of the third generations an average value of 57 M total reads were obtained, but only 55 M were accepted for analysis, of which 54 M mapped on TAIR10 release (Nov 2010). The mean value of unmapped reads was 33.5 M. Quality of sequencing was very high, as resulted from the FASTQC analysis and as previously reported for the first two generations.

Since plants of the third generation are grown under control conditions, the main objective of the transcriptomic analysis was to identify genes showing positive or negative modulation due to the treatments received during the first and the second generation. In agreement with this idea various analyses using Venn diagrams were performed in order to pinpoint genes involved in a possible transgenerational memory. Firstly, each treated sample of the first and the second generation and the TTC condition of the third generation were compared with their respective controls (C0, CC, CCC). As shown in Venn figure 3.35 (a) in the intersection between all the three comparisons (yellow, blue and green zone) there was an abundant subset of modulated genes. 14 genes were up-regulated, 22 genes displayed a negative modulation and 75 changed the direction of their modulation. In particular among the 22 down-regulated genes (Table 9), 6 genes were components of photosystem I and II, like for example *LHCB2* (#2) and *LHCA3* (#6), which showed the same intensity of repression in the three generations. Another negative modulated gene was thiamine pyrophosphate dependent pyruvate decarboxylase (#44). Such gene was weakly down-regulated in the first generation, whereas the repression heavily increased from the second generation.

Among the 14 genes up-regulated there were 2 genes belonging to the disease resistance protein class (#14 - #15), where the up-regulation was the same in all the three generations.

Furthermore, 2 genes were involved in the lipid metabolism (#9 - #11) and 3 genes were members of signaling receptor kinases (#22 - #23 - #24).

Two other intersections were considered among TT vs CC, CT vs CC, TTC vs CCC, CTC vs CCC (figure 3.35, b) and among TT vs CC, TC vs CC, TTC vs CCC, TCC vs CCC (figure 3.35, c), respectively. In the first intersection (TT vs CC, CT vs CC, TTC vs CCC, CTC vs CCC) only 1 gene was down-regulated, that of the ribulose bisphosphate carboxylase small chain 3b (RBCS1A-#7), a gene maintained repressed also in intersection between TTC vs CCC, TT vs CC; T0 vs C0 already considered. In addition, in the second intersection, 2 genes were over-expressed (#17 - #34) and 9 genes were repressed, of which CYP702A1 (#17) is a member of cytochrome P450 family, a wide gene superfamily of heme-thiolate proteins involved in a variety of metabolic reactions (Xu et al., 2001). Among the genes whose expression similarly dropped in the two generations, there were 2 transcription factors (#20 - #21); the latter is *LBD41*, which is involved in lateral organ development. Finally, alcohol dehydrogenase 1 (*ADH*-#45) and pyruvate carboxylase (#44) showed the same behavior.

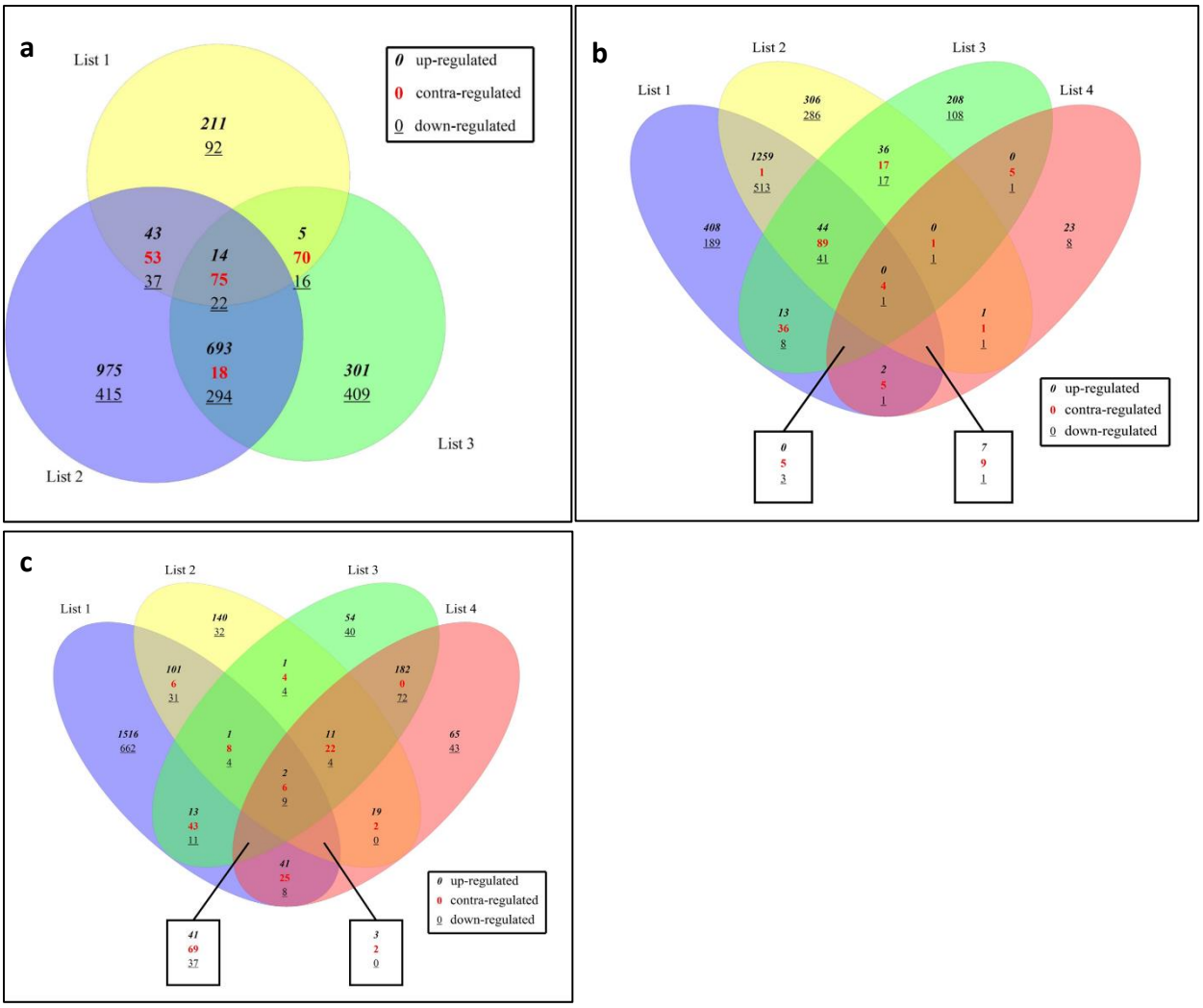

Figure 3.35. Schematic representation of genes potentially involved in a transgenerational "memory". a) Venn diagrams show the number of shared genes among comparison TTC vs CCC (list 1), TT vs CC (list 2), T0 vs C0 (list 3). **b**) Intersection among TT vs CC (list 1), CT vs CC (list 2), TTC vs CCC (list 3), CTC vs CCC (list 4). **c**) Common genes of differentially expressed genes among comparisons TT vs CC (list 1), TC vs CC (list 2), TTC vs CCC (list 3), TCC vs CCC (list 4). Venn diagrams were obtained with VennPlex 1.0.0.2 software (Cai et al., 2013).

Table 9. List of genes potentially involved in a transgenerational memory similarly modulated in the three generations. The bold transcripts are discussed in detail.

#	Gene_ID	symbol	log2-ratio T0 vs C0	p-value	log2-ratio TT vs CC	p-value	log2-ratio TC vs CC	p-value	log2-ratio CT vs CC	p-value	log2-ratio TCC vs CCC	p-value	log2-ratio TTC vs CCC	p-value	log2-ratio CTC vs CCC	p-value	description
1	AT1G29930	AB140	-2,003	0,00005	-2,978	0,00005					-1,03136	0,00045	-1,880	0,00005	-2,695	0,00005	chlorophyll a-b binding protein 2 3
2	**AT2G05100**	**LHCB2**	**-2,750**	**0,0033**	**-2,276**	**0,00695**							**-1,791**	**0,0001**	**-2,405**	**0,0048**	**photosystem ii light harvesting complex protein**
3	AT4G10340	LHCB5	-1,938	0,00005	-1,625	0,00145							-1,414	0,00005	-1,067	0,00695	chlorophyll a-b binding protein chloroplastic-like
4	AT4G12800	PSAL	-1,887	0,00005	-1,893	0,0147							-2,237	0,00055			photosystem i reaction center subunit xi
5	AT4G02770	PSAD-1	-1,775	0,002	-1,764	0,0135			-2,170	0,012			-1,857	0,00585			photosystem i reaction center subunit ii-1
6	**AT1G61520**	**LHCA3**	**-1,850**	**0,00005**	**-1,197**	**0,0026**							**-1,190**	**0,0006**			**light-harvesting complex i chlorophyll a b binding protein 3**
7	**AT1G67090**	**RBCS1A**	**-1,213**	**0,00025**	**-3,996**	**0,00005**			**-2,987**	**0,00005**			**-1,639**	**0,00005**	**-2,788**	**0,00055**	**ribulose bisphosphate carboxylase small chain 3b**
8	AT4G28850	ATXTH26	1,484	0,00005	2,735	0,00005			2,341	0,00005	1,263	0,00005	1,005	0,00005	0,947	0,00005	probable xyloglucan endotransglucosylase hydrolase protein 26-like
9	**AT5G24220**		**1,441**	**0,00005**	**2,383**	**0,00005**			**2,286**	**0,00005**	**1,026**	**0,00005**	**1,140**	**0,00005**			**lipase class 3-related protein**
10	AT1G52700		-1,453	0,0001	-2,421	0,00005			-2,422	0,00005	-1,202	0,00005	-1,222	0,00005			phospholipase carboxylesterase family protein
11	**AT2G11810**	**ATMGD3**	**3,341**	**0,00005**	**2,571**	**0,00005**			**3,250**	**0,00005**	**1,682**	**0,00205**	**1,669**	**0,0017**			**monogalactosyldiacylglycerol synthase 3**
12	AT1G56430	ATNAS4	-3,990	0,00005	-2,294	0,00005			-4,701	0,00005			-1,155	0,00005			nicotianamine synthase
13	AT5G23220	NIC3	-3,592	0,00005	-4,377	0,00005			-5,451	0,00005	-1,146	0,00005	-1,104	0,00005			nicotinamidase 3
14	**AT3G51570**		**1,096**	**0,0001**	**1,656**	**0,00005**			**1,417**	**0,00005**			**1,042**	**0,00085**			**tir-nbs-lrr class disease resistance protein**
15	**AT5G18350**		**1,382**	**0,00005**	**1,605**	**0,00005**			**1,470**	**0,00005**	**1,406**	**0,00005**	**1,347**	**0,00005**			**tir-nbs-lrr class disease resistance protein**
16	AT3G20087		-2,212	0,00615	-2,442	0,00215					-3,357	0,0022	-3,369	0,0022			cytochrome family subfamily polypeptide 15
17	**AT1G65670**	**CYP702A1**			**1,730**	**0,00065**	**1,119**	**0,01625**	**1,378**	**0,00425**	**1,230**	**0,0102**	**1,611**	**0,00285**			**cytochrome family subfamily polypeptide 5**
18	AT3G50560		1,195	0,0011	2,650	0,0001			3,159	0,00005	1,081	0,00525	1,233	0,00245			3-ketoacyl- reductase
19	AT3G27220		-1,332	0,00005	-1,717	0,00005					-1,154	0,00005	-1,148	0,00005			kelch repeat-containing protein
20	**AT3G10040**				**-3,518**	**0,00005**	**-2,360**	**0,00005**			**-2,159**	**0,00005**	**-2,411**	**0,00005**			**sequence-specific dna binding transcription factor**
21	**AT3G02550**	**LBD41**			**-1,873**	**0,00005**	**-1,385**	**0,00005**			**-1,115**	**0,00005**	**-1,415**	**0,00005**			**lob domain-containing protein 41-like**
22	**AT3G46240**		**1,984**	**0,00005**	**3,412**	**0,00005**			**2,646**	**0,00005**	**2,104**	**0,0004**	**1,760**	**0,00065**			**receptor protein kinase-like protein**
23	**AT1G63560**		**2,341**	**0,00005**	**3,462**	**0,00005**			**3,089**	**0,00005**	**1,113**	**0,0013**	**1,516**	**0,00005**			**receptor-like protein kinase-related family protein**
24	**AT3G46370**		**1,470**	**0,00005**	**2,853**	**0,00005**			**2,174**	**0,00005**	**1,178**	**0,00025**	**1,007**	**0,00135**			**leucine-rich repeat protein kinase-like protein**
25	AT1G16230		1,272	0,0019	2,073	0,00005			1,615	0,0003			1,378	0,00635			syntaxin of plants syp5
26	AT3G57157		-2,402	0,00125	-3,785	0,00005			-4,470	0,00005	-1,412	0,00005	-1,073	0,0004			
27	AT1G66725				-2,129	0,00005	-1,743	0,00005	-3,043	0,00005	-1,320	0,0018	-1,607	0,00005			
28	AT2G31083	CLE5	-5,681	0,00005	-3,210	0,00005			-5,270	0,00005	-1,311	0,00005	-1,336	0,00005			protein clavata3 esr-related 5
29	AT3G56620		1,558	0,00005	2,357	0,00005			2,335	0,00005			1,199	0,00005			nodulin 21 -like transporter protein
30	AT4G16620		1,212	0,00405	1,108	0,0001					1,396	0,00005	1,126	0,0001			nodulin 21 -like transporter family protein
31	AT4G19690	ATIRT1	-3,048	0,00005	-3,076	0,00005					-2,407	0,00005	-2,569	0,00005	8,693	0,00005	fe(2+) transport protein 1
32	AT5G44110	ATNAP2			-1,454	0,00005	-1,530	0,00005	-2,312	0,00005	-1,467	0,00005	-1,628	0,00005	0,629	0,0002	abc transporter i family member 19
33	AT4G14980				-1,138	0,00005	-1,356	0,00005			-1,052	0,00005	-1,066	0,00005	1,071	0,00005	cysteine histidine-rich c1 domain-containing protein
34	**AT5G54585**				**2,823**	**0,00005**	**1,037**	**0,00165**	**4,660**	**0,00005**	**1,900**	**0,00005**	**1,806**	**0,00005**			
35	AT3G60700		-2,763	0,00005	-3,053	0,00005			-3,178	0,00005			-1,226	0,00005			uncharacterized protein
36	AT5G24920	AtGDU5	-2,432	0,00005	-1,262	0,00005			-2,580	0,00005	-2,083	0,00005	-2,450	0,00005			glutamine dumper 5
37	AT5G39890		-1,764	0,00005	-3,752	0,00005	-1,949	0,00005					-2,354	0,00005	1,361	0,00005	2-aminoethanethiol dioxygenase-like
38	AT1G04330		1,109	0,0001	3,401	0,00005			2,497	0,00005			1,018	0,00065			uncharacterized protein
39	AT5G54790		1,241	0,0001	2,522	0,00005			2,193	0,00005	1,268	0,00035	1,188	0,00075			uncharacterized protein
40	AT1G47400		-1,770	0,014	-2,818	0,00005			-3,184	0,00005	-4,912	0,00005	-4,985	0,00005	3,183	0,00035	
41	AT1G47395		-1,761	0,00115	-2,304	0,00005			-2,693	0,00005	-3,594	0,00005	-3,668	0,00005	2,524	0,00005	
42	AT3G56290				-3,334	0,00005	-1,528	0,00005	-3,936	0,00005	-2,406	0,00005	-2,687	0,00005			uncharacterized protein
43	AT4G33560		-2,634	0,00005	-4,701	0,00005	-3,239	0,00005	-2,051	0,00005	-2,727	0,0001	-3,234	0,00005	1,850	0,00365	wound-responsive family protein
44	**AT4G33070**		**-1,720**	**0,00005**	**-3,858**	**0,00005**	**-2,361**	**0,00005**			**-3,163**	**0,00005**	**-3,477**	**0,00005**	**0,767**	**0,0007**	**pyruvate decarboxylase**
45	**AT1G77120**	**ADH**			**-2,201**	**0,00005**	**-2,591**	**0,00005**			**-1,753**	**0,00005**	**-2,499**	**0,00005**	**1,469**	**0,00005**	**alcohol dehydrogenase 1**

4. DISCUSSION

In both natural and agricultural ecosystems, the environment is rarely optimal for plant growth. Furthermore, environmental stresses limit the overall productivity of agriculture. Plants usually experience large seasonal fluctuations in light, temperature, and nutrients, often to levels that are suboptimal for their growth, thus they are continuously exposed to new combinations of environmental stresses. Moreover, most natural environments are continuously suboptimal with respect to one or more environmental parameters, such as water or nutrient availability (Chapin, 1991).

The interest in guaranteeing plant growth in suboptimal environments is great, because these are the only habitats in most developing countries into which agriculture can expand (Chapin, 1991); this aspect is of relevance due to the increasing food demand of growing global population. Many lands and agricultural soils are lacking or insufficient in one or more of the essential nutrients to support healthy and productive plant growth, also due to human activities and farming practices. Consequently, nutritional stress is one of the most diffused types of stresses for plants. The lack of macronutrients like nitrogen, phosphorus and potassium can have devastating effects on plant growth, although a common perception is that plants can respond to insufficient nutrient supply involving physiological changes that are unique to nutrient stress (Chapin, 1991; Medici and Krouk, 2014; Krapp et al., 2014). Indeed, when deprived of external nitrogen source, barley plants and many other species (Harrison and Helliwell 1979; Krapp et al., 2014; Castaings et al., 2011) increase their potential to absorb nitrogen. One of the leading macronutrient often lacking is nitrogen, essential for adequate plant growth and constituent of several primary metabolites, such amino acids, nucleic acids, pigments as well as secondary metabolites, such as amines, phytohormones, alkaloids. Symptoms of nitrogen deficiency are slow growth, chlorosis of leaves and their fall off and ultimately woodiness of stems and accumulation of anthocyanin pigments.

To date, despite the massive use of N-fertilizers in food production, the Nitrogen Use Efficiency (NUE) of crops remains still very low (i.e. 30-35% for cereal crops) (Eickhout et al., 2006). Nowadays it is crucial to develop new strategies to increase NUE. According to this idea the study of physiological, transcriptional and genetic mechanisms involved in plant response to stress plays a relevant role.

Recently it has been demonstrated that plants can "learn" from past environmental events and use these "memories" to support responses when these events occur again. (Kinoshita and Seki, 2014). A common argument pointing up responses to a range of biotic and abiotic stresses is the phenomenon of *priming* through which previous exposure provides to plants a large resistance to future exposure. Primed plants show either faster and/or stronger activation of the various defense responses that are induced following attack by either pathogens or insects, or in response to abiotic stress (Conrath et al., 2006). The benefit to the plant in being primed for specific stress responses is in aiding a more rapid response if the stress reappears (Ton et al., 2007). Responses to abiotic stresses are known as acclimation or hardening; these responses can also be reinforced by *priming* treatments. *Priming* can be elicited by exogenous application of chemical treatments as well as by exposure to the stress signals themselves (Jakab et al., 2005; Heil and Bueno, 2007).

For example, in *Arabidopsis* drought signals are converted into effects on gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). Such expression changes are commonly accompanied by variations in the chromatin status (Campos and Reinberg, 2009; Kim et al., 2008). Moreover, also low humidity conditions represent a usual stress event for plants, in which changes in methylation status are involved (Tricker et al, 2012). Recently, the transposition of a retrotransposon in *Arabidopsis* has been reported to involve an epigenetic mechanism in response to elevated temperatures (Ito et al., 2011). From these observations it follows that multiple exposures to stresses enable plants to respond to a new stress by more rapid adaptive changes to gene expression patterns compared with plants not previously exposed (Ding et al., 2012). For instance, in *Arabidopsis* previous exposition to either osmotic or oxidative stress can markedly alter subsequent osmotic stress-induced Ca^{2+} responses, indeed the nature of the alterations in Ca^{2+} response depends on the identity and severity of the previous stress, suggesting that there is an imprint of previous stresses (Knight et al., 1998). Consequently, it is reasonable to expect that also in response to nutritional stress some epigenetic mechanisms could be involved; by this mechanism the responses could be transmitted to the progenies stabilizing stress-dependent gene expression changes. In favor of this idea evident heritable epigenetic modifications induced by different nutritional stresses in mammals have been already

reported (McMillen et al., 2008; Vanhees et al., 2013, Mehedint et al., 2010; Niculescu, 2012). In spite of these promising observations, the role of epigenetics in "*transgenerational stress memory"* is still controversial in plants.

4.1 Transgenerational physiological effects of nitrogen deprivation

Higher plants have evolved tangled mechanisms enabling them to respond to environmental changes (Bruce et al., 2007).

However, many of these kind of responses can be inherited by successive generations of plants, stabilizing a "*stress memory*", even if it has not yet been clarified the mechanisms involved (Bruce et al., 2007).

In order to confirm this phenomenon, a first series of experiments was performed using three successive generations of *Arabidopsis* plants grown in hydroponic conditions and exposed for 1 week to nitrogen deprivation in the first two generations. At the end of the experiment, differences were reported in the root architecture and in shoot biomass in both generations (see figure 3.2-3.3-3.4), especially in second generation. The morphological observations on roots and leaves gave an indication that *Arabidopsis* plants are able to adapt to nitrogen starvation and this capability seems to be reinforced in the second generation. In particular, plants subjected to a continuous supply of nitrogen during the first generation and exposed for 1 week to nitrogen deficiency in the second one (CT), appeared more suffering as compared to the other treatments (see figure 3.3, A-B, b). Conversely, the root system of plants subjected to nitrogen starvation in first generation and in the second one (TT) seemed to have maintained a sort of "*memory*" of treatment in the first generation, showing more extended and dense roots. Indeed, they appeared less suffering in comparison with CT plants and with a root system more extended and longer. In addition, they showed a reduced biomass of shoots (figure 3.4, a), even if differences were not statistically significant.

According to these observations, an increase in the root-to-shoot biomass ratio was demonstrated by Remans and coworkers (2006). This ratio was found to increase gradually from 0.35 to 0.71 in plants supplied with decreasing nitrate concentrations from 10 to 0.05 mM (Remans et al., 2006). Lateral roots growth was significantly promoted by nitrogen limitation because of both an accelerated appearance of visible lateral roots and

an increase in the mean length of individual ones. Nevertheless, the positive effect of low nitrate concentration on the increase of roots biomass was limited to the first days of deprivation (Remans et al, 2006).

In addition to morphological variations, short-term uptake experiments were performed to characterize in detail the mechanism of nitrate acquisition in *Arabidopsis* roots of the two generations. It is well known that nitrate is a signaling molecule and that the exposure of the roots to the anion induces an increased uptake of the anion itself; this behavior is largely due to the induction of genes involved in nitrate transport and assimilation and to the parallel regulation of other metabolic processes (e.g. carbon metabolism) (Stitt, 1991). This kind of responses is termed "*primary nitrate response*" (Redinbaugh and Campbell, 1991; Medici and Krouk, 2014).

In order to test the transgenerational effect during the uptake experiment, control plants and stressed plants of both generations were exposed for 24 hours to a nutrient solution containing 1 mM nitrate as nitrogen source ("*induction"*) and uptake was evaluated in the high affinity range of transport system (iHATS). Results were comparable to those reported in previous works (Okamoto et al., 2003; Liu et al., 1999), with *Arabidopsis* plants treated with a period of nitrogen deprivation (T0i, CTi, TTi), showing an increased capability to take up nitrate when exposed to the anion (figure 3.4; figure 3.5 A). Particularly, this capability reached a peak after 8 h of the "*induction"* treatment, as reported by Okamoto and coworkers (Okamoto et al., 2003).

Afterwards, time-course experiments showed that a down-regulation of uptake rates occurred prolonging exposition of the roots to nitrate; this would indicate the involvement of a feedback regulation by end-products of nitrate metabolism or nitrate itself, similarly to what has been observed by Glass and coworkers (Glass et al., 2001).

The down-regulation of nitrate influx by ammonium (Aslam et al., 1996) or by amino acids (Doddema and Otten, 1979; Breteler and Siegerist, 1984; Muller and Touraine, 1992.; Muller et al., 1995) has also been advanced. Moreover, effects of ammonium on nitrate uptake seem to be more complex, considering the possibility of affecting nitrate uptake at different levels (transcript abundance, protein level, or direct effects of ammonium on the nitrate transporters) (Vidmar et al., 2000). High affinity transport systems are also diurnally regulated in response to carbohydrates availability (Gazzarrini et al., 1999).

However, the effect of "*induction*" rapidly dropped in the first generation (T0i); this decrease appeared to be slower in the second generation (CTi, TTi) (see figure 3.6, a). This latter result might indicate that an adaptation mechanism to nitrate deprivation was inherited from the first generation.

Comparing physiological data of uptake experiments of the first generation (T0i) with those of the second generation (CTi, TTi) it was evident that changes following nitrate exposure (*induction*) were about two times higher in plants with a double treatment of deprivation (TTi) (figure 3.7). On the other hand, plants with a single treatment of nitrogen deficiency showed the same induction potential in the first (T0i) as in the second generation (CTi). These findings were described for the first time in this work, supporting the idea that a potential "memory" of past events and transgenerational adaptation to nutritional (nitrogen) stress conditions may be occur in higher plants.

On the other hand control plants showed, within the limit of the assay method, an efflux of nitrate, confirming the idea that plants with optimal nutrient status do not need to absorb excess nitrate.

4.2 Transgenerational transcriptomic analyses

With the aim to clarify if the physiological responses could be related to variations at transcriptomic level, genome-wide analyses with RNA-sequencing were performed on *Arabidopsis* roots.

To date various microarray and RNA-sequencing studies on the modulation of gene expression by nitrate in plants, including *Arabidopsis* (Wang et al., 2000; Wang et al., 2003; Vidal et al., 2013; Scheible et al., 2004) and maize (Liu et al., 2008), are available.

In this work, the RNA-sequencing analyses revealed that the transcriptional modulation induced by nitrogen deprivation treatments concerned a huge amount of genes (over 3000 genes), but here only the most interesting differentially expressed genes are discussed (about 400) in details considering only three comparison: T0 vs C0, TT vs CC and finally CT vs CC (see Table 2 and Table S1). The distribution of principal ontological (GO) classes did not show significant changes in comparison with *Arabidopsis* reference, between the first and the second generation. The only considerable difference was observed in cellular and metabolic process for the comparison TT vs CC in the second

generation, where the difference between samples and reference decreased, probably as a consequence of a prolonged stress.

Findings reported in this work corroborated experimental evidences claimed in previous transcriptional studies on *Arabidopsis* (Gojon et al., 2011; Kiba et al., 2012, Lezhneva et al., 2014)**.** In particular the nitrogen deprivation strongly down-regulated *NRT1.1*, a nitrate transporter acting as a sensor of nitrate concentration in the external medium and defined as a transceptor (Gojon et al., 2011); this repression was even stronger in the second generation. On the other hand transporters involved in the adaptation to long-term nitrogen starvation, like *NRT2.5* and *NRT2.4*, were similarly up-regulated in deficient plants, according to results reported in the literature (Kiba et al., 2012, Lezhneva et al., 2014). Furthermore, enzymes involved in the nitrate reduction pathway (*NIA* and *NiR*) were more down-regulated in the second generation; glutamine syntethase isoforms, glutamate synthase 2 (*GLU2*) as well as specific isoform of glutamate dehydrogenase were up-regulated by nitrogen starvation and generally showed a higher induction in the second generation. Also asparagine synthase was down-regulated by a single treatment of nitrogen starvation. Together these results suggest that nitrogen deprivation shuts off nitrate reduction pathway possibly activating a nitrogen recycling pathway, starting from the recovery of ammonium from all possible resources, like for example glutamate or glutamine (see figure 3.11-3.12, Table 2). Concerning this point it is noteworthy that the up-regulated isoform of glutamate dehydrogenase (*GDH3*) is normally expressed at very low levels of nitrate (Thimm et al., 2004; Scheible et al., 2004).

Supporting this idea, various ammonium transporters were up-regulated in the two generations; according to previous findings four ammonium transporters have been found to be up-regulated under nitrogen deficiency (Gazzarrini et al., 1999; Sohlenkamp et al., 2000) in *Arabidopsis* roots (see figure 3.11-3.12, Table 2). Ammonium transporter genes have been shown to have a variety of expression modulation also in rice, lotus and tomato, being up-regulated either under nitrogen deprivation or after ammonium re-supply, whereas the expression of some genes is unaffected by nitrogen levels (d'Apuzzo et al., 2004; Sonoda et al., 2003; Suenaga et al., 2003).

It may be tempted to speculate that N-deprived plants try to recover any possible source of nitrogen and, interestingly this behavior appears to be accentuated in plants with a

prolonged stress. The urea transporter, *DUR3*, as well as an accessory protein, which regulates urease activity, were strongly induced by N-starvation, especially in the second generation. Indeed, in *Arabidopsis*, the amount of DUR3 protein in roots was strongly induced after 3 days of nitrogen starvation (Kojima et al., 2007).

Another important source of nitrogen can be amino acids, as reported in several studies (Persson and Näsholm, 2001; Forsum et al., 2008). Results of the present work corroborate this idea; indeed various transmembrane amino acids transporters as well as amino acids permeases and specific amino acid transporters were mainly up-regulated in second generation. The specific lysine histidine transporter 1 (*LHT1*) was induced in the second generation; Hirner (Hirner et al., 2006) and Svennerstam (Svennerstam et al., 2007) suggest that root uptake of a number of amino acids in *Arabidopsis* is mediated, at least partly, by this transporter. Plants over-expressing the *LHT1* gene displayed increased growth with L-glutamine, L-glutamate and L-asparagine, as demonstrated by Forsum and coworkers (Forsum et al., 2008).

In addition to serving as a nutrient, nitrate also provides many signals in order to modulate a lot of other primary and secondary metabolic pathways; indeed nitrate induces reprogramming not only of nitrogen metabolism but also of carbon metabolism, resource allocation and root development (Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004; Zangh and Forde, 1998). In this regard nitrogen deprivation treatments modulated some genes involved in TCA cycle, especially in the second generation with a longer stress, as well as repressed principal components of electron transport chain. Interestingly an alternative NAD(P)H dehydrogenase and an alternative oxidase, which introduce electrons at the level of ubiquinone pool and provide an alternative route for electrons from the ubiquinone pool to oxygen respectively, were progressively activated through the generations and by the duration of the stress. The presence of alternative enzymes is not linked to proton translocation and, hence, is non-phosphorylating (Moore et al., 2003). As reported by Møller, these alternative enzymes have the potential to catalyze wasteful respiration but can also decrease the production of reactive oxygen species in the respiratory chain (Møller, 2001) and they are commonly activated by N-deprivation (Moore et al., 2003). However, knowledges on the significance of the non-phosphorylating NAD(P)H DH family in plant mitochondria has been limited by the lack of

a clear relation between genomic evidences and biochemical data and may deserve further investigations (Moore et al., 2003).

Nitrate deficiency caused the down-regulation of many genes of oxidative pentose phosphate pathway, according to Bussel (Bussel et al., 2013). On the other hand, glycolysis, strictly related with oxidative pentose phosphate pathway, appeared to be weakly up-regulated considering the activation of a phosphoglycerate mutase and phosphoenolpyruvate carboxylase 2, especially in plants subjected to a prolonged stress.

Other interesting data concern the production of secondary metabolites, like phenylpropanoids via the shikimic acid pathway. Several genes were modulated by nitrogen deficiency towards an increased production of these compounds that are involved in many functions in plants (Cho et al., 2007). This activation seemed to be weaker in the second generation in comparison with the first one. Conversely, regarding the polyamine production, other secondary metabolites subjected to changes in response to a variety of abiotic stresses (Alcazar et al., 2006), data support a nitrogen recovery strategy of N-starved plants, by blocking the biogenesis of polyamine and releasing nitrogen from various N-containing compounds. However, the physiological significance of the role of polyamines is still not clear. Further molecular studies are necessary to understand the function of polyamines in stress tolerance, as claimed by Alcazar (Alcazar et al., 2006). Concerning this, in the last decade many genes associated with polyamine metabolism have been cloned from several species and their expression profiles under several stress conditions have been analyzed (Kakkar and Sawhney, 2002).

Generally, data of the present work suggest a high modulation of genes involved in cell wall modification by N-deprivation, especially in the second generation, possibly relating to the observed morphological modification of roots (see § 3.1.1 and 3.1.2, figure 3.3, B – 3.4, b). Moreover, production of new cells is strictly related with replication of genome. In this regard the synthesis of new histones and the introduction of histone modifications play a leading role in order to make DNA accessible by replication machinery (Sequeira-Mendes and Gutierrez, 2015). For this reason it seemed to be impressive the up-regulation of 10 histone proteins, suggesting a proper relation with replication and organogenesis.

Aquaporins and tonoplast intrinsic proteins were classes mainly repressed in N-deprived plants in this study, as resulted from a general clustering analysis. The high repression of two specific tonoplast intrinsic proteins in starved plants is consistent with the operation of nitrogen recycling pathways. On the other hand, the higher over-expression of peroxidase and metallothioneins suggest an involvement of these genes in responses to oxidative stress. However regarding all transcripts identified in the present work, further analysis is needed to clarify their involvement in the response mechanism of N-deprivation.

In order to confirm the physiological results real time RT-PCR analyses were performed. Plants were grown with the same nitrogen treatments as for the measurements of nitrate uptake (see § 3.2.1-3.2.2) and a 24-h time-course experiment with or without nitrate supply was performed to follow the expression dynamics of selected genes. As expected, data showed a strong up-regulation by nitrate of genes involved in the uptake of the anion and in its assimilatory pathway. Furthermore, this up-regulation was also displayed by other genes involved in primary and secondary metabolisms, highlighting the fundamental role of nitrate in cellular processes.

Concerning nitrate uptake transcript levels of *NRT2.1, NRT2.2*, *NRT2.4* and *NRT2.5* genes were evaluated. Principally *NRT2.1* expression was induced by nitrate supply in treated plants (CTi - TTi). Surprisingly, induction peaked earlier in plants subjected to a double N-deficiency (TTi). This behavior was also observed for *NRT2.2*, although the amount of induction was much lower and not statistically significant. These changes suggest an adaptation of plants in order to react earlier to nitrate re-supply.

Okamoto and coworkers reported that *NRT2.1* showed the strongest induction by nitrate in roots after only 3h of nitrate provision. Differently from the results presented here, those authors demonstrated that also *NRT2.2* was induced by nitrate after 3 h of root exposure to the anion, but this level of induction was 4-fold higher than in shoot (Okamoto et al., 2003). These differences might be due to the sensitivity of the RT-PCR method and/or to the plants growth conditions. Evidence that *NRT2.1* and *NRT2.2* encode for the iHATS was supported by the demonstration that high-affinity nitrate uptake was reduced to 27% of wild-type values in a T-DNA mutant (Filleur et al., 2001). Moreover, disruption of *NRT2.1* caused substantial impairment of the inducible high-affinity nitrate

influx, whereas disruption of *NRT2.2* caused only a modest reduction of influx (Li et al., 2007); *NRT2.2* seems to serve a small, but important, contributory and compensatory role in iHATS nitrate influx in *Arabidopsis* when *NRT2.1* is lacking (Li et al., 2007).

The modulation of *NRT1.1* expression was also investigated by real time RT-PCR. As observed by other authors (Liu et al., 1999; Okamoto et al., 2003), gene expression reached a peak after 4h of root exposure to nitrate in plants subjected to nitrogen deprivation either in the second generation (CTi) or in both generations (TTi). Interestingly, the level of induction was higher in TTi plants, possibly indicating an adaptation to a prolonged stress. Contrary to previous observations (Okamoto et al., 2003), the expression of this transporter progressively decreased after 4 h of induction, instead of remaining induced from 12 h to 48 h.

However *NRT2.4, NRT2.5, NRT1.3*, that are induced by a severe nitrate starvation (Kiba et al., 2012; Lezhneva et al., 2014; Okamoto et al., 2003), showed a strong down-regulation upon supply of the anion.

Similarly, a stronger and earlier induction of expression of two proton pumps by nitrate exposure in sample TTi, may further suggest a transgenerational adaptation mechanism to N-deprivation.

Genes involved in nitrate metabolism were also investigated in this study. According to the literature (Rastogi et al., 1993; Taiz and Zeiger, 2002) *NIA* and *NiR*, belonging to the reduction pathway, were strongly induced by nitrate supply, except for NiR in CTi sample.

Another investigated gene, *NADH-GOGAT*, was weakly induced (not statistically significant) upon nitrate supply in samples that experienced longer deprivation stress. *NADH-GOGAT* is expressed at higher levels in roots than in leaves, whereas in leaves Fd-GOGAT is the predominant form (Lancien et al., 2002). *NADH-GOGAT* and cytosolic glutamine synthetase have been proposed to play a concerted role in the synthesis of Glu from Gln during nitrogen transport via the vascular bundle from roots or senescing tissues (Tobin and Yamaya, 2001). As claimed by Lancien the NADH-GOGAT enzyme may play a minor accessory role in photorespiratory ammonium re-assimilation (Lancien et al., 2002).

Surprisingly, a transcript of unknown function, encoding for a glutamine amidotransferase was dramatically over-expressed, especially in TTi plants (see figure 3.22). A possible role

of this protein in the adaptation to nitrogen starvation deserves further analysis at the physiological and molecular level.

Two other genes involved in secondary metabolism showed to be putatively part of an adaptation mechanism to N-starvation: arginine decarboxylase 2 (*ADC2*) showed the typical temporal shift of induction from 8h to 4h when comparing CTi with TTi. *PAL3* displayed a very high over-expression in plants treated subjected to a prolonged N-deprivation (TTi).

Finally, genes coding for an amino acids transporter, a malate synthase and a sulfate transporter showed an increased expression in plants stressed for two generations (TTi), further supporting the idea of a transgenerational adaptation mechanism.

In order to evaluate the establishment of a putative "*transgenerational memory*" RNA-sequencing analyses were also performed on third generation, maintained under control condition. Various genes of photosystems and light harvesting complexes as well as ribulose bisphosphate carboxylase small chain 3b were maintained strongly down-regulated in roots also in third generation in absence of stress, but the meaning needs to be further evaluate. Opposite to these results, Geider and coworkers demonstrated that in *Phaeodactylum tricornutum* the light-harvesting complex proteins remained a constant proportion of total cell protein during nutrient starvation (Geider et al., 1993).

Finally, expression of alcohol dehydrogenase 1 (*ADH*) and pyruvate decarboxylase (*PDC1*) also dropped in three generations. Concerning this, increased H3 acetylation was found after submergence of rice plants, which led to increasing expression of these two stress responsive genes (Tsuji et al., 2006); this result might imply a putative epigenetic mechanism involved in the repression described above for N-deprived plants.

4.3 Stress effects on methylation status

DNA sequence variation can be a slow process and is therefore not ideal for an organism or population to adapt and survive in a dynamic environment (Heard and Martienssen, 2014), as is the case of plants, forced to live permanently in the same location. Some epigenetic mechanisms (i.e. DNA methylation), modulated by different environmental signals, have been proposed to enable ''soft inheritance,'' permitting adaptation to fluctuating environments and nutrition (Richards, 2006).

Many efforts to demonstrate adaptive epigenetic changes in plants have been made. Most of them have focused on biotic and abiotic stress for a good reason, although these approaches presented some caveats (Heard and Martienssen, 2014). Indeed, attempts to experimentally find evidence for adaptive epigenetic variations in stress tolerance have previously met with very limited success (Slaughter et al., 2012), as intergenerational maternal effects on seeds, similar to maternal effects in mammals, are difficult to rule out (Pecinka and Mittelsten Scheid, 2012).

Several recent studies have reported that different environmental stresses bring about altered methylation states of DNA as well as modifications of nucleosomal histones. In one of these studies, when maize seedlings were exposed to cold stress, genome-wide 5-methylcytosine demethylation occurred predominantly at the nucleosome core regions in root tissue (Steward et al., 2002). A noteworthy aspect was that, even after the seedlings were returned to normal growth conditions, the decreased methylation level did not reverse.

Furthermore, stress exposure of parent plants can even lead to "*stress memory*" that is carried forward to the next generation of unstressed plants, a phenomenon that is different from the priming effect, already discussed (see above) but equally interesting, especially for this work. This "*transgenerational stress memory*" was indeed observed in wild radish, *Raphanus raphanistrum*, responding to herbivore damage (*Pieris rapae*) and treatment with jasmonic acid (Agrawal, 2002). The authors demonstrated that the progeny of treated plants was more resistant to herbivory than control plants.

In order to investigate the establishment of a "*transgenerational memory*" of N-deprivation stress and to assess the implication of changes in methylation status, a genome wide analysis was performed on first and second generation, using bisulfite sequencing. For time limitation, the third generation has not yet been analyzed at the time of the present report, but it represents a compelling step forward that is expected to deliver a better understanding of the epigenetic changes observed in this study. Methylomes obtained in this work presented general features similar to those described by Lister and coworkers (Lister et al., 2008). The level of methylation in all the three methylable contexts (CG, CHG, CHH) was higher in pericentromeric regions, where the presence of TEs was more evident compared with the chromosome arms. The frequency

of methylated cytosines was higher in the GC context and similar in all chromosomes of all samples. Data from clustering analyses revealed that the treatment of N-deficiency made treated samples (T0-1, T0-2, TT-1, TT-2) much more homogenous than plants maintained in control conditions (C0-1, C0-2, CC-1, CC-2) (see figure 3.28-3.29). The latter were much more distant and different among each other at the CG methylation level (see figure 3.29) as if the nutritional stress had levelled differences in some particular way. In contrast, in the CHG and CHH contexts the effect of generations, rather than the effect of treatment appeared prevalent.

A meta-analysis of DNA methylation levels in GC context (gene-body methylation) across the total number of genes of *Arabidopsis*, showed a clear reduction of methylation in treated samples relative to control samples (see figure 3.32), confirming the segregation of the two conditions previously suggested by clustering analyses. Thus, based on these results, the expression level of genes involved in the methylation pathway was investigated in order to verify a possible decrease in the expression of genes involved in methylation maintenance. Changes in expression profiling, if existing, resulted to be under the threshold considered in transcriptomic analyses (2-fold change).

Afterwards, differentially methylated regions (DMRs) shared by all treated samples were investigated by comparing the treated samples (T0-1, T0-2, TT-1, TT-2) with their respective controls in the same generation (C0-1, C0-2, CC-1, CC-2) in all the three methylation contexts. DMRs were rarely spread through the genome in these samples, especially in first generation, whereas in the second generation an increase in DMRs was registered (see figure 3.33), in particular for the CG context.

Furthermore, many genes were identified within or in the surrounding of these DMRs. Some of these genes are involved in transport of nitrate, nitrogen and ammonia metabolism, secondary metabolism of phenylpropanoids, abiotic stress and cell wall modification as discussed in the RNA-seq results. Interestingly, a subset of such genes were also identified as changing in expression levels, as previously reported (see § 3.3.1 and Table S2). Two genes, members of photosystem I and II (*PSAL* and *LHCA2*), maintained down–regulated in the first and second generation (T0 vs C0, TT vs CC) showed associated DMRs. According to the transcriptional analysis, other members of the photosystem gene family were down-regulated in the two generations, and

interestingly they remained so in the third generation, in absence of stress (see § 4.2). Thus, this class of genes in *Arabidopsis* may deserve further investigations at biochemical, genomic and epigenetic level to correlate their biological significance to stress responses. In particular, *PSAL* showed an associated DMR and down-regulation of gene expression in T0 vs C0, TT vs CC, TTC vs CCC as reported above (see § 4.2) and appeared completely switched off in the third generation, without the N-deprivation conditions. It could be speculated that this strong down-regulation may be inherited thanks to an epigenetic change, possibly in the methylation status or at the nucleosome level as already reported for *ADH1* by Tsuji (Tsuji et al., 2006) in response to low oxygen conditions. Obviously, this gene needs further investigations related to stress responses and acquired "transgenerational memory".

Indeed, in literature there are evidences that epigenetic modifications of chromatin, both at the level of DNA and nucleosomes, are implicated in plant stress responses (Bruce et al., 2007; Campos and Reinberg, 2009; Kim et al., 2008; Tricker et al, 2012). Epigenetic control of transcription may allow the alteration of gene expression after stress events and there is already evidence that this occurs in some circumstances. The role of epigenetic modifications involved in the transcription of stress responsive genes is presumably to favor variations that switch on gene expression when stress is sensed and then reestablish repression, once the stress signal is removed, according to the priming effect. In fact, Bruce and coworkers hypothesized that exposure to a priming agent may induce a gene or a subset of genes but instead of reverting to the transcriptionally silent state once the stimulus is removed, retaining the region in a "permissive" state may facilitate faster and more potent responses to subsequent attacks (Bruce at al., 2007).

In addition also transposable elements can regulate genes epigenetically, but only when inserted within or very close to them (Lippman et al., 2004). In our study, in each generation and for each methylation context, DMRs were found within annotated TE elements and some genes were also identified in proximity of these TEs. In some cases, we verified that genes previously selected for being in association with DMRs actually presented the DMR in a TE element located in the promoter or trailer. Among these there was *LHCA2*, with a DMR located in a COPIA68 LTR-retrotransposon. However, despite significant differential methylation in that 500 bp region(~ - 40%, $P \leq 0.05$), visual

inspection of methylation levels in all the samples by a genome browser did not show a convincing change in the shape of the methylation pattern, indicating that the biological significance of such changes may be questionable and deserves further investigation.

Finally, at least one gene (*APR3*) was found to show a reproducible methylation change in samples subjected to a repeated stress (TT). The identified gene belongs to the thioredoxin family, suggesting an involvement in stress response (see § 3.3.1.7) and showed a strong down-regulation at the expression level in second and third generation (see Table 8) consistent with epigenetic suppression. The modification at the methylation level mainly involved the gene body compartment and was prominent especially in the CHH context (see figure 3.34).

It should be noticed that the few cases of DNA methylation changes physically associated with regulated genes sequences and/or transposable elements appeared dramatically underrepresented if confronted with the global reduction of DNA methylation observed in association with the applied stress. The computational methodology used for detecting differential methylation at local sequence scale, albeit up to date, may not be capable of identifying, in our hands, subtle differences. Nevertheless, genes such as *LHCA2* represent suitable candidates for follow-up investigations of epigenetic effects resulting from nitrogen deprivation.

5. CONCLUSION

The work presented in this thesis aimed at demonstrating for the first time, to the best of our knowledge, the establishment of a "*transgenerational stress memory*" in response to nitrogen deficiency at physiological, transcriptional and epigenetic level in *Arabidopsis thaliana*, the model plant used in this work and subjected to nitrogen deprivation for three successive generations.

This study showed that not only did nitrogen deficiency induce changes in root and shoot morphology, as expected, but that a sort of transgenerational mechanism of adaptation also seemed to be established when the stress was repeatedly imposed to the plants.

In addition, the nitrate uptake system was confirmed to be inducible by the exposure of roots to the anion and feedback-regulated by the anion itself; however, this capability appeared to be increased in plants subjected to a N-deprivation during two generations, while feedback-regulation was slightly delayed.

Furthermore, transcriptional analyses showed that many genes involved in nitrate metabolism as well as carbon and secondary metabolism, were modulated by nitrogen starvation. This modulation of gene expression in some cases was more pronounced through successive generations. In particular, some genes of plants in the third generation maintained this modulation, even if the stress stimulus (N-deprivation) was absent. These data support the idea that a "*transgenerational memory of stress"* in plants might occur.

In order to understand the bases of this mechanism an analysis of epigenetic modification in the DNA methylation status was performed using an up-to-date protocol for BS-seq.,

Under the experimental conditions employed in this work, differentially methylated regions (DMRs) appeared to be conserved in treated (N-deprived) plants in the first and in the second generation, especially in the CG context. Various DMRs found in different methylation contexts and generations were found within or in proximity of genes and TEs. Some of these genes were also pinpointed from independent transcriptional analyses and resulted to be differentially expressed. One of this (*PSAL*) belongs to a subset of genes modulated also in the third generation when the stress was interrupted, suggesting a possible epigenetic variation involved in the "*transgenerational stress memory"* observed at other levels.

This work highlights the presence of some transgenerational mechanisms of adaptation to stress conditions carried out by plants, improves the knowledge in the context of nitrogen requirement, useful for crop science, and may delineate new interesting lines of research in the future.

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7. SUPPLEMENTARY MATERIALS

Table S1. List of main transcripts modulated in *Arabidopsis* roots in response to different N-deprivation treatments. In the table are shown: transcript number (*#*), the *Arabidopsis* gene identifier (*Gene_ID*); the common name of gene product (*Symbols*); for each comparison, Log2 FC (Fold change) and relative p-value; the description of the function. Each transcript is discussed in detail in the text. With asterisks, infinite expression values have been replaced with|12.00| or |-7|, as theoretical maximal or minimal value.

#	Gene_ID	Symbols	log2-ratio T0 vs C0	p-value	log2-ratio TT vs CC	p-value	log2-ratioCT vs CC	p-value	Description
1	AT5G52560	ATUSP			1.174	0.0001	1.080	0.0001	UDP-sugar pyrophosphorylase
2	AT1G63000	NRS/ER			1.193	0.0001			nucleotide-rhamnose synthase/epimerase-reductase
3	AT4G23920	ATUGE2	1.228	0.00005					UDP-D-glucose/UDP-D-galactose 4-epimerase 2
4	AT4G10960	UGE5	1.000	0.00005					UDP-D-glucose/UDP-D-galactose 4-epimerase 5
5	AT1G12780	ATUGE1	-1.024	0.00005					UDP-D-glucose/UDP-D-galactose 4-epimerase 1
6	AT1G67070	DIN9	-1.050	0.0002	1.324	0.0001			Mannose-6-phosphate isomerase, type I
7	AT3G02570	MEE31			1.046	0.0115			Mannose-6-phosphate isomerase, type I
8	AT1G08200	AXS2			1.068	0.0001			UDP-D-apiose/UDP-D-xylose synthase 2
9	AT1G26570	ATUGD1			1.151	0.0001	1.036	0.0001	UDP-glucose dehydrogenase 1
10	AT5G15490				1.282	0.0001			UDP-glucose 6-dehydrogenase family protein
11	AT5G39320				1.196	0.0001			UDP-glucose 6-dehydrogenase family protein
12	AT3G29360				1.025	0.0001			UDP-glucose 6-dehydrogenase family protein
13	AT5G66280	GMD1			1.100	0.0001			GDP-D-mannose 4,6-dehydratase 1
14	AT2G34850	MEE25			1.247	0.0009	1.249	0.0009	NAD(P)-binding Rossmann-fold superfamily protein
15	AT4G15290	ATCSLB05	1.178	0.00005	1.014	0.0001	1.264	0.0001	Cellulose synthase family protein
16	AT4G33330	GUX2	1.667	0.00005	2.073	0.0001	2.064	0.0001	plant glycogenin-like starch initiation protein 3
17	AT5G33290	XGD1	1.368	0.00005					xylogalacturonan deficient 1
18	AT2G20520	FLA6			1.741	0.0001	1.232	0.0002	FASCICLIN-like arabinogalactan 6
19	AT4G40090				2.241	0.0001	1.194	0.0001	
20	AT1G55330						1.090	0.0001	
21	AT5G44130	FLA13	-1.107	0.00005			-1.683	0.0001	FASCICLIN-like arabinogalactan protein 13 precursor
22	AT1G35230	AGP5	-1.428	0.00005					arabinogalactan protein 5
23	AT5G65390				1.236	0.0001			
24	AT2G04780	FLA7			1.178	0.0001			FASCICLIN-like arabinoogalactan 7
25	AT3G13520	AGP12			1.122	0.0001			arabinogalactan protein 12
26	AT5G06390	FLA17			1.041	0.0001			FASCICLIN-like arabinogalactan protein 17 precursor
27	AT4G12730	FLA2			1.018	0.0001			FASCICLIN-like arabinogalactan 2
28	AT3G52370	FLA15	1.045	0.00005	1.005	0.0001			FASCICLIN-like arabinogalactan protein 15 precursor
29	AT3G62680	ATPRP3			2.066	0.0001	1.241	0.0001	proline-rich protein 3
30	AT1G54970	ATPRP1	-2.070	0.00005					proline-rich protein 1
31	AT1G12040	LRX1			1.990	0.0001	1.471	0.0001	leucine-rich repeat/extensin 1
32	AT2G15880		-1.732	0.00005	-2.126	0.0001	-1.030	0.0001	Leucine-rich repeat (LRR) family protein
33	AT4G33970		-2.073	0.00005					Leucine-rich repeat (LRR) family protein
34	AT2G19780				1.044	0.0001			Leucine-rich repeat (LRR) family protein
35	AT4G08400		1.047	0.00005	2.867	0.0001	2.148	0.0001	Proline-rich extensin-like family protein
36	AT4G08410		1.178	0.00005	2.516	0.0001	1.908	0.0001	Proline-rich extensin-like family protein
37	AT3G54590		1.274	0.00005	2.408	0.0001	1.669	0.0001	
38	AT1G76930	ATEXT1	-1.542	0.00005			-1.004	0.0001	extensin 4
39	AT2G32990	AtGH9B8	1.219	0.00005	1.792	0.0001	1.573	0.0001	glycosyl hydrolase 9B8
40	AT1G19940	AtGH9B5			1.560	0.0001	1.357	0.0003	glycosyl hydrolase 9B5
41	AT3G62740	BGLU7	-1.390	0.0003	1.363	0.0005			beta glucosidase 7
42	AT5G64570	ATBXL4			1.046	0.0004	1.020	0.0009	beta-D-xylosidase 4
43	AT3G52840	BGAL2	-1.570	0.00005	-1.263	0.0001	-1.521	0.0001	beta-galactosidase 2
44	AT4G33810				-1.399	0.0007	-1.733	0.0001	Glycosyl hydrolase superfamily protein
45	AT4G30270	MERI-5	-1.041	0.00005					xyloglucan endotransglucosylase/hydrolase 24
46	AT3G19620		-1.666	0.00215					Glycosyl hydrolase family protein
47	AT1G78060				1.103	0.0001			Glycosyl hydrolase family protein
48	AT5G49360	ATBXL1	-1.592	0.00005					beta-xylosidase 1
49	AT2G43890		1.843	0.0003	4.339	0.0001	2.540	0.0001	Pectin lyase-like superfamily protein
50	AT3G07970	QRT2	1.479	0.00005	2.428	0.0001	2.215	0.0001	Pectin lyase-like superfamily protein
51	AT4G22080	RHS14	1.075	0.00005	2.713	0.0001	2.127	0.0001	root hair specific 14
52	AT1G11920				1.722	0.0001	1.307	0.0001	Pectin lyase-like superfamily protein
53	AT2G43880				1.900	0.0001	1.260	0.0001	Pectin lyase-like superfamily protein
54	AT2G22620				1.271	0.0001	1.064	0.0001	Rhamnogalacturonate lyase family protein
55	AT3G09540				1.180	0.0001	1.006	0.0001	Pectin lyase-like superfamily protein
56	AT1G02460						-1.002	0.0001	Pectin lyase-like superfamily protein
57	AT5G14650		-1.099	0.00005			-1.017	0.0001	Pectin lyase-like superfamily protein
58	AT1G09890						-1.073	0.0045	Rhamnogalacturonate lyase family protein
59	AT4G24430				-1.049	0.0067	-1.596	0.0003	Rhamnogalacturonate lyase family protein
60	AT3G26610				-1.700	0.0001	-1.757	0.0001	Pectin lyase-like superfamily protein
61	AT3G15720		-1.646	0.00005					Pectin lyase-like superfamily protein
62	AT2G33160		-2.318	0.00355					glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein
63	AT1G05650		2.134	0.00005	1.667	0.0001			Pectin lyase-like superfamily protein
64	AT3G59850				1.436	0.0001			Pectin lyase-like superfamily protein
65	AT2G43870				1.285	0.0027			Pectin lyase-like superfamily protein
66	AT5G48900				1.185	0.0001			Pectin lyase-like superfamily protein
67	AT3G07010				1.170	0.0001			Pectin lyase-like superfamily protein
68	AT1G10640				1.151	0.0068			Pectin lyase-like superfamily protein
69	AT4G24780				1.086	0.0001			Pectin lyase-like superfamily protein
70	AT3G27400		-1.659	0.00005					Pectin lyase-like superfamily protein

71	AT2G19150		1.294	0.00005	2.424	0.0001	2.608	0.0001	Pectin lyase-like superfamily protein
72	AT5G20860		1.379	0.00005	1.964	0.0001	2.066	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
73	AT5G04960				2.142	0.0001	1.724	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
74	AT3G10710	RHS12			1.701	0.0001	1.457	0.0001	root hair specific 12
75	AT5G09760				-1.410	0.0001	-1.694	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
76	AT5G19730		-1.313	0.00005	-1.405	0.0001	-1.786	0.0001	Pectin lyase-like superfamily protein
77	AT1G05310				-1.874	0.0001	-2.457	0.0001	Pectin lyase-like superfamily protein
78	AT5G47500		-2.157	0.00005					Pectin lyase-like superfamily protein
79	AT4G19420				1.416	0.0001	1.728	0.0001	Pectinacetylesterase family protein
80	AT1G57590				1.008	0.0001			Pectinacetylesterase family protein
81	AT1G23200		2.228	0.00005	2.519	0.0001	2.443	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
82	AT5G64640				1.458	0.0001	1.388	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
83	AT2G45220				1.166	0.0001	1.079	0.0001	Plant invertase/pectin methylesterase inhibitor superfamily
84	AT5G51500		-1.039	0.01045	1.247	0.0058			Plant invertase/pectin methylesterase inhibitor superfamily
85	AT5G51490				1.182	0.0001			Plant invertase/pectin methylesterase inhibitor superfamily
86	AT4G07960	ATCSLC12	1.204	0.00005	2.740	0.0001	2.639	0.0001	Cellulose-synthase-like C12
87	AT5G22740	ATCSLA02	1.135	0.00005	1.020	0.0001	1.127	0.0001	cellulose synthase-like A 2
88	AT5G03760	ATCSLA09	1.170	0.00005	1.387	0.0001	1.082	0.0001	Nucleotide-diphospho-sugar transferases superfamily protein
89	AT2G15350	ATFUT10	1.364	0.0014	1.917	0.0001	1.367	0.0001	fucosyltransferase 1
90	AT5G20260		-1.943	0.00005					Exostosin family protein
91	AT2G15370	ATFUT5	1.181	0.00005	1.043	0.0001			fucosyltransferase 5
92	AT5G34940	AtGUS3			1.010	0.0001	1.085	0.0001	glucuronidase 3
93	AT4G28850	ATXTH26	1.484	0.00005	2.735	0.0001	2.341	0.0001	xyloglucan endotransglucosylase/hydrolase 26
94	AT3G44990	ATXTR8			2.295	0.0001	2.150	0.0001	xyloglucan endo-transglycosylase-related 8
95	AT5G57530	AtXTH12	1.463	0.00005	2.296	0.0001	1.952	0.0001	xyloglucan endotransglucosylase/hydrolase 12
96	AT4G28250	ATEXPB3			2.252	0.0001	1.714	0.0001	expansin B3
97	AT4G25820	ATXTH14			2.189	0.0001	1.636	0.0001	xyloglucan endotransglucosylase/hydrolase 14
98	AT1G12560	ATEXP7			1.809	0.0001	1.552	0.0001	expansin A7
99	AT5G57540	AtXTH13			1.474	0.0002	1.497	0.0001	xyloglucan endotransglucosylase/hydrolase 13
100	AT1G62980	ATEXP18			2.129	0.0001	1.440	0.0001	expansin A18
101	AT4G17030	AT-EXPR					1.257	0.0001	expansin-like B1
102	AT2G28950	ATEXP6			1.391	0.0001	1.226	0.0001	expansin A6
103	AT2G03090	ATEXP15			1.888	0.0001	1.176	0.0001	expansin A15
104	AT2G20750	ATEXPB1			1.814	0.0001	1.117	0.0001	expansin B1
105	AT5G57560	TCH4			-1.239	0.0001	-1.637	0.0001	Xyloglucan endotransglucosylase/hydrolase family protein
106	AT2G18660	PNP-A	-1.615	0.0134	-2.363	0.0017	-2.711	0.0034	plant natriuretic peptide A
107	AT2G36870	XTH32	-1.124	0.0006					xyloglucan endotransglucosylase/hydrolase 32
108	AT1G69530	AT-EXP1	-1.272	0.00005					expansin A1
109	AT3G55500	ATEXP16	-1.599	0.00155					expansin A16
110	AT4G01630	ATEXP17			1.416	0.0001			expansin A17
111	AT5G48070	ATXTH20			1.412	0.0001			xyloglucan endotransglucosylase/hydrolase 2
112	AT3G45960	ATEXLA3			-1.603	0.0001			expansin-like A3
113	AT1G64670	BDG1			1.154	0.0004	1.012	0.0011	alpha/beta-Hydrolases superfamily protein
114	AT5G22630	ADT5			1.084	0.0001	1.177	0.0001	arogenate dehydratase 5
115	AT1G11790	ADT1			1.252	0.0001	1.137	0.0001	arogenate dehydratase 1
116	AT3G29200	ATCM1	1.217	0.00005			1.030	0.0001	chorismate mutase 1
117	AT1G24735		1.896	0.006	2.327	0.0004	2.400	0.0003	caffeoyl- o-methyltransferase
118	AT4G26220		1.519	0.00005	1.305	0.0001	1.717	0.0001	probable caffeoyl- o-methyltransferase at4g26220-like
119	AT4G34050	CCoAOMT1	1.281	0.00005	1.728	0.0001	1.632	0.0001	caffeoyl- o-methyltransferase
120	AT2G37040	ATPAL1	1.564	0.00005	1.558	0.0001	1.597	0.0001	PHE ammonia lyase 1
121	AT1G65060	4CL3	1.433	0.00085	1.853	0.0001	1.589	0.0006	4-coumarate:CoA ligase 3
122	AT1G51680	4CL.1	1.771	0.00005	1.672	0.0001	1.547	0.0001	4-coumarate:CoA ligase 1
123	AT3G21240	4CL2	1.457	0.00005	1.420	0.0001	1.449	0.0001	4-coumarate:CoA ligase 2
124	AT4G36220	CYP84A1			1.462	0.0001	1.432	0.0001	ferulic acid 5-hydroxylase 1
125	AT2G40890	CYP98A3	1.453	0.00005	1.244	0.0001	1.269	0.0001	cytochrome P45 , family 98, subfamily A, polypeptide 3
126	AT5G04230	ATPAL3	-1.678	0.00005	-1.533	0.0001	-2.425	0.0001	phenyl alanine ammonia-lyase 3
127	AT1G67980	CCOAMT	-2.275	0.00005	-2.593	0.0001	-3.440	0.0001	caffeoyl-CoA 3-O-methyltransferase
128	AT5G14700		1.023	0.00005					NAD(P)-binding Rossmann-fold superfamily protein
129	AT4G30470		1.100	0.00005	1.100	0.0001			NAD(P)-binding Rossmann-fold superfamily protein
130	AT5G48930	HCT	1.381	0.00005	1.023	0.0001			hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
131	AT3G19450	ATCAD4	1.397	0.00005					GroES-like zinc-binding alcohol dehydrogenase family protein
132	AT3G21230	4CL5	1.320	0.00005					4-coumarate:CoA ligase 5
133	AT5G54160	ATOMT1	1.276	0.00005					O-methyltransferase 1
134	AT1G15950	ATCCR1	1.265	0.00005					cinnamoyl coa reductase 1
135	AT3G53260	ATPAL2	1.207	0.00005					phenylalanine ammonia-lyase 2
136	AT3G10340	PAL4	1.157	0.00005					phenylalanine ammonia-lyase 4
137	AT2G30490	ATC4H	1.034	0.00005					cinnamate-4-hydroxylase
138	AT4G34230	ATCAD5	1.020	0.00005					cinnamyl alcohol dehydrogenase 5
139	AT5G66690	UGT72E2	1.487	0.00005					UDP-Glycosyltransferase superfamily protein
140	AT5G16410				2.492	0.0001	2.873	0.0001	HXXXD-type acyl-transferase family protein
141	AT5G47980				2.723	0.0001	1.753	0.0001	HXXXD-type acyl-transferase family protein
142	AT5G07860				1.148	0.0002	1.602	0.0001	HXXXD-type acyl-transferase family protein
143	AT1G28680		1.051	0.00005	1.470	0.0001	1.234	0.0004	HXXXD-type acyl-transferase family protein
144	AT5G07870				1.205	0.0001	1.223	0.0001	HXXXD-type acyl-transferase family protein
145	AT5G23220	NIC3	-3.592	0.00005	-4.377	0.0001	-5.451	0.0001	nicotinamidase 3
146	AT4G35160				1.410	0.0001			O-methyltransferase family protein
147	AT1G77520				1.165	0.0001			O-methyltransferase family protein
148	AT1G77530				1.013	0.0001			O-methyltransferase family protein
149	AT4G31910		2.267	0.00005					HXXXD-type acyl-transferase family protein
150	AT1G69880	ATH8			3.533	0.0001	3.626	0.0001	thioredoxin H-type 8
151	AT5G16400	ATF2					1.593	0.0001	thioredoxin F2
152	AT5G61440	ACHT5					1.572	0.0001	atypical CYS HIS rich thioredoxin 5
153	AT1G76080	ATCDSP32	-1.847	0.00005			-1.034	0.0001	chloroplastic drought-induced stress protein of 32 kD
154	AT5G06690	WCRKC1	-1.369	0.00005	-1.045	0.0161	-1.622	0.0012	WCRKC thioredoxin 1
155	AT2G16060	AHB1	-2.030	0.00005	-3.443	0.0001	-2.217	0.0001	hemoglobin 1
156	AT5G11930		3.674	0.00005	4.025	0.0001	4.026	0.0001	Thioredoxin superfamily protein
157	AT4G33040		3.994	0.00005	3.422	0.0001	3.866	0.0001	Thioredoxin superfamily protein
158	AT3G62960				2.726	0.0098	2.712	0.0098	Thioredoxin superfamily protein
159	AT1G06830		1.030	0.0008	3.985	0.0001	2.358	0.0001	Glutaredoxin family protein
160	AT1G03850		-1.098	0.00005			1.870	0.0001	Glutaredoxin family protein
161	AT1G28480	GRX480			2.138	0.0001	1.779	0.0001	Thioredoxin superfamily protein
162	AT5G06470				1.294	0.0001	1.013	0.0016	Glutaredoxin family protein
163	AT3G62930		-3.077	0.00005	-2.107	0.0013	-1.448	0.0057	Thioredoxin superfamily protein
164	AT1G64500				-3.194	0.0001	-4.159	0.0001	Glutaredoxin family protein
165	AT3G62950		-4.544	0.00095					Thioredoxin superfamily protein
166	AT2G47880				12*	0.0001			Glutaredoxin family protein
167	AT2G30540		-1.293	0.0135	1.274	0.0038			Thioredoxin superfamily protein
168	AT3G57070				1.156	0.0001			Glutaredoxin family protein
169	AT5G14070	ROXY2	-1.965	0.00005					Thioredoxin superfamily protein
170	AT4G25100	ATFSD1	-2.609	0.00005	-1.630	0.0001	-1.115	0.0001	Fe superoxide dismutase 1

171	AT3G56350		-1.693	0.00055					Iron/manganese superoxide dismutase family protein
172	AT3G02470	SAMDC					-1.107	0.0018	S-adenosylmethionine decarboxylase
173	AT3G25570		-1.524	0.00785	-2.372	0.0020	-1.456	0.0043	Adenosylmethionine decarboxylase family protein
174	AT4G34710	ADC2	-1.083	0.00005	-1.257	0.0001	-1.505	0.0001	arginine decarboxylase 2
175	AT5G08170	ATAIH			1.125	0.0001	1.080	0.0001	agmatine deiminase
176	AT5G13700	APAO			1.366	0.0043	1.370	0.0042	polyamine oxidase 1
177	AT1G05250				3.259	0.0100	3.110	0.0120	Peroxidase superfamily protein
178	AT2G35380		2.594	0.00005	2.938	0.0001	2.796	0.0001	Peroxidase superfamily protein
179	AT5G14130				2.430	0.0028	2.619	0.0017	Peroxidase superfamily protein
180	AT2G38390		1.920	0.00005	2.589	0.0001	2.479	0.0001	Peroxidase superfamily protein
181	AT1G68850		2.139	0.00005	2.327	0.0001	2.362	0.0001	Peroxidase superfamily protein
182	AT5G15180		2.025	0.00005	2.084	0.0001	2.271	0.0001	Peroxidase superfamily protein
183	AT4G37520				2.110	0.0001	2.058	0.0001	Peroxidase superfamily protein
184	AT3G49960		1.349	0.00005	2.411	0.0001	1.960	0.0001	Peroxidase superfamily protein
185	AT1G34510				2.361	0.0001	1.635	0.0001	Peroxidase superfamily protein
186	AT1G30870				1.659	0.0001	1.517	0.0001	Peroxidase superfamily protein
187	AT5G67400	RHS19			2.363	0.0001	1.505	0.0001	root hair specific 19
188	AT5G22410	RHS18			1.475	0.0001	1.474	0.0001	root hair specific 18
189	AT4G37530				1.650	0.0001	1.242	0.0001	Peroxidase superfamily protein
190	AT4G26010				1.549	0.0001	1.155	0.0001	Peroxidase superfamily protein
191	AT5G42180				1.270	0.0001	1.023	0.0001	Peroxidase superfamily protein
192	AT4G11290						-1.129	0.0001	Peroxidase superfamily protein
193	AT5G39580				-1.448	0.0001	-1.360	0.0001	Peroxidase superfamily protein
194	AT4G33420				-1.996	0.0001	-1.435	0.0001	Peroxidase superfamily protein
195	AT2G37130				-1.362	0.0001	-1.556	0.0001	Peroxidase superfamily protein
196	AT5G64100				-1.431	0.0001	-1.572	0.0001	Peroxidase superfamily protein
197	AT3G49120	ATPCB	-2.164	0.00005			-1.882	0.0001	peroxidase CB
198	AT1G49570		-2.319	0.00005	-1.647	0.0001	-2.067	0.0001	Peroxidase superfamily protein
199	AT5G64120				-1.130	0.0001	-2.261	0.0001	Peroxidase superfamily protein
200	AT5G64110		-2.692	0.00005	-1.916	0.0008	-2.578	0.0008	Peroxidase superfamily protein
201	AT5G19880		-1.721	0.0015					Peroxidase superfamily protein
202	AT2G18140				3.054	0.0133			Peroxidase superfamily protein
203	AT5G05340				1.606	0.0001			Peroxidase superfamily protein
204	AT5G51890				1.349	0.0001			Peroxidase superfamily protein
205	AT1G44970				1.124	0.0001			Peroxidase superfamily protein
206	AT5G66390				1.093	0.0001			Peroxidase superfamily protein
207	AT4G16270				1.075	0.0014			Peroxidase superfamily protein
208	AT5G40150				1.012	0.0001			Peroxidase superfamily protein
209	AT2G34060		-1.038	0.00005					Peroxidase superfamily protein
210	AT4G28110	AtMYB41	2.670	0.00005	3.515	0.0001	2.676	0.0001	myb domain protein 41
211	AT3G12720	ATMYB67	1.534	0.00005	2.452	0.0001	2.613	0.0001	myb domain protein 67
212	AT5G14340	AtMYB40	2.343	0.00005	2.570	0.0001	2.585	0.0001	myb domain protein 4
213	AT3G24310	ATMYB71	1.376	0.00005	1.879	0.0001	2.574	0.0001	myb domain protein 3 5
214	AT5G16770	AtMYB9	1.673	0.00005	2.647	0.0001	2.501	0.0001	myb domain protein 9
215	AT1G16490	ATMYB58	1.805	0.00005	2.295	0.0001	2.249	0.0001	myb domain protein 58
216	AT3G02940	AtMYB107	1.355	0.00005	2.105	0.0001	2.135	0.0001	myb domain protein 1 7
217	AT1G17950	ATMYB52	2.174	0.00005	1.688	0.0001	2.124	0.0001	myb domain protein 52
218	AT4G33450	ATMYB69	1.136	0.00005	1.769	0.0001	2.092	0.0001	myb domain protein 69
219	AT2G23290	AtMYB70			1.340	0.0001	2.020	0.0001	myb domain protein 7
220	AT1G34670	AtMYB93	2.081	0.00005	2.170	0.0001	2.019	0.0001	myb domain protein 93
221	AT4G17785	MYB39			2.049	0.0001	1.956	0.0001	myb domain protein 39
222	AT4G12350	AtMYB42	1.697	0.00325	2.096	0.0003	1.931	0.0006	myb domain protein 42
223	AT1G57560	AtMYB50			2.173	0.0001	1.794	0.0001	myb domain protein 5
224	AT3G48920	AtMYB45	2.233	0.00005	2.040	0.0001	1.737	0.0001	myb domain protein 45
225	AT1G73410	ATMYB54	1.479	0.00005	1.770	0.0001	1.698	0.0001	myb domain protein 54
226	AT4G09460	AtMYB6					1.555	0.0001	myb domain protein 6
227	AT1G09540	ATMYB61			1.175	0.0001	1.255	0.0001	myb domain protein 61
228	AT1G79180	ATMYB63	1.375	0.00005	1.072	0.0001	1.188	0.0001	myb domain protein 63
229	AT5G57620	AtMYB36			1.103	0.0001	1.152	0.0001	myb domain protein 36
230	AT4G37260	ATMYB73					1.046	0.0001	myb domain protein 73
231	AT5G52260	AtMYB19	1.188	0.00005	1.330	0.0001	1.006	0.0001	myb domain protein 19
232	AT4G22680	AtMYB85					1.005	0.0002	myb domain protein 85
233	AT1G48000	AtMYB112					-1.073	0.0022	myb domain protein 112
234	AT5G54230	AtMYB49			-1.536	0.0001	-1.133	0.0001	myb domain protein 49
235	AT2G02060						-1.140	0.0001	Homeodomain-like superfamily protein
236	AT4G01680	AtMYB55					-1.150	0.0001	myb domain protein 55
237	AT3G28910	ATMYB30			-1.535	0.0001	-1.539	0.0001	myb domain protein 3
238	AT3G06490	AtMYB108	1.132	0.00005					myb domain protein 1 8
239	AT4G38620	ATMYB4	1.103	0.00005					myb domain protein 4
240	AT4G34990	AtMYB32	1.092	0.00005					myb domain protein 32
241	AT1G66230	AtMYB20	1.059	0.00005					myb domain protein 2
242	AT5G15310	ATMIXTA	-1.280	0.01235					myb domain protein 16
243	AT3G53200	AtMYB27	-1.780	0.00425	2.056	0.0050			myb domain protein 27
244	AT4G25560	AtMYB18	1.401	0.00005	1.312	0.0001			myb domain protein 18
245	AT5G65230	AtMYB53	1.262	0.00005	1.188	0.0001			myb domain protein 53
246	AT2G31180	ATMYB14			1.020	0.0001			myb domain protein 14
247	AT3G47600	ATMYB94			-1.030	0.0002			myb domain protein 94
248	AT5G62470	ATMYB96			-1.600	0.0001			myb domain protein 96
249	AT3G27220		-1.332	0.00005	-1.717	0.0001			Galactose oxidase/kelch repeat superfamily protein
250	AT4G05100	AtMYB74	3.192	0.00005					myb domain protein 74
251	AT5G16600	AtMYB43	1.055	0.00005					myb domain protein 43
252	AT5G56840		4.366	0.00005	4.702	0.0001	5.055	0.0001	myb-like transcription factor family protein
253	AT4G36570	ATRL3	2.095	0.00005	5.087	0.0001	4.503	0.0001	RAD-like 3
254	AT2G21650	ATRL2			4.061	0.0002	4.081	0.0002	Homeodomain-like superfamily protein
255	AT1G01380	ETC1	3.064	0.00005	2.284	0.0001	2.704	0.0001	Homeodomain-like superfamily protein
256	AT1G19000						1.137	0.0001	Homeodomain-like superfamily protein
257	AT5G37260	CIR1			-1.759	0.0001	-1.027	0.0001	Homeodomain-like superfamily protein
258	AT3G09600		-1.245	0.00005			-1.029	0.0001	Homeodomain-like superfamily protein
259	AT2G38090				-1.492	0.0001	-1.043	0.0001	Duplicated homeodomain-like superfamily protein
260	AT3G16350		-1.362	0.00005	-1.338	0.0001	-1.205	0.0001	Homeodomain-like superfamily protein
261	AT5G58900				-1.344	0.0001	-1.690	0.0001	Homeodomain-like transcriptional regulator
262	AT1G01060	LHY	-2.062	0.00005					Homeodomain-like superfamily protein
263	AT1G75250	ATRL6	-7*	0.00005					RAD-like 6
264	AT1G71030	ATMYBL2	-3.032	0.00005	-1.871	0.0051			MYB-like 2
265	AT1G70000		1.480	0.00005					myb-like transcription factor family protein
266	AT2G46830	CCA1	-1.733	0.00005					circadian clock associated 1
267	AT2G33710		1.700	0.00005	3.094	0.0001	2.941	0.0001	Integrase-type DNA-binding superfamily protein
268	AT1G03800	ATERF10					2.453	0.0032	ERF domain protein 1
269	AT2G38340						2.212	0.0114	Integrase-type DNA-binding superfamily protein
270	AT3G16280				1.683	0.0001	1.777	0.0001	Integrase-type DNA-binding superfamily protein

271	AT4G36900	DEAR4	1.166	0.00005			1.745	0.0001	related to AP2 1
272	AT3G25730	EDF3	1.269	0.00005	1.517	0.0001	1.684	0.0001	ethylene response DNA binding factor 3
273	AT4G16750						1.519	0.0001	Integrase-type DNA-binding superfamily protein
274	AT1G13260	EDF4					1.196	0.0001	related to ABI3/VP1 1
275	AT1G22985						1.034	0.0001	Integrase-type DNA-binding superfamily protein
276	AT5G52020						-1.064	0.0001	Integrase-type DNA-binding superfamily protein
277	AT5G13330	Rap2.6L	-1.631	0.00005			-1.106	0.0001	related to AP2 6l
278	AT2G46310	CRF5			-1.047	0.0001	-1.131	0.0001	cytokinin response factor 5
279	AT1G43160	RAP2.6			-1.146	0.0001	-1.157	0.0001	related to AP2 6
280	AT1G68550						-1.396	0.0001	Integrase-type DNA-binding superfamily protein
281	AT4G25470	ATCBF2					-1.405	0.0060	C-repeat/DRE binding factor 2
282	AT2G20880				-3.403	0.0001	-1.516	0.0001	Integrase-type DNA-binding superfamily protein
283	AT4G28140		-1.158	0.0014	-1.897	0.0001	-1.591	0.0001	Integrase-type DNA-binding superfamily protein
284	AT4G25480	ATCBF3			-1.266	0.0001	-1.643	0.0001	dehydration response element B1A
285	AT3G54990	SMZ			-1.046	0.0001	-1.795	0.0001	Integrase-type DNA-binding superfamily protein
286	AT1G64380		-1.188	0.00045	-1.530	0.0001	-1.955	0.0001	Integrase-type DNA-binding superfamily protein
287	AT2G47520	HRE2			-2.530	0.0025	-2.149	0.0161	Integrase-type DNA-binding superfamily protein
288	AT4G13620				-2.436	0.0001	-2.562	0.0001	Integrase-type DNA-binding superfamily protein
289	AT2G22200				-2.158	0.0001	-2.858	0.0001	Integrase-type DNA-binding superfamily protein
290	AT4G06746	DEAR5	-1.801	0.00005	-4.037	0.0001	-3.932	0.0001	related to AP2 9
291	AT1G22810		2.537	0.00015					Integrase-type DNA-binding superfamily protein
292	AT5G51990	CBF4	2.046	0.00005					C-repeat-binding factor 4
293	AT3G25890		1.337	0.00005					Integrase-type DNA-binding superfamily protein
294	AT1G71450		1.310	0.0069					Integrase-type DNA-binding superfamily protein
295	AT5G64750	ABR1	1.062	0.0082					Integrase-type DNA-binding superfamily protein
296	AT4G11140	CRF1	-1.149	0.01375					cytokinin response factor 1
297	AT1G36060		-1.447	0.00005					Integrase-type DNA-binding superfamily protein
298	AT5G65130		-1.596	0.00235					Integrase-type DNA-binding superfamily protein
299	AT2G40340	AtERF48			1.649	0.0001			Integrase-type DNA-binding superfamily protein
300	AT5G25810	tny			1.055	0.0001			Integrase-type DNA-binding superfamily protein
301	AT4G39780				-1.056	0.0001			Integrase-type DNA-binding superfamily protein
302	AT1G46768	RAP2.1	-1.184	0.00005	-1.190	0.0001			related to AP2 1
303	AT1G22190				-1.341	0.0001			Integrase-type DNA-binding superfamily protein
304	AT1G25560	EDF1			-1.395	0.0001			AP2/B3 transcription factor family protein
305	AT1G72360	HRE1			-1.675	0.0001			Integrase-type DNA-binding superfamily protein
306	AT2G44940		1.307	0.00005					Integrase-type DNA-binding superfamily protein
307	AT1G64000	ATWRKY56	2.493	0.00005	3.064	0.0001	3.444	0.0001	WRKY DNA-binding protein 56
308	AT4G22070	ATWRKY31			3.784	0.0001	3.292	0.0001	WRKY DNA-binding protein 31
309	AT5G41570	ATWRKY24	2.494	0.00005	2.797	0.0001	2.759	0.0001	WRKY DNA-binding protein 24
310	AT4G04450	AtWRKY42	2.413	0.00005	4.589	0.0001	2.746	0.0007	WRKY family transcription factor
311	AT2G46130	ATWRKY43	3.011	0.00005	2.469	0.0001	2.649	0.0001	WRKY DNA-binding protein 43
312	AT2G40740	ATWRKY55	1.818	0.00005	1.567	0.0008	1.982	0.0001	WRKY DNA-binding protein 55
313	AT2G40750	ATWRKY54	1.481	0.00005	1.212	0.0001	1.505	0.0001	WRKY DNA-binding protein 54
314	AT5G64810	ATWRKY51					1.422	0.0001	WRKY DNA-binding protein 51
315	AT1G29860	ATWRKY71					1.250	0.0007	WRKY DNA-binding protein 71
316	AT4G18170	ATWRKY28			-1.233	0.0004	-1.027	0.0011	WRKY DNA-binding protein 28
317	AT2G47260	ATWRKY23					-1.077	0.0001	WRKY DNA-binding protein 23
318	AT5G01900	ATWRKY62	-1.285	0.00005			-1.122	0.0051	WRKY DNA-binding protein 62
319	AT4G01250	AtWRKY22					-1.133	0.0001	WRKY family transcription factor
320	AT1G66600	ABO3			-1.278	0.0030	-1.337	0.0017	ABA overly sensitive mutant 3
321	AT5G24110	ATWRKY30			-2.239	0.0001	-1.366	0.0007	WRKY DNA-binding protein 3
322	AT5G26170	ATWRKY50					-1.477	0.0041	WRKY DNA-binding protein 5
323	AT1G69310	ATWRKY57			-1.343	0.0001	-1.809	0.0001	WRKY DNA-binding protein 57
324	AT1G80590	ATWRKY66					-7*	0.0001	WRKY DNA-binding protein 66
325	AT5G46350	ATWRKY8	-1.130	0.01545					WRKY DNA-binding protein 8
326	AT3G01970	ATWRKY45	-1.494	0.00005					WRKY DNA-binding protein 45
327	AT5G13080	ATWRKY75	-1.637	0.00005					WRKY DNA-binding protein 75
328	AT2G21900	ATWRKY59			1.145	0.0001			WRKY DNA-binding protein 59
329	AT5G22570	ATWRKY38			1.102	0.0162			WRKY DNA-binding protein 38
330	AT2G46400	ATWRKY46			-1.878	0.0001			WRKY DNA-binding protein 46
331	AT3G53650				1.829	0.0001	1.748	0.0001	Histone superfamily protein
332	AT2G37470						1.183	0.0001	Histone superfamily protein
333	AT3G20670	HTA13					1.164	0.0001	histone H2A 13
334	AT2G28740	HIS4					1.100	0.0001	histone H4
335	AT5G10400						1.074	0.0001	Histone superfamily protein
336	AT3G09480				1.280	0.0002	1.023	0.0018	Histone superfamily protein
337	AT3G46320						1.021	0.0001	Histone superfamily protein
338	AT3G27360						1.003	0.0098	Histone superfamily protein
339	AT2G28720		1.016	0.00005					Histone superfamily protein
340	AT2G18050	HIS1-3	1.252	0.00005					histone H1-3
341	AT3G09340		4.443	0.0042	5.932	0.0001	4.403	0.0001	Transmembrane amino acid transporter family protein
342	AT1G61270		1.650	0.00005	2.445	0.0001	2.412	0.0001	Transmembrane amino acid transporter family protein
343	AT5G09220	AAP2	1.772	0.00005	1.843	0.0001	1.722	0.0001	amino acid permease 2
344	AT5G63850	AAP4	1.438	0.00005	2.104	0.0001	1.677	0.0001	amino acid permease 4
345	AT5G40780	LHT1			1.299	0.0001	1.568	0.0001	lysine histidine transporter 1
346	AT5G49630	AAP6	1.321	0.00005	1.743	0.0001	1.564	0.0001	amino acid permease 6
347	AT3G09330				1.962	0.0001	1.561	0.0001	Transmembrane amino acid transporter family protein
348	AT4G21120	AAT1			1.442	0.0001	1.409	0.0001	amino acid transporter 1
349	AT5G41800				1.399	0.0001	1.341	0.0001	Transmembrane amino acid transporter family protein
350	AT5G23810	AAP7			1.766	0.0001	1.212	0.0001	amino acid permease 7
351	AT2G39130		1.258	0.00005	1.105	0.0001	1.086	0.0001	Transmembrane amino acid transporter family protein
352	AT3G10600	CAT7					-1.115	0.0017	cationic amino acid transporter 7
353	AT5G15240				-2.011	0.0001	-1.534	0.0001	Transmembrane amino acid transporter family protein
354	AT3G56200				-1.497	0.0001	-1.909	0.0001	Transmembrane amino acid transporter family protein
355	AT3G54830				-2.417	0.0001	-3.818	0.0003	Transmembrane amino acid transporter family protein
356	AT2G33260				1.103	0.0064			Tryptophan/tyrosine permease
357	AT5G02170		-1.009	0.00015	1.074	0.0018			Transmembrane amino acid transporter family protein
358	AT5G04770	ATCAT6	-1.435	0.00005					cationic amino acid transporter 6
359	AT5G04120		6.302	0.00005	9.507	0.0001	9.757	0.0001	Phosphoglycerate mutase family protein
360	AT3G49160						1.051	0.0001	pyruvate kinase family protein
361	AT2G42600	ATPPC2			1.136	0.0001			phosphoenolpyruvate carboxylase 2
362	AT5G13110	G6PD2			-1.112	0.0001	-1.220	0.0001	glucose-6-phosphate dehydrogenase 2
363	AT5G35790	G6PD1	-1.176	0.0011			-1.561	0.0066	glucose-6-phosphate dehydrogenase 1
364	AT1G24280	G6PD3	1.129	0.00005	-1.755	0.0001	-2.539	0.0001	glucose-6-phosphate dehydrogenase 3
365	AT1G13700	PGL1			1.690	0.0002	1.859	0.0001	6-phosphogluconolactonase 1
366	AT1G64190				-1.348	0.0001	-1.950	0.0001	6-phosphogluconate dehydrogenase family protein
367	AT5G41670				-1.194	0.0001	-1.999	0.0001	6-phosphogluconate dehydrogenase family protein
368	AT2G11270						12*	0.0006	citrate synthase-related
369	AT5G25880	ATNADP-ME3	1.396	0.00015	1.703	0.0001	1.517	0.0001	NADP-malic enzyme 3
370	AT1G70410	ATBCA4					-1.175	0.0001	beta carbonic anhydrase 4

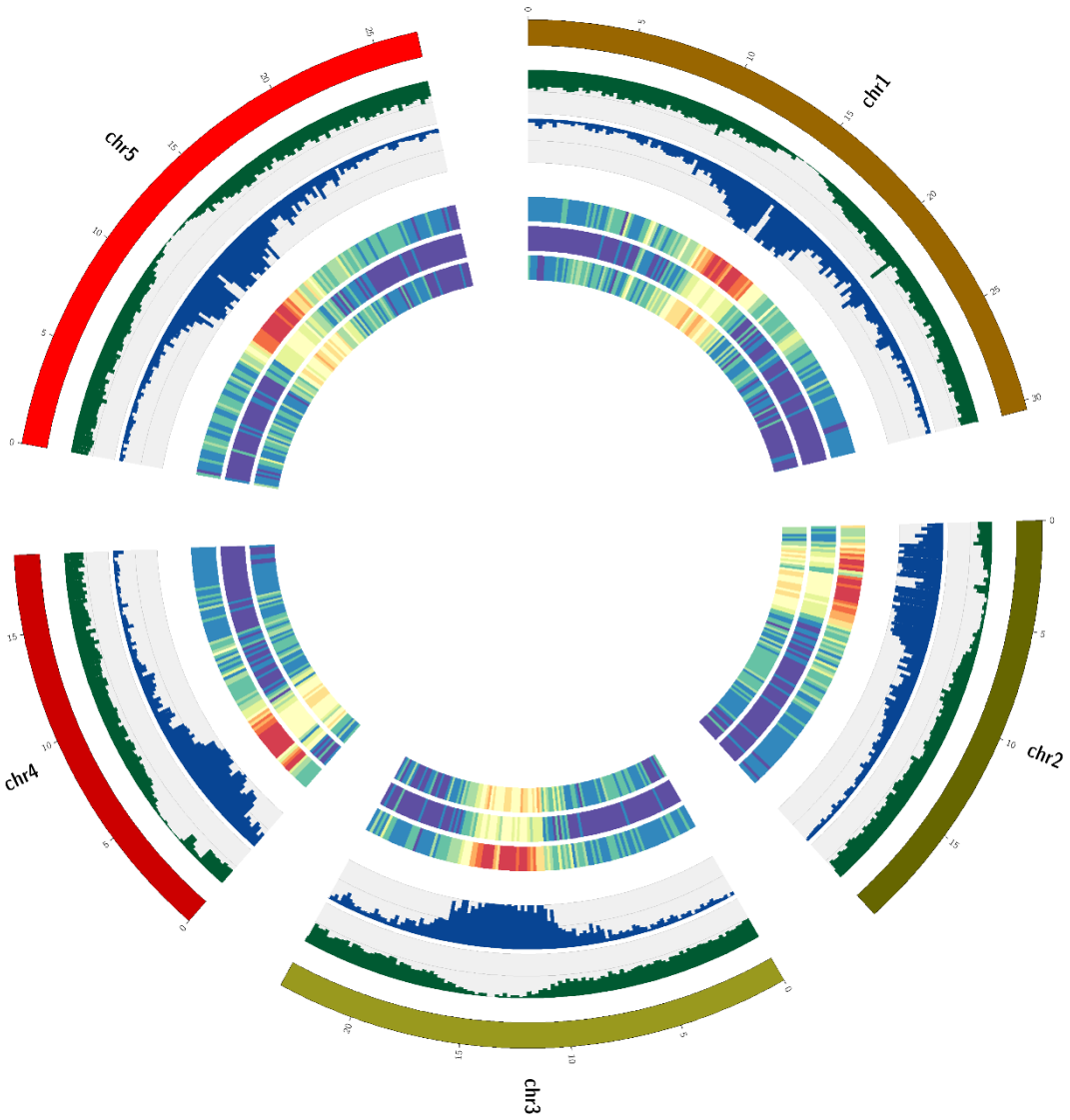

Figure S1. Schematic representation of *Arabidopsis* methylome in control roots of first generation (N sufficiency, C0). The graph shows, from the outer to the inner circle, chromosome ideograms of the *Arabidopsis* genome, gene density, frequency of transposable elements (TE), methylation in the CG context, methylation in the CHG context and methylation in the CHH context. From blue to red color there is an increment in methylation. The graph was produced with the Circos software (www.circos.ca - Krzywinski M. et al., 2009) and represented the average of two replicates (C0-1, C0-2).

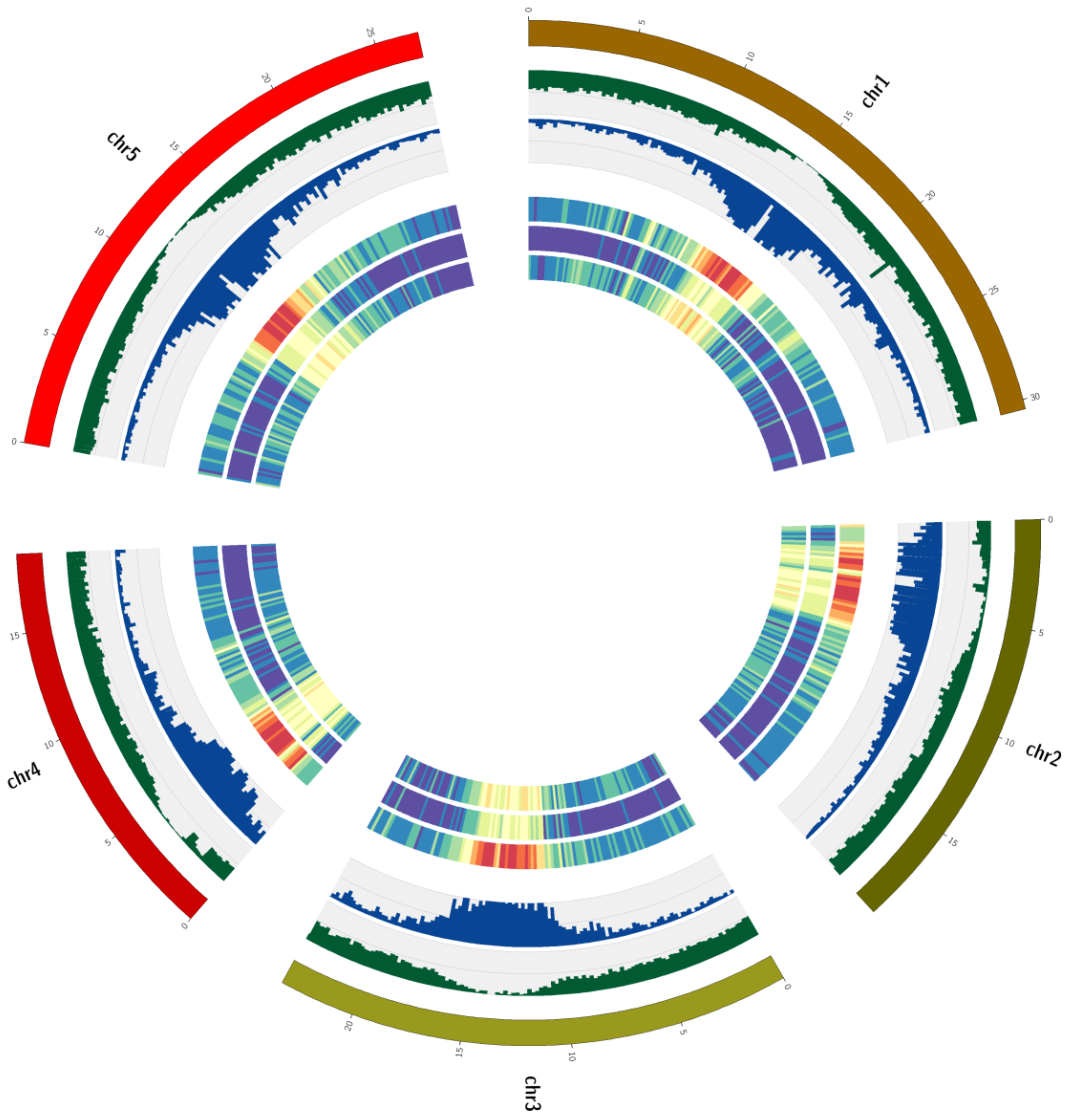

Figure S2. Schematic representation of *Arabidopsis* methylome in roots of plants grown under N deprivation during first and second generations (TT). The graph shows, from the outer to the inner circle, chromosome ideograms of the *Arabidopsis* genome, gene density, frequency of transposable elements (TE), methylation in the CG context, methylation in the CHG context and methylation in the CHH context. Methylation in the CG, CHG, CHH contexts are shown with a color scale from blue (-100%) to red (+100%). The graph was produced with the Circos software (www.circos.ca - Krzywinski et al., 2009) and represented the average of two replicates (TT-1, TT-2).

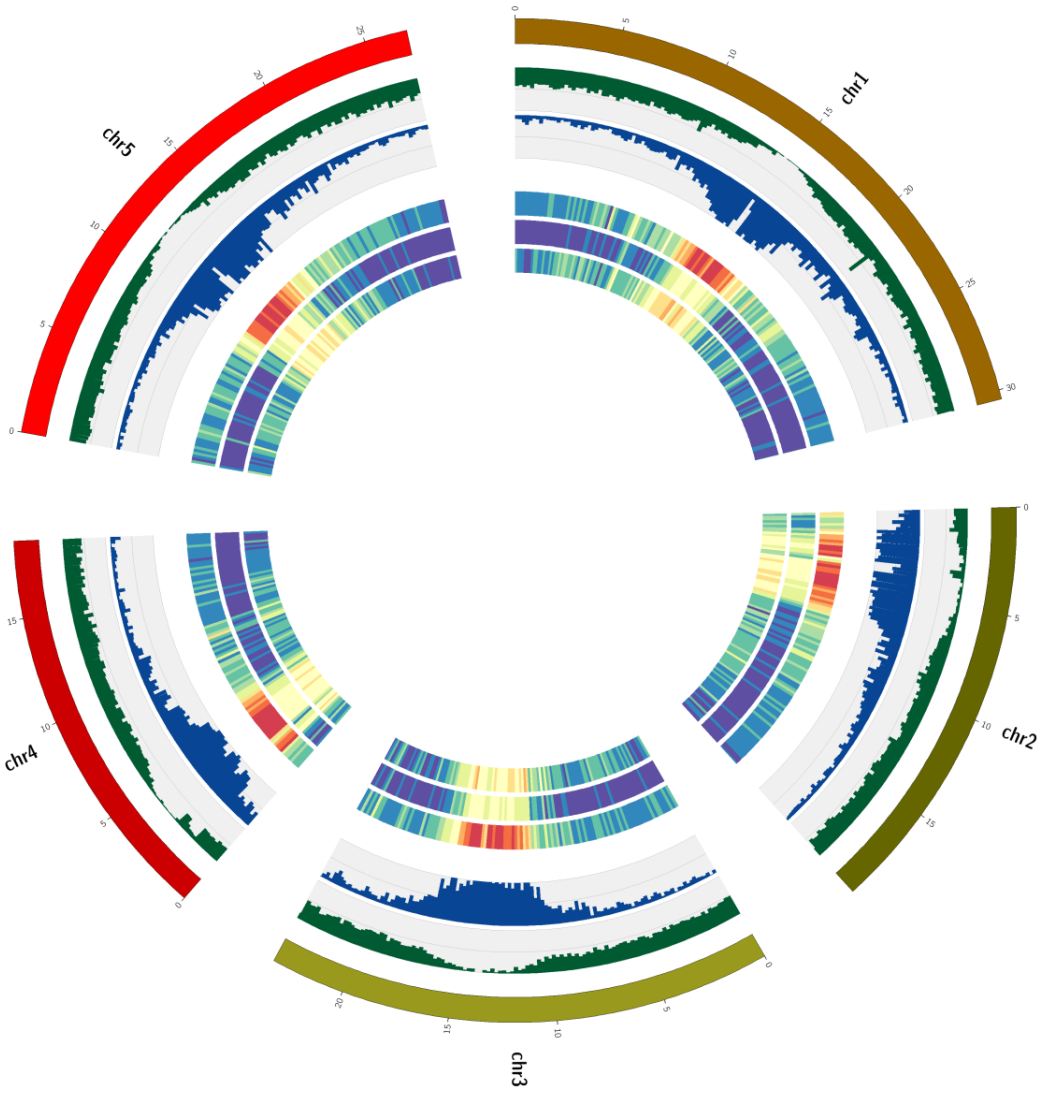

Figure S3. Schematic representation of *Arabidopsis* methylome in roots of plants grown under N sufficiency during first and second generations (CC). The graph shows, from the outer to the inner circle, chromosome ideograms of the *Arabidopsis* genome, gene density, frequency of transposable elements (TE), methylation in the CG context, methylation in the CHG context and methylation in the CHH context. Methylation in the CG, CHG, CHH contexts are shown with a color scale from blue (-100%) to red (+100%). The graph was produced with the Circos software (www.circos.ca - Krzywinski et al., 2009) and represented the average of two replicates (CC-1, CC-2).

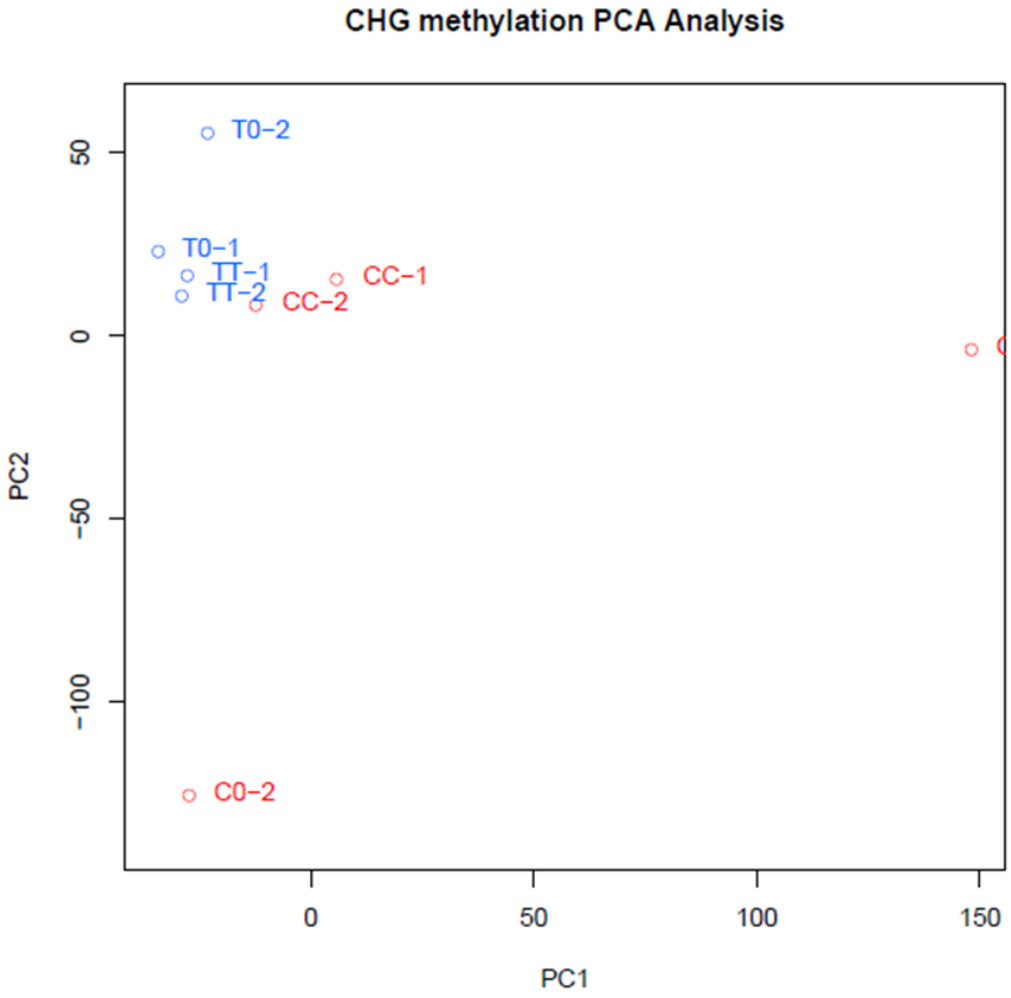

Figure S4. Principal component analysis (PCA) of methylation in CHG context. A cluster analysis based on PCA method was performed in all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and in all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012).

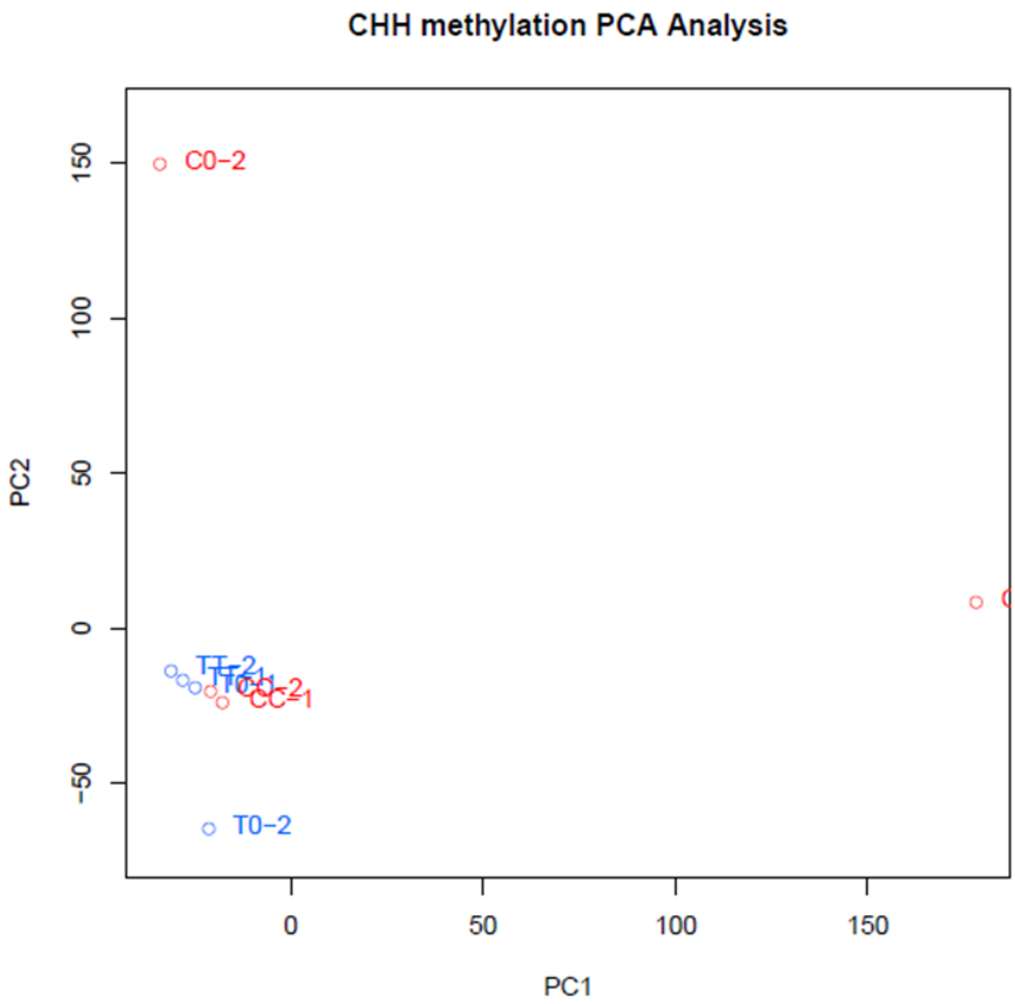

Figure S5.Principal component analysis (PCA) of methylation in CHH context. A cluster analysis based on PCA method was performed in all biological replicates of the N-deprived samples in first and second generation (T0-1; T0-2; TT-1, TT-2) and in all biological replicates of the control samples of both generations (C0-1, C0-2, CC-1, CC-2). The analysis was performed with MethylKit R Package (Akalin et al., 2012)

Table S2. List of genes found in DMRs in different contexts and generations modulated in *Arabidopsis* roots in response to different N-deprivation treatments. In the table are shown: gene number (*#*), the *Arabidopsis* gene identifier (*Gene_ID*); the common name of gene product (*Symbols*); for each comparison, Log2 FC (Fold change) and relative p-value; the description of the function.

			CHG 2nd generation						
#	**Gene_ID**	**Symbol**	**log2-ratio T0 vs C0**	**p-value**	**log2-ratio TT vs CC**	**p-value**	**log2-ratio CT vs CC**	**p-value**	**Description**
1	AT3G61470	LHCA2	-2,100	0,00005					photosystem I light harvesting complex gene 2
2	AT2G21100		1,923	0,00005	2,61146	0,00005	2,657	0,00005	Disease resistance-responsive (dirigent-like protein) family protein
3	AT5G38450	CYP735A1	-1,542	0,00005	-1,902	0,00005	-2,447	0,00005	cytochrome P45 , family 735, subfamily A, polypeptide 1
4	AT1G14120				1,893	0,00005			2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
5	AT2G04070		1,129	0,0006	1,914	0,00005			MATE efflux family protein
			CHH 1st generation						
6	AT1G33320						-1,501	0,00005	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein
7	AT1G70880				-1,104	0,00005			Polyketide cyclase/dehydrase and lipid transport superfamily protein
8	AT3G55940		2,264	0,00005	2,638	0,00005	2,270	0,00005	Phosphoinositide-specific phospholipase C family protein
9	AT1G01010	ANAC001			-1,107	0,00005			NAC domain containing protein 1
10	AT5G22530				-3,801	0,00085	-2,664	0,00005	Protein of unknown function
11	AT5G24600						-1,846	0,00045	Protein of unknown function, DUF599
			CHH 2nd generation						
12	AT4G12800	PSAL	-1,887	0,00005	-1,893	0,0147			photosystem I subunit l
13	AT4G12330	CYP706A7	-1,013	0,00005	-3,143	0,00005	-2,823	0,00005	cytochrome P45 , family 7 6, subfamily A, polypeptide 7
14	AT1G60730				-1,063	0,00005			NAD(P)-linked oxidoreductase superfamily protein
15	AT3G26460		1,363	0,00005	2,199	0,00005	2,026	0,00005	Polyketide cyclase/dehydrase and lipid transport superfamily protein
16	AT5G52060	ATBAG1	1,375	0,00005					BCL-2-associated athanogene 1
17	AT3G26720				-1,103	0,00005			Glycosyl hydrolase family 38 protein
18	AT1G78340	ATGSTU22	1,191	0,00005	2,565	0,00005	2,749	0,00005	glutathione S-transferase TAU 22
19	AT5G46870		1,745	0,00005	2,142	0,00005	1,993	0,00005	RNA-binding (RRM/RBD/RNP motifs) family protein
20	AT3G46340		1,341	0,00005	3,169	0,00005	2,411	0,00005	Leucine-rich repeat protein kinase family protein
21	AT3G49260	iqd21	2,233	0,00115					IQ-domain 21
22	AT1G04425		-1,374	0,00005					micro RNA
23	AT1G31770	ABCG14					-1,022	0,00005	ATP-binding cassette 14
24	AT3G56200				-1,497	0,00005	-1,909	0,00005	Transmembrane amino acid transporter family protein
25	AT5G26200		-2,260	0,0072					Mitochondrial substrate carrier family protein
26	AT2G04070		1,129	0,0006	1,914	0,00005			MATE efflux family protein
27	AT1G31200	ATPP2-A9					-1,076	0,01115	phloem protein 2-A9
28	AT3G33528		-1,693	0,0108				0	unknown
29	AT4G08360		-3,229	0,00005			-3,015	0,00385	KOW domain-containing protein
30	AT1G66420		2,290	0,01095				0	DNA-binding storekeeper protein-related transcriptional regulator
31	AT2G20080						1,602	0,00005	unknown
32	AT3G02410	ICME-LIKE2	2,110	0,0004	2,490	0,00005	2,229	0,00005	alpha/beta-Hydrolases superfamily protein
33	AT3G41762		-1,518	0,0001					unknown
34	AT5G22555				1,621	0,00005			unknown
35	AT5G40470		1,044	0,00005					RNI-like superfamily protein
36	AT5G66800						-1,094	0,0101	unknown

Table S3. Percentage of genes associated with differentially methylated regions (DMRs) located in the promoter (2000 bp upstream region), the trailer (2000 bp downstream region) or the gene body.

	% promoter	% trailer	% gene body
CG 1st generation	35,6	43,8	37,0
CG 2nd generation	33,1	38,3	42,2
CHG 1st generation	50	25	50
CHG 2nd generation	21,7	54,3	41,3
CHH 1st generation	54,8	35,7	28,6
CHH 2nd generation	50,6	39,3	27,5

Table S4. List of genes found near TE in DMRs in different contexts and generations modulated in *Arabidopsis* roots in response to different N-deprivation treatments. In the table are shown: gene number (*#*), the *Arabidopsis* gene identifier (*Gene_ID*); the common name of gene product (*Symbols*); for each comparison, Log2 FC (Fold change) and relative p-value; the description of the function. With asterisks, infinite expression values have been replaced with|12.00|, as theoretical maximal value.

CG 1st generation									
#	**Gene_ID**	**Symbol**	**log2-ratio T0 vs C0**	**p-value**	**log2-ratio TT vs CC**	**p-value**	**log2-ratio CT vs CC**	**p-value**	**Description**
1	AT1G21050		-1,920	0,00005	-1,140	0,00005	-1,040	0,00005	Protein of unknown function, DUF617
2	AT1G79160		1,442	0,00005	1,926	0,00005	1,911	0,00005	not assigned unknown
3	AT1G79170				12*	0,0017			not assigned unknown
CG 2nd generation									
4	AT3G61470	LHCA2	-2,100	0,00005					photosystem I light harvesting complex gene 2
5	AT4G11320				1,121	0,00005	1,276	0,00005	Papain family cysteine protease
6	AT5G44572		-1,832	0,00005					not assigned no ontology
7	AT3G06435		-1,104	0,00005	-2,539	0,00005			Expressed protein
CHG 2nd generation									
8	AT3G61470	LHCA2	-2,100	0,00005					photosystem I light harvesting complex gene 2
9	AT2G04070		1,129	0,0006	1,914	0,00005			MATE efflux family protein
CHH 1st generation									
10	AT5G22530				-3,801	0,00085	-2,664	0,00005	not assigned unknown
CHH 2nd generation									
11	AT3G49260	iqd21	2,233	0,00115					IQ-domain 21
12	AT5G26200		-2,260	0,0072					Mitochondrial substrate carrier family protein
13	AT2G04070		1,129	0,0006	1,914	0,00005			MATE efflux family protein
14	AT1G31200	ATPP2-A9					-1,076	0,01115	phloem protein 2-A9
15	AT1G66420		2,290	0,01095					DNA-binding storekeeper protein-related transcriptional regulator
16	AT5G22555				1,621	0,00005			not assigned unknown

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