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**Molecular and physiological traits related to Nitrogen Use Efficiency (NUE) in eggplant  
(*Solanum melongena* L.)  
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## Abstract

Eggplant (*Solanum melongena* L.) yield is highly sensitive to N fertilization, whose excessive use is responsible of environment and human health damages. Lowering N input together with the selection of improved Nitrogen-Use-Efficiency (NUE) genotypes, more able to uptake (NUpE), utilize (NUtE) and remobilize N available in soils, could be challenging to maintain high yields. The aim of this study was to explore the natural variation among 19 eggplant accessions in response to low and high  $\text{NO}_3^-$  supply to identify NUE-contrasting genotypes, in hydroponic and greenhouse pot experiments. AM222 and AM22 accessions were identified in both growing systems as N-use efficient and inefficient, respectively. In addition, another pair of NUE-contrasting genotypes were also chosen (67-3 and 305E40) being parents of available segregant populations (RIL). Overall, the results indicated the key role of N-utilization component (NUtE) to confer high NUE. The higher N-content in AM222 fruits suggesting the efficient N-remobilization as a strategy to enhance NUtE, suggesting GS as a key enzyme.

The role of the main nitrate transporter (NRT2.1) in our system was also evaluated by the assessment of related gene expression trend, a functional assay and allelic variant analysis. For the first time in eggplant, the protein-protein interaction SmNRT2.1-SmNRT3.1 was demonstrated. Interestingly, a SNP *CLCa* variant in the vacuole N-storage chloride channel protein was identified, determining a missense mutation from a TAT/tyrosine to TGT/cysteine codon change. In *silico* analysis demonstrated *CLCa* allelic variants determined structural protein changes whose different functionality in N-storage will be demonstrated by further experiments.

To deeply understanding the physiological and genetic mechanisms underlying NUE complex trait, the four selected eggplant genotypes (AM222, AM22, 67-3 and 305E40) were employed for both transcriptomics and metabolomics approaches. In particular, taking into account both short- and long-term N-stress, the same experimental design was adopted to explain the differences in NUE phenotype between both genotype pairs. Metabolomics results highlighted that alanine, aspartate and glutamate metabolism was highly affected in the efficient genotypes, while RNA-seq analysis was able to underlined differential gene transcripts involved in processes of reactive oxygen species (ROS) rate-limiting production that could be attributed to a higher antioxidant activity ability of the N-use efficient genotypes.

Overall, here we demonstrated the predominant role of NUtE to confer high NUE in eggplant; indeed the role of NUpE related genes did not show significant differences among genotypes. More interestingly, transcriptomics and metabolomics were able to find a novel integrated

network of differential expressed genes and metabolites belong the NUtE component between N-use efficient and inefficient genotypes.

**Keywords:** Enzyme activities, Nitrogen Balance Index (NBI), root morphology, plasticity, heritability, Nitrogen Use Efficiency (NUE); Nitrogen Uptake Efficiency (NUpE), Nitrogen Utilization Efficiency (NUtE), Metabolomics, Transcriptomics.

## CHAPTER 1. THE EGGPLANT CROP

### 1.1. Origins, historical background in the taxonomic context

The *Solanaceae* family comprises about 3500 species, some of them are economically important and widely cultivated such as eggplant, tobacco, pepper, potato and petunia. Many of the cultivated species are of food and ornamental interest, while others are source of chemical compounds and plant models for biotechnological studies.

The classification of the members of this family dates back to the 18th century thanks to the interest of famous botanists such as Linneaus, Tournefort, Miller, Jacquin etc., based mainly on flower structure and several other morphological traits.

In 1789, Jussieu published his “Genera Plantarum” where, for the first time, the *Solanaceae* family was described. In the mid-nineteenth century, Dunal proposed a large rearrangement within *Solanaceae*, while, at the end of the same century, two new treatises on vascular plants were published, "Bentham and Hooker's Genera Plantarum" and "Die Naturlichen Pflanzenfamilien of Engler and Prantl", respectively, in which the *Solanaceae* were included. Over the years, the classification was enriched based on new species identification and for new chemical, floral, embryological and anatomical features.

In the 20<sup>th</sup> century, Bitter (1912), with the contribution of other botanists, examined the current largest family of *Solanaceae* and the genus *Solanum*, using modern criteria such as karyotype, ploidy level, seeds, crossability, flower vasculature and proteins.

In the second half of the 20th century (D'arcy 1972, 1979, 1991), the genus *Solanum* was subdivided into seven subgenres as *Leptostemonum* (to which eggplant belong), *Potatoae* (to which potato, tuberous and tomato belong), *Solanum* (nightshades), *Minon*, *Bassovia*, *Lycia solanum*, *Archae solanum*.

Since taxonomic groupings depend on new factors, such as the availability of new analytical tools, the classification of taxa could evolve over the years, being the subject of debate in the scientific world.

The Russian pioneer Vavilov, who in the 1920s and '30s founded the concept of the "centers of origin of the genetic diversity of cultivated plants" or the species diversification centers, recognizes three different subspecies of eggplant, native to different geographical areas of the South Asia:

- subsp. *agrestis* native of India,
- subsp. *west asian* native of the west
- subsp. *oriental-asiaticus* native of Japan-China.

It is well known that the eggplant was domesticated in the Indus-China region in ancient times, and the center of origin is uncertain yet. The first brinjal-eggplant diversification center is considered the whole South-East Asia, the place where the greatest morphological diversity of eggplant was found, with small fruits (a few tens of grams) in the wild eggplant with oblong fruits and various shapes, sizes and different colors. The diversity also concerns other morphological features such as plant height and the presence of thorns as forms of adaptation to different climates (temperate or tropical) and different biotic or abiotic stresses.

The first recorded history of the eggplant has been found in an ancient Chinese agricultural treatise, the *Qimin Yaoshu*, that was completed in 544 BC. The plant has been cultivated in eastern and southern Asia since prehistory (Fuchsia Dunlop 2006).

Eggplant has been cultivated for a long time in India and the first records of culinary use date back to Sanskrit texts (Khan 1979), around the Christian era. It was introduced in Japan from China in the 8th century AD, where eggplant was common and popular for a long time. In India, it is considered a national vegetable with a daily consumption among the poor people, roots, fruits and leaves are known for various medical properties (Khan, 1979) against otitis, dental abscesses, cholera, bronchitis, dysentery, diabetes, asthma and cholesterol excess.

The Mediterranean basin may be considered a secondary diversification center, due to the introduction of eggplant following the 7th century of the Arab conquest.

Cultivars from Mediterranean regions are vigorous plants, characterized by large fruits with different shapes commonly of dark purple color but sometimes white. Nowadays, the eggplant is widespread and cultivated all over the world.

## **1.2. Economic importance**

There are two leading countries in eggplant production, about 90% of world production is from China and India (about 10 million tons and 6 million tons, respectively) (FAO 2013), Japan, (490,000 tons), Philippines (170,000 tons), Indonesia (145,000 tons) and Thailand (64,000 tons). Turkey, Egypt and Italy are the largest producers in the Mediterranean basin with 850,000, 560,000, 362,000 tons, respectively. The practice of growing of  $F_1$  commercial hybrids that replace traditional varieties is very widespread, indeed in Japan, 100% of commercial cultivars are currently  $F_1$  hybrids. In Europe, this percentage decreases, but about 80%, and the old European cultivars are commercially grown only on a small-scale supply to local markets. However, in some countries such as China, local varieties are

produced and utilized preferably for color, shape and size features. However, the spread and distribution of commercial F<sub>1</sub> hybrid cultivars over the world bring significant risk of biodiversity erosion.

### 1.3. Genetics and diversity

*Solanum melongena* is a diploid species ( $2n = 24$ ), preferentially autogamous, although the allogamy is strongly dependent on environmental conditions.

Many studies on variability, using markers such as proteins, isozymes and allozymes in the eggplant were carried out, and related species were made for taxonomy targets. Dendrograms based on cultivated and wild species diversity of proteins shown robust consistency with previous classifications obtained using morphological criteria (Issiki et al., 1994; Karihaloo and Gottlieb, 1995).

Genetic diversity in *S. melongena* cultivars is very low due to the genetic bottlenecks that occurred during its domestication, cultivation and breeding. More recently, novel traits are often bred into commercial varieties from landraces or wild relatives with higher genetic diversity (Muñoz-Falcón et al., 2011).

DNA polymorphisms, based on molecular markers and used to calculate the genetic distances among taxa, are an excellent tool for clarifying the classification and the relationships among the species within the subgenera.

Some authors used chloroplast DNA (cpDNA) for maternal inheritance widely preserved in plant phyla, not highly informative within the species, slightly variable, and therefore more informative among species, very variable among distant species and therefore mainly suitable for studying the subgenus (eg. *Leptostemonum*) (Sakata and Lester, 1994; 1997; Olmstead and Palmer, 1992).

In a context of intraspecific investigation, nuclear DNA markers (as RAPD, AFLP, SNP and SSR), based on coding and non-coding DNA sequence, may be used for the molecular mapping of important agronomic traits and for the comparison of genomes of the same or different species.

Complex agronomic traits such as vigor, number of fruits, and tolerance to biotic and abiotic stresses, are mainly quantitative and under multiple genes control. However, gene control of the development and determination of a specific trait is complex depending on epistatic, pleiotropic and linkage effects.

#### 1.4. The development of advanced genetic tools in the *Solanaceae* family

The *Solanaceae* family includes many members of which only a few are currently used in human food (potato, tomato, pepper, eggplant), others are used as ornamental plants (petunia, *Datura* etc) or medicinal drugs (tobacco, *Atropa belladonna* L., *Hyoscyamus niger* L., *Mandragora* spp.).

In the early twentieth century, the *Solanaceae* were used for research in traditional genetics and in molecular genetics, while in the second half, especially, the tomato was used in genome analysis, linkage maps, positional cloning, and loci of quantitative traits (QTL). By contrast, tobacco was used for the somatic cells study, *in vitro* cells and tissues propagation, genetic transformation and doubled haploids production. *Petunia* was considered a plant model to highlight the biochemical and molecular processes of flower color development, while potato was important for the understanding of physiology, biochemistry and genetics of the early stage of tuber development since the beginning of the 20<sup>th</sup> century (Bernard 1901). During this period, research valuable information was provided on complex agronomic traits using all these plants. Furthermore, the comparative analysis of the tomato genome contributed to the genetic and genomic research of peppers and aubergines and helped to understand the evolution of plant genome.

Finally, the oldest research on the haploid embryos regeneration from pollen grains or the beginning of the doubled haploid (DH) plant production is attributed to *Datura innoxia* (Guha and Maheshwari 1966). DH plant production is now an important biotechnological application in breeding programs of all autofertile species, because homozygous plants can be obtained in a single step and in much shorter time compared to several generations of selfing.

**Tomato.** In the early 1900s, morphological traits such as plant dwarfism, color, fruit and leaf shape were the object of attention in tomato to understand the link between phenotype and genome and the first genetic map was defined (Craig 1907, Halsted 1905, Hedrick and Booth 1907; Drinkard, 1908; Jones 1917).

In the second half of the 20<sup>th</sup> century, the tomato genetic map was increased to 320 morphological and isoenzymatic loci (Mutschler et al., 1987), thanks to the introduction of new mutant phenotypes obtained by radiation-induced mutagenesis (Stubbe 1957, 1972).

Around the 1970s, linkage maps based on morphological and iso-enzymatic markers were set aside because of the isolation of markers based on restriction fragment length (RFLP) DNA polymorphisms, which contributed to the construction of a much faster map than the

previous approaches. RFLP-based map was the first molecular tool for linkage analysis of gene and quantitative loci mapping (QTL), cloning genes with unknown sequence and position of known map associated with a phenotypic effect, introgressive reproduction programs assisted by markers for the introduction of new traits that provide environmental benefits (eg. resistance to diseases).

Among the first QTLs, traits involved in sugars concentration and fruit weight appeared the most meaningful (Osborn et al 1987, Paterson et al., 1988). Many mapping studies were carried out in various interspecific populations since the progeny of interspecific crosses provided greater genetic variation than the intraspecific crosses, making RFLP detection more informative. Once the presumed QTL was mapped, the positional cloning of genes underlying QTL was proceeded, an example was the QTL including fw2.2 on chromosome 2, important for tomato fruit weight, the gene was of unknown function, but involved in cell division (Frary et al., 2000).

Introgression lines (ILs) of *S. brushes* in *S. lycopersicum* were developed based on the selection assisted by markers with RFLP (Eshed and Zamir 1995). This segregant population finally enabled to the identification of an important QTL involved in the fruit sugar content, an invertase gene (Fridman et al., 2004).

However, in recent decades, next generation sequencing technologies have improved the detection of DNA variation allowing the maps construction in intraspecific populations based on single nucleotide polymorphisms (SNP) (Shirasawa et al 2010).

Since the early 2000s, the use of multi-parent inter-croos populations (MAGIC populations) represents as alternative genetic resources for mapping and QTL analysis. MAGIC populations were developed by crossing multiple founder lines, selected to include a wide range of genetic diversity, where the obtained linkage maps showed a high percentage of recombination frequencies compared to the biparental populations. (Pascual et al., 2015).

**Tobacco.** In the scientific community of plants, tobacco is one of the most used plant model for genetic engineering, gene transformation and somatic cell development.

Using tobacco cultures, the totipotency of a single somatic cell able to regenerate roots, sprouts and a functional plant was demonstrated. For this goal, the importance of hormones supply in the culture medium to induce plant organs differentiation were widely reported (e.g. Muir et al. 1954; Vasil and Hildebrandt 1965a, b; Nagata and Takebe 1971, 1968).

With the setting up of the “Murashige and Skoog” (MS) culture medium, it was possible to guide the development of tobacco cell cultures as well as of other species, widely applied for *in vitro* propagation yet (Murashige and Skoog 1962).

Several authors used tobacco for *in vitro* anther culture to regenerate from pollen grains haploid plants (Bourgin and Nitsch 1967, Nitsch and Nitsch 1969, Vasil and Nitsch 1975) setting up an important technique for the development of double haploid plants (Negrutiu et al., 1984).

Another important step in scientific research was the transformation of tobacco cells with T-DNA or Ti-plasmid through *Agrobacterium tumefaciens*, which allowed both the stable integration and the inheritance of foreign genes (Chilton et al 1977, De Block et al 1984)

Today, *Nicotiana benthamiana* is the plant model for the study of host-pathogen interactions, becoming an important experimental system for functional analysis on protein localization and protein-protein interactions (Goodin et al., 2008). This is due to the susceptibility of *N. benthamiana* to infection with most viruses, and the ease with which is transformed by leaf infiltration with *Agrobacterium tumefaciens* (agro-infiltration).

**Potato.** Potato genetic analyses are difficult and limiting, due to the tetraploidy and the tetrasomic inheritance of species that generates Mendelian segregation ratios only for two of the twelve possible allelic states of heterozygous parents (Aaaa x aaaa and Aaaa x Aaaa) (Cadman 1942).

The simplification of potato genetics is due to the reduction of ploidy (from tetraploid to diploid) obtained from the parthenogenesis of female pollinated gametes crossing potato with *S. phureja* genotypes (Hougas et al., 1958) or alternatively from another culture of 4n cultivars and the regeneration of 2n male gamete plants (Dunwell and Sunderland 1973).

The first potato linkage maps were constructed using RFLP markers (Bonierbale et al., 1988; Gebhardt et al., 1989, 1991), using interspecific and intraspecific crosses. The construction of linkage maps in two related, although sexually incompatible, species (such as tomato and potato) with the same set of RFLP allowed to study, for the first time, the comparison of genome structure, with the first deduction of models for genome evolution and speciation.

These comparisons were effective because between distant species such as potato-tomato (Bonierbale et al.1988) or potato-arabidopsis (Gebhardt et al., 2003) there are many blocks of synteny. The RFLP linkage maps were milestones for mapping the resistance to Y potato virus (PVY) and the root cyst nematode *Globodera* (Barone et al., 1990; Leonards-

Schippers et al., 1992; Ritter et al., 1991; Kasai et al., 2000; Sattarzadeh et al., 2006). In the first decade of 21<sup>st</sup> century, a first draft of genome sequence was obtained by using a homozygous diploid genotype of *S. phureja*, cultivated in Colombia and Peru (Potato Genome Sequencing Consortium PGSC 2011; Sharma et al., 2013).

Tuberization is a complex trait and a number of functional genes in tuberization were identified, including two genes that control length-dependent tubing (Prat 2010). StSP6A gene encodes a protein similar to FLOWERING LOCUS T (FT), which controls flowering depending on the day-length in *Arabidopsis thaliana* (Navarro et al., 2011), while StCDF1 gene encodes a member of the DOF (DNA-binding with one finger) transcription factor family (Kloosterman et al., 2013).

**Pepper.** The first genetic studies on chili were focused on fruit color and shape, the pungency and the sweet taste (Webber 1912, Hurtado-Hernandez and Smith 1985, Peterson 1959).

Only in the last twenty years, genes controlling these traits were identified, for example in the pungency, a presumed acyltransferase is involved in the final phase of capsaicin biosynthesis (Stewart et al., 2005).

Interspecific crosses (*C. annuum* x *C. chinense*), to construct the first linkage map using tomato RFLP markers, were used, highlighting synteny between tomato and pepper as the basis for describing the evolution between these two species (Prince et al 1993, Tanksley et al., 1988). Later, thanks to the development/use of different molecular DNA-markers, linkage analysis for different qualitative and quantitative traits were developed such as the anthocyanins, carotenoids, capsaicinoids content, the resistance to pathogens, the male fertility (Römer et al., 2007; Tsaballa et al., 2011; Nimmakayala et al., 2014; Ramchiary et al., 2014).

Recently, Kim et al. (2014) reported the sequencing and assembly of the spicy chilli genome (Mexican landrace of *C. annuum* cv. CM334), used as a reference genome for comparison with the "Perennial" and "Dempsey" cultivars and the *C. chinense* species and as a platform to improve the nutritional and medicinal values of the *Capsicum* species. Genomic and transcriptomic analysis clarified how the change in gene expression and the neo-functionalization of capsaicin synthase modeled capsaicinoid biosynthesis.

**Eggplant.** It is also known as aubergine (UK) or brinjal (Africa and Asia), the main breeding aims include qualitative and quantitative traits, ranging from aesthetic and culinary

preferences, such as the improvement of post-harvest performances that could facilitate transport over long distances and storage time, to a better adaptation at limiting growth conditions in different climates. The classical breeding goals have led to important results, such as the reduction of acidity, diminishing the bitter taste of fruits. Today on the seed market, cultivars adapted to different environmental conditions are available providing good yield and quality.

Regarding biotic stress, there is progress in pathogens resistance, frequently utilizing wild related species as a source of resistance through interspecific hybridization (Ano et al., 1991; Robinson et al. 2001; Acciari et al., 2001; Toppino et al. al., 2008).

Compared to other *Solanaceae* species, the knowledge of genetic control mechanisms of quantitative traits, such as the environmental stresses tolerance is rather poor in eggplants. To date, one of the most studied aspects is probably the anthocyanins control and accumulation (Nolla et al., 1932; Janick and Topoleski 1963; Tigchelaar et al., 1968).

Molecular analysis helped and simplified breeding by determining those genomic regions, including QTLs, which have a greater effect in the phenotype determination. Marker-assisted selection has become an ideal tool for genetic improvement programs including the eggplant: molecular markers associated with some phenotypic traits of interest may be useful for pre-filtering the seedling phase to eliminate unwanted genotypes with the advantage of limiting the genotypes to be breed, reducing the time for phenotyping.

The first genetic study on the eggplant dates back to 1918 (Halsted) and was focused on the heritability of fruit color, then resumed and refined in the following decades (Tatebe 1939, Tigchelaar et al., 1968), while the inheritance of male infertility was also studied (Nuttall 1963).

Over the decades, several encouraging results led eggplant breeders to applications in marker-assisted selection: a molecular linkage map was published adopting a segregant population between susceptible and resistant cultivar to bacterial disease (Nunome et al., 1998), in which resistance and fruit shape were mapped.

Frary et al. (2000) developed another genetic map based on an interspecific F<sub>2</sub> progeny (*S. linnaeanum* Hepper and Jeger x *S. melongena*), with RFLP and cDNA tomato markers, which allowed mapping of the first traits of interest such as anthocyanins content in different organs and fruits and leaves shape. In addition, this interspecific map also allowed the comparison with tomato, which should further facilitate genetic analysis and eggplant breeding.

A first intraspecific linkage map was constructed by using 233 tomato RFLP markers mapped on F<sub>2</sub> progeny from an interspecific cross between *S. melongena* x *S. linnaeum* (Doganlar et al., 2002). More recently, an intraspecific map based on 415 SNP (single nucleotide polymorphism) and SSR (simple sequence repeat) markers, was established (Barchi et al., 2009). The construction of maps is a prerequisite for QTL mapping of biochemical, morphological and yield traits (Toppino et al., 2008; Portis et al., 2014).

Gramazio et al. (2014) had developed another interspecific map (*S. melongena* x *S. incanum*) with RFLP markers with the aim to map genes involved in the chlorogenic acid biosynthesis pathway (polyphenol with nutraceutical potential). In more recent years, association mapping experiments have made possible the association of molecular markers (SNPs and SSRs) with plant traits, useful to find different QTLs (Ge et al., 2013; Cericola et al., 2014; Portis et al., 2015).

A first draft of the whole genome sequence of the typical Asian aubergine "Nakate-Shinkuro" has been recently published (Hirakawa et al., 2014). In 2014, an Italian consortium (including the Universities of Verona and Turin, CREA and ENEA) had used a combination of Illumina sequencing and optical mapping to produce a high-quality eggplant draft genome by using '67/3' inbred line (unpublished data).

## CHAPTER 2. FERTILIZER NITROGEN USAGE

### 2.1. Nitrogen, macroelement for plant: nitrate, ammonium, urea.

Nitrogen (N) is an important element for the development and growth of plants, able to stimulate growth and photosynthesis. Nitrogen in the atmosphere (air is 79% N<sub>2</sub> gas) cannot be easily absorbed by plants except legumes via biological N fixation (nodule-located Rhizobium bacteria convert atmospheric N<sub>2</sub> to a form the plant can use). As nitrogen in the earth's crust is less than 1%, N in the soil, it is a limiting factor for agricultural production.

Lack of N results in declining soil fertility, low yield and low crop quality, therefore it needs to be restored by organic or mineral N sources. Fertilizers, whether applied as manure or as mineral N, are therefore important for agriculture, but their excessive amounts in the soil may cause pollution and climate warming, moving into the ground water, eutrophicate surface water or escape to the atmosphere.

Nitrogen exists in the soil system in different forms changing very easily from one form to another. These several N sources can be used by plants such as mineral form of nitrate ions (NO<sub>3</sub><sup>-</sup>); ammonium (NH<sub>4</sub><sup>+</sup>); and to a lesser extent gaseous ammonia (NH<sub>3</sub>); nitrogen oxide (NO); amino acids and organic peptides (von Wiren et al., 1997; Miller et al., 2007).

Nitrogen is absorbed during plant growth and then exported from the fields by harvesting. The specific absorption of N forms depends on environmental conditions and soil.

Since the early twentieth century, the nitrification process was substantially investigated in soils, showing that specific group of microorganisms serving as the dominating ammonia oxidizers to environments. Previously, several applications have highlighted the widespread presence of ammonia-oxidizing bacteria (AOB) that play a role in nitrification activity but this ability was limited at below pH 5.5. However, variables such as pH, temperature, sufficiently available soil moisture, oxygen and bacterial populations can influence nitrification process in the soil (Hu et al., 2014). Therefore, in tropical soils, even in conditions of neutral pH, ammonium can accumulate because of the low nitrification rate. By contrast, soils saturated with water, slightly oxygenated, with acidic pH, low temperatures and lack of organic matter, were characterized by a greater amount of NH<sub>4</sub><sup>+</sup> (Mengel and Kirkby, 1987).

Plants absorb nitrogen from the soil both in the form of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>. However, plants might be decided in relation to NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> availability in soil and many other factors.

Moreover, nitrate limits the uptake of harmful elements, such as chloride, into large quantities; furthermore, unlike ammonium, nitrate is not volatile, being mobile in the soil. In

addition, nitrate promotes synergistically the intake of cations, such as  $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$ , while ammonium competes for uptake with these cations.

Legaz et al (1996) showed a greater efficiency of nitrogen uptake in *Citrus* by using nitrate labeled with isotope  $^{15}N$ , compared to ammonium fertilization applied on different soils. The differences in N-absorption were higher in sandy soil with 60% absorption efficiency, when N was applied as potassium nitrate and only 40% when N was applied as ammonium sulphate.

Ammonium ( $NH_4^+$ ) is another important N-source for plant growth but also important for N transformation processes in soil. It can be lost through volatilization under high pH conditions or under certain conditions, through nitrification can be oxidized into nitrate. Plants can directly uptake  $NH_4^+$  and, other cationic minerals in soil replace the positive ion charge. Thus,  $NH_4^+$ , being less mobile of  $NO_3^-$ , does not move down into the underground water supplies.  $NH_4^+$  that is not absorbed by plants is subject to other changes in soil system. The nitrification is the conversion of  $NH_4^+$  into  $NO_3^-$ . It depends on temperature and takes place from one to several weeks. The soil microbes, released over longer periods (soil organic matter), also immobilize ammonium that is held by the soil and it does not readily leach. Ammonium sources are therefore suitable for autumn applications. Commonly N applied forms include ammonium sulfate, diammonium phosphate, and monoammonium phosphate. Ammonia fertilizers, in particular urea, are among the most economical fertilizers on the market and are easily synthesized, transported and distributed (Hageman, 1984).

Urea, such as ammonium, is subject to volatilisation when it is not immediately incorporated into the soil; therefore, significant quantities can be lost through this process (Chen et al., 2008). To limit the loss of fertilizer, controlled release fertilizers based on urea with polymer coating (PCU) have been marketed (Landels 2010a). They are considered ecologically intelligent nitrogen fertilizers (ESN), permeable to water with gradual N release in response to increase in temperatures during the growing season (Agrium Inc. 2011) but with costs ranging from 15% to 30% compared to uncoated urea, which makes it more interesting product for use in field crops.

Urea can be also coated with nitrification inhibitor (NIU), 3,4-dimethylpyrazole phosphate (DMPP) to form a stabilized granule. This class of N-fertilizers is a great promise because of its positive effect on crop yield and the reduction of N loss at low concentrations, due to its stability and the limited movement in the soil (Azam et al., 2001; Pasda et al., 2001; Zerulla

et al., 2001b). However, their use may cause phytotoxicity, which could affect marketability of product, e.g. of leaf vegetables (Reeves and Touchton 1986).

## **2.2. Nitrogen loss from soil system: consequences associated with N loss in the environment**

N fertilizers are lost by volatilization of ammonia by nitrification or leaching, erosion, runoff and denitrification, and these processes can vary widely depending on the agroecosystem, the chemistry of fertilizer and its application method, the matrix and soil composition.

Physical and chemical events responsible for N loss from soil are well known (Sánchez Pérez et al., 2003). The loss of nitrate by leaching is a physical event: it occurs when the N input exceeds N demand for plant harvest or when the N-content of the residual soil is high. Leaching depends mainly on irrigation systems and in regions with humid climate and soil matrix (coarse grained soils).

Loss of  $\text{NO}_3^-$  moves with soil water, usually excess water, below the rhizosphere zone and has the potential to enter into ground- or surface water. Considerable N amounts are lost from the soil system through crop removal.

Denitrification can be a major loss mechanism of  $\text{NO}_3^-$ , it takes place under anaerobic conditions when  $\text{NO}_3^-$  is converted into  $\text{N}_2$  gas by denitrification bacteria, which uses, at low  $\text{O}_2$  conditions,  $\text{NO}_3^-$  rather than  $\text{O}_2$  for respiration.

N from fertilizers can be dispersed into the atmosphere through ammonia volatilization. Loss rates depends on the type of fertilizer and manure, the application method, N speed in the soil and the pH, the structure and the cation exchange capacity, the climatic conditions (Piccini et al., 2016). Cycling N is a dynamic process in nature; some of these processes like leaching, denitrification, ammonia volatilization, soil erosion, etc. also lead to serious consequences of environmental pollution.

N fertilizer losses with outflow of water from agricultural land can be an important factor in eutrophication, which causes an excessive growth of aquatic algae together with anoxia producing toxic substances to the aquatic environment and the organisms living in that ecosystem (Baligar et al., 2001).

Finally, N deposition and greenhouse effect are caused by the return to the earth's surface as co-deposition of N ammonia released into the atmosphere through volatilization (Buresh et al., 1987). The aquatic and forest ecosystems are more prone to N deposition, and as a consequence, this can also lead to global warming due to possible emissions of nitrogen-based greenhouse gases (Mosier et al., 1998; 1999).

### 2.3. Yield and Fertilizer

Intensive agriculture requires large fertilizer amounts that can considerably increase crop yields (Matson et al., 1997; Tilman et al., 2002). However, this large utilization also increased the risk of negative impact on the environment, climate, human and animal health (Delgado and Scalenghe, 2008, Tschardt et al., 2012). Strategies to optimize the availability of fertilizer per plant are well documented; N fertilizer can be locally applied around the sowing area, especially in the early stages of plant growth compared to the application on the whole soil land area (Grant et al., 2001; Lu and Miller, 1993; Valluru et al., 2010).

Fertilizer applications have been studied to evaluate crop performance or root growth and nutrient uptake (Hodge, 2004; Rose et al., 2009; Weligama et al., 2008) and environmental aspects such as  $\text{NO}_3^-$  leaching (Baker, 2001; Ruidisch et al., 2013; Zhou et al., 2008).

Nitrogen is the most important nutrient, and the productivity of aubergines is strongly dependent on this macronutrient (Oliveira et al., 1971). Several authors highlighted the strong dependence on nitrogen and water irrigation. In large scale experiments, the maximum yield of eggplant fruit was obtained with 125 kg of N  $\text{ha}^{-1}$  (Aujla et al., 2007), while fruit production of aubergines continued to increase until N applications of 187.5 kg N  $\text{ha}^{-1}$  (Pal et al., 2002).

Increasing N concentration, in the nutritive solution, determined an increase in dry leaf weight due to N accumulation and photosynthates (Takebe et al., 1995), in stem elongation and leaf expansion rate, accompanied by a decrease in root biomass (Balliu et al., 2008), an improvement in plant height, leaves number and yield (74%) (Wange & Kale, 2004).

In the last four decades, the doubling in food production over the world has been associated with a significant increase in N fertilizers use. This intensification in N applications generated agro-ecosystem pollution and led to higher production costs (EL-shobaky, 2002; Rizk, 2002). The more frequent consequences are the eutrophication of freshwater (Carpenter, 2005) and marine ecosystems (Beman et al., 2005) because of leaching when high N fertilizer rates are applied in the agro-ecosystems (Tilman, 2002). In addition, gaseous emissions of N-oxides can react with stratospheric ozone, generating toxic ammonia in the atmosphere (Ramos, 1996; Stulen et al., 1998).

Through the improvement of agronomic practices, applied fertilizers use efficiency, the understanding of mechanisms involved in plant nutrient pathways and the selection of fertilizers use efficient genotypes will be possible to define more appropriate management practices for a sustainable agriculture.

## CHAPTER 3. NITROGEN USE EFFICIENCY (NUE) IN PLANTS

In the last five decades, N fertilizers have contributed to maximize crop yields, however 50–70% of N provided to the soil is lost (Hodge et al., 2000), and thus improving plant NUE is an essential strategy to reduce environmental damage due to nitrate leaching, ecosystem saturation and water pollution.

Nowadays, the goal of a sustainable agricultural research is to produce "More from less", therefore the applied strategies aim to maximize the efficiency of N use (NUE), maintaining (or decreasing) N fertilizer applications with the increase of crops yields.

Here, the definition of NUE, the selection techniques and establishment of high NUE genotypes and the interacting factors with NUE are described. In particular, we emphasize the challenges for enhancing NUE.

### 3.1. Nitrogen Use Efficiency and its components

The assessment of genetic variability for plant NUE performances required evaluating germplasm in low and high N supply to identify NUE-efficient and inefficient genotypes. There are many different NUE definitions and calculations.

In the past, nitrogen use efficiency (NUE) was defined “as a flow ratio between dry mass and N” (Hirose 1971, 1978), some definitions include the N use, content and availability as components of NUE equation (Good et al., 2004).

However, the simplest NUE definition is by Moll et al. (1982), who stated N use efficiency “as grain production per unit of N available in the soil”. NUE is a complex trait comprising two key major components, N Uptake Efficiency (NUpE) and N utilization efficiency (NUtE), and calculated as a direct product of NUpE and NUtE.

Another method to describe NUE is the utilization index (UI), which influences the amount of biomass produced per NUE unit can also be described as NUEg, which is the production of grain per unit of N available. However, crop could produce large quantities of biomass per unit N (high UI) without converting the acquired N into seed production and then have a low NUEg. Other NUE calculations take into account agronomic and physiological variations described in table 1.

Some authors point out that NUE can be improved by increasing NUpE, NUtE or both. Further, NUE is also defined as the fresh matter (FM) or dry matter (DM) produced per N content, in plants used for biomass production (Good et al., 2004).

| <b>Term</b>             | <b>Formula</b>          | <b>Definition</b>                                           | <b>Comments</b>                             |
|-------------------------|-------------------------|-------------------------------------------------------------|---------------------------------------------|
| Nitrogen use efficiency | $NUE = Sw/N$            | Sw, shoot weight (DW); N, nitrogen content of shoots (DW)   | Does not account for biomass increases      |
| Usage index             | $UI = Sw \times (Sw/N)$ |                                                             | Takes into account biomass increase         |
| Nitrogen use efficiency | $NUE = Gw/Ns$           | Gw, grain weight; Ns, nitrogen supply; Nt, total N in plant | Reflects increased yield per N unit applied |
| Uptake efficiency       | $UpE = Nt/Ns$           |                                                             | Measures efficiency of uptake N into plant  |
| Utilization efficiency  | $UtE = Gw/Nt$           |                                                             | Fraction of N converted to grain            |

**Table 1.** From Good et al., 2004, definitions and formulae used to describe nutrient use efficiency in plants.

### 3.2. Nitrogen Uptake Efficiency and root system

Roots are fundamental for acquiring water and mineral nutrients including N.

Nitrogen Uptake Efficiency (NUpE) is defined the root ability to uptake N available from the soil (Good et al., 2004), which depends on its absorption area (rhizosphere) and lateral roots (more than 90% of the total length of root system) playing an important role in nutrients absorption (Jungk et al., 2015).

Therefore, improving the current knowledge on the relationship between plant growth and root dynamics is of a great importance (Wu et al., 2005). Among root qualitative and quantitative traits, the most important are those involved in response to N-limiting conditions (Guingo et al., 1998; Kamara et al., 2003; Gallais and Coque, 2005).

A strategy for improving NUpE would be to increase uptake through the expression of more efficient N transporters, although this in some cases can only improve N uptake but not NUE (Fraisier et al., 2001; Garnet et al., 2009, Masclaux-Daubresse et al., 2010).

A second opportunity could be the identification of genes directly involved in root architecture (Julkowska et al., 2015; Slovak et al., 2016; Wang et al., 2017, 2018) and the use of mutants to putative genes target involved in root development (Hochholdinger et al., 2004).

However, the study on NUpE and root system, especially in the soil, is a topic that remains relatively unexplored. For this purpose, root systems measurement, under control conditions, have been developed throughout rhizotrons (Devienne-Barret et al., 2006; Laperche et al., 2007) or hydroponic systems (Tuberosa et al., 2003) allowing an easier dissection of root analysis and, above all, a better understanding of root dynamics.

Most of our current knowledge on the regulation of inorganic N absorption is the result of studies conducted on model organisms. During the last decade, molecular biology studies demonstrated the importance of high affinity (HATS) and low affinity (LATS) transport systems (Glass et al., 2002; Orsel et al., 2002) in the environmental responses for N acquisition. Indeed,  $\text{NO}_3^-$  transport system in maize has been hypothesized be a physiological indicator for adaptation to low N input, demonstrated by a higher *ZmNRT2.1* transcript accumulation as a key regulation step for iHATS (Inducible High-Affinity System) (Quaggiotti et al., 2003).

### 3.3. Nitrate sensing and signaling

Nitrate is not just a macronutrient necessary for plant growth, but it is also considered a signal molecule that plants perceive to regulate their metabolism and physiology. Understanding how plants perceive and respond to different N levels is essential for the future goals of a sustainable agriculture, reducing its environmental and economic costs.

The only nitrate transporter sensor proposed so far is NRT1.1, a dual-affinity nitrate transporter that triggers N responses and is required for the normal expression of nitrate-sensitive genes. Furthermore, NRT1.1 shows a high or low affinity for the nitrate transport, depending on the phosphorylation status of a threonine residue 101. The phosphorylated form of NRT1.1 is a high affinity transporter, whereas the non-phosphorylated form behaves as a low affinity transporter (Liu and Tsay, 2003). CIPK23-CBL9 protein complex (CIPK, CBL-Interacting Protein kinase, CBL, calcineurin-like protein B) were implicated in NRT1.1 double-affinity transition changes through phosphorylation (Noguero and Lacombe, 2016). Furthermore, auxin transport is also mediated by NRT1.1 (Bouguyon et al., 2015).

Recently, the important role played by  $\text{Ca}^{2+}$  on the nitrate signal transduction as second messenger downstream of the perception of nitrate in the roots and shoots was demonstrated (Riveras et al., 2015). Indeed, some studies based on reporter aequorin (a calcium-activated photoprotein) demonstrated a rapid transient increase in  $\text{Ca}^{2+}$  ions at the cytosolic level because of the absorbed nitrate mediated by the NRT1.1/NFP6.3 transporter.

In addition, a rapid increase in inositol 1,4,5-trisphosphate (IP3) was also recorded, and the IP3 and cytosolic  $\text{Ca}^{2+}$  levels were inhibited by the blockage of phospholipase C (PLC) activity and also by the blockage of the NRT1.1/ NFP6.3 transporter. The inhibited phospholipase C significantly reduced the expression of nitrate-sensitive genes such as NRT2.1, NRT3.1, NiR, TGACG SEQUENCE-SPECIFIC BINDING PROTEIN1 (TGA1) genes (Riveras et al., 2015).

Among other consequences related to the increase of  $\text{Ca}^{2+}$  ions in response to nitrate, the increased activity of the CPK kinases was highlighted, the CPKs (CPK10, CPK30 and CPK32) were hypothesized are key regulators of primary nitrate responses (Liu et al., 2017). More recently, studies showed that there are other proteins in plants that can play a role as nitrate signaling, such as NLP7, which should function as transcriptional activator in the regulation of nitrate expression of *Arabidopsis* genes (Konishi and Yanagisawa, 2013; Marchive et al., 2013).

Alignment of nine *Arabidopsis* NLP protein sequences and four orthologous sequences from *Lotus japonicus*, unveiled a conserved Ser205 residue, has been suggested as a possible

target of CPK phosphorylation (Liu et al., 2017). It is important to note that NLP7 has been shown to be phosphorylated by CPK10 and the interaction of both proteins was also shown in the nucleus of protoplasts in the presence of nitrate (Liu et al., 2017). NLP7 is an early nitrate dependent regulator of gene expression changes and can translocate into the nucleus in response to nitrate treatment (Marchive et al., 2013).

In addition to NLP7, other transcription factors known to participate in the regulation of gene expression in N response were studied in *Arabidopsis*. The interaction with the targets of the affine promoter of nitrate-responsive genes was verified only for TGA1, NLP6 / 7, bZIP1 and TCP20 (reviewed in Undurraga et al., 2017). TCP20 was involved in the *NRT1.1* expression, induction only under low nitrate, whereas TGA1 regulated gene expression in response to high nitrate and it was involved in the nitrate transport and metabolic agents.

Finally, the understanding of nitrate signaling remains a major challenge for the scientific community because of many questions remain unanswered. Understanding how nitrate can stimulate the increase in cytosolic  $\text{Ca}^{2+}$  and how many possible nitrate sensors exist, could be an important goal towards developing better strategies to increase NUE.

### 3.4. Nitrate transporters in plants

Many studies, focused on the regulation and function of nitrate transporters, were performed on *Arabidopsis*, and comparative genetic approaches and functional genomics were needed to identify homologues in other plant species (barley, rice, maize, wheat). The transporter system was classified according to protein regulation and activity:

- Inducible High Affinity Transport System (iHATS) and Constitutive High Affinity Transport System (cHATS) that operates at low nitrogen external concentrations (millimolar concentrations <1mM).
- Inducible Low Affinity Transport System (iLATS) and Constitutive Low Affinity Transport System (cLATS) that operates at high external nitrogen levels (millimolar concentrations > 1 mM).

Four gene families of  $\text{NO}_3^-$  transporters, NRT1, NRT2, NRT3, CLC were identified. NRT1 family is responsible for low transport affinity (LATS), whereas the members of NRT2 family are responsible for high affinity (HATS). In the NRT2 family, several members require an additional protein from the NRT3 family also known as the 'Nar2' family.

(protein:protein interaction); for example *AtNRT3.1* showed a similar expression pattern of *AtNRT2.1*.

In nitrate starvation, HATS system is reduced to its constitutive level (cHATS), with N addition the inducible system (iHATS) is activated. It is down regulated by the accumulation of N endogenous levels such as  $\text{NH}_4^+$  or amino acids (Gansel et al., 2001; Lejay et al. 2003; Nazoa et al. 2003; Okamoto et al. 2003; Wang et al. 2018; Tegeder and Masclaux-Daubresse, 2018). The nitrate transporters (listed in table 2) are located on the plasma membrane. Both high and low affinity transporters mediate nitrate transport together with protons ( $\text{1NO}_3^-/\text{2H}^+$ ) and the energy is provided by the proton-motive force (PMF).

The CLC gene family (Chloride Channel Family, seven CLCa-g members) located on the tonoplast (CLCa, CLCb and other endomembranes), are proposed to be anion channels involved in accumulation of vacuolar nitrate by means of a proton exchange mechanism.

| <b>NRT1 family</b> | <b>NRT1.1</b>                                 | <b>NRT1.2</b>                                                  | <b>NRT1.3</b>                                | <b>NRT1.4</b>                                  |
|--------------------|-----------------------------------------------|----------------------------------------------------------------|----------------------------------------------|------------------------------------------------|
|                    | nitrate uptake<br>Liu et al.,1999             | nitrate uptake<br>Kanno Y. et al., 2013                        | polyamine transport<br>Tong et al., 2016     | discharges from the xylem<br>Chiu et al., 2004 |
|                    | <b>NRT1.5</b>                                 | <b>NRT1.6</b>                                                  | <b>NRT1.7</b>                                | <b>NRT1.8</b>                                  |
|                    | nitrate xylem loading<br>Lin et al., 2008     | nitrate for seed development<br>Almagro et al., 2006           | nitrate remobilization<br>Fan et al., 2009   | Xylem unloading<br>Li et al., 2010             |
|                    | <b>NRT1.9</b>                                 | <b>NRT1.10</b>                                                 | <b>NRT1.11</b>                               | <b>NRT1.12</b>                                 |
|                    | loading into root phloem<br>Wang et al., 2011 | bidirectional long-distance transport<br>Andersen et al., 2013 | xylem-to-phloem transfer<br>Hsu et al., 2013 | xylem-to-phloem transfer<br>Hsu et al., 2013   |
| <b>NRT2 family</b> | <b>NRT2.1</b>                                 | <b>NRT2.2</b>                                                  | <b>NRT2.3</b>                                | <b>NRT2.4</b>                                  |
|                    | nitrate uptake<br>Orsel et al 2004            | nitrate uptake<br>Orsel et al 2004                             | long distance<br>Feng et al., 2011           | double role<br>Kiba et al., 2012               |
|                    | <b>NRT2.5</b>                                 | <b>NRT2.6</b>                                                  | <b>NRT2.7</b>                                |                                                |
|                    | phloem loading<br>Kechid et al., 2013         | nitrate uptake<br>Kechid et al., 2013                          | nitrate in seeds<br>Chopin et al., 2007      |                                                |
| <b>NRT3 family</b> | <b>NRT3.1</b>                                 | <b>NRT3.2</b>                                                  |                                              |                                                |
|                    | dual component<br>Okamoto et al., 2006;       | dual component<br>Okamoto et al., 2006;                        |                                              |                                                |
| <b>CLC family</b>  | <b>CLCa</b>                                   | <b>CLCb</b>                                                    |                                              |                                                |
|                    | proton-exchange<br>Geelen et al., 2000        | proton-exchange<br>Von Der Fecht-Bartenbach et al., 2010       |                                              |                                                |

**Table 2.** *Arabidopsis* genes involved in nitrogen uptake, translocation and storage.

#### 3.4.1. Low-affinity transport system (LATS): NRT1/PTR FAMILY (NPF)

In *Arabidopsis*, 53 genes were identified in the NRT1 family, of which 13 were involved in nitrate transport.

The first nitrate transporter identified was NRT1.1 (CHL1), which encodes a protein with 12 trans-membrane segments (TMS), able to perceive large external nitrate levels because it is a double affinity transporter. The NRT1.1 may be phosphorylated by the protein kinase CIPK23 in threonine 101 (Thr101) to form the plasma membrane dimer. When the external nitrate concentrations are low making the protein in high affinity mode; by contrast, at high external nitrate concentrations, the NRT1.1 remains in the un-phosphorylated state and the protein shows a low affinity mode.

Therefore, the NRT1.1 is considered a double affinity transporter, acting as an inducible low affinity transporter (except in rice where it has been found to be constitutive) (Tsay et al., 1993; Tabuchi et al., 2007; Cerezo et al., 2001). The NRT1.1 is generally expressed in the tips of primary and secondary roots, in pericellular and epidermal cells, in root hairs and in the quiescent centre, resting center of root meristems (Krouk et al., 2010).

The NRT1.2 is a low affinity constitutive nitrate transporter, dominant in roots, expressed in rhizoderma and this expression is independent from root age (Tsay et al., 1993; Huang et al., 1999; Filleur et al., 2001). Recently, Kanno et al. (2013) also suggested a role as an importer of ABA, while Tal et al., (2016) suggested a role in gibberellin transport too.

#### 3.4.2. High-affinity transport system (HATS): NRT2 and NRT3 family

The NRT2 family contains several members in higher plants. In *Arabidopsis*, seven NRT2 genes are identified: the NRT2.1 is the main and inducible nitrate transporter and together with the NRT2.2 are localized mainly in roots. The NRT2 transporters (like many NRT1 transporters) contain putative twelve transmembrane domains and the members did not carry out anything beyond the nitrate. Approximately 80% of wild type in nitrate uptake was observed when the genes coding for these two proteins were unfunctional.

The NRT2.4 gene transporter is expressed in young root and it is induced by N limiting conditions (Masclaux-Daubresse et al., 2010) and might be involved in nitrate transport from stored pools to cytoplasm.

The NRT3-type genes characterized in plant cells were previously called WR3 and subsequently NAR2 family. Early studies showed their expression induced by wounds and pathogens (Titarenko et al., 1997). Thereafter, the involvement of NRT3, in the high affinity

transport system (HATS), was initially identified using mutants in *Chlamydomonas reinhardtii* (Quesada et al., 1994).

Numerous genes belonging to NRT3 (NAR2) family were identified in several plant species. Until now, three NAR2 have been characterized in barley (HvNAR2.1-HvNAR2.3) (Tong et al., 2005), two in *Arabidopsis* (AtNAR2.1 and AtNAR2.2) (Orsel et al., 2006) and two in rice (OsNAR2.1 and OsNAR2.2) (Yan et al., 2011).

However, not all the members of the NAR2 family have demonstrated an involvement in the HATS for nitrate, for example, among the three NAR2 isoforms in barley, only HvNAR2.3 constituted a functional unit with HvNRT2.1 (Tong et al. 2005), while in rice only OsNAR2.1 and not OsNAR2.2 (Yan et al 2011). Kotur et al. (2012), using yeast two-hybrid assays, showed that all the NRT2, with the exception of AtNRT2.7, strongly interact with AtNAR2.1. The results of *Xenopus* oocyte system instead revealed that all the NRT2 polypeptides (with the exception of AtNRT2.1) can sustain a small flow of nitrate, although, when co-expressed with AtNAR2.1, this flow was greatly increased.

To clarify the function of members of the NAR2 family on nitrate transport it is necessary to identify the possible partner pairs and the specificity of their interaction. In *Arabidopsis*, HATS for nitrate required two genes expression: AtNRT2.1 and AtNAR2.1 (AtNRT3.1) (Filleur et al., 2001; Orsel et al., 2004, 2006; Okamoto et al., 2006; Li et al., 2007). In mutants with AtNRT2.1 interrupted, the functionality of iHATS was reduced by 70% (Filleur et al., 2001; Li et al., 2007), while the AtNAR2.1 unfunctionality caused 95% reduction of iHATS (Okamoto et al., 2006; Orsel M. et al., 2006). Furthermore, the growth of impaired mutants in AtNRT2.1, AtNRT2.1 / AtNRT2.2 or AtNAR2.1 was strongly limited at low external nitrate concentration (Okamoto et al., 2006; Orsel et al., 2006; Li et al. 2007). By contrast, AtNRT1.1 did not seem to require concurrent expression of AtNAR2.1, as the AtNAR2.1 mutant exhibited a normal transport function of low affinity nitrate (LATS) (Tsai et al., 1993; Okamoto et al., 2006).

Orsel et al. (2006) using knock-out mutant plants, compared in tests for the response to Lateral Root (LR) growth at low N conditions, *atnrt2.1-1* and *atnar2.1-1N* (without NRT2.1 and NAR2.1 genes, respectively), showing significant differences between the two mutants. For both mutants, root system responses can be attributed to low nitrate intake rates that determined the N deprivation. Furthermore, the *atnar2.1-1* mutant showed the same inhibition on LR initiation on the primary root as the mutant *atnrt2.1-1*. This phenotype could result from the decrease of AtNRT2.1 expression in the *atnar2.1-1* mutant.

Furthermore, in tobacco NpNRT2.1, an orthologue of AtNRT2.1, can replace the mutant *atnrt2.1-1*, but not *atnar2.1*. This suggests that NpNRT2.1 may functionally restore HATS activity under nitrogen limitation (N), but it may not work for LR response. Thus, NpNRT2.1 needs an accessory protein for its function, but this synergistic action has to be well explained (Orsel et al., 2007).

*NAR2.1* and *NRT2.1* expression level in *Arabidopsis* and barley were reported significantly repressed by high nitrate concentrations (Ishikawa et al., 2009; Yong et al., 2010), conversely similar *NAR2.1* and *NRT2s* expression levels were observed in rice roots treated with both low and high nitrate concentrations (Yan et al., 2011).

The over-expression of NpNRT2.1 in tobacco did not improve production and the effects are limited to a slight increase in the nitrate absorption, hypothesizing that *NRT2.1* over-expression, not sustained by *NAR2.1*, had limited effects (Filleur et al., 2001).

The mutant *atnrt2.1-1* instead showed more marked growth phenotype than the mutant *atnar2.1-1* (Orsel et al., 2006), at low nitrogen limitations, underlining a more suffering from the N deficiency of mutant *atnar2.1-1* than *atnrt2.1-1* (Orsel et al., 2006).

### **3.5. Nitrogen Utilization Efficiency: Nitrate translocation and assimilation**

The complexity of the NUtE is partially highlighted by numerous scientific papers from the application of transcriptomics, metabolomics, functional genomics, quantitative genetics, electrophysiology that implemented hypothetical elucidatory models of the behavior of the whole plant during nitrogen metabolism. Quantitative Trait Loci (QTLs) involved in the nitrogen assimilation pathways were identified in both model and non-model organisms.

Several components have been studied, involved in carbohydrate partitioning, N storage and N rebalancing, root-to-leaf rebalancing, ability to keep the leaves green longer than standard genotypes during the fruit or seed filling phase.

#### **3.5.1. Long distance nitrate translocation**

Nitrate is translocated in different plant tissues through the transporters that load and unload nitrate into the xylem and phloem. The AtNRT1.5 and AtNRT2.3 transporters upload nitrate from root to the xylem. In particular, AtNRT1.5 is a bi-directional inductive nitrate-regulated pH transporter, while the isoform OsNRT2.3a were reported as long-distance

high-affinity transporter in rice that charges nitrate into the xylem for the translocation to the shoot (Yan et al, 2011; Tang et al., 2012).

The NRT1.4 and NRT1.8 are carriers for downloading nitrate from the xylem (Chiu et al., 2004; Lin et al., 2008; Li et al., 2010). In particular, the NRT1.4 downloads nitrate from the xylem in leaves, showing a role in their maintenance in nitrate homeostasis. The NRT1.8 is the main way of effluxing nitrate from the xylem, but its expression is in parenchyma cells. In the phloem, the NRT1.9 transporter plays a role in nitrate loading (Wang et al., 2011), while NRT1.11 and NRT1.12 are involved in xylem-to-phloem transport for redistributing nitrate into the developing leaves for plant growth (Hsu et al., 2014).

Furthermore, the NRT2.4 and NRT2.5 plays a double role in root and shoot transport, contributing to phloem loading of nitrate in shoots during N long-term starvation (Lezhneva et al., 2014; Kiba et al., 2012).

### 3.5.2. Nitrate assimilation pathway

The assimilation of nitrate provides for the reduction of nitrate to ammonium, in a path that will bring the ammonium assimilation into amino acids.

Nitrate reduction occurs both in roots and shoots, the first step is the reduction of nitrate to nitrite, catalyzed in the cytosol by the enzyme nitrate reductase (NR).

Nitrite is translocated to the plastids / chloroplasts where it is reduced to ammonium by the nitrite reductase (NiR). In the plastids/chloroplasts, ammonium (from nitrate reduction, from photorespiration or amino acid recycling), is assimilated by the so-called GS/GOGAT cycle. The glutamine synthetase (GS) fixes ammonium on a glutamate molecule to form glutamine, glutamine reacts with 2-oxoglutarate to form two molecules of glutamate, this reaction is catalysed by the glutamate synthase (or glutamine 2-oxoglutarate amino transferase, GOGAT).

Two families of multigenes for GS are known, GLN1 and GLN2. GLN1 encodes cytosolic GS1 isoforms while GLN2 encodes a chloroplastic GS2. Two isoforms were identified in plants also for GOGAT: Fd-GOGAT (localized in photosynthetic tissues) and NADH-GOGAT (localized in non-photosynthetic tissues) using ferredoxin and NADH as electron donors, respectively.

Three other main enzymes participate in the ammonium assimilation; the ATP-dependent asparagine synthase (AS) in the cytosol catalyzes the transfer of the glutamine amino group into an aspartate molecule to generate the amino acids glutamate and asparagine, it can also use ammonia as a substrate (Masclaux-Daubresse et al., 2006). The amino acid asparagine is

recognized as an amino acid transport and storage (Rochat and Boutin 1991, Lam et al., 2003) and could counteract for the reduced activity of ammonia assimilation dependent on the GS because of N/C ratio higher than glutamine. Finally, carbamoyl phosphatase synthase (CPSase) is an enzyme that operates in the plastids, catalyzing the synthesis of carbamoyl phosphate, a precursor of the amino acids citrulline and arginine, which use bicarbonate, ATP and ammonium or the amide group of glutamine.

In the mitochondrion, NADH-glutamate dehydrogenase (GDH) has a catalytic activity that determines deamination of glutamate (Masclaux-Daubresse et al., 2006; Purnell and Botella, 2007). However, under stress conditions, it can incorporate ammonium in glutamate in response to high levels of ammonium (Skopelitis et al., 2006).

The assimilation of nitrogen requires the availability of macromolecules such as FADH and NADH (reducing power used by the enzymes NR, NiR, GOGAT), energy consumption provided by the ATP (GS, AS) and carbon skeletons necessary for the synthesis of amino acids, all these products were supplied by the photosynthetic machinery.

### 3.5.3. *Senescent leaves like nitrate source*

Nitrate transporters like NRT1.7 (specific in phloem), NRT1.11, NRT1.12 appear to be also involved in N remobilization from older leaves to younger tissues during the vegetative phase or to the storage organs during reproductive phase (Fan et al., 2009; Hsu et al., 2013; Wu et al., 2014). This is considered another key factor for the increase of N use efficiency and the ability of rebalancing N from senescent tissues.

An important N source are the proteins involved in the photosynthesis that during senescence processes are used as a source for growing organs. Scientific evidence from maize, wheat and barley demonstrated that N grain content is related to leaves appearance. Moreover, leaf senescence is not only important for the N re-stabilization but also for crop yield.

The first organelles prone to deterioration during senescence are the chloroplasts, where the main form of N for re-mobilization comes from the Rubisco.

In the young leaves, N recycling is due to enzymes like GS2 and Fd-GOGAT, and the N-recycled is reduced and assimilated by the GS/GOGAT cycle, before processing inside the chloroplast (Masclaux-Daubresse et al., 2008). In the senescent leaves, N recycling and re-assimilation are catalyzed by GS1 to produce glutamine and, to a lesser extent, by GDH (Kichey et al., 2007; Habash et al., 2007). These enzymes could not be the only limiting factors in N mobilization (Masclaux et al., 2001), indeed other enzymes such as AS may be

involved, depending on plant species (Lam et al., 2003; Buchanan-Wollaston et al., 2005; Herrera-Rodriguez et al., 2006).

However, the GS1 role is complex and several authors emphasized the importance of this enzyme in N management (Hirel et al., 2001; Obara et al., 2004; Bernard and Habash, 2009). Other authors underlined that GS1 isoforms are found in different plant tissues and do not have the same kinetic properties and therefore not all the GS1 isoforms participate in the same way in N re-mobilisation (Ishiyama et al., 2004; Guo et al., 2004).

#### 3.5.4. Vacuolar nitrate storage

In mature plant cells, the largest volume is occupied by the vacuole, which is an organelle for different functions: it is an important buffer system that regulates cytoplasmic homeostasis, preventing toxicity, useful for hydrolyzing enzymes in acidic environment and finally it is a storage site for different N forms. The main vacuolar nitrate transporters are members of the CLC multigene family and the NRT2.7 high-affinity transporter.

The ATPase/vacuolar pyrophosphatase that generates a proton-motive force provides the energy required for solute transport. The tonoplast is rich in aquaporins and selective ion channels that mediate the movement of vacuolar ions. The nitrate storage capacity in the vacuole is ten times greater compared to the cytoplasm (about  $30\text{-}50 \text{ mol} \cdot \text{m}^{-3}$  of the vacuole versus  $3\text{-}5 \text{ mol} \cdot \text{m}^{-3}$  of the cytoplasm) and the volume of the vacuole is much larger.

In many plant species, CLC are the main protein route for vacuolar nitrate. CLC proteins were found in all living realms. Initially, it was thought that all known CLC transporters were channels or antiporters purely for transport of  $\text{Cl}^-$  (Lisal and Maduke, 2008; Bergsdorf et al., 2009).

Studies on the physiological function of AtClCa, through homozygous knock-out mutants, showed, through the loss of functionality, a 50% reduction of total  $\text{NO}_3^-$  content. However, the mutants did not show phenotypic differences when compared to the wild type, indicating a specific role of AtClCa: greater affinity for  $\text{NO}_3^-$  but not  $\text{Cl}^-$  ions (Geelen, et al., 2000). As identified in model plants, also in cereals and other species, CLC proteins are the main transporters of vacuolar nitrate (Dechorgnat, 2011).

The selectivity for nitrate of CLC proteins is based on the presence of conserved amino acids (Picollo et al., 2009; De Angeli et al., 2009). A serine residue (Ser107) is conserved in human and bacterial CLC proteins while in plants a conserved proline (Pro160) residue has

been identified, this residue is presumably responsible for the higher selectivity for nitrate and not for chloride (Geelen et al., 2000; Nguitragool et al., 2006).

Seven members were identified in the CLC family (CLCa-g) but only CLCa and CLCb have greater selectivity for nitrate than chloride, showing also a positive regulation with nitrate and light.

Given the recognized role of CLCa in plant tissues for nitrate use efficiency, Hans et al. (2016) argued that this transporter can not be ignored for improving NUE.

Ammonia-inducible aquaporins can also mediate vacuolar storage and the proteins characterized for this role are called aquaporin TIP1.2, TIP2.1 and TIP2.3, which are able to both storage and preventing excess ammonium in the cytoplasm (introduced by roots from AMT carriers or as product of reduced nitrate) (Reddy et al., 2015).

Transport of ammonium inside the vacuole is driven by electrochemical gradient generated by the deprotonation of ammonium in the cytoplasm, which causes a difference in pH between cytoplasm and vacuole. The ammonia inside the vacuole is useful to protect cells from toxic effects by storing (Loqué et al., 2005).

### **3.6. C/N balance in NUE**

The environmental variables such as light, carbon dioxide (CO<sub>2</sub>), water, macro and micronutrients are important factors for plant growth. Nitrogen, together with carbon, are essential elements because they are constituents of the most important biological macromolecules such as amino acids, nucleotides, chlorophyll and metabolites.

Plants invest a large N amount for photosynthetic compounds, such as Rubisco carboxylase and Phosphoenolpyruvate PEP proteins for sugars production (Zhu et al., 2008a). Furthermore, the increase of PEP carboxylase gene expression was shown to cause an increase in glutamine, influencing the reactions that serve to replenish the Krebs cycle of the subtracted intermediates for the synthesis of various compounds in turn involved in those important processes in both energy and biomass production (Foyer et al., 2003).

The link between C and N has been recognized as fundamental for routine cellular activity, the ability to uptake and use N could be compromised in case of scarce C availability. A correct C/N balance is essential for growth, development and plant responses to a wide range of stress and, finally, for completing life cycle and harvested production.

The CO<sub>2</sub> through photosynthesis is processed into sucrose and glucose, and then converted into 2-oxoglutarate (2OG) or  $\alpha$ -ketoglutarate through the glycolysis and tricarboxylic acid

cycle. The 2OG acts as a C skeleton for the synthesis of glutamate (Glu) incorporating  $\text{NH}_4^+$  derived from the reduction of  $\text{NO}_3^-$ . An additional  $\text{NH}_4^+$  with glutamine production (Gln) is incorporated into the glutamate (Kaur et al., 2009). Glutamine and glutamate can donate  $\text{NH}_4^+$  for biosynthesis of all the amino acids.

The  $\text{NO}_3^-$  and/or  $\text{NH}_4^+$  are usually uptake by root system and transported into the leaf. The N uptake and assimilation are partially regulated by photosynthetic rates (Zheng 2009). At the uptake level, NRT2.1 transporter also acts as a C/N sensor, signaling the imbalance of C-relations with N when C increase was determined and root formation became an answer to this imbalance (Little et al., 2005; Zheng, 2009). In *Arabidopsis*, a second component was identified as an important regulator of high C/low N metabolism, the glutamate receptor 1.1 (AtGLR1.1) (Kang et al., 2003).

### 3.7. Genetic improvement for NUE

Many attempts were made to identify novel genes and/or to manipulate the expression of those involved in N uptake, assimilation, translocation, recycling and remobilization to increase NUE in crops, especially under limited N supply (Kant et al. 2011; Xu et al. 2012). The first approach was focused on  $\text{NO}_3^-$  transporter genes, but their multiplicity or the existence of many unknown feedback pathways regulating influx and efflux, represents a major problem. As reported previously, NRT1 gene family is assumed to mediate the low-affinity transport system (LATS), with the exception of NRT1.1, which is a dual affinity transporter (Wang et al. 1998). NRT2 gene family were demonstrated to encode high-affinity transport system HATS components by a correlation between the regulation of iHATS for  $\text{NO}_3^-$  uptake and NRT2.1 expression in root (Forde 2000; Orsel et al. 2002). Later, the protein encoded by NAR2.1 was demonstrated to be essential for high-affinity  $\text{NO}_3^-$  uptake because its disruption reduces plant performance more than NRT2.1 (Orsel et al. 2006). They also suggested that AtNAR2.1 gene could be considered in genetic modification to improve NUE in plants. In rice, NRT2.1 over-expression did not show any effect on N uptake when the protein partner (NAR2.1) was limited. Indeed, NRT2.1 and NRT2.2 transporters required NAR2.1 products for their function (Yan et al. 2011). However, at high N soil concentrations, NRT2 transporters as well as Nia and Nii genes were transcriptionally repressed by N metabolites such as amino acids like glutamine. On the other hand, in response to nitrate deprivation, NRT2 genes were up- and down-regulated

(Tsay et al. 2007; Meyer and Stitt 2001). Further, NRT1.1 and NRT2.1 were demonstrated to be up-regulated by glucose-6-phosphate, sugar and phytochrome (Lillo et al. 2008).

Over-expression of the key N assimilatory enzymes, either NR or NiR genes or GS, and GOGAT was considered for NUE improvement. Although a variation of the NR expression provoked insignificant or no changes in plant growth, GS gene expression modulation modified plant development (Masclaux-Daubresse et al. 2010; Brauer et al. 2011). Conversely, GOGAT over-expression was rare, probably due to the large size of the GOGAT mRNA making it technically difficult to achieve.

In recent years, many studies were focused on transcription factors implicated in NUE. The ectopic expression of transcription factors could produce a significant effect on plant's phenotype. The transcription factor Dof1, involved in the activation of non-photosynthetic, C4-related PEPc, as well as other proteins related to organic acid metabolism, affected NUE (McAllister et al., 2012).

Yanagisawa et al. (2004) and, more recently, Kurai et al. (2011) demonstrated that the over-expression of Dof1 in Arabidopsis and rice increased amino acid content, carbon skeleton production and caused a reduction in glucose, suggesting that Dof1 may be an important factor in plant NUE.

Lillo et al. (2008) demonstrated as two transcription factors, HY5 and HYH from the bZIP family, were essential for NR gene expression, and correlated with NRT1.1 expression. Furthermore, another transcription factor HAP3, a member of a large protein family known as haeme activator proteins (HAP), involved in regulating flowering time in plants, has been implicated in plant NUE (Cai et al. 2007). The over-expression of HAP in tomato and Arabidopsis resulted in early or delayed flowering time, respectively (Ben-Naim et al. 2006; Wenkel et al. 2006; Cai et al. 2007), suggesting that HAP proteins/complexes may interact with N assimilation enzymes as well. More recently, Araus et al. (2016) demonstrated as the BTB and TAZ DOMAIN PROTEIN 2 (BT2) plays a central role in NUE network under low N status.

Finally, post-translation regulation was also involved in  $\text{NO}_3^-$  uptake and assimilation. Noticeably, protein phosphorylation, i.e. CDPK, SnRK1, represents the major regulation factors at post-translation level (Lillo et al. 2008; Marchive et al. 2013).

### 3.8. Strategies to improve NUE in crops

As NUE involves many different genes and morphological and physiological traits, there are potentially many strategies for NUE improvement in crops. All of these require an increased knowledge of how plants respond to different N regimes as well as to environmental limitations to be better used to improve NUE.

In general, NUE can be improved by i) rationalizing N supply and demand; ii) maximizing crop  $\text{NO}_3^-$  uptake and assimilation and minimizing N losses from soils; iii) manipulating root growth, iv) using of N use efficient genotypes to exploit the biological diversity for an efficient nutrient acquisition/utilization from soils both spatially and temporally (Shen et al. 2013).

Based on these knowledge, two molecular approaches were considered to increase NUE in crops: 1) conventional breeding aided by marker-assisted selection (MAS) to cumulate QTLs and alleles improving NUE in a single genotype; 2) the transgenic/genome editing approach by manipulating (e.g. over-expression) candidate genes designed as responsible to improve specific NUE components (Good et al. 2004).

In the last decade, Quantitative Trait Loci (QTL) studies were employed to identify molecular markers useful to improve NUE by MAS, as example below are reported identified QTL related to NUE until to 2012 (Table 3).

| <i>Reference</i>       | <i>Species</i> | <i>Traits analysed</i>                        | <i>Mapping population</i>    | <i>Candidate genes</i> |
|------------------------|----------------|-----------------------------------------------|------------------------------|------------------------|
| Agrama et al. (1999)   | Maize          | Plant biomass, kernel weight and grain yield  | F2:3 families population     |                        |
| Hirel et al. (2001)    | Maize          | Grain yield, grain N yield,                   | RIL population               | GS1                    |
| Obara et al. (2001)    | Rice           | Grain yield, GS1, and NADH-GOGAT              | Backcross inbred lines (BIL) | GS1 and NADH-GOGAT     |
| Loudet et al. (2003)   | Arabidopsis    | Plant biomass, N content                      | RIL population               |                        |
| Obara et al. (2004)    | Rice           | Panicle weight, NADH-GOGAT and GS1            | Backcross inbred lines (BIL) | GS1 and NADH-GOGAT     |
| Charmet et al. (2005)  | Wheat          | Grain dry matter, N accumulation              | RIL population               |                        |
| Lian et al. (2005)     | Rice           | Root, shoot, and plant weight                 | RIL population               |                        |
| Laperche et al. (2007) | Wheat          | Grain yield, total N amount, NHI              | Double haploid lines         | Rht-B1, Ppd-D1, and B1 |
| Habash et al. (2007)   | Wheat          | Grain yield, N content of grain               | Double haploid lines         | GS2                    |
| Coque et al. (2008)    | Maize          | Grain yield, plant dry matter, N uptake, etc. | RIL population               | GS and GDH             |
| N. Zhang et al. (2010) | Maize          | Ten enzyme activities and seedling biomass    | Inbred RIL population        | GS and NR              |
| Quraishi et al. (2011) | Wheat          | Meta-analysis of QTLs for NUE                 | Various populations          | GOGAT                  |

**Table 3.** Main NUE studies on NUE in plants (modified from Chardon et al., 2012).

Obara et al. (2001) carried out one of the first study on NUE variation in rice segregant populations. Afterwards, the identified QTL were mapped in maize, barley and Arabidopsis (Mickelson et al. 2003).

Based on high-density molecular linkage maps, a Quantitative trait locus (QTL) analysis makes possible to understand the genetic basis for such complex traits in different crops (Ikram and Chardon 2010), and to develop molecular markers effective for improving NUE. Through the transgenic approach, the transformation of Alanine aminotransferase gene (AlaAT) allowed plants to over-express AlaAT and consequently NUE (Good et al. 2007; Shrawat et al. 2008). By contrast, genes codifying for enzymes involved in N assimilation did not show a higher NUE phenotype. Differences in NUE in such plants appeared correlated with increased levels and mobilization of alanine as well as increased nitrate uptake in roots, associated with higher N uptake efficiency during vegetative growth. These responses were observed in canola under N-limiting conditions (Good et al. 2007) and in rice where it increased biomass and yield (Shrawat et al. 2008).

Furthermore, the over-expression of either NR or NiR genes was shown to increase mRNA levels, and often affected N uptake, but it was not correlated to increased yield or growth plants regardless the N source available (Good et al. 2004).

Controversial results were reported for the GS/GOGAT altering pathway. Although several studies demonstrated that in transgenic plants, the enhanced GS activity was not associated with higher NUE, others showed a significant increase in plant biomass upon incorporating a novel GS1 gene construct. For example, it has been reported that over-expression of *ZmGS1.3* in maize led to an increase of 30% in kernel number (Martin et al. 2006). Until now, there are not successful transgenic commercial lines where this gene has been over-expressed.

On the other hand, few data were reported showing the production of transgenic plants over-expressing GOGAT genes. Recently, Quraishi et al. (2011) demonstrated that the genome regions containing GS and GOGAT genes in wheat were linked to NUE. Furthermore, these authors found that these regions are syntenic among cereal genomes (maize, sorghum, rice), underlying the existence of conserved genome regions responsible for the genetic control of NUE trait.

More recently, rice plants transformed with NRT2.1 driven by the promoter NAR2.1 exhibited, in field trials, an improved NUE compared both with wild-type and with pUbi:NRT2.1-plants. Interestingly, the expression of NRT2.1 was specifically increased in

roots, leaf sheaths and internodes in the pNAR2.1:NRT2.1-plants, while an increase in all organs was evidenced in the pUbi:NRT2.1-plants (Chen et al. 2016). In conclusion, integrate marker-assisted breeding (MAS) and the transgenic approach could be crucial to identify genes, QTL and strategies to improve a complex trait as NUE.

## **AIMS AND OBJECTIVES OF THIS WORK**

Crop productivity depends heavily on nitrogen (N) fertilization. Plant roots acquire N from the soil mainly as ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ) or amino acids, but  $\text{NO}_3^-$  is the predominant N form in aerobic soils and the most limiting nutrient for plant growth and productivity. Thus, massive N-based fertilizer use to increase crop yield was adopted resulting detrimental to the environment, with elevated economic and ecological costs. N-fertilization practices are important for reducing the N-use in excess, but N-use efficiency (NUE) breeding is becoming essential for the development of genotypes able to maintain high crop yield under lower N input in a sustainable agriculture.

However, plant NUE is inherently complex trait as each step, including N-uptake, translocation, assimilation, and remobilization, governed by multiple interacting genetic and environmental factors. In particular, NUE included two components: Nitrogen Uptake Efficiency (NUpE), the plant's ability to take up N from soil, and Nitrogen Utilization Efficiency (NUtE), the ability to make high yield per unit of assimilated N.

Eggplant (*Solanum melongena* L.) is a vegetable crop of increasing importance during the last years. It is a warm-weather crop mostly cultivated and consumed in many tropical and subtropical regions of the world. Like tomato, potato and pepper, also in eggplant now biotechnological and genomic tools are available for aiding breeding. Improving NUE in eggplant is particularly important, as large amounts of N-fertilizer are required to maximize yield. Several studies on different crops highlighted natural variation for NUpE and NUtE, thus the identification of NUE-contrasting eggplant genotypes and the underlied physiological and genetic traits could provide tools for developing marker-assisted selection (MAS).

The present PhD thesis aimed to provide knowledge and strategies for an ecologically and sustainable cropping system by the reduction of N fertilizer, using eggplant as target for vegetable species. In particular, the main goal was to identify eggplant efficient genotypes in the N-use compatible with the sustainability of the EU agricultural practices. Indeed, the

development of high NUE genotypes is more challenging than targeting N applications, as a part of an integrated nutrient management.

The main goal and potential deliverables of this project have been 1) the selection of eggplant genotypes contrasting for NUE; 2) the improvement of knowledge on morpho-physiological and genetic responses in NUE-contrasting eggplants to isolate flag traits useful for MAS; 3) the integrated metabolomics and transcriptomics analyses to reveal key metabolites and genes enhancing N-metabolism cycle in *Solanum melongena*; 4) the cloning, functionally assay and allelic variant analysis of genes involved in N-uptake, transport and assimilation.

## CHAPTER 4. NATURAL VARIATION IN EGGPLANT (*Solanum melongena* L.) HIGHLIGHTED KEY MORPHO-PHYSIOLOGICAL TRAITS USEFUL FOR IMPROVING NITRATE USE EFFICIENCY

### 4.1. Introduction

Nitrogen (N) is a major limiting factor for plant growth and productivity in both natural and agricultural environments, being an essential component of proteins, secondary metabolites and nucleic acids (Brady and Weil, 1999). As a result, over the past decades, N fertilization has increased more than 20-fold, in excess compared to crop requirement (35%) (Glass, 2003; Shen et al., 2003; Good et al., 2004; Sarasketa et al. 2014; Wang et al., 2014), causing massive environment and human health damages (Good and Beatty, 2011). Lowering N fertilizer input together with the selection of genotypes more capable to uptake, utilize and remobilize N available in soil (McAllister et al, 2012), could be an important challenge on plant nutrition research (Hirel et al., 2007) and pivotal to sustain high crop yields reducing environmental, economic and health costs (Good et al., 2004; Sebilio et al., 2013). Nitrogen Use Efficiency (NUE), generally referred as “grain yield production per unit of N available in soils” (Moll et al., 1982), is a complex genetic trait influenced by several environmental factors. As a rule, NUE has two main components: Nitrogen Uptake Efficiency (NUpE), which describes the ability of plant to take up N from soil, and Nitrogen Utilization Efficiency (NUtE), which indicates the ability of plants to convert in grain the assimilated/remobilized N (Good et al., 2004; Xu et al. 2012).

Among N forms present in soil solutions, nitrate ( $\text{NO}_3^-$ ) is the primary N-source in aerobic soils, distributed unevenly both in space and time (Crawford, 1995; Miller and Cramer, 2004; Sorgonà et al., 2011), whose uptake by roots is the first step for N metabolism in plant. However, the root ability to use  $\text{NO}_3^-$  efficiently depends on morphological and physiological features underlying a complex genetic control (Lynch, 1995; Sorgonà et al., 2006; Ruffel et al., 2011). In particular, an efficient  $\text{NO}_3^-$  uptake, especially at low concentrations in soils, depends on root length and surface area, required to exploit a larger total soil volume, together with a higher number of nitrate transporters per unit of root surface (Lawlor, 2002). Plants have developed three  $\text{NO}_3^-$  uptake systems, a Low-Affinity Transport System (LATS) and two High-Affinity Transport Systems, a constitutive (cHATS) and an inducible (iHATS) (Forde and Clarkson 1999; Forde 2000; Glass et al. 2002). LATS is constitutively expressed, operating at high external  $\text{NO}_3^-$  concentrations ( $> 1 \text{ mM}$ ), and its uptake activity is linear depending on the external  $\text{NO}_3^-$  level (Glass, 2009). Conversely, both the HATS operate at

low  $\text{NO}_3^-$  concentrations ( $< 0.5 \text{ mM}$ ), however, while cHATS is active in plants never provided with  $\text{NO}_3^-$  having a greater affinity for this anion, the iHATS is induced by  $\text{NO}_3^-$  supply (Glass et al. 2002; Glass 2009).

NUpE performance is associated with root growth and ability to increase  $\text{NO}_3^-$  uptake rates, thus several strategies to improve root traits were developed (Lynch, 2007; Smith and De Smet, 2012; Gregory et al., 2013). However, since  $\text{NO}_3^-$  concentration in soils is often at low millimolar ranges (Miller et al., 2007), the selection for a more efficient HATS system could be of a particular importance for plant nutrient uptake.

Several reports showed that *NRT2.1*, member of NRT2 nitrate transporter family, expression is highly synchronized with iHATS regulation in different plant species (Filleur and Daniel-Vedele, 1999; Fraiser et al., 2000; Orsel et al., 2006; Cai et al., 2007; Sorgonà et al., 2011). Another member of the HATS, displaying the same pattern of *NRT2.1* and belonging to NAR2 family (annotated also as NRT3 according to Leràn et al., 2014), were identified in different plant species (Tong et al., 2005; Okamoto et al., 2006; Orsel et al., 2006; Gu et al., 2014). In *Arabidopsis*, a co-expression of both *NRT2.1* and *NAR2.1* highly sustains the activation of the HATS (Okamoto et al., 2006; Orsel et al., 2006).

Once  $\text{NO}_3^-$  has been absorbed, it is reduced to ammonium, through Nitrate and Nitrite Reductase (NR and NiR, respectively), in leaves or roots, and then assimilated into amino acids through Glutamine Synthetase/Glutamate Synthase (GS/GOGAT) enzymes and successively remobilized, used and/or stored in vacuolar system (McAllister et al., 2011). These activities contribute to the NUtE physiological component in crops (Kant et al., 2011; Xu et al., 2012).

Eggplant (*Solanum melongena* L.) is the third most important Solanaceous vegetable crop (Barchi et al., 2011), cultivated and consumed worldwide, especially in India and China (Hazra et al., 2003). Despite the relevance and complexity of plant NUE, essential for the development of a sustainable agriculture, limited information on genetic variation for this trait is available for eggplant, whose productivity is highly sensitive to N fertilizer (Pal et al., 2002). Several studies on model and crop species highlighted a large natural variation for quantitative traits such as root morphology, nitrogen uptake and assimilation, related to both genetic control and environmental adaptation (Chardon et al., 2010; 2012; De Pessemier et al., 2013; Walch-Liu et al., 2008). Beyond the dissection of the phenotypic variability, these genetic resources could represent a pivotal tool for selecting high-NUE genotypes (Chardon et al., 2010; Coque et al., 2008; Bi et al., 2007; Han et al., 2015).

The aim of this study was to investigate the natural variation for NUE-related traits in several eggplant accessions from different geographic origin, grown for 18 days in hydroponic system under low and high  $\text{NO}_3^-$  levels, to identify contrasting NUE genotypes through a morphological, physiological and molecular approach. Root morphology and plant biomass (root and shoot dry weight) were measured and considering the two physiological components, N-uptake efficiency (NUpE) and N-utilization efficiency (NUtE), NUE were calculated. Afterwards, four NUE-contrasting eggplants, selected in hydroponics system, were grown in soil pots in a greenhouse till fruit harvests to confirm their different N-use efficiency.

## 4.2. Materials and Methods

### 4.2.1. Plant material

Nineteen eggplant (*Solanum melongena* L.) accessions were selected from a large germplasm collection (Table 4, Cericola et al., 2013) which included genotypes of different geographic origin from Mediterranean Basin [Turkey (5), Italy (3), Spain (1)] and Asia [Thailand (4), China (2), India (2)], and a wide variability for fruit size, shape, color and plant growth habit. Furthermore, two breeding lines were included (67-3 and 305E40), parents (male and female, respectively) of a segregant population already established and characterized as  $F_2$  (Toppino et al., 2016), which nowadays become a Recombinant Inbred Lines (RIL) population.

| ID     | Accession Name                         | Origin        | Areal          |
|--------|----------------------------------------|---------------|----------------|
| AM22   | Angiò 4                                | China         | EA             |
| AM069  | Bin 6                                  | Italy         | WE             |
| AM116  | N 220-A                                | India         | EA             |
| AM148  | Slim Jim                               | India         | EA             |
| AM151  | Violetta                               | Lunga Italy   | WE             |
| AM158  | Ank2                                   | India         | EA             |
| AM177  | FiL withe                              | Turchia       | WE             |
| AM194  | TAI 455                                | Thailand      | EA             |
| AM199  | TAI 470                                | Thailand      | EA             |
| AM209  | 19 CN                                  | China         | EA             |
| AM218  | CGN17451(Dolmalik; PI 176758)          | Turkey        | WE             |
| AM222  | CGN23346 (Topatan; PI 169649)          | Turkey        | WE             |
| AM229  | CGN17568 (Yuvorlak Patlican; PI167373) | Turkey        | WE             |
| AM241  | CGN23348 (PI 169658)                   | Turkey        | WE             |
| AM246  | CGN18602 (PI 173107)                   | Turkey        | WE             |
| AM260  | CGN17479(Semiredonda Jaspeada)         | Spain         | WE             |
| AM266  | Dingaras                               | China         | EA             |
| AM_210 | 67-3                                   | Breeding line | WE $\times$ EA |
| AM_211 | 305E40                                 | Breeding line | WE             |

**Table 4.** Germplasm used for NUE characterization.

#### 4.2.2. Hydroponic system growth conditions

Seeds, surface sterilized with 5% (v/v) NaClO for 15 minutes and rinsed with deionized water, were then germinated in Petri dishes (Ø 90 mm) on filter paper with 0.1 mM CaSO<sub>4</sub>. After 10 days, seedlings with fully expanded cotyledons, were selected by uniform size and transferred in hydroponic tanks (4 L, ten seedlings for tank) containing 2.5 mM K<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 46 µM H<sub>3</sub>BO<sub>3</sub>, 9 µM MnCl<sub>2</sub>, 0.76 µM ZnSO<sub>4</sub>, 0.32 µM CuSO<sub>4</sub>, 0.11 µM Na<sub>2</sub>MoO<sub>4</sub>, and 20 µM Fe-EDTA. Nitrate was added to solution as Ca(NO<sub>3</sub>)<sub>2</sub> at 0.5 mM (Low Nitrate, LN) or 10 mM (High Nitrate, HN) concentrations. Furthermore, in order to balance Ca<sup>2+</sup>, 4.75 mM CaSO<sub>4</sub> were added in the LN solution. Then, the growing units were transferred to a growth chamber at 24°C, 65% relative humidity and 14 h photoperiod with 350 µmol m<sup>2</sup> s<sup>-1</sup> light intensity for 18 days. The nutrient solution was renewed every three days and the pH was adjusted to 5.8 with 1 N KOH.

#### 4.2.3. Morphological root analysis

Five plants (28 days old) for each genotype, exposed to LN and HN for 18 days, were collected and divided into shoots and roots. Roots were dipped in 0.1 % (w/v) toluidine blue (Sigma Aldrich, #89160) for 5 min and then scanned at 300 dpi resolution (WinRhizo STD 1600, Instruments R gent Inc., Quebec, Canada) to determine Primary (PRL, cm), Lateral (LRL, cm) and Total Root Length (TRL, cm), and volume (cm<sup>3</sup>) using WinRhizo Pro System v. 2002a software (Lupini et al., 2016; 2017). Lateral Root Number (LRN, #) was manually counted as reported by Lupini et al. (2014). Shoots and roots were then dried at 72°C for 48 h to determine their dry weight (SDW and RDW, respectively, g). Based on the above measurements, Root Length Ratio (RLR, root length/whole dry weight, cm g<sup>-1</sup>), Root Mass Ratio (RMR, root dry weight/whole dry weight, g g<sup>-1</sup>), Specific Root Length (SRL, root length/root dry weight, cm g<sup>-1</sup>), Root Fineness (RF, root length/root volume, cm cm<sup>-3</sup>), and Root Tissue Density (RTD, root dry weight/root volume, g cm<sup>-3</sup>) were also calculated (Lupini et al., 2016; 2017).

#### 4.2.4. Nitrogen concentration and Nitrogen Use Efficiency

Total nitrogen content (Nc, mg N) was determined by combustion method through a LECO-CNS-1000 analyzer (LECO Instruments Ltd., Mississauga, ON) as reported by Lupini et al. (2017).

Nitrogen Use Efficiency (NUE,  $\text{SDW N\%}^{-1}$ , where N% is the g N (100 g DW) $^{-1}$ ) (Chardon et al., 2010) and Nitrogen Utilization Efficiency (NUtE,  $\text{SDW}^2 \text{Nc}^{-1}$ ) (Siddiqi and Glass, 1981) were calculated. Nitrogen Uptake Efficiency (NUpE) was also estimated as total (shoot + root) dry weight (TDW) x N concentration (g N g TDW $^{-1}$ ) (Chardon et al., 2010).

#### 4.2.5. Enzyme activities

Enzyme activities of related-nitrogen metabolism (Nitrate Reductase - NR, Glutamine Synthetase - GS, and Glutamine Oxoglutarate Aminotransferase - GOGAT) were assayed on root and shoot of the selected genotypes grown at both N levels.

Nitrate reductase (NR) enzyme was extracted using 50 mM MOPS–KOH buffer (pH 7.8) containing PVP 0.5% (w/v), 5 mM NaF, 1  $\mu\text{M}$  Na<sub>2</sub>MoO<sub>4</sub>, 10  $\mu\text{M}$  FAD, 1  $\mu\text{M}$  leupeptin, 2 mM  $\beta$ -mercaptoethanol, 5 mM EDTA. Then, the homogenate was centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatant was immediately incubated at 30°C for 35 min in a solution containing 50 mM MOPS–KOH buffer (pH 7.6), supplemented with 5 mM NaF, 10 mM KNO<sub>3</sub>, 155  $\mu\text{M}$  NADH, and 5 mM EDTA. After, the reaction was stopped with 5.8 mM sulfanilamide and 0.8 mM *N*-naphthyl-ethylene-diamine-dichloride (NNED). The absorbance was measured at 540 nm (Foyer et al., 1998).

Glutamine Synthetase (GS) enzyme was extracted using 25 mM Tris–HCl buffer (pH 7.6), 1 mM MgCl<sub>2</sub>, 1 mM EDTA–Na<sub>2</sub>, 14 mM  $\beta$ -mercaptoethanol and PVP 1% (w/v), and the activity was measured according to Debouba et al. (2007), using hydroxylamine as substrate (Wallsgrave et al., 1979). Finally, GOGAT activity was measured by the decrease in absorbance at 340 nm of the NADH oxidation according to Groat and Vance (1981). All procedures of enzymes extraction were carried out at 4°C. Total soluble protein was estimated using bovine serum albumin as standard, according to Bradford (1976). The measurement of absorbance was performed using a UV-Vis spectrophotometer (Perkin Elmer Lambda 35, Waltham, MA, USA).

#### 4.2.6. RNA isolation and qRT-PCR

Shoots and roots of selected genotypes (28 days old), grown in hydroponic and exposed to LN and HN for 18 days, were collected (three independent biological replicates for each sample) and immediately frozen in liquid nitrogen.

Total RNA was extracted from 100 mg of eggplant roots and shoots using “RNeasy Plant Mini Kit” (Qiagen) according to the technical bulletin. RNA was subjected to DNase

treatment using the “Precision DNase Kit” (Primer Design) and then reverse transcribed into cDNA with “QuantiTect Reverse Transcription Kit” (Qiagen) following the manufacture’s protocol. Gene expression was analyzed using qRT-PCR, which was performed with a Rotor-Gene RG-6000 thermal cycler (Corbett Research). To amplify the gene fragments, cDNA template diluted 1:10 was used in a 15 µl reaction with 7.5 ul of SYBR Green (IQTM Supermix Master Bio-Rad) and 0.5-1.2 uM of gene-specific primer listed in Table 5. The amplification reactions were carried out as follows: 95°C for 5 min, followed by incubation for 15s at 95°C and denaturation for 15 s at 95°C, annealing for 60 s at 59°C for 40 cycles, followed by elongation at 72°C for 20 s. Specificity of amplification was assessed by melt curves analyzed for the presence of a single peak. The analysis was done on three biological replicates and in technical triplicate. A relative standard curve for each gene was generated using a 2-fold serial dilution of pooled cDNA (obtained mixing equal proportion of all cDNA samples) using the Rotor gene software (Q-Rex Software version 1.0, QIAGEN). PCR efficiency of primer pairs was optimized to be in the range 92-100% with  $R^2$ -values of 0.996. The DNA sequences of candidate NUE-related genes were kindly provided by the Italian Eggplant Genome Consortium (Rotino et al., 2014). *Adenine phosphoribosyl transferase* (*SmAPRT*) and *Glyceraldehyde 3-phosphate dehydrogenase* (*SmGAPDH*) from eggplant were used as reference genes (Gantasala et al., 2013; Barbierato et al., 2016). The relative quantification of each gene in different samples was performed using the geometric averaging method (geNorm) (Vandesompele et al., 2002). Gene expression heatmap was obtained by using gplots R package version 3.5.0 (Warnes et al., 2005).

| ID<br>Solgenomics.net | ID Eggplant Genome Consortium (EGC) | Gene   | Primer Forward 5'→3'     | Primer Reverse 5'→3'     |
|-----------------------|-------------------------------------|--------|--------------------------|--------------------------|
| Solyc08g078950.3      | SMEL_008g317030.1                   | NRT1.1 | TAGCGCTGCCATGATTTTAGGA   | TGTAACGTTGTTGGCTGCACCTGC |
| Solyc06g074990.1.1    | SMEL_006g266950.1                   | NRT2.1 | AGTACTTCTTCGACAGGTTTGA   | ATCCGAAGAAAATCCTCCGAA    |
| Solyc03g112090.2      | SMEL_003g189360.1                   | NRT3.1 | TTATGACTCTGCTGGTGAAATTG  | ATGGAGACGATGGAGAAGGC     |
| Solyc03g112100.2      | SMEL3Ch00.09477                     | NRT3.2 | GAGTATTGATGGCCGGGGA      | GGTGCGTAACATAGTCGCAC     |
| Solyc02g094060.2      | SMEL_002g168160.1                   | CLCa   | CAGTGGTGGAAGCATGTC       | GGAAGTACCCCTGCTGCTT      |
| Solyc11g013810.1      | SMEL_004g210000                     | NR     | AGTTCTCCCTCCACACGTTGAC   | TCTGGCTTGTACCACCATGCTTCA |
| Solyc10g050890.1.1    | SMEL_012g384590.1.01                | NiR    | TTGATGTTAGGCTCAAGTGC     | CGCATCATAAATCGGCCATAGT   |
| Solyc04g014510.2.1    | SMEL_011g362580.1                   | GS1    | ACGTTCAAATGGGGTGTTC      | TTCTCCGTGTCTCTTCCAC      |
| Solyc01g080280.2.1    | SMEL_001g138750.1                   | GS2    | ATCACATGAAGCAGCATCGT     | CCAGTTGCCATGTTCCCAAA     |
|                       | Gantasala et al,2013                | APRT   | GAGATGCATGTAGGTGCTGTGCAA | GGCCCTTCAATTCTGGAACCTCA  |
|                       | Barbierato et al., 2017             | GAPDH  | GGTGCCAAGAAGTTGTGAT      | GGTTACGATCAACGTGTTGC     |

**Table 5.** Target genes designed according to gene sequences

#### 4.2.7. Eggplant yield and Nitrogen Use Efficiency under greenhouse condition

The performance of four NUE-contrasting eggplant accessions was evaluated in greenhouse experiment at the CREA-Center for Genomics and Bioinformatics Research in Montanaso Lombardo (45° 20'N, 9° 26'E, Italy) from 15<sup>th</sup> June to 26<sup>th</sup> October 2017. The AM22, AM222, 67-3 and 305E40 accessions were sown in a 54-holes tray filled with peat (Technic N° 3, from Free peat B.V. Sluiskade NZ 79/80 Vriezenveen –Holland). Plantlets, at the third leaves, were transplanted in plastic pots (13 L) containing 7 kg of sterilized soil: peat: perlite (1:1:1, v:v:v) mixture. The soil was a medium-silty soil collected at the experimental CREA center, and perlite was Agrilit® (Perlite Italiana srl, Corsico - Milano, Italy). Beyond N concentration in soil that was 1.39%, after 28 days 0 and 30 kg ha<sup>-1</sup> of calcium nitrate were added (LN and HN treatment, respectively), dissolved in irrigation water, split in six sub-applications taking care to maintain the field capacity. Two fertilizer additions with liquid Specialfos from Alfe srl (Pomponesco-MN, Italy), which contains 30% P and 20% K as potassium phosphonate and potassium phosphite were performed by using 1 mL/pot dissolved in 500 ml of water, after 69 and 116 days.

Fruits were harvested when they reached the commercial ripeness stage; 15 harvests were performed and number, fresh and dry weight determined. At the end of the pot experiment (120 days), leaves and stems were separately collected to determine fresh and dry weight as well as nitrogen content as reported above. NUE and its components were calculated as Yield/Nitrogen supply – Ns (NUE), Nitrogen content/Nitrogen supply (NUpE) and Yield/N content (NUE), according to Moll et al. (1982).

#### 4.2.8. Nitrogen Balance Index

A DUALEX instrument (Force A, Orsay, France) was used for the determination of index values for leaf chlorophyll and epidermal flavonoid contents (Goulas et al. 2004, Cartelat et al. 2005).

The same day for each plant (at first fruit cluster when the first fruit has reached typical size – BBCH-scale 71-701, Feller et al., 1995), measurements were taken on the central parts of the last fully developed leaf avoiding major veins, abaxially and adaxially. The chlorophyll index used subsequently was based on the measurement on the adaxial leaf surface, the flavonoid index was calculated as the sum of the index values from measurements on the abaxial and adaxial surface. After the DUALEX reading, a sample from the leaf surrounding, the measurement site, was extracted with a borer, immediately transferred to liquid N and later on

used for determination of C and N contents. The values of leaf chlorophyll index were linearly correlated with leaf chlorophyll content (Cerovic et al. 2012). The ratio of chlorophyll to flavonoid index values was defined as N balance index (NBI), an indicator of C/N allocation by Cartelat et al. (2005), following the theory that flavonoid synthesis increases when the use of carbon assimilates for growing new organs was limited by N availability.

#### 4.2.9. Statistical analysis

The hydroponic experiments were set up in a completely randomized design with at least five replications. All data were checked for normality (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Leven median test). The data were analyzed by two-way ANOVA (genotype and nitrate as main factors), and means were separated by Tukey's honest significant difference (HSD) test ( $p < 0.05$ ). In addition, hierarchical classification of accessions was carried out according to the Ward's method (Ward, 1963). Statistical analysis was employed using Systat software (Systat Software Inc., Chicago, IL, USA).

Phenotypic plasticity (PL), defined as the change in phenotype caused by the environment variation (Bradshaw, 1965), was calculated as the plastic variance ( $\sigma^2_{PL}$ ), which is the sum of the environment and genotype-environment interaction variances:  $\sigma^2_E + \sigma^2_{G \times E}$ . On the other hand, plasticity (pl) is a quote of phenotypic variance ( $\sigma^2_P$ ), calculated as  $\sigma^2_{pl} = \sigma^2_{PL}/\sigma^2_P$  (Scheiner and Lyman, 1989). Finally, the heritable component of the plastic variation due to the genotype-environment interaction ( $h^2_{PL}$ ), was estimated as  $\sigma^2_{G \times E}/\sigma^2_P$  (Scheiner and Lyman, 1989). The “lmer” function of the lme4 package (Bates et al., 2013) in R v.2.15.1 (Development Core Team R, Vienna, Austria, 2012) was employed and fit a REML-based analysis of variance (ANOVA) model: Phenotype = Genotype + N level + Genotype-by-N Level + Residuals, where N level (low or high) was treated as a fixed effect, and Genotype and Genotype-by-N level were treated as random effects (Corbeil and Searle, 1976).

Conversely, trait broad sense heritability ( $h^2_B$ ) was calculated as  $\sigma^2_G/\sigma^2_P$ , where  $\sigma^2_G$  is the genetic variance component (attributable to variation among genotypes), while  $\sigma^2_P$  is the total phenotypic variance, as previously defined. The same function in R, as reported above, was used to fit a REML based ANOVA model: Phenotype = Genotype + Residuals, where Genotype was treated as a random effect.

Greenhouse pot experiment was set up as completely randomized blocks with six replicates for each accession and N-treatment. After checking for normality and homogeneity of

variance, the data were analyzed by two-way ANOVA (genotype and nitrate as main factors), and means separated by Tukey's honest significant difference (HSD) test ( $p < 0.05$ ).

Data of relative gene expression were analyzed by two-way ANOVA based on three biological replicates for each treatment by using R software version 3.5.0.

## 4.3. Results

### 4.3.1. Growth chamber experiments

#### 4.3.1.1. Eggplant natural variation in response to nitrate

Eggplant biomass, in terms of SDW and RDW, among 19 eggplant accessions in response to low (0.5 mM, LN) and high (10 mM, HN)  $\text{NO}_3^-$  supplies, was firstly investigated. For each trait, biplot graphs showed the average values of each accession obtained at LN (plotted along the vertical axis) compared to those at HN (plotted along the horizontal axis) levels (Figure 1). The biplot analyses allowed us to understand which trait was mostly involved in response to  $\text{NO}_3^-$  supply. Indeed, the genetic variation among genotypes was explained by their distribution along the diagonal bisector of biplots, while the distance from the bisector indicated the adaptive response to the anion of each genotype. RDW showed a higher Coefficient of Variation (CV) compared to SDW (0.44 vs 0.37) depending on both N supply (especially at LN) and genotype (Figure 1). In particular, RDW showed a higher average (0.012 g) at LN compared to HN (0.0026 g), on the contrary, SDW average was higher (0.067 g) at HN. The deviation from the bisector of genotypes for RDW appeared to be more variable at LN (from 0.004 to 0.025 g), while it was more marked at HN (from 0.02 to 0.13) for SDW parameter (Figure 1).

The high variability highlighted in RDW suggested investigating more deeply on the other root morphological parameters (Figure 2). In general, the traits associated to root length had higher values under LN supply. For example, TRL (0.49 CV) and LRL (0.54 CV) showed the higher variation compared to the others, due to both N supply and genotype (Figure 2). A similar trend was observed in RLR, LRN and SRL, which showed lower CV values (0.41, 0.33 and 0.35, respectively). Conversely, both RMR and RTD were strongly influenced by both LN and HN supplies, respectively (Figure 2). Finally, RF showed a low variability due to both N supplies (mainly at high HN) and genotype (Figure 2). Among the physiological traits, NUpE showed the highest coefficient of variation ( $\text{CV} = 0.47$ ), while NUE and NUtE exhibited a lower CV, highlighting a similar distribution along the bisector, and their scarce response to nitrate supply compared to NUpE (Figure 3). In addition, NUpE average was

significantly higher at HN respect to LN (4.12 vs 3.02 mg N). NUE average was 0.011 (g SDW N<sup>-1</sup>) at LN, ranging from 0.0043 to 0.019, while at HN, no significant differences were recorded (0.010). A similar trend was observed for NUtE, which exhibited average values of 0.0011 and 0.0010 (g SDW<sup>2</sup> mg N<sup>-1</sup>) at LN and HN, respectively. Overall, the accessions showed highly significant ( $P<0.0001$ ) differences in all the traits at both nitrate supply, except NUE and RTD.

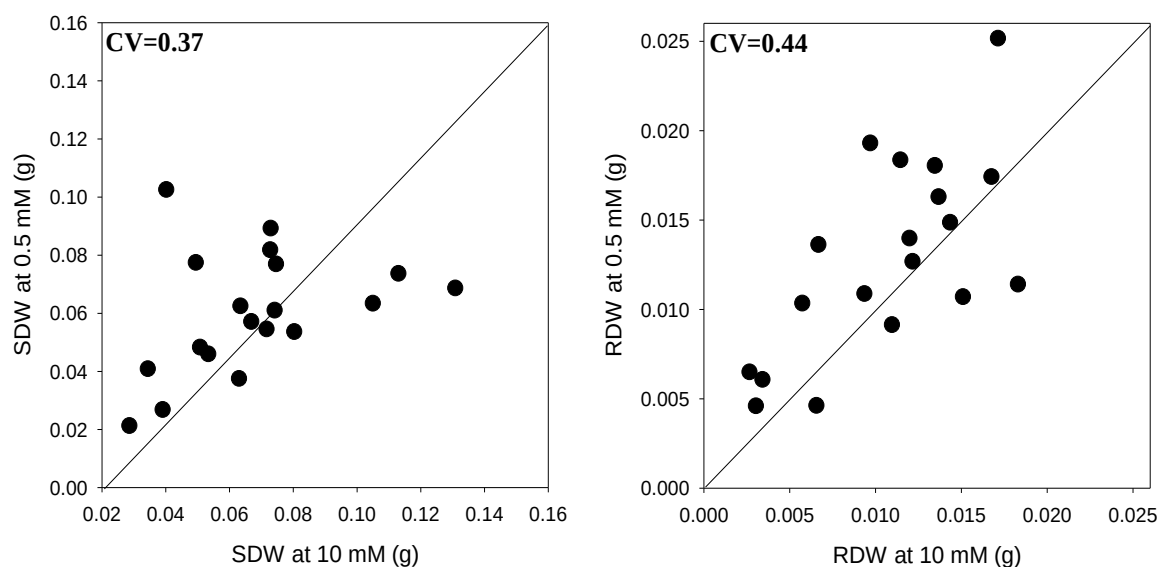


**Figure 1.** Natural variation in shoot (A) and root dry weight (B) of the 19 eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days. CV= coefficient of variation.

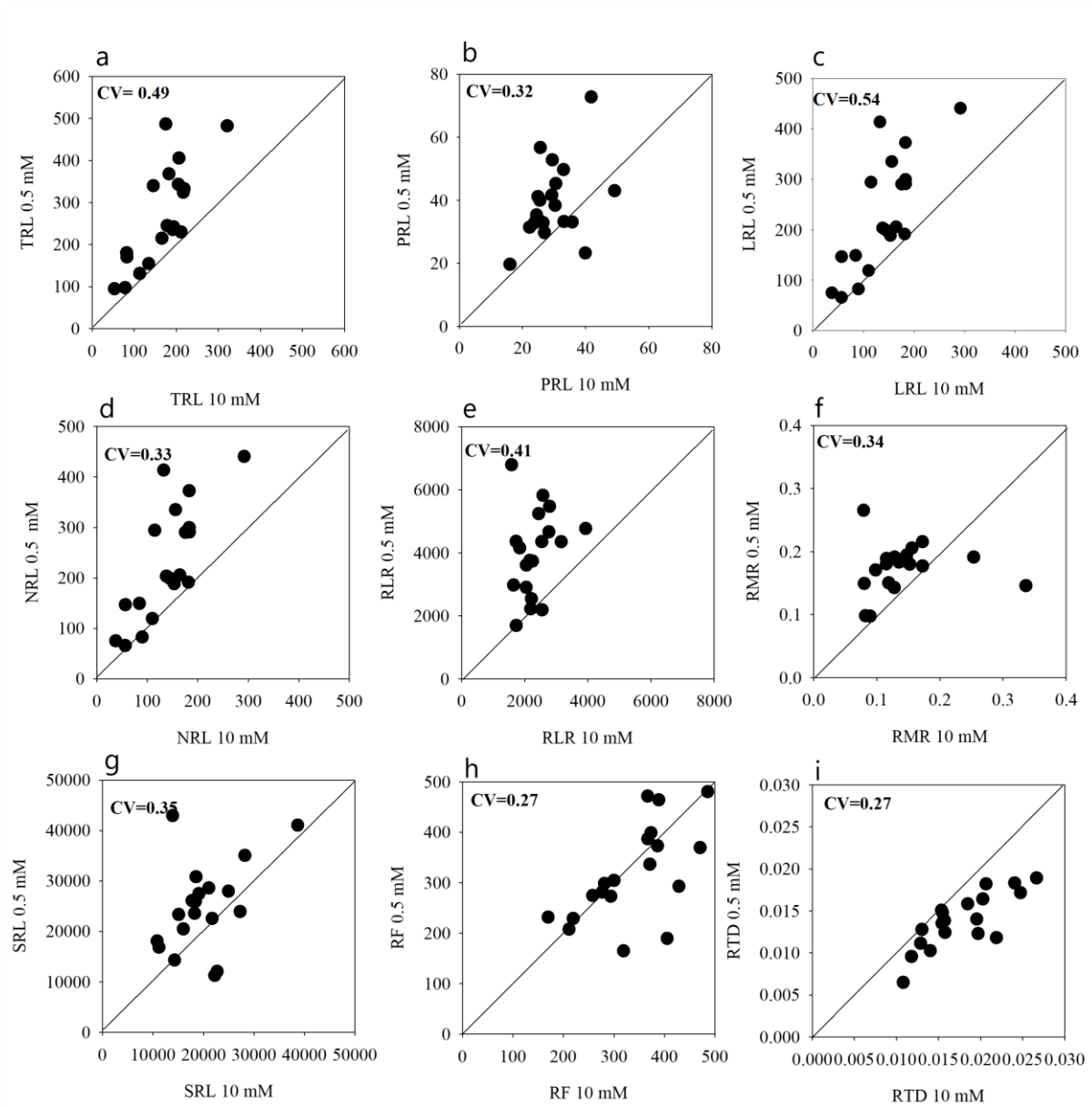


**Figure 2.** Root morphology traits of the eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days. TRL, total root length; PRL, primary root length; LRL, lateral root length; NRL, number of lateral roots;

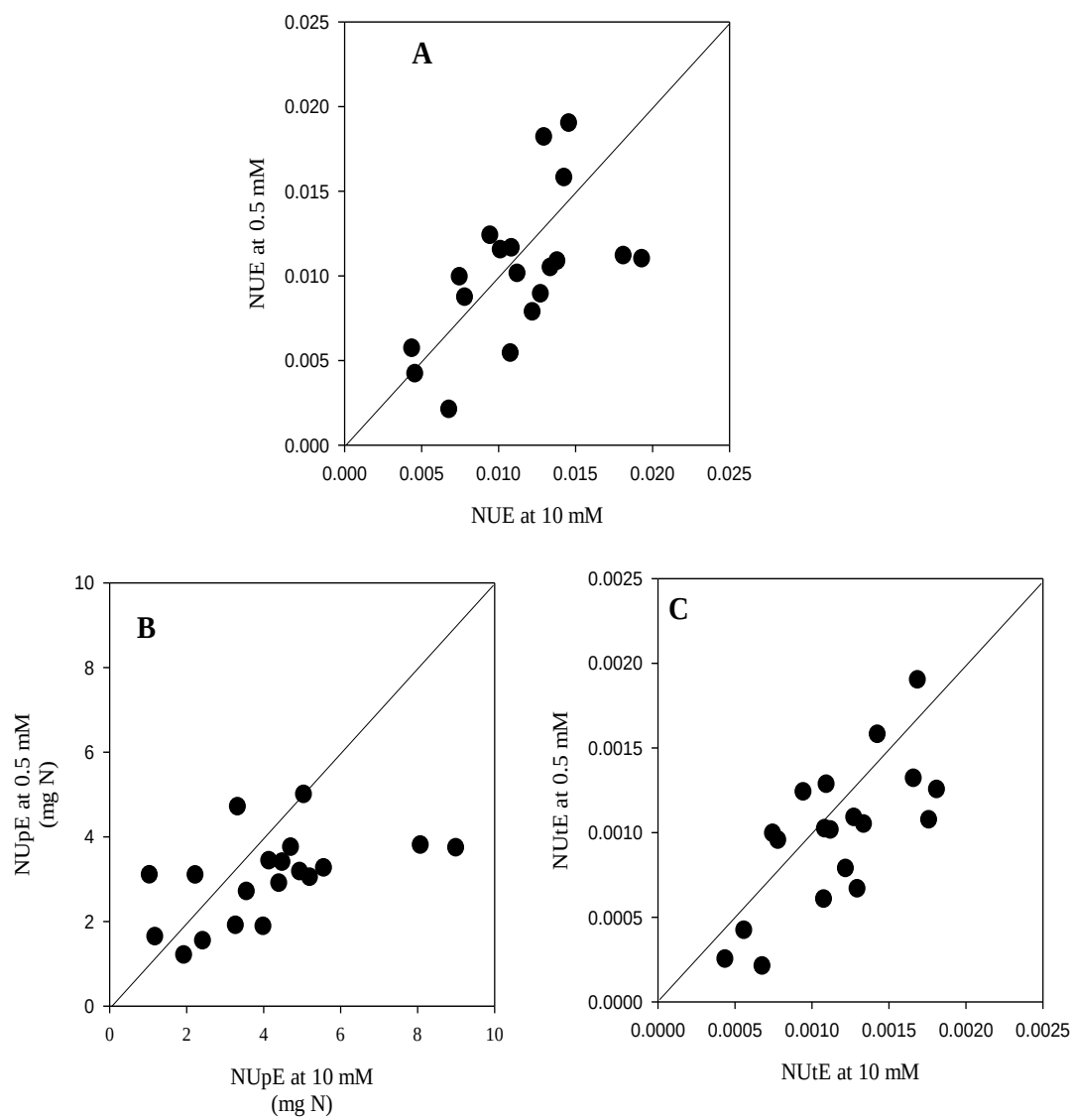


**Figure 3.** Nitrate use efficiency (A, NUE), Uptake Efficiency (b, NUpE) and Utilization Efficiency (C, NUtE) of the eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days.

#### 4.3.1.2. Global ANOVA, plasticity, genetic variation and heritability

To determine the percentage of explained variance due to genetic, environment and their interaction, global ANOVA was performed for all the traits under both N supplies (Figure 4). The genotype resulted the higher cause of variation, reaching values overcoming 50% in RF, NUtE and SDW, while the lowest values were found in root traits (RLR, RMR and SRL) (Figure 4).

The eggplant collection showed a high level of plasticity (PL) for many traits, ranging from 0.382 to 0.807 (Table 6). RMR, SRL, LRL exhibited the highest PL values, while the lowest ones were observed in RF, followed by NUtE and SDW. Conversely, broad sense heritability ( $h^2_B$ ) ranged from 0.376 to 0.771 and 0.242 to 0.791 at LN and HN, respectively (Table 6). At LN, the highest values of  $h^2_B$  were detected in TRL, LRL, RF and NUtE; by contrast, RLR, RMR and SRL exhibited the lowest ones. Lowest  $h^2_B$  values for the same traits were also observed at HN, while NUtE and NUtE showed high  $h^2_B$  values and RF maintained the highest one (Table 6). Finally, the plastic heritability ( $h^2_{PL}$ ), was calculated according to Scheiner and Lyman (1989) and showed a range from 0.085 (NUtE) to 0.227 (RTD) (Table 6).

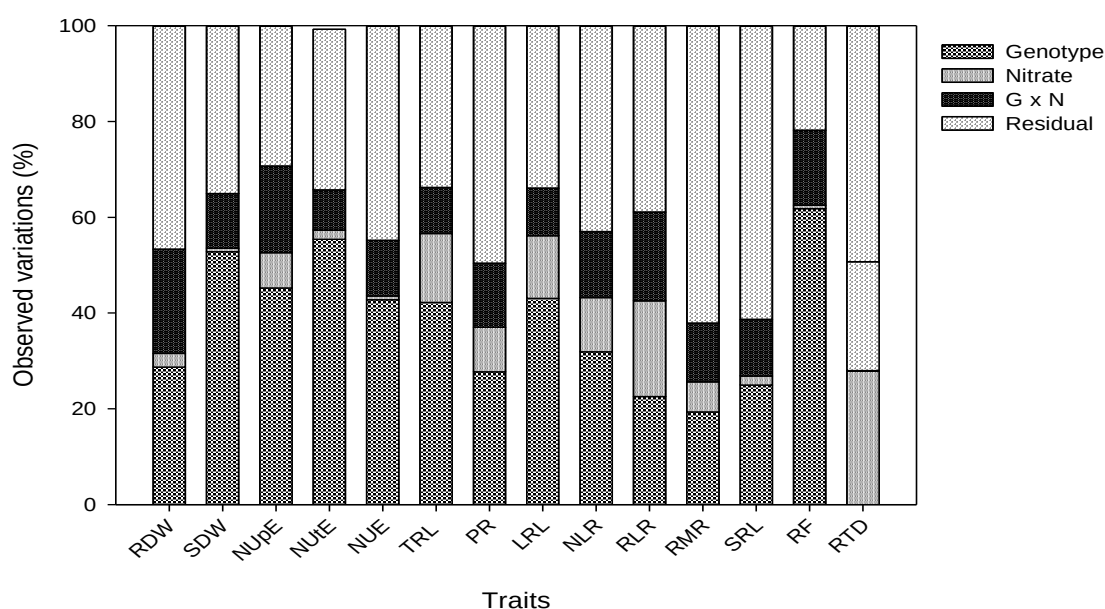


**Figure 4.** Global ANOVA of biomass, root morphology and NUE definition traits of the eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days. Different grid indicates effect of the genotype (accession), nitrate and their interaction as percentage of the observed variation.

| TRAIT                                                                        | Broad sense heritability ( $h^2_B$ ) <sup>1</sup><br>at LN/HN <sup>2</sup> | Plasticity (PL) <sup>3</sup> | Plastic heritability ( $h^2_{PL}$ ) <sup>4</sup> |
|------------------------------------------------------------------------------|----------------------------------------------------------------------------|------------------------------|--------------------------------------------------|
| Shoot dry weight (SDW, g)                                                    | <u>0.480</u> /0.565                                                        | 0.472                        | 0.114                                            |
| Root dry weight (RDW, g)                                                     | <u>0.509</u> /0.489                                                        | 0.713                        | 0.217                                            |
| Total root length (TRL, cm)                                                  | <u>0.634</u> /0.533                                                        | 0.578                        | 0.096                                            |
| Primary root length (PRL, cm)                                                | <u>0.459</u> /0.493                                                        | 0.723                        | 0.134                                            |
| Lateral root length (LRL, cm)                                                | <u>0.643</u> /0.542                                                        | 0.569                        | 0.099                                            |
| Number lateral root (N#)                                                     | <u>0.551</u> /0.448                                                        | 0.681                        | 0.138                                            |
| Root length ratio (RLR, root length/whole dry weight, cm g <sup>-1</sup> )   | <u>0.494</u> /0.632                                                        | 0.774                        | 0.186                                            |
| Root mass ratio (RMR, root dry weight/whole dry weight, g g <sup>-1</sup> )  | <u>0.376</u> /0.242                                                        | 0.807                        | 0.122                                            |
| Specific root length (SRL, root length/root dry weight, cm g <sup>-1</sup> ) | <u>0.425</u> /0.300                                                        | 0.750                        | 0.118                                            |
| Root fineness (RF, root length/root volume, cm cm <sup>-3</sup> )            | <u>0.771</u> /0.791                                                        | 0.382                        | 0.156                                            |
| Root tissue density (RTD, root dry weight/root volume, g cm <sup>-3</sup> )  | <u>0.520</u> /0.409                                                        | 0.721                        | 0.227                                            |
| Nitrogen uptake efficiency (NUpE, mg N)                                      | <u>0.517</u> /0.751                                                        | 0.547                        | 0.181                                            |
| Nitrogen utilization efficiency (NUE, SDW <sup>2</sup> TNA <sup>-1</sup> )   | <u>0.620</u> /0.706                                                        | 0.442                        | 0.085                                            |
| Nitrogen use efficiency (NUE, SDW N% <sup>-1</sup> )                         | <u>0.553</u> /0.527                                                        | 0.576                        | 0.115                                            |

**Table 6.** Heritability (broad sense,  $h^2_B$ ) of each trait calculated at LN and HN; plasticity (PL) and plastic heritability ( $h^2_{PL}$ ) were also estimated taking into account G x N interaction.

#### 4.3.1.3. Trait correlation and cluster analysis

Pearson's correlation analysis among morpho-physiological traits was carried out at LN and HN (Table 7). At LN supply, NUE resulted highly correlated with NUtE (0.9723) and NUpE (0.6201) components together with LRN (0.8396) and SDW (0.6267); NUpE exhibited correlation coefficients  $> 0.7$  with SDW and RDW but also with TRL, LRL and LRN, while NUtE showed the highest values of correlation for LRN (0.7954) (Table 7). At HN, NUE and its components showed the highest correlation with SDW (0.9564, 0.8091 and 0.8610, respectively), TRL and LRL (Table 7).

Further, an ascendant hierarchical cluster analysis was performed to identify contrasting NUE-genotypes taking in to account all the morpho-physiological traits. Three clusters were defined by using Ward's method (Ward, 1963) (Figure 5). Cluster 1 contained the accessions AM22, AM194, 67-3 and AM151, showing the lowest SDW at HN compared to cluster 3 (data not shown). Interestingly, at LN, these genotypes exhibited a marked reduction for several root traits, such as TRL, LRL and NLR, as well as for NUE and its components, compared to the other clusters (data not shown). Cluster 2, including 8 accessions, exhibited intermediate values for NUE and its components as well as for several root traits (Figure 5). Additionally, cluster 3, composed by seven accessions, including AM222 and AM241, was characterized by the highest SDW and RDW values, NUE and its components and root traits such as TRL, LRL, NLR and RLR, particularly at LN (data not shown).

The N-use efficient contrasting genotypes were selected, considering at least one representative genotype from clusters 1 and 3, which showed the highest phenotypic diversity. In particular, in cluster 3, characterized by accessions with similar high NUE, AM222 was chosen as N-efficient genotype for its highest values in root traits such as TRL and LRL compared to AM241 (data not shown). Among the genotypes included in cluster 1, AM22 was chosen as the N-inefficient for its lowest values in NUE and for its different geographic origin respect to AM222 (data not shown). Finally, 67-3 (cluster 1) and 305E40 (cluster 3) accessions were also selected for NUE-contrasting performance and because they are parents of a RIL segregant population recently developed from a F<sub>2</sub> population (Toppino et al., 2016). Moreover, the genotype 67-3 was also sequenced (Rotino et al., 2014; <http://www.eggplantgenome.org/>). The genotype 67-3 showed a rather similar phenotype to AM22, while 305E40 showed a root morphology similar to AM222.

| Traits <b>LN</b> | RDW            | SDW            | TRL           | PRL      | LRL           | LRN            | NUpE          | NUE           | NUtE          | RLR           | RMR      | SRL            | RF            | RTD           |
|------------------|----------------|----------------|---------------|----------|---------------|----------------|---------------|---------------|---------------|---------------|----------|----------------|---------------|---------------|
| RDW              | <b>1</b>       | <b>0,5791</b>  | <b>0,5396</b> | 0,3542   | <b>0,5206</b> | <b>0,5253</b>  | <b>0,7355</b> | <b>0,5454</b> | <b>0,5077</b> | 0,0955        | 0,4534   | -0,4515        | -0,2108       | 0,2732        |
| SDW              | <b>0,5791</b>  | <b>1</b>       | 0,4345        | 0,1734   | 0,4340        | <b>0,6734</b>  | <b>0,7946</b> | <b>0,6267</b> | <b>0,5795</b> | -0,1483       | -0,1081  | <b>-0,4743</b> | -0,2299       | 0,4278        |
| TRL              | <b>0,5396</b>  | 0,4345         | <b>1</b>      | 0,4237   | <b>0,9942</b> | <b>0,5517</b>  | <b>0,7090</b> | 0,4137        | 0,4158        | <b>0,7318</b> | 0,2072   | 0,2788         | 0,4211        | 0,1791        |
| PRL              | 0,3542         | 0,1734         | 0,4237        | <b>1</b> | 0,3313        | 0,1523         | 0,2482        | 0,0261        | -0,0718       | 0,3061        | -0,0616  | 0,0440         | -0,1021       | -0,2204       |
| LRL              | <b>0,5206</b>  | 0,4340         | <b>0,9942</b> | 0,3313   | <b>1</b>      | <b>0,5555</b>  | <b>0,7105</b> | 0,4166        | 0,4324        | <b>0,7331</b> | 0,2260   | 0,2814         | 0,4427        | 0,2074        |
| NLR              | <b>0,5253</b>  | <b>0,6734</b>  | <b>0,5517</b> | 0,1523   | <b>0,5555</b> | <b>1</b>       | <b>0,6802</b> | <b>0,8396</b> | <b>0,7954</b> | 0,1605        | 0,0215   | -0,1472        | -0,0097       | 0,3783        |
| NUpE             | <b>0,7355</b>  | <b>0,7946</b>  | <b>0,7090</b> | 0,2482   | <b>0,7105</b> | <b>0,7102</b>  | <b>1</b>      | <b>0,6201</b> | <b>0,6143</b> | 0,1572        | 0,1115   | -0,3174        | -0,0391       | 0,4040        |
| NUE              | <b>0,5454</b>  | <b>0,6267</b>  | 0,4137        | 0,0261   | 0,4166        | <b>0,8396</b>  | <b>0,6201</b> | <b>1</b>      | <b>0,9723</b> | -0,0926       | -0,1034  | -0,3217        | -0,1366       | 0,4376        |
| NUtE             | <b>0,5077</b>  | <b>0,5795</b>  | 0,4158        | -0,0718  | 0,4324        | <b>0,7954</b>  | <b>0,6143</b> | <b>0,9723</b> | <b>1</b>      | -0,0317       | -0,0713  | -0,2487        | -0,1157       | 0,4004        |
| RLR              | 0,0955         | -0,1483        | <b>0,7318</b> | 0,3061   | <b>0,7331</b> | 0,1605         | 0,1572        | -0,0926       | -0,0317       | <b>1</b>      | 0,2547   | <b>0,7545</b>  | <b>0,5952</b> | -0,1894       |
| RMR              | 0,4534         | -0,1081        | 0,2072        | -0,0616  | 0,2260        | 0,0215         | 0,1115        | -0,1034       | -0,0713       | 0,2547        | <b>1</b> | -0,0177        | -0,0370       | -0,2230       |
| SRL              | -0,4515        | <b>-0,4743</b> | 0,2788        | 0,0440   | 0,2814        | -0,1472        | -0,3174       | -0,3217       | -0,2487       | <b>0,7545</b> | -0,0177  | <b>1</b>       | <b>0,5972</b> | -0,4406       |
| RF               | -0,2108        | -0,2299        | 0,4211        | -0,1021  | 0,4427        | -0,0097        | -0,0391       | -0,1366       | -0,1157       | <b>0,5952</b> | -0,0370  | <b>0,5972</b>  | <b>1</b>      | 0,3840        |
| RTD              | 0,2732         | 0,4278         | 0,1791        | -0,2204  | 0,2074        | 0,3783         | 0,4040        | 0,4376        | 0,4004        | -0,1894       | -0,2230  | -0,4406        | 0,3840        | <b>1</b>      |
| Traits <b>HN</b> | RDW            | SDW            | TRL           | PRL      | LRL           | LRN            | NUpE          | NUE           | NUtE          | RLR           | RMR      | SRL            | RF            | RTD           |
| RDW              | <b>1</b>       | <b>0,7130</b>  | <b>0,6794</b> | 0,2359   | <b>0,7275</b> | <b>0,6940</b>  | <b>0,5496</b> | <b>0,6247</b> | <b>0,6335</b> | -0,3020       | 0,4530   | <b>-0,7694</b> | -0,0325       | <b>0,5297</b> |
| SDW              | <b>0,7130</b>  | <b>1</b>       | <b>0,7651</b> | -0,0038  | <b>0,7993</b> | <b>0,6029</b>  | <b>0,8091</b> | <b>0,9564</b> | <b>0,8610</b> | -0,4360       | -0,2099  | <b>-0,4777</b> | 0,3584        | <b>0,5911</b> |
| TRL              | <b>0,6794</b>  | <b>0,7651</b>  | <b>1</b>      | 0,4032   | <b>0,9892</b> | <b>0,6951</b>  | <b>0,7055</b> | <b>0,7623</b> | <b>0,7584</b> | -0,0114       | -0,0654  | -0,3137        | 0,3612        | <b>0,5387</b> |
| PRL              | 0,2359         | -0,0038        | 0,4032        | <b>1</b> | 0,3086        | 0,4226         | 0,0205        | 0,1105        | 0,2101        | 0,3443        | 0,2456   | -0,0939        | -0,1638       | -0,1002       |
| LRL              | <b>0,7275</b>  | <b>0,7993</b>  | <b>0,9892</b> | 0,3086   | <b>1</b>      | <b>0,6956</b>  | <b>0,7240</b> | <b>0,7631</b> | <b>0,7530</b> | -0,0807       | -0,0299  | -0,3732        | 0,3547        | <b>0,5952</b> |
| NLR              | <b>0,6940</b>  | <b>0,6029</b>  | <b>0,6951</b> | 0,4226   | <b>0,6956</b> | <b>1</b>       | 0,3955        | <b>0,6258</b> | <b>0,5705</b> | -0,2027       | 0,0806   | <b>-0,5746</b> | 0,1990        | <b>0,7061</b> |
| NUpE             | <b>0,5496</b>  | <b>0,8091</b>  | <b>0,7055</b> | 0,0205   | <b>0,7240</b> | 0,3955         | <b>1</b>      | <b>0,7442</b> | <b>0,9134</b> | -0,3723       | -0,3002  | -0,2948        | 0,3387        | 0,3353        |
| NUE              | <b>0,6247</b>  | <b>0,9564</b>  | <b>0,7623</b> | 0,1105   | <b>0,7631</b> | <b>0,6258</b>  | <b>0,7442</b> | <b>1</b>      | <b>0,8869</b> | -0,4096       | -0,3287  | -0,3980        | 0,3530        | <b>0,5170</b> |
| NUtE             | <b>0,6335</b>  | <b>0,8610</b>  | <b>0,7584</b> | 0,2101   | <b>0,7530</b> | <b>0,5705</b>  | <b>0,9134</b> | <b>0,8869</b> | <b>1</b>      | -0,4509       | -0,2922  | -0,3785        | 0,2780        | 0,3605        |
| RLR              | -0,3020        | -0,4360        | -0,0114       | 0,3443   | -0,0807       | -0,2027        | -0,3723       | -0,4096       | -0,4509       | <b>1</b>      | 0,2806   | 0,3720         | 0,0447        | -0,2355       |
| RMR              | 0,4530         | -0,2099        | -0,0654       | 0,2456   | -0,0299       | 0,0806         | -0,3002       | -0,3287       | -0,2922       | 0,2806        | <b>1</b> | -0,4479        | -0,4131       | 0,0187        |
| SRL              | <b>-0,7694</b> | <b>-0,4777</b> | -0,3137       | -0,0939  | -0,3732       | <b>-0,5746</b> | -0,2948       | -0,3980       | -0,3785       | 0,3720        | -0,4479  | <b>1</b>       | 0,2713        | -0,4539       |
| RF               | -0,0325        | 0,3584         | 0,3612        | -0,1638  | 0,3547        | 0,1990         | 0,3387        | 0,3530        | 0,2780        | 0,0447        | -0,4131  | 0,2713         | <b>1</b>      | <b>0,5947</b> |
| RTD              | <b>0,5297</b>  | <b>0,5911</b>  | <b>0,5387</b> | -0,1002  | <b>0,5952</b> | <b>0,7061</b>  | 0,3353        | <b>0,5170</b> | 0,3605        | -0,2355       | 0,0187   | -0,4539        | <b>0,5947</b> | <b>1</b>      |

**Table 7.** Pearson's correlation between traits calculated at LN and HN. Bold values indicated significance at  $p < 0.05$

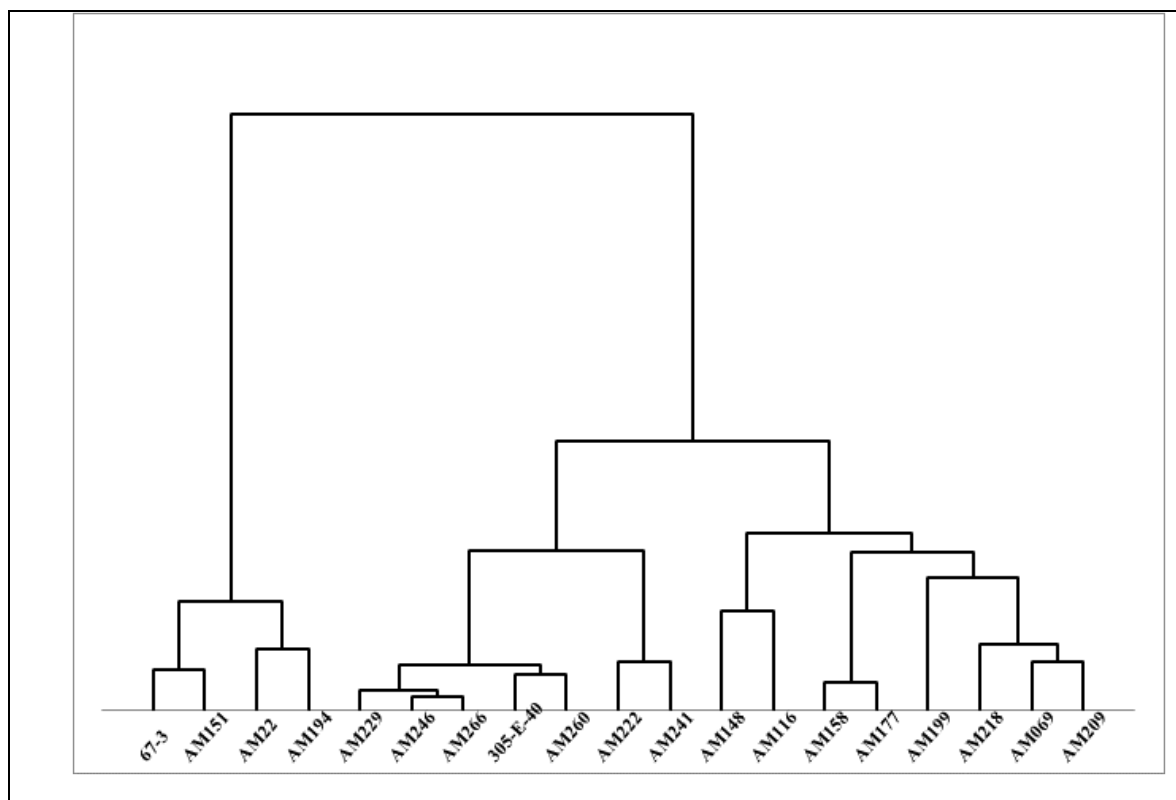


**Figure 5.** Clustering of the 19 eggplant accessions based on biomass, root morphology and NUE definition traits exposed to 0.5 or 10 mM nitrate for 18 days.

#### 4.3.1.4. Enzyme activities

The activity of closely related NUTe enzymes NR, GS and GOGAT was determined in roots and shoots of the selected genotypes, at both LN and HN levels (Figure 6).

At HN, all the accessions showed a higher NR activity in shoots without significant differences among them; while, at LN, 67-3 exhibited a significant higher activity compared to the others and to its own activity at HN level (Figure 6. a). Conversely, in roots, NR activity was influenced only by nitrate supply in all the accessions (Figure 6. b).

Different responses for GS activity were observed in shoots, among genotypes (Figure 6. c-d). In particular, at LN, 67-3 and AM222 showed a significant higher GS activity compared to the others, whereas AM22 and 305E40 showed a significant higher activity at HN (Figure 6. c). In roots, AM222 exhibited a strong GS activity at HN, while it was higher in 67-3 at LN; AM22 and 305E40 did not showed any difference between N levels (Figure 6. d).

Finally, in both plant tissues, GOGAT activity pointed out a higher activity in all the accessions at HN, showing the higher activity in 305E40 shoots. No difference was evident among the accessions at LN (Figure 6. e-f).

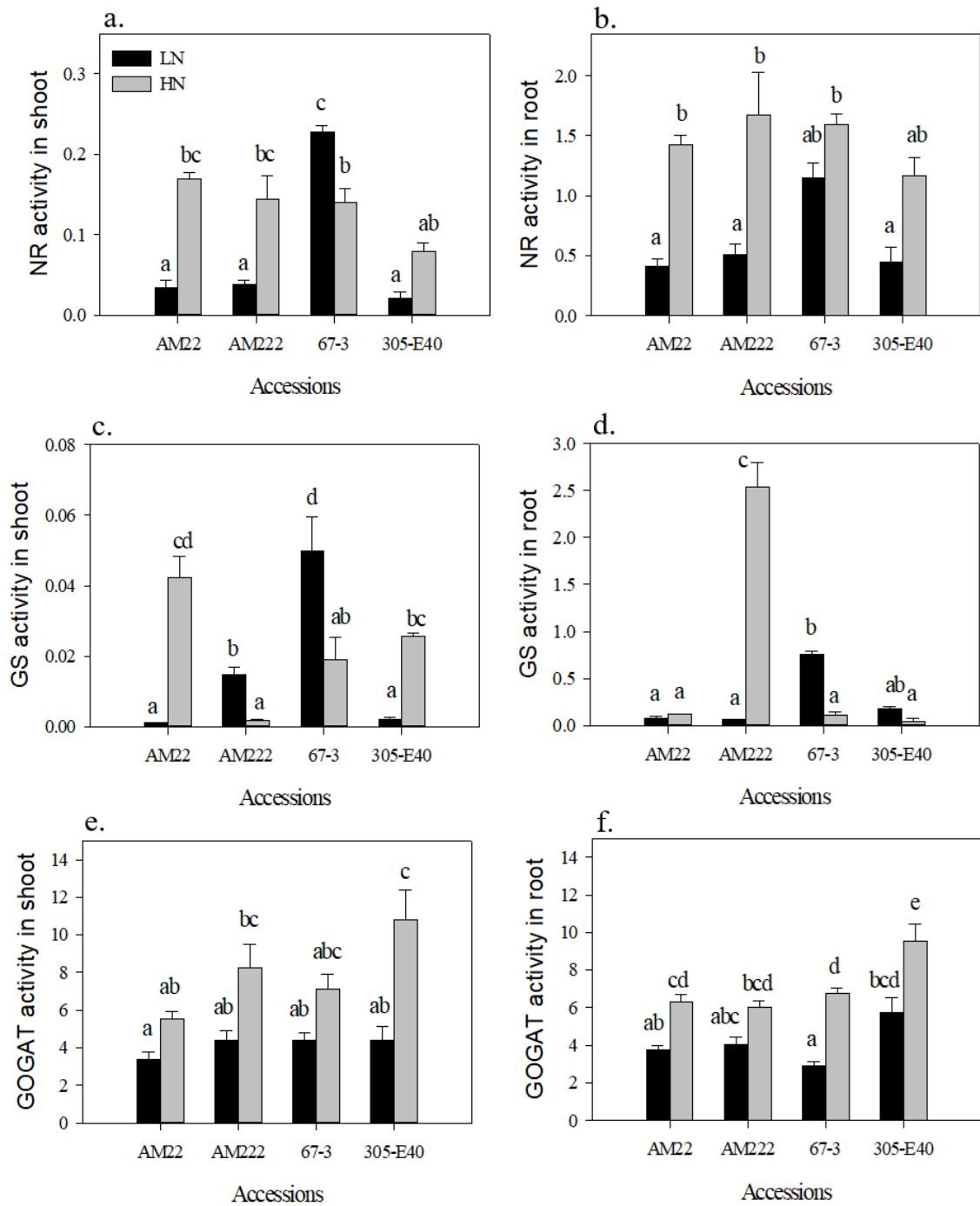


**Figure 6.** Nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT) activities in shoot (A, C and E, respectively) and root (B, D and F, respectively) of the eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days. The values are mean  $\pm$  SE ( $n = 8$ ). Different letters indicate means that differ significantly, according to Tukey's HSD test at  $P < 0.05$ .

#### 4.3.1.5. Expression analysis of candidate genes for nitrate uptake and assimilation in eggplant

Gene expression data were analyzed by two-way ANOVA (data not shown). Distinct patterns of nitrate transport and N metabolism key genes were observed in both roots and shoots in NUE contrasting eggplants (Figure 7). In roots, the transcription levels of N uptake and assimilation related genes underlined different responses of genotypes to LN and HN. In particular, at LN, 67-3 showed a different gene expression pattern compared to AM22, AM222 and 305E40, with a significant higher transcript level of *SmCLCa*, *SmGS2*, *SmNR* and *SmNiR* (Figure 7. a). Furthermore, AM222 displayed a distinguishable pattern due to higher expression of *SmNRT2.1*, *SmNRT3.1*, *SmGS1* and *SmGOGAT*, compared to AM22 and 305E40 (Figure 7. a). At HN, 67-3 confirmed its different behavior compared to the others, due to the higher and lower expression levels of *SmCLCa* and *SmNRT1.1*, *SmNRT2.1*, *SmNRT3.1*, respectively. AM222 exhibited a high expression level of *SmGS1* and *SmGOGAT*, while AM22 showed a marked expression of *SmNRT1.1*, *SmNRT3.2*, *SmNR* and *SmNiR* genes (Figure 7. a).

In shoots, at LN, AM22 was characterized by the lowest expression level in all the genes (Figure 7B). By contrast, 67-3 showed a significant higher expression of *SmNR*, *SmNiR*, *SmGS2* and *SmCLCa*; while *SmGOGAT* and *SmGS1* expressions were higher in AM222 and 305E40, respectively (Figure 7. b). Interestingly, considering the shoots at HN, 67-3 and AM22 were grouped in the same cluster as consequence of a higher expression level in all the genes. In addition, *SmCLCa* appeared significantly higher expressed in AM22 respect the other genotypes (Figure 7. b). Remarkably, AM222 showed a higher expression of both *SmGS1* and *SmGOGAT* compared to AM22 and 67-3 (Figure 7. b).

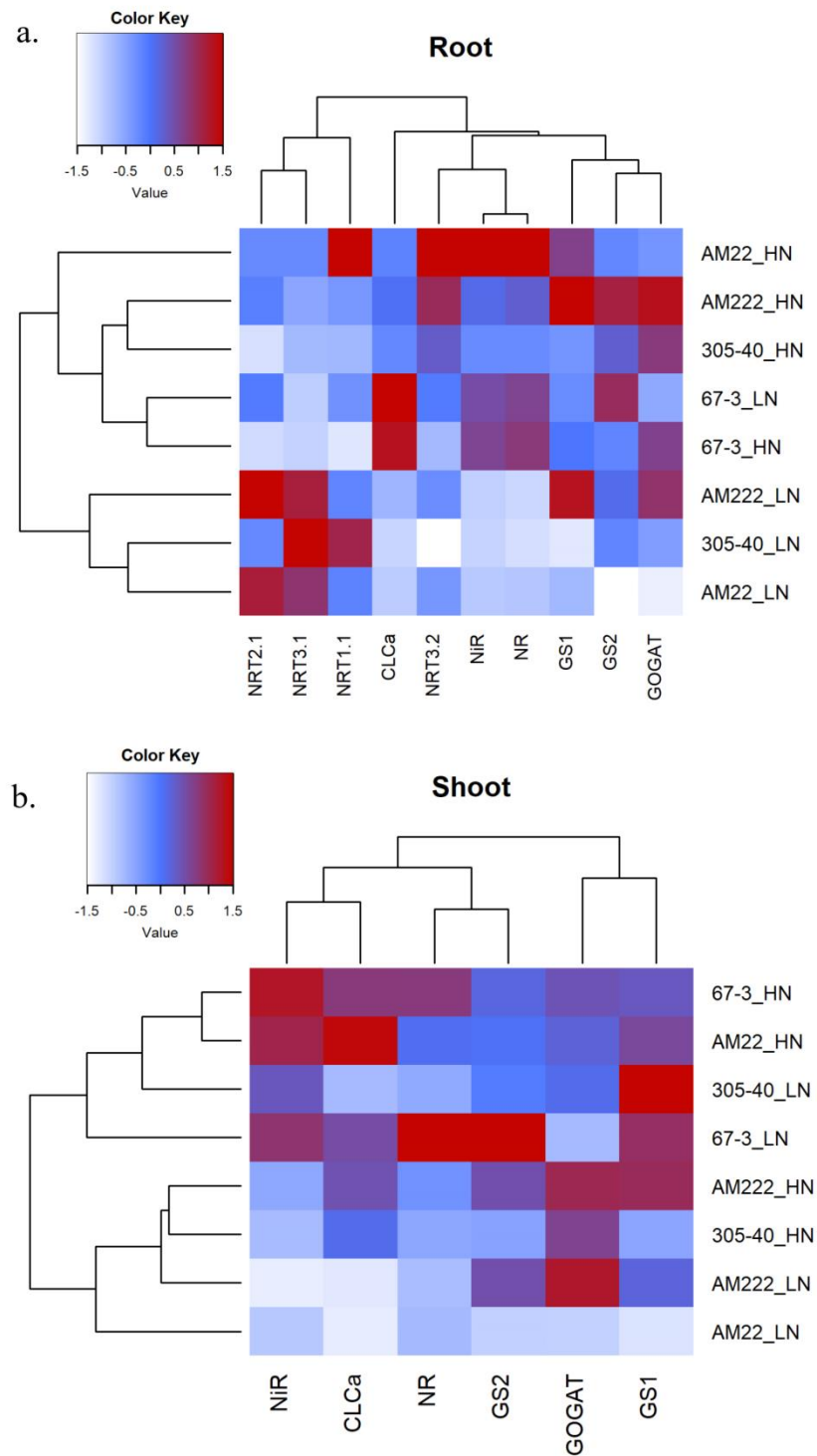


**Figure 7.** Heatmap of N-related gene expression in root and shoot of the eggplant accessions exposed to 0.5 or 10 mM nitrate for 18 days.

#### **4.4. Greenhouse pot experiment**

##### *4.4.1. Biomass and yield production*

AM22, 67-3, AM222 and 305E40 eggplant genotypes were grown in greenhouse pots experiment until berries reached commercial ripening. Genotypes exhibited different responses to HN and LN levels in terms of biomass production. Fruit, leaf and stem dry weights (FDW, LDW and SDW, respectively) showed a significant increase in all the genotypes grown at HN compared to LN (Figure 8). At HN, AM222, 67-3 and 305E40 exhibited similar performances for FDW, but significantly higher than AM22. However, AM22 appeared more sensitive to nitrate, increasing its FDW by 6-fold from LN to HN treatment (Figure 8. a).

By contrast, AM22 displayed the highest LDW value at HN, whereas no significant differences were observed among the other accessions. Interestingly, no differences among accessions were observed at LN (Figure 8. b). Finally, 305E40 showed the highest SDW compared to the other accessions at HN, while at LN no significant differences were observed (Figure 8. c).

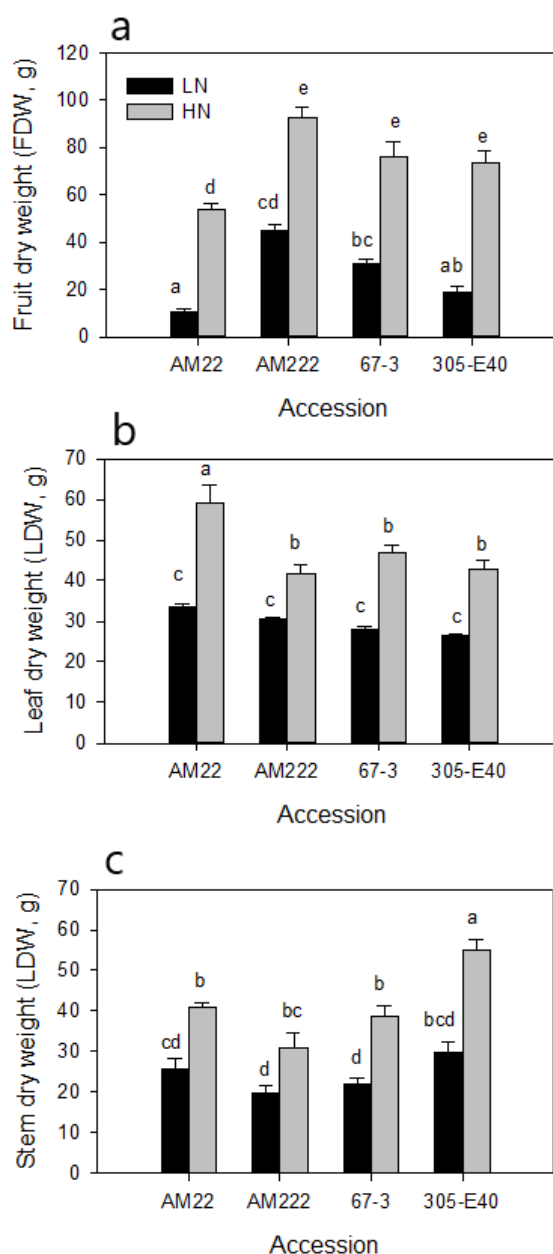


**Figure 8.** Fruit, leaf and stem dry weight (g) of the eggplant accessions grown in microcosm system and exposed to low (LN) or high (HN) nitrate. The values are mean  $\pm$  SE ( $n = 6$ ). Different letters indicate means that differ significantly, according to Tukey's HSD test at  $P < 0.05$ .

#### 4.4.2. Nitrogen content

The N content in fruits, leaves and stems was significantly increased in all the accessions at HN compared to LN treatment. In detail, AM222 showed the higher fruit N content at HN compared to the other accessions, which in contrast, did not exhibit significant differences among them (Figure 9. a). At LN, a similar trend was observed among genotypes. Interestingly, a significant difference was evident between AM22, the N-inefficient genotype, having a lower N content than AM222, the N-efficient one (Figure 9. a). By contrast, AM22

showed a significant higher leaf N content at HN, whereas at LN the accessions did not show any significant differences (Figure 9. b). Moreover, 305E40 showed the highest stem N content at HN, while at LN no differences among accessions were exhibited (Figure 9. c).

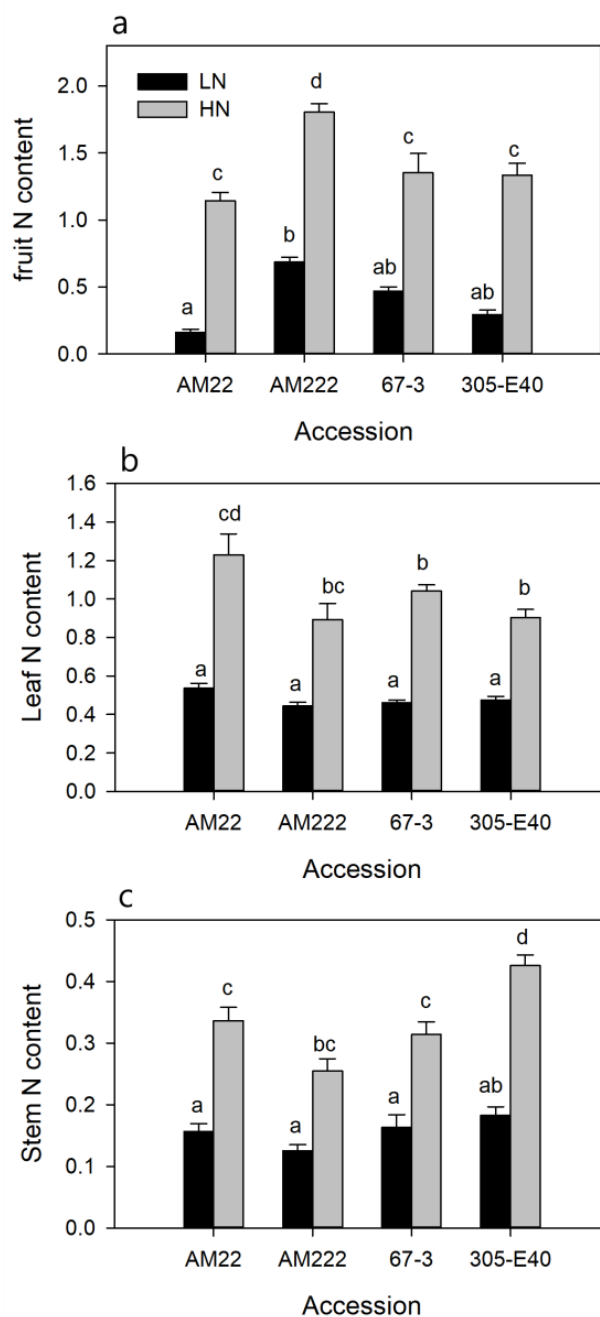


**Figure 9.** Fruit, leaf and stem N content (g) of the eggplant accessions grown in microcosm system and exposed to low (LN) or high (HN) nitrate. The values are mean  $\pm$  SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at  $P < 0.05$ .

#### 4.4.3. Nitrogen balance index

Leaf chlorophyll content index, estimated with the DUALEX instrument, was higher at HN compared to LN, regardless genotypes, which did not significantly differ among them (Figure 10. a). Conversely, flavonoids content index in leaf epidermal showed an inverse trend, resulting higher at LN (Figure 10. b). Therefore, Nitrogen Balance Index (NBI) showed a similar pattern to the chlorophyll index (Figure 10. c). Correlations between Genotype x N-level, C and N leaf contents highlighted significant values between chlorophyll and N content ( $r = 0.85$ ,  $p < 0.008$ ), flavonoids and N content ( $r = -0.74$ ,  $p < 0.04$ ) as well as NBI and N content ( $r = 0.80$ ,  $p < 0.02$ ). Significant correlations resulted also for chlorophyll, flavonoids and NBI related to the leaf N/C ratio,  $r = 0.85$ , ( $p < 0.008$ ),  $r = -0.75$ , ( $p < 0.04$ ) and  $r = 0.81$  ( $p < 0.02$ ), respectively. Although not significantly different, chlorophyll content index at HN tended to be lower in 67-3 and AM222 genotypes, while NBI was significantly lower in 67-3 compared to the other genotypes. At LN, flavonoid content index was significantly lower in AM222 compared to 305E40 (Figure 10. c).

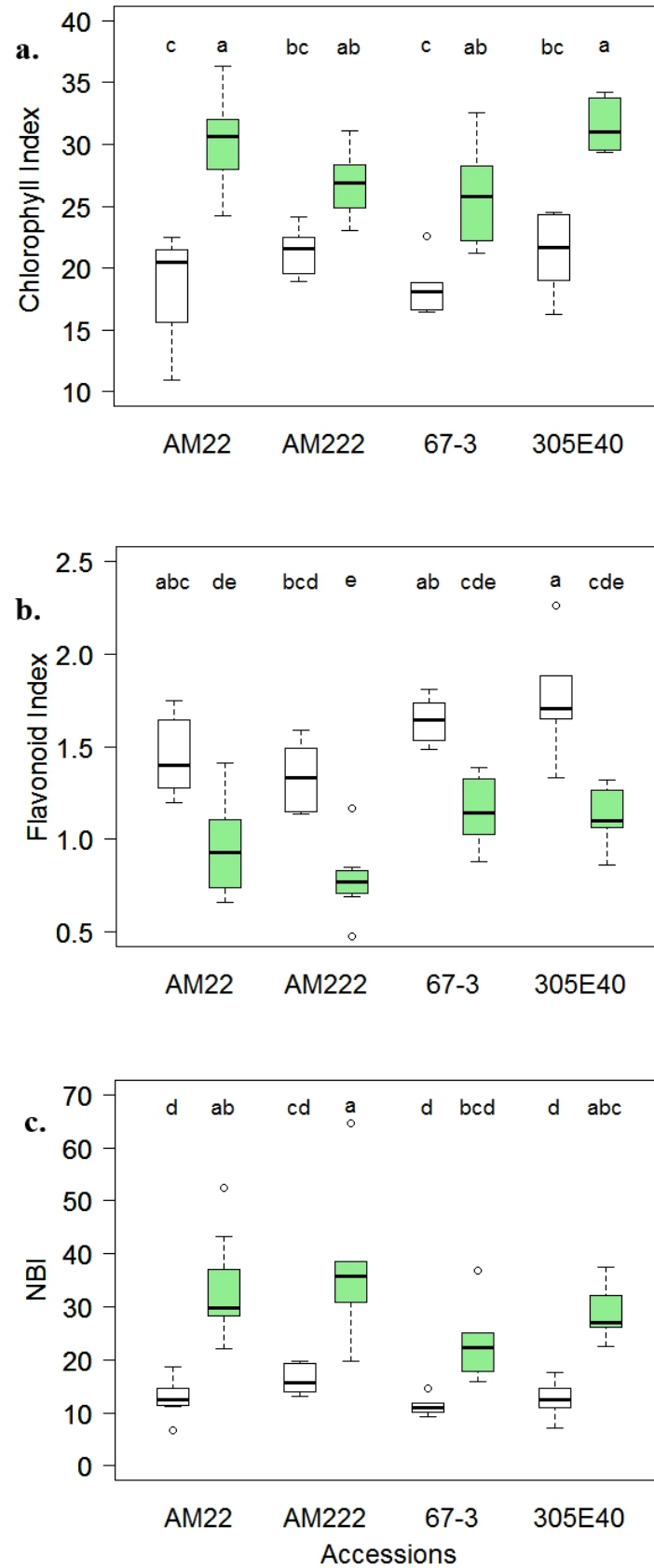


**Figure 10.** Chlorophyll index (A), Flavonoid index (B) and Nitrogen Balance Index (C, NBI) of the eggplant accessions grown in pots and exposed to low (LN, white) or high (HN, green) nitrate. The values are mean  $\pm$  SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at  $P < 0.05$ .

#### 4.4.4. Nitrate use efficiency in pot experiment

NUE calculation confirmed significant differences among contrasting genotypes, as already observed in hydroponic experiment. At LN, AM222 was characterized by a significant highest NUE compared to the other accessions, whereas, at HN, AM22 showed the lowest NUE compared to AM222, 67-3 and 305E40, among which differences were not detected (Figure 11 .a). Thus, at LN, AM22 together with 305E40 confirmed their N-use inefficiency as observed in hydroponic; at HN, only AM22 showed the lowest performance compared to the other genotypes. Furthermore, at LN, AM222 and 67-3 pointed out significant higher NUtE compared to AM22 and 305E40, while, at HN, no differences among the accessions were evident (Figure 11. b). Finally, NUpE was strongly dependent on nitrate level, being highest at HN in all the genotypes. As a rule, no significant differences among the genotypes within each N level were observed (Figure 11. c).

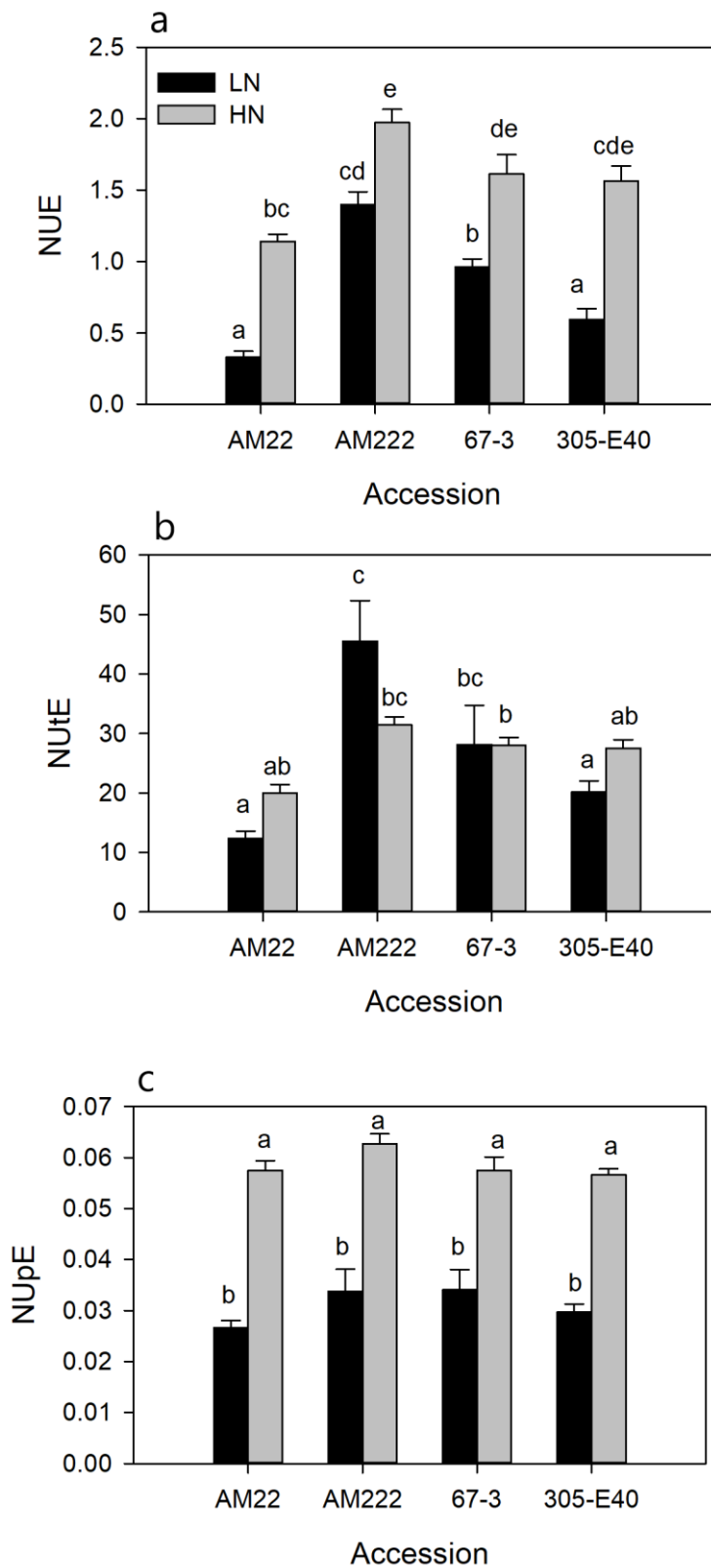


**Figure 11.** Nitrate Use efficiency (NUE), Utilization Efficiency (NUtE) and Uptake Efficiency (NUpE) of the eggplant accessions grown in microcosm system and exposed to low (LN) or high (HN) nitrate. The values are mean  $\pm$  SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at  $P < 0.05$ .

## 4.5. Discussion

Eggplant yield is strongly dependent to N fertilizer input, but to date the adaptive plant responses to N-limited fertilization are still poorly understood and disregarded to genetic mechanisms underlying a complex trait like NUE. Given the spatial and temporal soil heterogeneity for nutrients, a significant amount of genetic variation and phenotypic plasticity for NUE appeared not so unexpected (Byers, 2005; Han et al., 2015) and consequently needs to be explored. Therefore, to improve NUE in crops became pivotal to estimate the genetic variability related to this complex trait. Further, to select for high NUE two more key issues should be addressed: i) to operate at limited N supply; ii) to detect flag traits highly correlated to yield and NUE. In this paper, for the first time, the genetic variation in response to nitrate supply was assessed in eggplant (*Solanum melongena* L.). A comprehensive framework, through a morpho-physiological and molecular approach, was carried out, highlighting the differences in N-use efficiency (NUE) among genotypes.

Nineteen eggplants, with different geographical origin and morphological features, were grown under low (LN) and high nitrate (HN) levels in hydroponic system. Morphological and physiological traits were analyzed allowing us to identify NUE contrasting eggplant accessions in response to LN and HN supply. Finally, the selected genotypes were grown in greenhouse pots experiment up to fruit ripening and harvesting, analyzing NUE and some related traits to confirm hydroponic data and NUE-contrasting genotypes.

In the last decades, NUE-related traits were isolated and mapped by Quantitative Trait Loci (QTL) analysis (Xu et al., 2012). Thus, 67-3 and 305E40 accessions were included in our collection, being the parents of a RIL segregant population already available, which may be employed in future experiments for isolating molecular markers linked to QTL of NUE-interest. In addition, the line 67-3 was subjected to genome sequencing by the “Italian Eggplant Consortium”.

### 4.5.1. Characterization of eggplants in hydroponic system

Biomass allocation (SDW and RDW) among accessions showed a more marked variability in root than shoot (CV 0.44 vs. 0.37), suggesting a different adaptive  $\text{NO}_3^-$ -dependent response in root traits. These results confirmed the role of nitrate as either nutrient or signal also in eggplant root development, as previously reported in other species (Crawford, 1995; Zhang and Forde, 2000; De Pessemier et al., 2013). As the root system is devoted to nutrient exploration and acquisition from soils (Lynch, 2013; Li et al., 2015; Mu et al., 2015),

particularly in N starved conditions, morphological root traits were investigated. Contrasting responses for root traits could depend on nutrient availability in soil and source allocation between roots and shoots (Ikram et al., 2012). Thus, the selection for high “root foraging” may represent a key point or flag trait to breed eggplants for higher NUE, based on the correlation between N uptake and QTLs for root morphological traits (Coque et al., 2008). Undeniably, eggplant roots exhibited a marked plasticity (phenotypic variation), mainly in Lateral Root Length (LRL) and their Number (LRN), characterized by higher coefficient of variation (CV), compared to other traits, mainly LN. Indeed, root growth increased along with lateral root branching to improve the foraging ability of the root system at N starved condition (Ikram et al., 2012). Among physiological traits, NUtE was more influenced by genotype than NUE and NUpE. Similarly, NUtE was recently demonstrated more important than NUpE in NUE features in tomato (Abenavoli et al., 2016; Lupini et al., 2017). Therefore perhaps representing a useful trait to improve the utilization efficiency in *Solanaceae*. It is noteworthy that the ability to utilize nitrate (NUtE) showed a significant phenotypic variation due to the genetic variance, underlined a rather high heritability (Table 1). Conversely, NUE appeared poorly affected by the nutrient conditions (N level) as already reported in other species, such as *Arabidopsis* and maize (Bertin and Gallais, 2001; Coque et al., 2008; Chardon et al., 2010). Through the hierarchical clustering, the pair of N-use efficient/inefficient genotypes AM222/AM22 based on their different origin (Turkey vs. China), genetic distance (Cericola et al., 2013) and their extreme contrasting root traits, were selected. In particular, AM222 exhibited high NUE, Lateral Root Length (LRL) as well as RLR and LRN, while AM22 showed low NUE and its components (NUpE and NUtE), associated with a reduced root system, low TNA and high root mass ratio. Further, other two contrasting lines, 67-3 and 305E40, parents of an already available RIL population, with a similar behavior to AM222 and AM22, respectively, were also included. Thus, the selected four genotypes were further analyzed for differences in N-metabolism enzymes as well as gene expression.

Differential gene expression levels among the contrasting genotypes of the nitrate transporters *SmNPF6.3* and *SmNRT2.1* involved in root formation and acquisition (Garnet et al., 2009), confirmed the role of root system to confer N-use efficiency (Figure 7). Indeed, root size plays a critical role in nutrient uptake in tomato (Abenavoli et al., 2016; Lupini et al., 2017), maize (Li et al., 2015) and rapeseed (Wang et al., 2017). In eggplants, some key gene expression and enzyme activities highlighted a strong correlation with N-efficiency. In particular, the efficient genotype (AM222) showed favorable features related to NUpE (*transporter gene expressions*) and NUtE (*enzyme activities*). Indeed, the efficient genotype

AM222 showed a marked higher expression of genes belonging to the high affinity transport system, *SmNRT2.1* together with *SmNAR2.1* (also named *NRT3.1*; L  ran et al., 2014), which encodes a protein partner needed for transporting nitrate (Quesada et al., 1994; Okamoto et al., 2006; Orsel et al., 2006; Lupini et al., 2016). In *Arabidopsis*, *AtNAR2.1* and *AtNRT2.1* resulted partner in HATS activity (Orsel et al., 2006; Okamoto et al., 2006) and the specific role of *AtNAR2.1* was later demonstrated by Yong et al. (2010). Recently, the pivotal role of *NAR2* for HATS activity was confirmed by an improved yield and NUE in transgenic rice harboring the construct *OsNAR2.1:OsNRT2.1* where *OsNAR2.1* promoter was able to enhance *OsNRT2.1* expression level, compared to its native promoter (Chen et al., 2017). In eggplants, for the first time, a co-functionality of *SmNRT3.1* (= *SmNAR2.1*) together with *SmNRT2.1* was highlighted, according to gene expressions. Indeed, *SmNRT3.1* was co-expressed with *SmNRT2.1*, and both genes were more expressed in AM222 when compared to the other accessions, resulting in a consistent increasing of shoot biomass (SDW). Interestingly, the functionality of *SmNRT3.2* (another member of NRT3 gene family), which was up regulated at HN yet remaining unclear. Focusing on NUtE, our result indicated that AM222 (together with 67-3) was also characterized by higher GS enzyme activity at N-limiting condition, as well as *SmGS2* transcript level in shoots. Functional genomics and QTL approaches have already showed a correlation between GS enzyme activity and N use efficiency (Bernard et al., 2009). Moreover, the variation of GS (either *GS1* or *GS2*) expression, as well as the enzyme activity were demonstrated to affect nitrate metabolism and NUE in several plants (Eckes et al., 1989; Miao et al., 1991; Fei et al., 2003; Brauer et al., 2011). However, further genomics studies may be useful to identify allelic variation or transcription factors to better understand the primary role of GS in NUtE.

#### 4.5.2. Plasticity, genetic variation and heritability

Since the beginning of last century, Wright (1931) suggested that, within a population, individual phenotypic plasticity could be “perhaps the chief object of selection”. More recently, the increasing attention to plant plasticity responses to complex environments was paid for deeper understanding the residual quote of genetic variability (G×E) in plasticity useful for selection, as complementary to genetic heritability (e.g., Hedrick, 1986, Schlichting, 1986). Thus, the development of methods for quantifying the genetic component of phenotypic plasticity became a precious key-point. Scheiner and Lyman (1989) indicated phenotypic plasticity and its heritability calculation, considering their variation among populations and environments. Knowing the features of a specific trait, it was important to

make a prediction about population response to selection (selection gain that is direct function of heritability) or to environmental variation (plant plasticity and its genetic component).

Here we reported broad sense heritability, plasticity and its genetic component related to shoot and root dry weights, root traits as well as N-use efficiency and its components (Table 6). Interestingly, our results underlined a high G×E interaction of target traits. By contrast, Han et al. (2015) recording a limited G×E interaction, obtained a weak selection gain. Frequently, high differences between  $h^2$  estimated at different conditions (here LN and HN) indicated traits with lower heritability and higher plasticity and vice versa. These contrasting features were already discussed between plasticity and its heritability (Scheiner and Lyman, 1989), leaving out the huge amount of genetic variability that is considered here. Among traits, Root Mass Ratio (RMR), Root Length Ratio (RLR) and Specific Root Length (SRL) showed the highest values of plasticity (PL). By contrast, Root Fineness (RF) and NUtE showed  $h^2_B$  values >0.7 together with the lowest PL values. Interestingly, traits with higher plasticity showed frequently high plastic heritability, which being due to a high G×N variance underlined an interesting residual quote of genetic variability useful for selection (Table 6). Furthermore, it is noteworthy that Total Root Length (TRL), Lateral Root Length (LRL), RF and NUtE, exhibiting  $h^2_B$  values >0.6, appeared significantly correlated with NUtE and NUE (Table 6).

These results agreed with the statement that Root System Architecture (RSA) is related to plant ability to uptake N from soils and might affect NUtE (Foulkes et al., 2009). Although RSA was a target trait involved in NUtE/NUE in model plant and crops (Zhang and Forde, 1998, Garnett et al., 2009), the manipulation of RSA-related genes affecting N-uptake and NUE in crops remain a challenge (McAllister et al., 2012). As alternative, the sole up-regulation of key N-transporters encoding genes would increase NUtE, as stated by Heidlebaugh et al. (2008).

#### 4.5.3. Greenhouse experiment

The four selected eggplant accessions were grown in plastic pots in greenhouse until yield production to calculate the agronomic NUE (Figure 12). First, the optimization of nitrogen (N) fertilization rate was deeply studied to avoid overfertilization, together with the monitoring of plant N status (Tremblay et al. 2012). Several strategies based on plant sensor diagnostic were proposed to define non-destructive methods. Fluorescence-based technologies provide new N status indicators by using direct measurements (chlorophyll and flavonoids) or its derived index (NBI, Cartelat et al., 2015). NBI in eggplant genotypes at different N supply

was monitored by DUALEX, and at the same time, C and N were analyzed by destructive method. Although a perfect correlation between DUALEX indexes and leaf C and N contents (C-N analyzer) were found (ref.), not significantly differences at each N level (LN and HN) among genotypes were highlighted. However, AM222 showed the highest NBI at both N levels (Figure 10. c), indicating the best N status compared to the others.

Based on N fruit content, NUE and NUtE component, AM222 was confirmed as the most efficient genotype. Interestingly, similar performance was observed in 67-3 that can be considered as N-use efficient genotype, while AM22 and 305E40 were confirmed as N-use inefficient ones. The variations observed in fruit production among accessions may be due to a different source-sink balance, determined by differing N translocation and remobilization, which strongly affected NUtE.

Differences in NUpE were nitrate dosage-dependent, regardless genotypes. By contrast, genotypic variations were displayed for NUtE, where AM222 appeared more efficient to utilize N, compared to the others. NUtE is highly related to N remobilization ability, and leaves are active for this physiological process (Masclaux-Daubresse et al., 2010). Our results displayed a lower N leaves content in the efficient genotype AM222, thereby indicating a high N-utilization and re-mobilization efficiency, compared to the others. By contrast, a high N allocation in leaves and stem of AM22 and 305E40, respectively, which confirmed their N-use inefficiency (Figures 9 and 11). Furthermore, the inefficient genotype AM22 showed a significant higher leaf dry weight (LDW), while AM222 produced a limited vegetative biomass (leaf and stem) addressing the resources to the fruits (Figure 8). Our results confirmed that differences in the N remobilization during the life cycle among the accessions might explain NUE different performances, mainly at N-limiting condition.

Different enzymes involved in N-translocation and remobilization could determine the differences in NUtE observed in both experiments. QTL analysis in maize revealed a co-segregation between GS genes and physiological traits related to productivity (Hirel et al., 2001), and more recently a linkage between NUE and GS/GOGAT was reported in wheat (Quraishi et al., 2011). AM222 exhibited higher GS activity and genes related expression, which might be responsible for high NUtE, confirming the results obtained in hydroponic system. Thus, NUtE, fruit dry weight and GS could be considered the best flag traits to identify rapidly high NUE eggplant genotypes, as observed in AM222.

In conclusion, NUE-contrasting genotypes were selected in growth chamber/hydroponic experiment considering simple key traits, confirmed in greenhouse pots experiment by crop yield evaluation. AM222 and AM22 showed NUE-contrasting performances, resulting N-use

efficient and inefficient, respectively. Noteworthy, in both experiments accessions 67-3 and 305E40 showed a significant contrasting values for NUE and many NUE-related traits, such as SDW, TRL, LRL, LRN and NUtE in hydroponic, confirmed in greenhouse. Based on NUE-contrasting performances, RILs might be phenotyped and genotyped to detect molecular markers (such as SSR and SNP) linked to major genes/QTL related to RSA and NUE. Interestingly, a high heritability ( $h^2_B$ ) and G×E interaction of target traits, such as LRL, RF and NUtE, were observed that may avoid a weak selection gain. Moreover, greenhouse pots experiments demonstrated that the remobilization of N stored from leaves to the fruits could be the key to enhancing NUE in eggplant. Then, molecular and physiological mechanisms indicated GS as a key enzyme influencing NUE, mainly its NUtE component. Further omics studies (transcriptomic and metabolomics) focused on C-N metabolism networks may pave the way towards the selection a of a more resilient eggplant with regard to N-requirement.



**Figure 12.** Four selected eggplant accessions were grown in plastic pots in the greenhouse until yield production to calculate the agronomic NUE.

## CHAPTER 5. MOLECULAR CLONING AND FUNCTIONAL ASSAY FOR GENES INVOLVED IN NITRATE TRANSPORT AND PLANT N-UPTAKE

### 5.1. Introduction

Root plasticity is a complex trait influenced by a multitude of genes, the amount of nitrate available in the soil and their interaction. The dynamic nature of the root system allows the plant to respond in a dependent way to the nutrients with alteration of the Root System Architecture (RSA) through the elongation of the primary root and the development of secondary Lateral Roots (LR) in the direction of the most abundant  $\text{NO}_3^-$  source (Giehl et al., 2014; Khan et al. 2016; Koevoets et al., 2016; Wu et al., 2016).

On the other hand, the role of many genes involved in sensing and nitrate transport, is closely related to development of RSA were extensively studied in model plants and crops (Vidal et al., 2010; O'Brien et al., 2016).

Two nitrate uptake systems were identified: Low and High Affinity Transport System (LATS and HATS, respectively) belonging to two distinct protein families, called NITRATE TRANSPORTER1 (NRT1)/ PEPTIDE TRANSPORTER (PTR) (except NRT1.1), which is a dual-affinity nitrate transporter (Liu et al., 1999) and NITRATE TRANSPORTER2. According to the recent nomenclature, NRT1 is now called NPF and NRT1.1 transporter was recalled NPF6.3 (Léran et al., 2014). Fifty-three NPF genes were identified in *Arabidopsis* but less than one-fifth were characterized as nitrate transporters (Tsay et al., 1993; Huang et al., 1999; Chiu et al., 2004; Almagro et al., 2008; Lin et al., 2008; Fan et al., 2009; Li et al., 2010; Wang and Tsay, 2011; Hsu and Tsay, 2013, Wang et al., 2018). NPF transporters are located in the plasma membrane and most were characterized as LATS nitrate transporters, while seven members of NRT2 family were classified as HATS nitrate transporters. Many NRT2 transporters are also located in the plasma membrane (except AtNRT2.7, instead located in the tonoplast as reported by Chopin et al., 2007). NRT2.1 is the leading NRT2 family transporter that participates in the nitrate uptake for high affinity when  $\text{NO}_3^-$  concentration in the soil is  $< 0.5 \text{ mM}$  (Remans et al., 2006).

However, in the last two decades limited information on functional genomics approaches to characterize nitrate transporters in *Solanaceae* were available and only recently the function and dual role of NRT2.3 in the  $\text{NO}_3^-$  acquisition and its long-distance transport was clarified (Fu et al. 2015).

In addition, the nitrogen use efficiency (NUE) is the result of two specific components: the utilization efficiency (NUtE) and the uptake efficiency (NUpE). The latter is strongly determined by the high affinity nitrate transporters, such as NRT2 and NRT3 families. Indeed, as nitrate is the major N source in the crop nutrition, enhancing nitrate uptake is one strategy for improving NUE (Schroeder et al., 2013). Thus, in this study, we focused on some members of the high affinity carriers (HATS) from eggplant for functional genomics. In particular, we cloned the putative *SmNRT2.1* transporter gene sequence and the two potential accessory proteins for HATS activity namely *SmNRT3.1* and *SmNRT3.2*. Further, a Protein-Protein Interaction assay (PPI) between *SmNRT2.1* and alternative members of NAR2/NRT3.1 family was carried out in order to investigate the ability of NRT2.1 to form complexes with NRT3 members. The constructs for overexpressing NRT2.1, NRT3.1, NRT3.2 genes were made, as well as the gene sequences for silencing NRT2.1 and CLCa genes that in the next future could provide new information on the uptake mechanisms in eggplant. Finally, the detection of allelic variants for the Chloride channel protein CLCa among different eggplant genotypes were assessed.

## 5.2. Materials and Methods

### 5.2.1. Plant Material and DNA constructs

The cDNA of the eggplant genotype 67-3 was used as template in all experiments. The plants were grown in hydroponic system as described in previous chapters.

To amplify the sequences of N-related genes, Phusion® DNA Polymerase (Invitrogen) was used by means of the thermalcycler Applied Biosystems 2720.

Component reaction mix and temperatures cycle are showed in Tables 8 and 9.

| Component        | Volume µl |
|------------------|-----------|
| Buffer [5x]      | 4         |
| dNTPs [10 mM]    | 0.4       |
| Primer F [10 µM] | 1         |
| Primer R [10 µM] | 1         |
| Taq Pol [2 U/µL] | 0.2       |
| DNA [500 ng]     | 2         |
| H <sub>2</sub> O | 11.4      |

**Table 8.** Component reaction mix to amplify the sequences of N-related genes.

| Step process     | Temperature (°C) | Time | Cycles |
|------------------|------------------|------|--------|
| Initialization   | 98               | 30 s |        |
| Denaturation     | 98               | 10 s |        |
| Annealing        | 63-66*           | 30 s | 30     |
| Elongation       | 72               | 15 s |        |
| Final elongation | 72               | 10 m |        |
| Final hold       | 4                | ∞    |        |

**Table 9.** PCR cycle, PCR steps of denaturation, annealing, and extension are repeated (or “cycled”) many times to amplify the target DNA. \*According to oligo melting temperatures (see table 11 primers list).

### 5.2.2. Elution of gel bands and DNA quantification

The amplified PCR fragments were separated by electrophoresis on 1% agarose gel, and excised products were purified by using the Wizard SV Gel kit and PCR Clean-up System (<http://www.promega.com/>). Finally, DNA quantification was determined as concentration in solution: - on agarose gel: load 1 µl of resuspended DNA (1 µl of DNA + 9 µl of H<sub>2</sub>O + 2 µl of 6x loading buffer) in a 1% agarose gel (1g of agarose per 100 ml of TAE buffer). Run one marker to one side (1Kb ladder, Fermentas) and at least three known concentrations of DNA for comparison to the standards, by sight or with an appropriate software (Molecular Analyst®, Bio-Rad), it was possible to determine the samples concentration.

### 5.2.3. Gateway system

Gateway® technology is a cloning method allowing us to transfer DNA fragments quickly and efficiently between different vectors, based on a site-specific recombination system. All the steps were carried out following the manual of pENTR™ Directional TOPO® Cloning Kits (Invitrogen). The first step involves the amplification of the gene sequence of interest by PCR (Table 10). Thereafter, amplified fragments were inserted into the pENTR™ / SD / D-TOPO® vector (Invitrogen) (Fig. 13).

| Reagents                  | µl |
|---------------------------|----|
| PCR product (20 ng / µl)  | 1  |
| Salt solution             | 1  |
| Sterile water             | 3  |
| TOPO® vector (20 ng / µl) | 1  |
| Final volume              | 6  |

**Table 10.** TOPO Cloning reaction.

The reaction mix should be gently shaken and incubated for 5 minutes at room temperature and finally transferred on ice. Thus, the competent cells are ready to be transformed.

#### 5.2.4. Competent cells transformation

In this step the construct (vector + fragment) is inserted into the competent cells One Shot® TOP10 (Invitrogen), following the protocol attached to One Shot® Chemical Transformation kit (Invitrogen).

The competent One Shot® TOP10 cells are inoculated on selective LB agar medium with 50 µg / mL Kanamycin (working concentration) placed in an incubator at 37 °C for 24 hours.

The colonies were taken from the plates and inoculated on liquid LB medium with the addition of kanamycin (50 µg /mL, working concentration), incubated at 37 °C for 24 hours and shaken at 170 rpm.

#### 5.2.5. Plasmid DNA extraction and digestion

This methodology allows us to extract DNA plasmid of interest from the bacterial cells. DNA extraction is carried out by Wizard® Plus SV Minipreps DNA Purification kit System (Promega).

To select the plasmid (pENTR) in which the fragment has to be correctly inserted, two different enzyme digestions were performed: and the restriction reactions were analyzed by electrophoresis to identify the clones in which the fragments were correctly inserted, based on their sizes.

The digestion mix was as follows: 1.5 µl DNA, 1 µl buffer [10x], Enzyme (1.U / µl) 0.5 µl, H<sub>2</sub>O 7 µl.

The total volume was 10 µl. The samples are then incubated 2h at 37 ° C. After digestion, the samples were resolved in electrophoresis 1% agarose gel and its image acquired with the GelDoc 1000 camera instrument (Bio-Rad) digitized with the Molecular Analyst® software (Bio-Rad).

Finally, a further control of the positive colonies was carried out by sequencing inserted cloned into the pENTR / SD / D-TOPO® vectors. Primers attL1 and attL2 were used and the samples were sent for sequencing in Institute “GATC - A Eurofins Genomics Company” (Ebersberg, Germany).

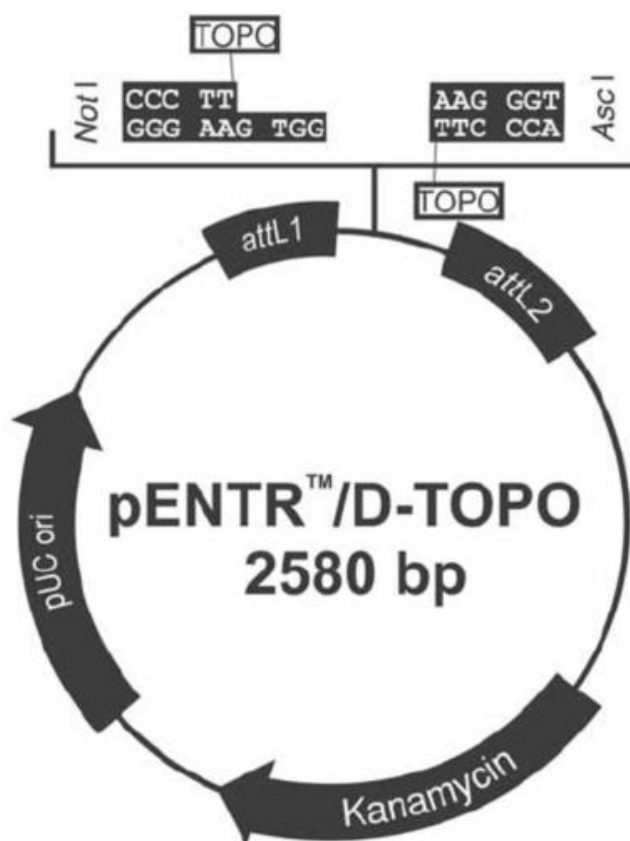


**Figure 13.** pENTR™/SD/D-TOPO® (Invitrogen)

#### 5.2.6. Expression plasmid for functional assays.

Genes target recombined between pENTR™ / SD / D-TOPO® and the cloning vectors (pK7GWIWG2 (II), pK2WG7, pYFC43 and pYFN43) are showed in Table 11.

The pENTR in which the sequence under analysis was inserted and recombined with pK7GWIWG2 (II), pK2WG7 (<https://gateway.psb.ugent.be/search>) (Fig. 14 and 15), binary vectors based on the Gateway® system that are able to replicate in *Agrobacterium tumefaciens*, and with the pYFC43 and pYFN43 vectors Gateway™-based BiFC binary vectors that efficiently allowed us the combination of candidate protein pairs with each possible N-or C-terminal to investigate the formation of protein complexes and the localization of protein-protein interactions in plant.

The recombination reaction was performed using the Gateway® LR kit CLONASE™ Enzyme Mix (Invitrogen™) is showed in Table 12.

The reaction was incubated in a thermocycler at 25 °C over night. Cells were transformed with the recombination reaction competent TOP10 for all constructs, following the protocol previously described.

| Gene application | PRIMER FORWARD<br>5'→3'         | PRIMER REVERSE<br>5'→3'           | GATEWAY<br>DESTINATION<br>VECTORS |
|------------------|---------------------------------|-----------------------------------|-----------------------------------|
| NRT2i            | caccCAAGTAATAGCCAGGGGAAGTG      | TGATTGCTGAGTACTTCTTT<br>GATAGG    | pK7GWIWG2 (II)                    |
| CLCai            | caccGAGAGTCGACGGAGAGCAACTC      | AGGCCGACTAGAAATGCCA<br>GTGT       | pK7GWIWG2 (II)                    |
| p35S:NRT2.1      | caccATGGCTGATGTGGAAGGAGAACC     | TCACTTTCCTGCCTTGGCCT<br>TTCTCTTCT | pK2WG7                            |
| p35S:NRT3.1      | caccATGGCAACCTTTTTCGTTGCCTCAGCA | TCATTTTCGCCCCGCAATTT<br>TGGCT     | pK2WG7                            |
| p35S:NRT3.2      | caccATGGCGATTAATACTCGTGCTAT     | TCACTTTCCTGCCTTGGCCT<br>TTCTC     | pK2WG7                            |
| YFPnt:NRT2.1     | caccATGGCTGATGTGGAAGGAGAACC     | TCACTTTCCTGCCTTGGCCT<br>TTCTCTTCT | pYFN43                            |
| YFPct:NRT3.1     | caccATGGCAACCTTTTTCGTTGCCTCAGCA | TCATTTTCGCCCCGCAATTT<br>TGGCT     | pYFC43                            |
| YFPct:NRT3.2     | caccATGGCGATTAATACTCGTGCTAT     | TCACTTTCCTGCCTTGGCCT<br>TTCTC     | pYFC43                            |

**Table 11.** Names and numbers of accession of the amplified genes to be inserted in the vectors and sequences of the primers used for amplification.

| Component                      | μl   |
|--------------------------------|------|
| pENTR + sequence (160 ng / μl) | 1 μl |
| target vector (160 ng / μl)    | 1 μl |
| H <sub>2</sub> O               | 4 μl |
| Reaction buffer                | 2 μl |
| LR Clonase <sup>TM</sup>       | 2 μl |

**Table 12.** LR recombination reaction between the entry clone and a Gateway® destination vector.

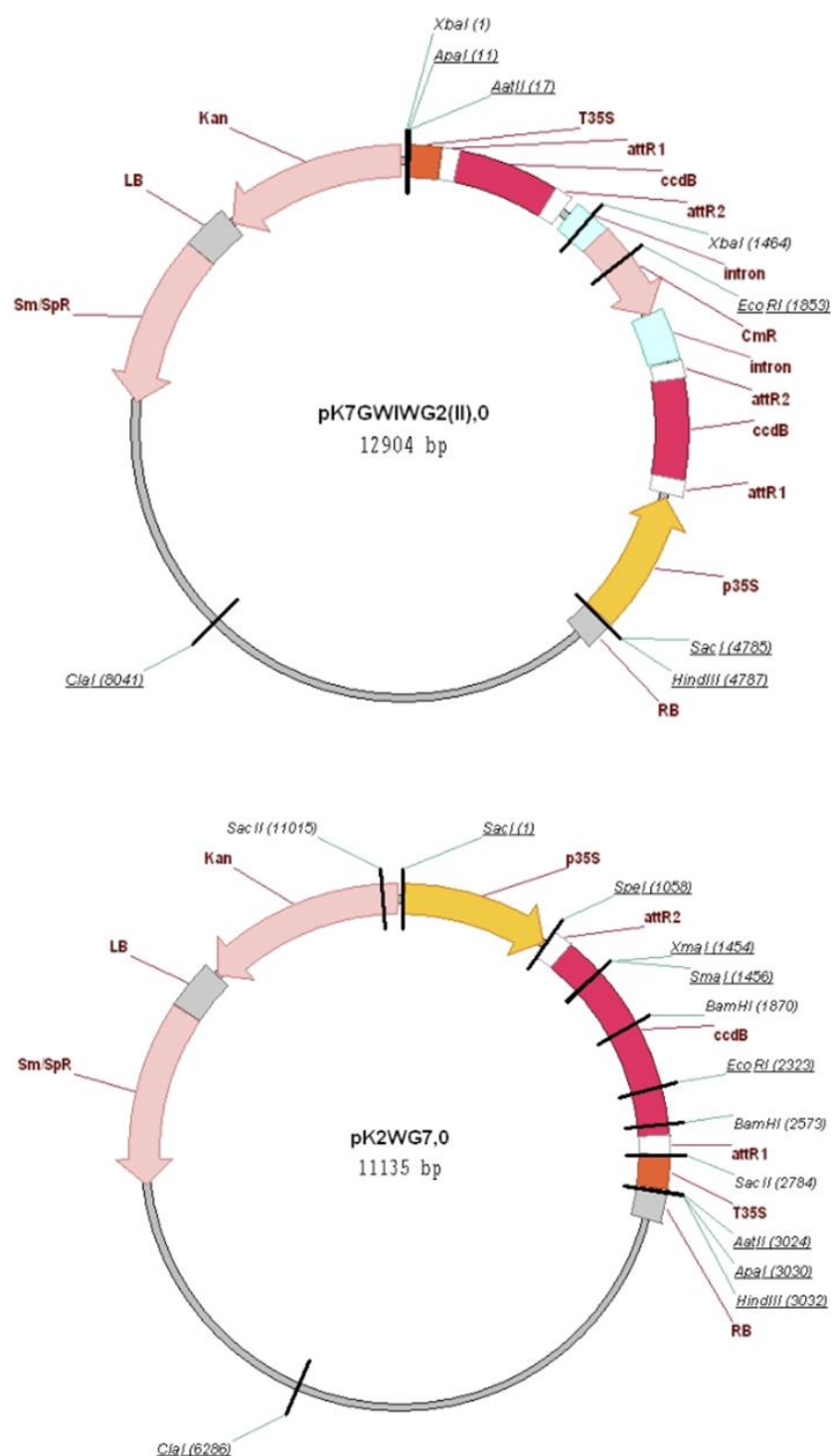


**Figure 14.** Gateway® binary vector pK7DGWIWG2D (II) and pK2WG7.0 used with *E.coli* and *Agrobacterium tumefaciens* ([www.psb.ugent.be](http://www.psb.ugent.be)).

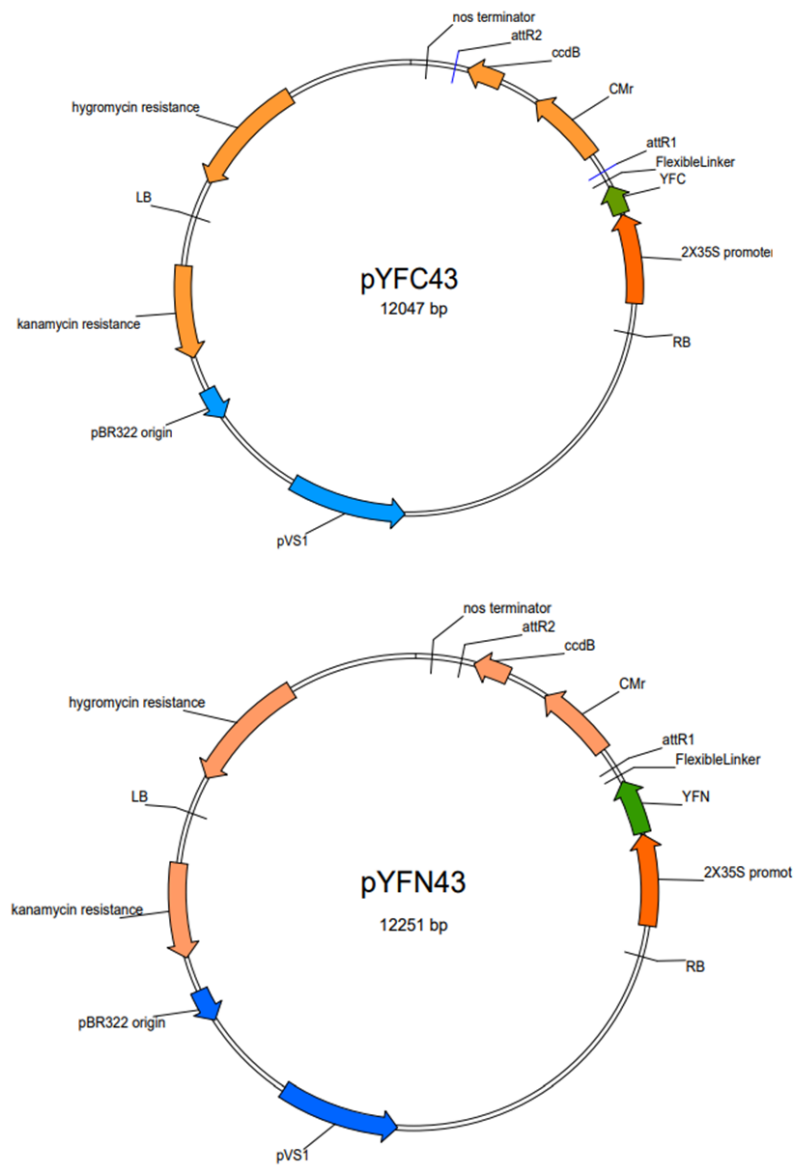


**Figure 15.** Gateway<sup>TM</sup>-based BiFC binary vectors pYFC43 and pYFN43 (<http://www.ibmcp.upv.es>), provided by dr. Simona Masiero (Università degli Studi di Milano).

### 5.2.7. *Agrobacterium tumefaciens* mediated transformation

After DNA miniprep, Agro-competent cells (heat-shock) were transformed at 42° for 30 s. One hundred and fifty microliters  $\mu$ l LB liquid medium was added before the incubation for 4 hours at 28° C. The putative transformed competent cells were spread in LB agar medium with selective antibiotics addition and finally incubated for 48 hours at 28° C.

*Agrobacterium* strains harbor resistance to rifampicin, while the vectors pK7GWIWG2 (II) and pK2WG7 to streptomycin and spectinomycin, respectively. Antibiotics were added under the hood after autoclaving LB agar medium and cooled to the following concentrations:

Streptomycin 50  $\mu$ g/mL;

Spectinomycin 50  $\mu$ g/mL;

Rifamycin 50  $\mu$ g /mL.

DNA plasmid extraction from *Agrobacterium* was performed as previously described, but the extraction yield is generally low. To verify the presence of recombinant plasmid the miniprep from *Agrobacterium* was used to re-transform the *E. coli* competent cells (as described above) because guarantee a higher yield useful for enzymatic digestion assays on the plasmid DNA extracted from *E.coli* (as shown in the Table 13 and Figure 16).

| GENE<br>TARGET | ENZYME  | CUTTING<br>SITE | GATEWAY<br>DESTINATION<br>VECTORS | APPLICATION                             |
|----------------|---------|-----------------|-----------------------------------|-----------------------------------------|
| NRT2           | KpnI    | G_GTAC^C        | pK7GWIWG2<br>(II)                 | Silencing;<br>Hairpin RNA<br>expression |
|                | SacI    | G_AGCT^C        |                                   |                                         |
| CLCa           | KpnI    | G_GTAC^C        | pK7GWIWG2<br>(II)                 | Silencing;<br>Hairpin RNA<br>expression |
|                | SacI    | G_AGCT^C        |                                   |                                         |
| NRT2.1         | HindIII | A^AGCT_T        | pK2WG7                            | Overexpression;                         |
|                | XbaI    | T^CTAG_A        |                                   |                                         |
| NRT3.1         | HindIII | A^AGCT_T        | pK2WG7                            | Overexpression;                         |
|                | XbaI    | T^CTAG_A        |                                   |                                         |
| NRT3.2         | HindIII | A^AGCT_T        | pK2WG7                            | Overexpression;                         |
|                | XbaI    | T^CTAG_A        |                                   |                                         |

**Table 13.** Enzymes used in this work, recognized cutting sites and application and type of destination vector.

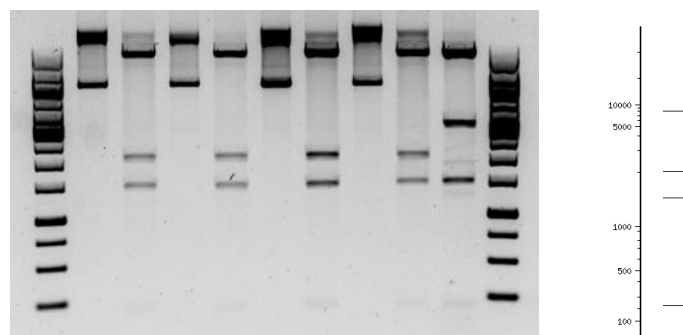


**Figure 16.** Double digestion with XbaI-HindIII restriction enzymes on the construct pK2WG7: NRT3.1.

#### 5.2.8. Plasmid DNA isolation (MaxiPREP)

*E. coli* cells containing the plasmid of interest previously transformed were inoculated in 100 ml LB medium and selective antibiotics as kanamycin for target genes and ampicillin for pAVA120 (used as positive control) before incubation over night at 37 °C. The bacterial cells were centrifuged at 4000 rpm for 5 minutes at 4° C. The pellet was then resuspended in 1.5 ml PLASMID PREP 1 and placed on ice for 30 minutes. After 2 ml PLASMID PREP2 addition, the solution was gently mixed and left on ice for 5 minutes. Two ml PLASMID PREP 3 was still added, the tubes inverted and put into ice for 30 minutes, followed they were centrifuged at maximum speed for 10 minutes and the supernatant transferred to new tube where 5 ul RNase were added before leaving at 37° C for 30 minutes (reagents composition are showed in Table 14). One volume of phenol (saturated with water or Tris) and 1 volume of chloroform:isoamyl alcohol 24:1 were added, vortexed and centrifuged for 3 minutes at maximum speed.

The aqueous phase was taken and transferred into new tube where 2 volumes of absolute ethanol was added before placing at -80° C for 30 minutes for DNA precipitation.

After 10 minutes of centrifugation at maximum speed, the supernatant was discarded and the pellet washed with 70% (v/v) ethanol, centrifuged for 5 minutes at maximum speed and again the supernatant discharged. The dry pellet was resuspended in 200 µL of TE, 1 volume 1.6 M NaCl containing 13% (w/v) PEG added, vortexed and placed in ice for 5 minutes before 10 minutes centrifugation at maximum speed. Once the supernatant was discarded, the pellet was washed with 70% ethanol and centrifuged for 5 minutes at maximum speed. The supernatant was discarded and the dried pellet resuspended in 100 ul of TE. The quantity and ratios 260/280, 260/230 were determined with the Nanodrop and finally DNA plasmid stored at -20° C.

| REAGENT                | COMPOSITION           |
|------------------------|-----------------------|
| PLASMID PREP 1         | 50 mM glucose         |
|                        | 10 mM EDTA            |
|                        | 25 mM Tris-HCL pH8    |
| PLASMID PREP 2         | 0,2 N NaOH            |
|                        | 1% SDS                |
| PLASMID PREP 3         | 2 M Na-acetate pH 4.8 |
| Ethanol absolute       |                       |
| 70% Ethanol            |                       |
| 1.6 M NaCl 13% w/v PEG |                       |

**Table 14.** Maxiprep reagents composition.

#### 5.2.9. Isolation of protoplasts from *Arabidopsis*

*Arabidopsis* seedlings were grown at 24° C under a photoperiod of 12 hours of light and dark, and 65% humidity. Four-week-old leaves of good health and cutted from the middle part into thin strips not exceeding 1 mm were selected, cutted and immediately transferred into Petri dishes containing enzyme solution (completely submerged).

Leaves were left in the dark under vacuum for 30 minutes, cell wall digestion was performed at room temperature in the dark for 3 hours, and thereafter the green color of Enzyme Solution indicated protoplasts release. Enzyme/protoplast solution was diluted with 1 volume W5 solution to remove undigested leaf tissue.

Nylon mesh 75 µm (previously maintained in 96% ethanol was washed first in water to remove excess of ethanol and washed with W5 solution) was used to filter the enzyme / protoplast solution and isolated the protoplasts from the digested plant material.

The solution was centrifuged at 100g for 2 minutes, the supernatant was removed and protoplasts resuspended gently in W5 solution.

The presence of protoplasts in solution was verified in the Bürker chamber by means of an optical microscope while the rest of the protoplasts was kept on ice for no more than 30 minutes. W5 solution was removed and protoplasts were resuspended in MMG solution and kept at room temperature. The protocol for protoplast preparation was performed according to Yoo et al. (2007) with some modifications.

#### 5.2.10. DNA-PEG-calcium transfection

The transformation reaction was set up in 2 ml tubes, with 20 µg of plasmid DNA (Maxiprep) in 100 µl of protoplasts and 110 µl of PEG solution and gently mixed. The transfection mixture was incubated at room temperature for 15 minutes and then diluted with 440 µl of W5 solution, gently mixed and centrifuged at 100 g for 2 minutes and the supernatant was removed. Protoplasts were resuspended with a 1 ml of WI solution and spreaded into Petri plates.

#### 5.2.11. Detection of protein interactions in plant using Bimolecular Fluorescence Complementation (BiFC) system.

The detection of protein-protein interaction was performed at the University of Pavia (Centro Grandi Strumenti, CGS) of the by means of a Leica TCS SP5 Fluorescence Microscopy Microscope allowing to exploit the specific spectral characteristics of the fluorescent proteins and to allow the fluorescence emission analysis.

### 5.3. Results

#### 5.3.1. Gene expression profile of putative high affinity nitrate transporter

As previously described in chapter 1, the panel of genes analyzed included *NRT2.1*, *NRT3.1* and *NRT3.2* that belong to the high affinity transport system involved in N-transport.

In the root of eggplants grown in hydroponic system under the conditions previously described, we assessed the expression level of two putative members of NRT3 gene family.

In Figure 17 the transcript levels of *SmNRT3.1* and *SmNRT3.2* normalized to the housekeeping *APRT* and *GAPDH* (reference Table 5) are shown. The *SmNRT3.1* gene showed a higher level of transcripts than *SmNRT3.2* at low N-condition (LN), while at high N (HN) the expression levels appeared inverted. As expected, we also found that the gene expression trend of *SmNRT2.1* and *SmNRT3.1* in eggplant was very similar, in agreement with the genes co-expression and proteins coupled described previously in the functional characterization of HATS in other plants (e.g. Okamoto et al., 2006; Orsel et al., 2006; Pii et al., 2016). *NRT2.1* and its partner protein *NRT3.1* (=NAR2.1) firstly described in *Arabidopsis*, but till now never described in the *Solanaceae*.

Interestingly, the 67-3 genotype showed at LN a significant reduced *SmNRT3.1* expression level compared to all others, by contrast in the same genotype the *SmNRT3.2* transcripts tended to be higher at LN compared to HN. As reported by Okamoto et al., 2006, “it is possible that *AtNRT3.2*, homolog of *AtNRT3.1*, might counteracted the loss of *AtNRT3.1* function in the *Atnrt3.1* mutant”. However, *AtNRT3.2* expression levels in root and shoot of *Atnrt3.1* mutant at LN, although somewhat higher than in wild-type" a similar hypothesis needs to be deepened in eggplant where 67-3 genotype could be a natural mutant impaired for *SmNRT3.1*.

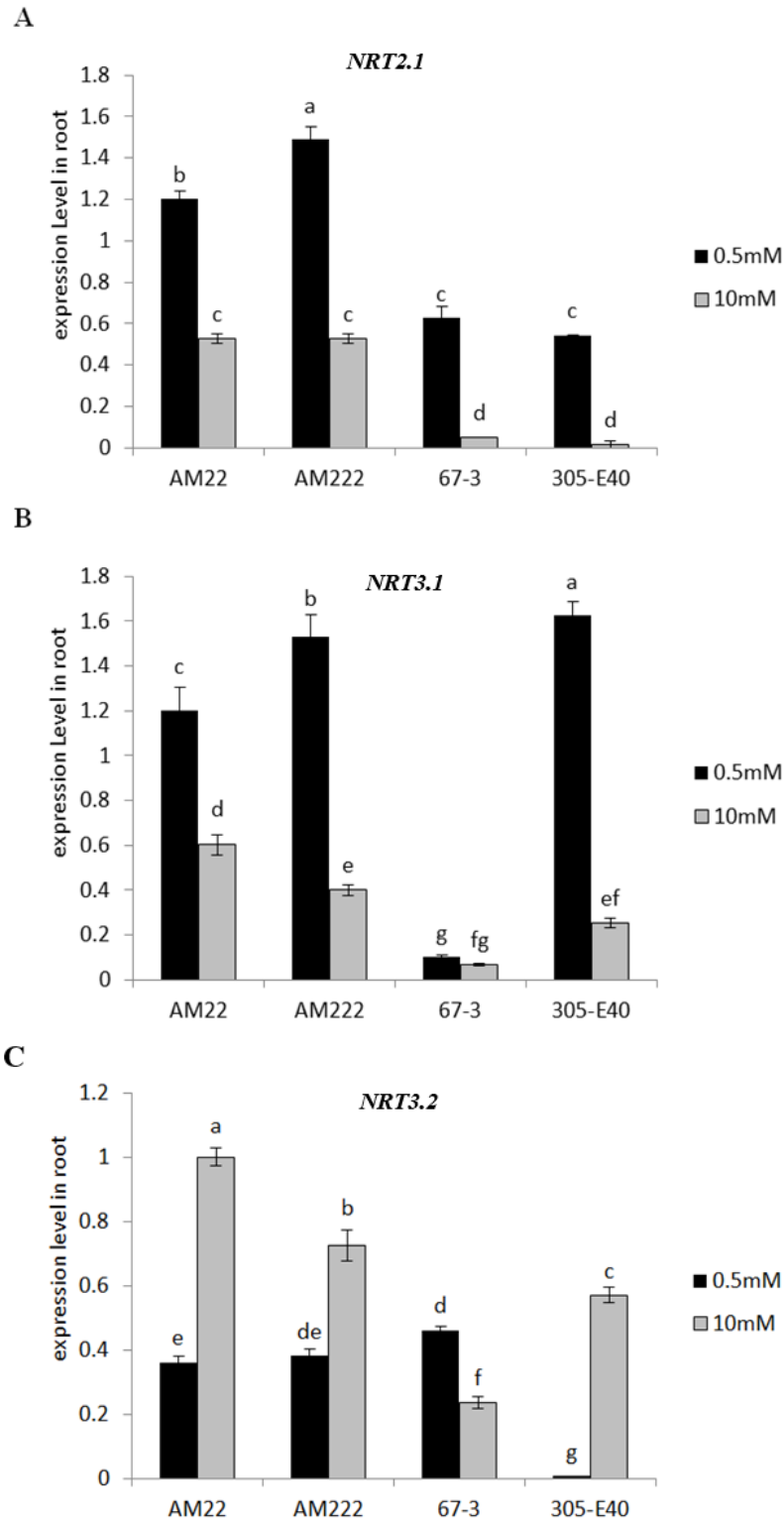


**Figure 17.** Expression analysis of A) *SmNRT2.1*, B) *SmNRT3.1* and C) *SmNRT3.2* genes in response to nitrate by relative quantitative real-time RT-PCR. The letters indicate the significant difference between genotypes\*treatment (Tukey,  $P < 0.05$ ). Each histogram represents the mean of 3 biological replicates  $\pm$  standard deviation.

### 5.3.2. Bimolecular fluorescence complementation (BiFC) analysis: *SmNRT2.1/ SmNRT3.1* and *SmNRT2.1/SmNRT3.2* proteins complexes interaction

The potential formation of proteins complexes NRT2.1/NRT3.1 and NRT2.1/NRT3.2 was studied in *Arabidopsis* leaf mesophyll protoplasts by *in vivo* transient expression by means of Bimolecular fluorescence complementation (BiFC) analysis, a high-throughput screening technologies widely used to detect protein-protein interactions (PPI) in living cells.

This technique is based on the reconstitution of an *in vivo* YFP fluorescent protein. The interaction of the complex was evaluated through protoplasts transformed with YFN:NRT2.1 and YFC:NRT3.1 or YFN:NRT2.1 and YFC:NRT3.2 (Figure 18).

The protoplasts transfected by YFN:NRT2.1/YFC:NRT3.1 and YFN:NRT2.1/YFC:NRT3.2 showed a similar fluorescence patterns.

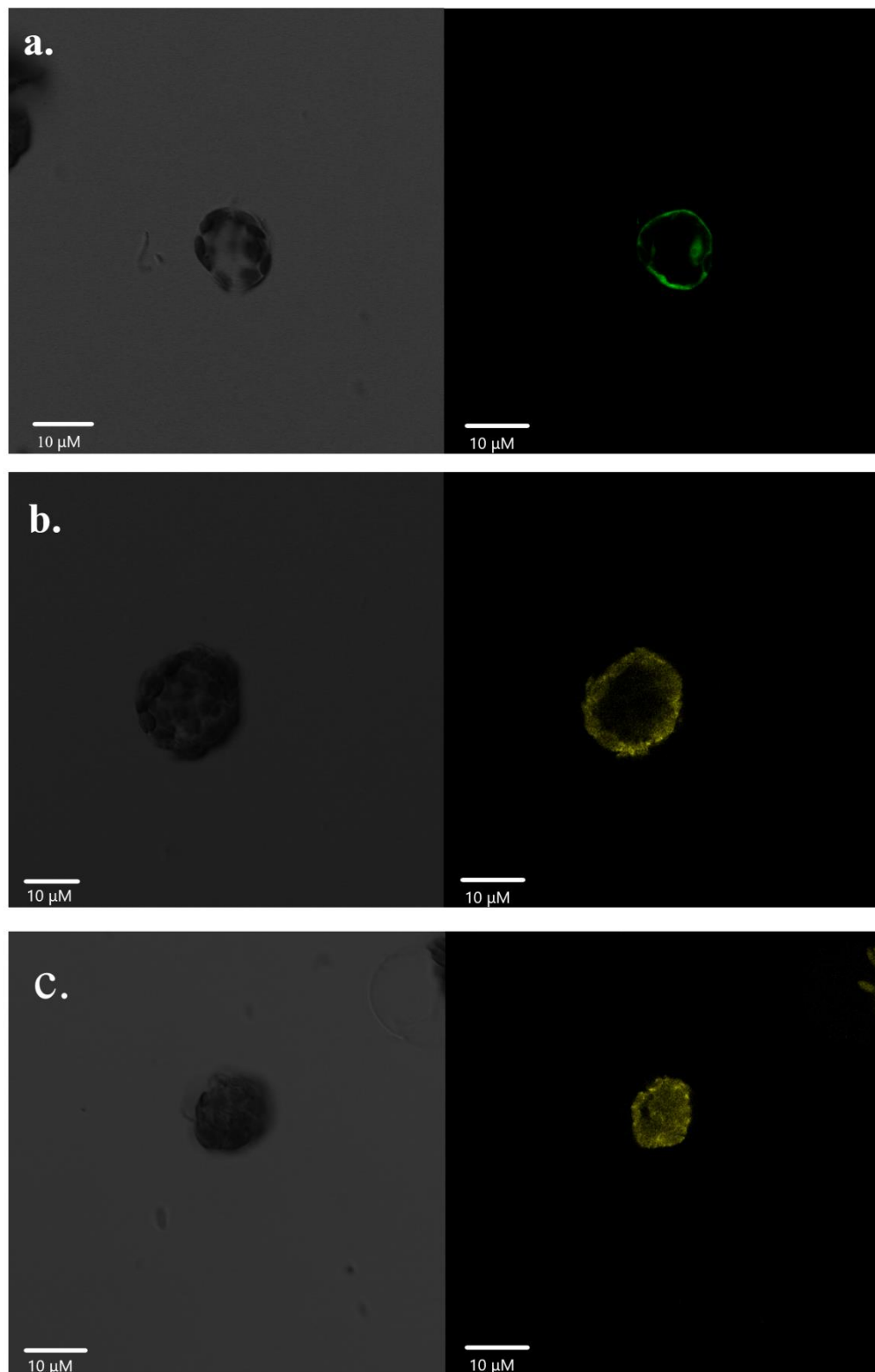


**Figure 18.** Transitional expression of split YFP constructs into protoplasts of *Arabidopsis* leaves. Protoplasts were transfected with (a) vector pAVA p35S-GFP provided by dr Laura Bassolino (CREA GB, Montanaso Lombardo, LO), (b) YFN:NRT2.1/YFC:NRT3.1, (c) YFN:NRT2.1/YFC:NRT3.2. Three protoplasts were sampled for each assay.

## 5.4. Discussion

The aim of this study was the assessment in NUE-contrasting eggplants of gene expression trend of the main N-transporter for high affinity *SmNRT2.1* and NRT3 family genes (*SmNRT3.1* and *SmNRT3.2*) encoding for its protein partner. Up- and down-regulation of these genes were detected at low and high N (LN and HN), for a first functional analysis in eggplant root. A specific assay was also set up to demonstrate the proteins complementation between *SmNRT2.1* and both members of NRT3 family *SmNRT3.1*, *SmNRT3.1* and *SmNRT3.2* transcript levels showed different pattern of expression induced by low and high N among genotypes. As expected by their HATS involvement, at LN *SmNRT2.1* showed a higher transcript level compared to HN in all genotypes. The same trend was observed for *SmNRT3.1*, except in 67-3 genotype, where the expression level resulted rather undetectable at both N levels. Interestingly, the opposite expression pattern was detected for *SmNRT3.2* which appeared higher expressed at HN, again except 67-3 genotype that showed a significant higher gene expression at LN. Thus, 67-3 genotype seemed impaired in *SmNRT3.1* (a putative mutant) compared to other genotypes and, as previously reported in other species, another gene of the same family can compensate the loss of function of the homologous gene (Wykoff and O'Shea, 2001, Okamoto et al., 2006, Kaiser et al., 2002). In 67-3 eggplant genotype, this case study appears possible between NRT3 homologous genes, making necessary and of interest a specific test of the functional role of both NRT3 genes when interact with *SmNRT2.1* N-transporter.

Thus, a functional assay as Bimolecular Fluorescence Complementation (BiFC) was set up to visualize the protein-protein interaction in living cells (*Arabidopsis* protoplasts) showed similar pattern of YFP fluorescence for both *SmNRT2.1*-*SmNRT3.1* and *SmNRT2.1*-*SmNRT3.2* pair protein complexes. Interestingly, the fluorescence complexes appeared located in different areas of the cell and these aspects should be deepen understood. Here, we demonstrated for the first time in eggplant the protein-protein interaction *SmNRT2.1*-*SmNRT3.1* as firstly reported in *Arabidopsis* (Okamoto et al., 2006).

The protein-protein interaction *SmNRT2.1*-*SmNRT3.2* has never been described before and opened a new scenario in a putative structure role, indeed NRT3.2 was previously described as not regulated by nitrate in *Arabidopsis* and Maize (Okamoto et al., 2006; Garnett et al., 2015).

Moreover, a previous RNA-seq eggplant experiment showed SmNRT3.2 20-fold more expressed in *Verticillium*-infected root compared to the uninfected control (Toppino et al., personal communication), and opened a putative response to biotic stress. Further questions could be interestingly addressed about crosstalk between abiotic/biotic stress responses in the eggplant, as already reported in tomato (AbuQamar et al. 2009). Other functional genomics studies were carried out for the gene cassettes development for *Agrobacterium*-mediated genetic transformation to study the gene in function in planta via over-expression or silencing of NRT2 and NRT3 genes. Indeed, the analysis of so obtained mutant phenotypes, will provide us an alternative and powerful tool for identifying morphological changes in root architecture and NUE. These mutants could help us to understand the flag genes more important for improving NUE.

Stable genetic transformation in eggplant via *A. tumefaciens* are underway at CREA-GB in Montanaso Lombardo (LO).

## CHAPTER 6. PUTATIVE CHLORIDE CHANNEL PROTEIN (CLCa) ALLELIC VARIANT AND SECONDARY TOPOLOGICAL SILICO ANALYSIS

### 6.1. Introduction

One or more alternative forms of a single gene locus are called “alleles”. All the alleles of a certain gene determine the same inherited trait, but each allele harbour a unique nucleotide sequence, which can produce different phenotypes. The domestication of plants not only modified the economic and agronomic phenotypes, but also leaves a genetic signature that influenced both the structure of the plant population and the genetic diversity of the domesticated species. During the domestication the number of alleles that are fixed within a crop is greatly reduced compared to the variation that is left behind in the genepool. Therefore, there may be many unadapted useful allelic variants that could be used to improve our home crop types. The importance of single nucleotide allelic variant were recently demonstrated with the advent of CRISPR/Cas9 gene editing technology. Scientific research is moving towards the goal of replacing an existing allele in commercial cultivars with an elite allele to improve agronomic performance. In rice, gene editing allowed to modify *NRT1.1B* from subsp. *japonica* form to the allele of subsp. *indica*, in which a single nucleotide polymorphism is responsible for increased efficiency of N-use (Li et al., 2018).

Moreover, NUE is strongly controlled by nitrate short-distance fluxes between vacuole and cytosol (Han et al., 2015a). Indeed, they (2015b) demonstrated that a higher nitrate concentration in cytoplasm compared to vacuole increased NUE in crops. However, although the energy required for vacuolar nitrate transport is provided by tonoplast proton pumps (Krebs et al., 2010), the channel CLCa is responsible for this transport (De Angeli et al., 2006). To pairs NUE-contrasting genotypes were selected in growth chamber/hydroponic experiment considering simple key traits, confirmed in greenhouse pots experiment by crop yield evaluation (Mauceri et al., *Journal of Integrative Plant Biology* submitted).

Here, we identified in eggplant a natural genetic variation in the chloride channel protein *CLCa* gene (carrier protein involved in the storage of nitrate in the vacuole) in position of the CDS 245A in 245G which determines a missense mutation for the change of the TAT/tyrosine codon in TGT/cysteine. Furthermore, the possible consequence of this variation in the protein was evaluated, highlighting interesting information about which further analysis could be done in the next future. In particular, attention should be paid to

characterize and clarify a possible functional role of the SNP identified on the function and performance of the transporter and its relationships to the improvement of NUE in eggplant.

## 6.2. Material and Methods

### 6.2.1. DNA extraction

Genomic DNA was isolate from AM22, AM222, 67-3, 305E40 eggplant genotypes. DNA was extracted from fresh leaves (10 days old) by using DNeasy Plant Mini Kit ([www.qiagen.com](http://www.qiagen.com)).

### 6.2.2. High-resolution melting curve PCR analysis

The HRM experiments for allelic discrimination were performed by the specific amplification of a 271 bp fragment with specific primers for the CLCa gene. Acquisition and analysis of the HRM curve were performed using Corbett Rotorgene 6000 for HRM analysis. EvaGreen® master mix (Cat. no. 172-5200; Bio-Rad) was used for genotyping of the locus, a double-stranded DNA-green fluorescent nucleic acid dye, which intercalated specifically with the double-stranded DNA for detecting DNA polymorphisms.

*SmCLCa\_F* TGTGTGTCGTCTCTGATTAGGA

*SmCLCa\_R* GGAGGATTTGCTCGGGGTAA

The fluorescence variation is plotted against the temperature, generating a typical amplitude fusion curve when the dsDNA dissociates into individual filaments, there is no longer any double-stranded DNA present and therefore the fluorescence is reduced.

The PCR cycling parameters are reported in Table 15.

|                              |                                             |
|------------------------------|---------------------------------------------|
| Initial denaturation cycle   | 94 ° C for 5 minutes                        |
| 40 cycles                    | 94 ° C for 10 s                             |
|                              | 56 ° C for 15s                              |
|                              | 72 ° C for 20 s                             |
| Preparation phase of the HRM | 72 ° C for 2 minutes                        |
|                              | 50 ° C for 1 minute                         |
| Melting phase increments of  | 0.07, 0.08, 0.09, 0.1, 0.15 and 0.2 ° C / s |

---

from 77 ° C at 95 ° C.

---

**Table 15.** PCR cycling parameters for the acquisition of the PCR and HRM curve in real time.

To check the quality of the data, PCR amplification graphs were analyzed by evaluating Ct values and fluorescence levels at the end-point.

The fusion temperature of the amplicon to which the two strands of DNA are separated depends on the sequence of the DNA bases. When two different samples were compared, they should give exactly the same thing melt-shaped curve in the absence of polymorphisms, by contrast, if one of the sequences under analysis harbour a mutation in the amplified DNA region, this will change the temperature at which the DNA strands merge and therefore the two melt curves appear different. The small differences even of a nucleotide can be highlighted because the HRM machine can monitor this process in high resolution then identifying whether the mutation is present or not.

### 6.2.3. Sequencing

PCR was performed using previous reported primer to amplify the fragment of 271bp that include SNP with Phusion® High-Fidelity DNA Polymerase in a final volume of 20 µl. PCR cycling parameters were:

|                            |                       |
|----------------------------|-----------------------|
| Initial denaturation cycle | 94 ° C for 5 minutes  |
| 40 cycles                  | 94 ° C for 10 s       |
|                            | 65 ° C for 15s        |
|                            | 72 ° C for 20 s       |
| Elongation                 | 72 ° C for 10 minutes |

---

PCR purified fragments were then sequenced to verify the identity of the amplified sequences and confirm the presence of polymorphism, the amplicons were sent to the Institute “GATC - A Eurofins Genomics Company” (Ebersberg Germany).

### 6.2.4. In silico analysis

Alignment of the sequences was generated as MUSCLE algorithm (Edgar, 2004) with the online tool <http://www.phylogeny.fr>. The relationships among eggplant genotypes were evaluated using the NETWORK 4.6.1.1 program (fluxus-engineering.com), which analyzed

nucleotide sequences, “collapses” into a single identical cluster (or nodes) and constructs a parsimonious network among genotypes (Bandelt et al, 1999). The nodes were interconnected by lines representing the mutational steps (number of SNPs) that exist between genotypes pair; the nodes within the network allowed us to understand the relationships among genotypes better than a phylogenetic tree, in which the genotype is found only at the end of each branch.

The evaluation of the biochemical properties of amino acids and the relative frequencies of the amino acids and their chances of substitution are based on the BLOSUM (BLOcks SUBstitution Matrix) matrix and the analysis were performed by [https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa\\_explorer.cgi](https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi).

The model to predict transmembrane helices from protein sequences was the Hidden Markov Model (HMM) of TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) as it has a specialized modeling of various regions of a membrane protein: helix capsule, helix center, regions close to the membrane and globular domains. The HMM is very suitable for the prediction of transmembrane helices because it can incorporate hydrophobicity, charge polarization, helix lengths and grammatical constraints.

## 6.3. Results

### 6.3.1. HRM analysis on gDNA

The HRM analysis performed on AM22, AM222, 67-3, 305E40 eggplant genotypes showed different fusion profiles only in 67-3, while AM22, AM222 and 305E40 showed a single melt profile (Figure 19).

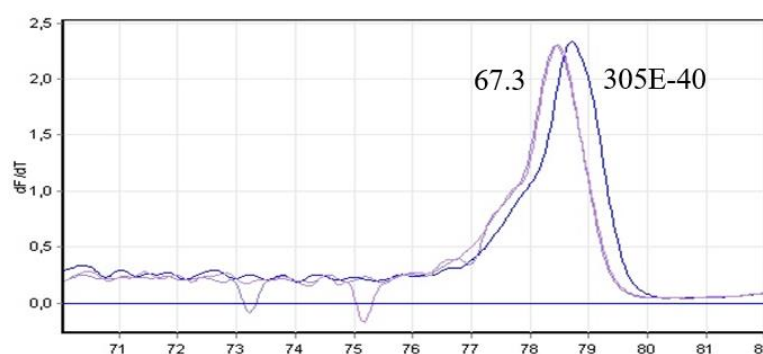


**Figure 19.** High Resolution Melt (HRM) analysis highlighted variations in coding sequences of CLCa gene. The SNP polymorphism was showed between 67-3 and 305E40 genotypes.

### 6.3.2. In silico analysis

Eleven putative *CLCa* alleles (DNA fragments) were sequenced, including the four selected genotypes and different eggplant-related wild species. The sequences multiple alignment showed a polymorphism in 245 position, where among our selected eggplants, 67-3 differs from the AM22, AM222 and 305E40 for an A instead of a G (Figure 20).

### 6.3.3. Network analysis

A network of different allele variants of the exon 2 fragment region (*CLCa*) was built through the Network software, underlining the relationships among eggplants and the related species, the matching sequences were clustered as single node (Figure 21). Each node represented the cluster of genotypes sharing the same sequence whose size is proportional to the number of samples, the node *mv1* was designed as a hypothetical allele sequence from an ancestral non-sampled genotype. The connection lines between the nodes represent the number of mutations (mutational steps). The largest group sharing the same sequence for *CLCa* included 305E40, Talina, DR2, AM222 and AM22 (all *S. melongena*), the second ones, separated by a SNP, included 67-3 and Dourga (*S. melongena*). A greater number of mutational steps separated *S. aethiopicum* and MSA genotype, *S. indicum*, *S. integrifolium*.

CLUSTAL multiple sequence alignment by MUSCLE (3.8)

```

22                -----NNNNNNNNANATCTCTTC-AGCATGATTGGAG-AA
222              -----NNNNNNNNANATCTCTTC-AGCATGATTGGAG-AA
DURGA-32076600   -----NNNNNNNNANNTCTCTTC-AGCATGATTGGAG-AA
32076294-INDICUM -----NNNNNNNNANNTCTCTTC-AGCATGATTGGAG-AA
32076183-305E40  -----NNNNNNNNANATCTCTTC-AGCATGATTGGAG-AA
32077677-INTEGRIFOLIUM -----NNNNNNANNTCTCTTC-AGCATGATTGGAG-AG
67/3-seq         -----NNNNNNANNTCTCTTC-AGCATGATTGGAG-AG
32076312-AETHIOPICUM -----TTAGGGTAGATCTCTTC-AGCATGATTGGAG-AA
67/3-consorsio   TGTGTGTCGTCTCTGATTAGGATCAACGAGAATGATCTCTTCAAGCATGGAGAAA
MSA              -----TTAGGGTAGATCTCTTC-AGCATGATTGGAG-AA
TALINA           -----NNNNNNNNANATCTCTTC-AGCATGATTGGAG-AA
DR2              -----NNNNNNNNANATCTCTTC-AGCATGATTGGAG-AA
                *****

22                AAGATCAAGAGTTCAAACATTACAATGTGTTTTCATCAAATGGACACTGGCATTCTAGT
222              AAGATCAAGAGTTCAAACATTACAATGTGTTTTCATCAAATGGACACTGGCATTCTAGT
DURGA-32076600   ANGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACACTGGCATTCTAGT
32076294-INDICUM AAGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACGCTGGCATTCTGGT
32076183-305E40  AAGATCAAGAGTTCAAACATTACAATGTGTTTTCATCAAATGGACACTGGCATTCTAGT
32077677-INTEGRIFOLIUM AAGATCAAGAGTTCAAACATTACCATATGTTTTCATCAAATGGACACTGGCATTCTGGT
67/3-seq         AAGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACACTGGCATTCTAGT
32076312-AETHIOPICUM AAGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACACTGGCATTCTGGT
67/3-consorsio   AAGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACACTGGCATTCTAGT
MSA              AAGATCAAGAGTTCAAACATTACAATATGTTTTCATCAAATGGACACTGGCATTCTGGT
TALINA           AAGATCAAGAGTTCAAACATTACAATGTGTTTTCATCAAATGGACACTGGCATTCTAGT
DR2              AAGATCAAGAGTTCAAACATTACAATGTGTTTTCATCAAATGGACACTGGCATTCTAGT
                * *****

22                CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAATATCTCTGG
222              CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAATATCTCTGG
DURGA-32076600   CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAATATCTCTGG

```

```

32076294-INDICUM      CGGCCTTGTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
32076183-305E40      CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
32077677-INTEGRIFOLIUM CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTTTCAATCGAAAAATATCTCTGG
67/3-seq              CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
32076312-AETHIOPICUM CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTTTCAATCGAAAAATATCTCTGG
67/3-consorsio       CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
MSA                   CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTTTCAATCGAAAAATATCTCTGG
TALINA                CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
DR2                   CGGCCTTCTTACCGGAGGTGTGGCCACCCTCATCAGTCTCTCAATCGAAAAATATCTCTGG
*****

22                    TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
222                   TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
DURGA-32076600       TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
32076294-INDICUM     TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
32076183-305E40     TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
32077677-INTEGRIFOLIUM TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
67/3-seq              TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
32076312-AETHIOPICUM TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
67/3-consorsio       TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
MSA                   TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
TALINA                TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
DR2                   TTACAAACTTCGGGCTACTGTGAACATATATTGATGACAAAAGGTTAATCACATTTAATCT
*****

22                    TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
222                   TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
DURGA-32076600       TTGANNTGTTTATGCNNCCGAGAAAATCATAAACAAATAAAGAT
32076294-INDICUM     TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAACTT-----
32076183-305E40     TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
32077677-INTEGRIFOLIUM TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
67/3-seq              TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
32076312-AETHIOPICUM TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAA-----
67/3-consorsio       TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCC-----
MSA                   TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAA-----
TALINA                TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
DR2                   TTGTTTTGTTTTTAC-CCCGAGCAAATCCTCCAAA-----
***      * * *      * * *      * * *

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**Figure 20.** Multiple sequence alignment of the CLCa sequences of 12 different genotypes. Polymorphism in position 245 is highlighted

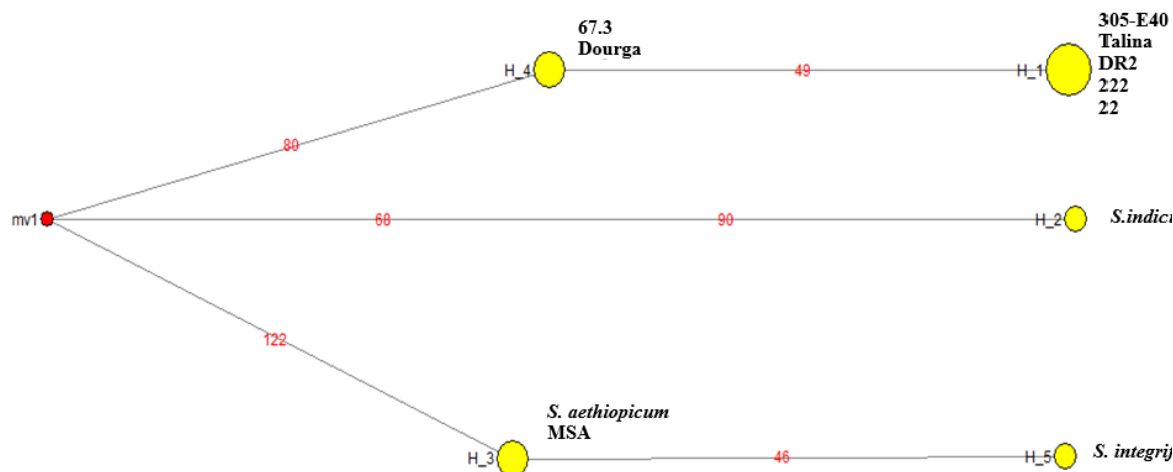


**Figure 21.** Relationships among eggplants genotype selected and the related species, the matching sequences were clustered as single node.

The prediction of the CLCa protein primary structure highlighted a missense mutation generated by the detected SNP, the codon TAT should translate for a tyrosine (82 Tyr, Y),

while the alternative codon TGT encodes for a cysteine (82 Cys, C). Through the tools Aminoacid explorer ([https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa\\_explorer.cgi](https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi)), it was possible to evaluate the biochemical properties of Tyr and Cys and the possible common substitutions (Table 16).

The relative frequencies of the amino acids and their chances of substitution are based on the BLOSUM (BLOcks SUBstitution Matrix) matrix. In bioinformatics, BLOSUM matrices are used to mark alignments between protein sequences, based on local alignments.

The BLOCKS database was scanned for many conserved regions of protein families (which do not have gaps in sequence alignment) and then count the relative frequencies of amino acids and their chances of substitution. The amino acids ordered according to their replacement scores in BLOSUM62 for tyrosine show a range of residues with positive (green) scores, those amino acids that frequently replace tyrosine in homologous proteins, while those with negative (red) scores as cysteine rarely replace (Table 16).

| Feature                      | Tyrosine                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Cysteine                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Synopsis                     | Tyrosine is both an aromatic and a hydroxyl amino acid, being equivalent to phenylalanine with the para position of the phenyl group substituted with a hydroxyl. This phenolic hydroxyl endows tyrosine with unique chemical properties, and allows it to serve as both a catalyst and a substrate to the vast array of kinases specific for this residue.                                                                                                              | Cysteine is unique in that it contains a sulfhydryl group, allowing it to form disulfide bonds with other cysteines or with any other molecules with free sulfhydryls. Cysteine is otherwise identical to serine, which has a hydroxyl in place of the cysteine sulfhydryl. Cysteine is also very uncommon in proteins, with only tryptophan being more rarely found.                                                                                                           |
| Side chain flexibility       | Moderate                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Low                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Interaction modes            | H-bonds, aromatic stacking, van der Waals                                                                                                                                                                                                                                                                                                                                                                                                                                | Covalent disulfide bonds, van der Waals                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| Potential side chain H-bonds | 3                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | 0                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| Residue molecular weight     | 163                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 103                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Isoelectric point            | 5.7                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 5.0                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Hydrophobicity               | 0.714                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 0.721                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Standard codon(s)            | UAY                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | UGY                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Properties                   | <div>  Polar         </div> <div>  Hydroxyl         </div> <div>  Aromatic         </div> <div>  Nonessential         </div> | <div>  Polar         </div> <div>  Sulfur         </div> <div>  Aliphatic         </div> <div>  Nonessential         </div> |

**Table 16.** Biochemical properties of Tyrosine and Cysteine. Output source from Aminoacid explorer ([https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa\\_explorer.cgi](https://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi))

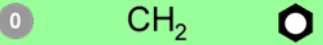
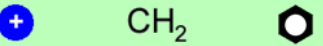
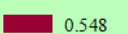
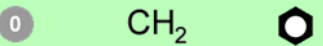
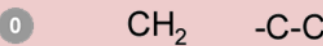
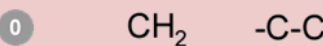
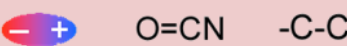
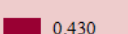
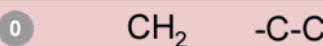
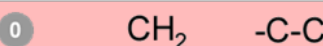
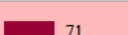
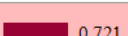
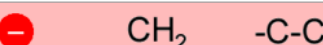
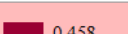
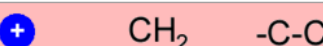
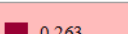
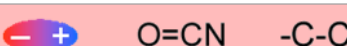
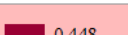
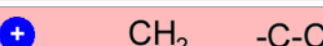
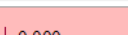
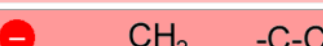
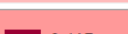
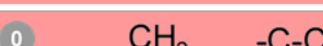
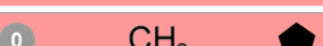
| Substitutions in BLOSUM62 for Tyrosine |                      |                                                                                     |                                                                                          |                                                                                             |
|----------------------------------------|----------------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| <u>1-letter code</u>                   | <u>3-letter code</u> | <u>Chemistry</u>                                                                    | <u>Molecular Weight</u>                                                                  | <u>Hydrophobicity</u>                                                                       |
| <u>Y</u>                               | Tyr                  |    |  163   |  0.714   |
| <u>F</u>                               | Phe                  |    |  147   |  0.951   |
| <u>H</u>                               | His                  |    |  137   |  0.548   |
| <u>W</u>                               | Trp                  |    |  186   |  0.854   |
| <u>I</u>                               | Ile                  |    |  113   |  1.000   |
| <u>L</u>                               | Leu                  |    |  113   |  0.918   |
| <u>M</u>                               | Met                  |    |  131   |  0.811   |
| <u>Q</u>                               | Gln                  |    |  128   |  0.430   |
| <u>V</u>                               | Val                  |   |  99    |  0.923   |
| <u>A</u>                               | Ala                  |  |  71  |  0.806 |
| <u>C</u>                               | Cys                  |  |  103 |  0.721 |
| <u>E</u>                               | Glu                  |  |  129 |  0.458 |
| <u>K</u>                               | Lys                  |  |  128 |  0.263 |
| <u>N</u>                               | Asn                  |  |  114 |  0.448 |
| <u>R</u>                               | Arg                  |  |  156 |  0.000 |
| <u>S</u>                               | Ser                  |  |  87  |  0.601 |
| <u>T</u>                               | Thr                  |  |  101 |  0.634 |
| <u>D</u>                               | Asp                  |  |  115 |  0.417 |
| <u>G</u>                               | Gly                  |  |  57  |  0.770 |
| <u>P</u>                               | Pro                  |  |  97  |  0.678 |



**Table 17.** The amino acids are reported according to their substitution scores in BLOSUM62 for tyrosine. Residues with positive scores (green) substitute frequently for tyrosine in homologous proteins, while those with negative scores (red) as cytosine substitute rarely. Sorted by BLOSUM62 score.

#### 6.3.4. Prediction of transmembrane helices in proteins

Because of CLCa protein is a multi-pass membrane vacuolar protein the TMHMM (transmembrane hidden Markov model algorithm) membrane topology prediction method was used, based on a hidden Markov model that correctly predicts 97-98% of transmembrane helices. Furthermore, TMHMM can discriminate between soluble and membrane proteins with 99% specificity and sensitivity.

The two CLCa protein variants were processed and the existence of 9 potential transmembrane sequences, 5 inside-loops and 5 outside loops was suggested for the protein harboured by 67-3. In this genotype, 82 Tyr appeared the first amino acid of a first transmembrane helix (Figure 22).

The architecture prediction of the transmembrane topology in the variant protein from 305E40 showed 10 TM domains and an alternative reorganization of the intra and extra-side loops between the first two transmembrane helices (Figure 23). In this case, the 82 Cys is not included in the first transmembrane helix but is located on the loop outside. In both genotypes, the transmembrane segments varying in length between 19 and 22 amino acids.

```
>CLCa_67-3
MEEPNLFATINDMEGQNNNEEERESTESNSSLLKRNRTLSSTSLAIVGTKVPHIESLDYEINENDLFKHDWRKR
SRVQTLQYVFIKWTLAFLVGLLTGGVATLISLSIENISGYKLRATVNYIDDKRYLMGFAFFGGANFLLTLVAAF
LTVYFAPTAAGPGVPEIKAYLNGVDTPNMFATTFLVKIIGSIGAVSAGLDLGKEEPLIHMGTCIASLLGQGGG
DSYKLSWHWLRYFNNDKRDRLITYGSSSGVCAAFGAPVAGVLFALAEVATWWRGALIWRFFSTAVVVVLRV
FIEYCKSGSCGLFGRGGLIMFDVSDVIVRYHVVDIIPVVTIGVIGGLLSLYNHVLRKVLRLYNLINEKGKLYK
LLLALSVSFLTISICIYGLPFLAKCKPCDSSLSESCPGTGETGNLQFNCPSGYNDLATLLLTKEETIRNIFS
QNTATEFQVASLLIFFLLYCILGVITFGIAPVSGLFIPILMGSGYGHLLGIAMKPYTKIDQRLYAVLGAASLM
AGSMRMTVTVCVIFLELTNNLLLLPITMLVLLIAKSVADCFNPSIYEIMLELKGLPFLEAHPEAWMRNITVGEL
ADVKKPPVVTLKGIKVRRIEVLKYTTNNGFPVVDGMIPELHGLVLRTHLLLVLKKKWFNLNRSRTEDWEVEEK
FTWIDLAEKWGRIEDIAITKDEMEMYVDLHHLINTTPHTVVESMSVAKAMVAFRQVGLRHMIIIVPKYQAAGVLP
VVGILTRQDLRACNILDVFPFLAKSKSSKKRH
```

```
# translated Length: 772
# translated Number of predicted TMHs: 9
# translated Exp number of AAs in TMHs: 227.29348
# translated Exp number, first 60 AAs: 0.00632
# translated Total prob of N-in: 0.20468
translated TMHMM2.0 inside 1 81
translated TMHMM2.0 TMhelix 82 104
translated TMHMM2.0 outside 105 130
translated TMHMM2.0 TMhelix 131 153
translated TMHMM2.0 inside 154 245
translated TMHMM2.0 TMhelix 246 268
translated TMHMM2.0 outside 269 277
translated TMHMM2.0 TMhelix 278 297
translated TMHMM2.0 inside 298 325
translated TMHMM2.0 TMhelix 326 348
translated TMHMM2.0 outside 349 367
```

|            |          |         |     |     |
|------------|----------|---------|-----|-----|
| translated | TMHMM2.0 | TMhelix | 368 | 390 |
| translated | TMHMM2.0 | inside  | 391 | 452 |
| translated | TMHMM2.0 | TMhelix | 453 | 475 |
| translated | TMHMM2.0 | outside | 476 | 478 |
| translated | TMHMM2.0 | TMhelix | 479 | 498 |
| translated | TMHMM2.0 | inside  | 499 | 527 |
| translated | TMHMM2.0 | TMhelix | 528 | 550 |
| translated | TMHMM2.0 | outside | 551 | 772 |

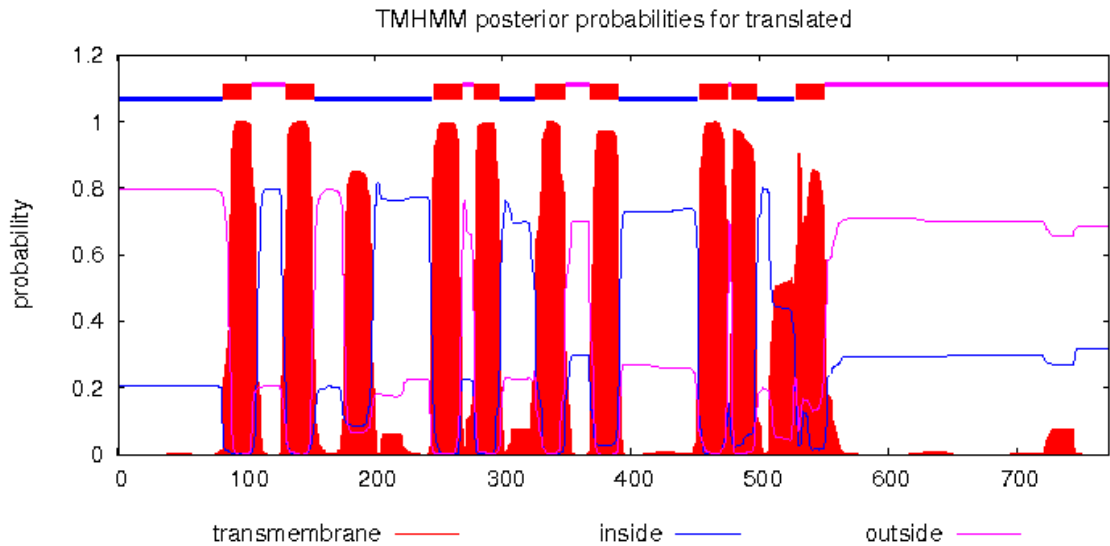


**Figure 22.** 67-3 CLCa prediction of transmembrane helices in proteins.

```
>CLCa_305E40
MEEPNI1FATINDMEGQNNNEEERESTESNSSLLKRNRTLSSTSLAIVGTKVPHIESLDYEINENDLFKHDWRKR
SRVQTLQ2CVFIKWTLAFLVGLLTGGVATLISLSIENISGYKLRATVNYIDDKRYLMGFAFFGGANFLLTLVAAF
LTVYFAPTAAGPGVPEIKAYLNGVDTPNMFGATTLFVKIIGSIGAVSAGLDLGKEEPLIHMGTCIASLLGQGS
DSYKLSWHWLRYFNNDNRDKRDLITYGSSSGVCAAFGAPVAGVLFAL3EEVATWWRGALIWR4TTFFSTAVVVVLRV
FIEYCKSGSCGLFGRGGLIMFDVSDVIVRYHVVDIIPVVTIGVIGLLGSLYNHVL5RKVLRLYNLINEKGKLYK
LLLALS6VS7LFTSICIYGLPFLAKCKPCDSS8LESCPGTGETGNL9KQFNCPSGYNDLATLLLT10TKETIRNIFS
QNTATEFQVASLLIFFLLYCILGVITFGIAVPSGLFIP11IILMGSGYGHLLGIAMKPYTKIDQRLYAVLGAASLM
AGSMRMTVTVCVIFLELTNNLLLLPITMLVLLIAKSVADCFNPSIYEIMLEL12KGLPFLEAHPEAWMRNITVGEL
ADV13KPPVVT14LK15GIEKVR16RIVEVLKYTT17HN18GFVVDGMIPELHGLVLRTHLLLV19LKKK20WFLNESRRTEDWEVEEK
FTWIDLAEKWGRIEDIAITKDEMEMYVDLHHLINTTPHTVVESMSVAKAMVAFRQVGLRHMIIVPKYQAAGVLP
VVGILTRQDLRACNILDVFP21HLAKSKSSKKRH
```

```
# translated Length: 772
# translated Number of predicted TMHs: 10
# translated Exp number of AAs in TMHs: 227.49256
# translated Exp number, first 60 AAs: 0.00517
# translated Total prob of N-in: 0.16542
translated TMHMM2.0 outside 1 88
translated TMHMM2.0 TMhelix 89 111
translated TMHMM2.0 inside 112 131
translated TMHMM2.0 TMhelix 132 154
translated TMHMM2.0 outside 155 175
translated TMHMM2.0 TMhelix 176 198
translated TMHMM2.0 inside 199 245
translated TMHMM2.0 TMhelix 246 268
translated TMHMM2.0 outside 269 277
translated TMHMM2.0 TMhelix 278 297
translated TMHMM2.0 inside 298 325
```

|            |          |         |     |     |
|------------|----------|---------|-----|-----|
| translated | TMHMM2.0 | TMhelix | 326 | 348 |
| translated | TMHMM2.0 | outside | 349 | 367 |
| translated | TMHMM2.0 | TMhelix | 368 | 390 |
| translated | TMHMM2.0 | inside  | 391 | 452 |
| translated | TMHMM2.0 | TMhelix | 453 | 475 |
| translated | TMHMM2.0 | outside | 476 | 478 |
| translated | TMHMM2.0 | TMhelix | 479 | 498 |
| translated | TMHMM2.0 | inside  | 499 | 527 |
| translated | TMHMM2.0 | TMhelix | 528 | 550 |
| translated | TMHMM2.0 | outside | 551 | 772 |

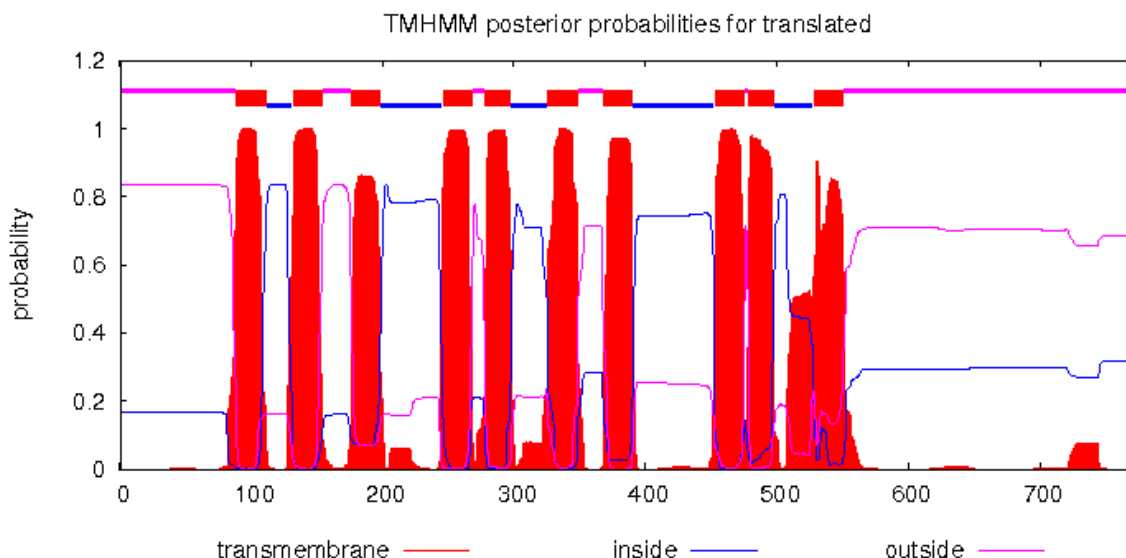


**Figure 23.** 305E40 CLCa prediction of transmembrane helices in proteins.

## 6.4. Discussion

The elucidation of the protein membrane secondary structure could determine a deep understanding of its role and function by defining protein regions that are intra or extracellular, attributing to these differences a putative change in protein function.

We used a computer-generated model as a basis for determining the secondary structure of the CLCa vacuolar nitrate transporter. TMHMM Server v. 2.0 generated a model in which both N- and C-terminal ends of CLCa protein were inside and outside alternatively in 67-3, while in 305E40 genotype both CLCa N- and C-terminal ends appeared outside. Differing numbers of TM domains and orientations are deduced for CLCa by computational analysis and structural analysis of the predicted protein sequence. Here, we showed two eggplants characterized by a variation in a single amino acid in the CLCa deduced protein sequence allowing us to predict a topology of 9 and 10 TM for 67-3 and 305E40 genotypes, respectively.

Taken together this descriptive information, we could resolve that CLCa protein variants due to a substitution of a tyrosine with a cytosine may have generated some structural and functional changes. To validate these conclusions *in vivo* determination of regions orientation

of the hypothetical loop should be necessary. In perspective, the hypothetical changes in protein functionality could involve the performance of vacuolar N-storage and N-metabolism, demonstrating the importance of allelic variations in NUE-related genes in eggplant.

## CHAPTER 7 THE METABOLOME PROFILING AND PATHWAY ANALYSIS REVEAL CHANGES ON THE PRIMARY METABOLISM OF EGGPLANTS (*Solanum melongena* L.) CHARACTERIZED BY CONTRASTING NITROGEN USE EFFICIENCY

### 7.1. Introduction

Nitrogen availability is one of the most limiting factors affecting plant growth and productivity, worldwide (Vitousek et al., 1997). For this reason, over the last four decades, the use of N fertilizers dramatically increased, allowing to satisfy agricultural production and consequently food supply (McArthur and McCord, 2017). On the other hand, this extensive use caused a negative impact on environmental, economic and human costs (Smolders et al 2010; Pingali et al., 2012; Behera et al., 2013; Rodriguez-Galiano et al., 2018).

Rationalizing the N fertilizers use together with the selection of genotypes with high Nitrogen Use Efficiency (NUE) may represent a possible strategy, especially for crops requiring high N input, either to preserve environment and human health or to maximize yield (Hendrickson, 1992; Pasda et al., 2001; Andraski and Bundy, 2005; Hirel et al., 2007; Correia et al., 2015).

NUE, defined as “the grain yield per unit of N available in the soil” (Moll et al., 1982), is a complex trait under physiological, biochemical and genetic control, therefore the challenge is to understand the mechanisms underlying plant NUE (Good et al., 2004; Xu et al. 2012).

Over the last years, an increasing number of omics studies, such as metabolomics, transcriptomics and proteomics, on model and crop plants, has been carried out to identify metabolites and/or gene transcripts and/or proteins involved in NUE control (Kusano et al., 2011). In particular, metabolic profiling is now becoming an important tool in understanding the changes in metabolites, composition and/or concentration, in response to environmental stress, biotic and abiotic, including N deficiency (Allwood et al., 2015; Ganie et al., 2015; Kurczyk et al., 2016; Mibei et al., 2018).

This research area has been more explored on cereals, such as rice, wheat and maize (Hirel et al, 2007), but should also be relevant in *Solanaceae*, since cultivation practices are highly dependent on N fertilizers (Oliveira et al., 1981; Pawar et al., 1997; Aujla et al., 2007; Zaho et al., 2015). In particular, limited information are available on eggplant (*Solanum melongena* L.), the third most important vegetables, after tomato and potato, and cultivated all over the world (Hazra et al., 2003).

In this study, a metabolomic analysis was conducted on four eggplant genotypes with contrasting NUE, to provide different profiling in response to low N supply at short- and

long-term exposure. GC-MS technique gives possibilities of analyzing different classes of compounds, including organic and amino acids, sugars (Wagner et al., 2003) and to detect changes in metabolite composition, in eggplant roots and shoots.

## 7.2. Materials and methods

### 7.2.1. Plant material and hydroponic growth conditions

Four eggplant (*Solanum melongena* L.) NUE-contrasting accessions were selected from a large collection of germplasm (Cericola et al., 2013). AM222 and AM22 showed high and low NUE, respectively, both in hydroponics and greenhouse pot experiments, while 67-3 and 305E40 exhibited contrasting behavior (Mauceri et al. *JIPB* submitted). In addition, 67-3 and 305E40 are two parents (males and females, respectively) of a segregating population already established as F<sub>2</sub> (Toppino et al., 2016), today became a RIL population of 156 individuals.

Seeds, sterilized surface with 5% (v / v) NaClO for 15 minutes and rinsed with deionized water, were germinated in a Petri dish (Ø 90 mm) on filter paper with 0.1 mM of CaSO<sub>4</sub>. After 10 days, seedlings selected by uniform size, were transferred in hydroponic boxes (4 L, twelve seedlings per box) containing a modified Hoagland solution (4.75 mM CaSO<sub>4</sub>, 2.5 mM K<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 46 µM H<sub>3</sub>BO<sub>3</sub>, 9µM MnCl<sub>2</sub>, 0.76 µM ZnSO<sub>4</sub>, 0.11µM Na<sub>2</sub>MoO<sub>4</sub> and 20µM Fe- EDTA) without NO<sub>3</sub><sup>-</sup>. The growing units were transferred to a growth chamber at 24°C, 65% relative humidity and 14 h photoperiod with 350 µmol m<sup>2</sup> s<sup>-1</sup> light intensity, for 2 days (T0). Then, plants were transferred in the same nutrient solution containing low NO<sub>3</sub><sup>-</sup> concentration (0.5 mM), for a short- (T1, 24 h) and long-term (T2, for 16 days). Nitrate was added to the solution as Ca(NO<sub>3</sub>)<sub>2</sub>, therefore in order to balance Ca<sup>2+</sup>, 4.75 mM CaSO<sub>4</sub> were added in the solution. The nutrient solution was renewed every three days and the pH was adjusted to 5.8 with 1 N KOH.

### 7.2.2. Extraction, derivatization and analysis of the profiling of metabolites in shoot and root of eggplants by GC-MS

Eggplant shoot and root tissues (100 mg of fresh weight) were manually homogenized (mortar and pestle) with liquid nitrogen and extracted in 700 µl of methanol, then 60 µl of ribitol (0.2 mg / ml, keep in 4°C) was added as an internal quantification standard.

The extracts were well vortexed at 4° for 15 minutes and subsequently sonicated for 20 minutes and vigorously mixed with 750 µl of double deionized water (DDW), and for separating polar and non-polar metabolites, 375 µl chloroform were added.

After vortexing, the mixture was centrifuged at 14000 rpm for 5 minutes at 4° C and 400 µl of supernatant was sampled and frozen in liquid nitrogen. The mixture of the samples was then lyophilized at -80° C. The derivatization provided the residues re-dissolving in 1 ml of pure pyridine enriched with 20 mg methoxyamine hydrochloride, in each sample 40 µl of the mixture were added and vortexed for 1.5 h at 37° C. In addition, a mix of alkanes and amino acids dissolved in pyridine was used. Finally, 70 µl MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide)] were added, vortexed and incubated at 37° C under stirring at 250 rpm for 0.5 h.

Each sample (110 µl) was transferred into vial and incubated at room temperature for 2 hours, before injection. One µl of each sample was then injected onto the GC column using a hot needle technique.

The metabolites were detected using a Trace GC Ultra coupled to a Thermo DSQ II mass spectrometer (Thermo Scientific, Waltham, MA, USA). The separation was performed using a 30 m TG-5MS column with a 0.25 µm film thickness (Thermo Scientific) and 1.2 mL per minute helium gas flow. The following temperature was programmed: 80 °C for 30 s, a ramp of 15 °C per minute at 330 °C, and an intake of 8 minutes.

Other specifications included the input temperature maintained at 280 °C and the auxiliary line at 300 °C. M/Z scan 40-600 (i.e. the mass-to-charge ratio) were scanned at five scans per second after ionization of the electronic impact.

The metabolites were identified using specific mass of the retention time index with reference spectra in NIST mass spectra libraries (2011, NIST/EPA/NIH Mass Spectral Library Version 2.0 g). The relative quantification of the metabolites was based on a pre-added internal standard (ribitol at 0.02 mg / ml) added during the extraction process. The amount for each compound were presented as log2 fold change of the relative response ratios (obtained by dividing the peak area of sample and the internal standard, the ribitol) between T1/T0 and T2/T0 and the results were expressed as mean values ± standard deviation.

For each genotype, three biological replicates were sampled, and each replicate consists of eight plants. Analysis of variance (ANOVA) was carried out by R software and the statistically differences were evaluated by Fisher's LSD test (P <0.05).

The list of metabolites of this dataset was analysed with MetaboAnalyst, using the option 'pathway analysis' (<https://www.metaboanalyst.ca/>).

### 7.3. Results and Discussion

#### 7.3.1. Metabolites profile in NUE contrasting genotypes

Metabolic profiling is now becoming an important tool to in understanding the N levels effect of N levels on the plant metabolome (Kusano et al., 2011; Suzuki et al., 2012), providing the actual information into what determines phenotypic traits. To get more insight into the effects of limited nitrate supply on the primary metabolism of NUE contrasting eggplants, roots and shoots were analyzed at short- (24 h, T1) and long-term (16 days, T2) treatment through GC-MS untargeted metabolomic to identify the metabolites and the pathways affected. In particular, 30 and 33 compounds in shoots and in roots, respectively, were identified including three main groups: amino acids, organic acids, sugars (Table 18). Although the same sugars were detected in both tissues, several differences were evident in organic acids and amino acids between roots and shoots. In particular, acetic and octadecanoid acids were detectable only in roots, by contrast glutamine, tryptophan and tyrosine were noticeable only in shoots (Table 18). Understanding the changes in amino acids is very important because they are not only the constituents of proteins, but also important signaling of regulatory molecules with significant impact on plant growth and abiotic stress response (Ganie et al., 2017). Under low N-supply, a decrease of amino acids content together with carbohydrates accumulation were observed in tobacco and oilseed rape (Paul and Driscoll, 1997; Tilsner et al., 2005). Changes in amino acid composition in leaf and root were also reported under N-limitation in maize genotypes having contrasting response to nitrogen deficit suggesting that plants trigger specific metabolic adaptive responses (Gaine et al., 2017). Scatter plot distribution of all the metabolites groups, in roots and shoots, revealed a linear positive correlation between the expression levels of metabolites at T1 and T2 in 305E40, and at less extent, in AM222. By contrast, AM22 and 67-3 showed a not linear metabolites expression. Furthermore, the distribution of outlier metabolites was more marked, in AM22 at T2, while in AM222 and 67-3 genotypes at T1. The best scatter of metabolites was differently localized among genotypes, showing a lower density (intensity gradient ranging from 0.005 to 0.025) in AM22 and 67-3 and a higher density (intensity gradient ranging from 0.01 to 0.05) in AM222 and 305E40 (Figure 24). The results suggested that AM22 and 305E40 did not appear responsive to N-treatment for metabolites changes, by contrast AM222 and 67-3 showed a significant variation in metabolites expression between N-term exposures.

The principal component analysis (PCA) highlighted a clear separation among eggplant accessions. The first two components, PC1 vs PC2, which explained 47.18 and 49.02% of total variability at T1 and T2, respectively, were showed (Figure. 25). After 24 hours of treatment

(T1), an evident clustering between a pair of NUE contrasting genotypes, AM22 and AM222, was showed, while 305E40 and 67-3 did not clearly cluster (Fig 25 A). After 16 days of treatment (T2), all the genotypes separately clustered, but AM 222 and 67-3 appeared more closed (Fig. 25 B). Overall, these results were consistent with the previous observation both in growth chamber and greenhouse experiments where NUE contrasting behavior between AM222 and AM22 were defined. Furthermore, the similar behavior at long-term N-exposure between AM222 and 67-3 was in accordance with physiological and molecular results (Mauceri et al. *JIPB* submitted).

| Shoots | Sugars       | Organic acids        | Amino acids   |
|--------|--------------|----------------------|---------------|
|        | Arabinose    | Glyceric acid        | Valine        |
|        | Myo-inositol | Pyroglutamic acid    | Isoleucine    |
|        | Fructose     | Citric acid          | Leucine       |
|        | Glucose      | Succinic acid        | Proline       |
|        | Sucrose      | Fumaric acid         | Glutamine     |
|        |              | Malic acid           | Glutamic acid |
|        |              | Oxalic acid          | Phenylalanine |
|        |              | Shikimic acid        | Serine        |
|        |              | Dehydroascorbic acid | Threonine     |
|        |              | Quinic acid          | Glycine       |
|        |              |                      | Alanine       |
|        |              |                      | Cysteine      |
|        |              |                      | Aspartic acid |
|        |              |                      | Asparagine    |
| Roots  | Sugar        | Organic acid         | Amino acid    |
|        | Arabinose    | Glyceric acid        | Valine        |
|        | Myo-inositol | Pyroglutamic acid    | Isoleucine    |
|        | Fructose     | Citric acid          | Leucine       |
|        | Glucose      | Succinic acid        | Proline       |
|        | Sucrose      | Fumaric acid         | Glutamic acid |
|        |              | Malic acid           | Phenylalanine |
|        |              | Oxalic acid          | Serine        |
|        |              | Shikimic acid        | Threonine     |
|        |              | Dehydroascorbic acid | Glycine       |
|        |              | Quinic acid          | Alanine       |
|        |              | Acetic acid          | Cysteine      |
|        |              | Octadecanoic acid    | Aspartic acid |
|        |              |                      | Asparagine    |
|        |              |                      | Tryptophan    |
|        |              |                      | Tyrosine      |

**Table. 18.** Metabolite composition in shoots and roots of NUE contrasting eggplant genotypes under low nitrate supply for 24 h (short-term, T1) and 16 days (long-term, T2) of treatment.

To identify common metabolite profiling patterns among genotypes, heat maps clustering compounds, in roots and shoots, for each experimental time (T0, T1 and T2) were created. For this purpose, fold expression ratio between metabolites concentration of each genotype (AM222, 67-3 and 305E40) to that detected in the N-use inefficient genotype AM22, at each experimental time was calculated (Figure 26 and 27). Overall, increasing (red, 0–1), unaffected (white, 0), and decreasing (blue, 0– -1) patterns of single compounds were found for both roots and shoots, at each experimental time.

Focusing on the extreme N-use efficient genotype AM222, it showed, at T0, a different metabolic profile in roots compared to 67-3 and 305E40. Further, a general more abundant of most amino acid, sugar and organic acid components, except for glucose, succinate, oxalate and leucine was highlighted. This behavior was also evident in shoots where a significant up-regulation of the same metabolites was showed with higher levels of sucrose, glucose and fructose. By contrast, some amino acids, such as pyroglutamate, proline, glutamine, leucine, fumarate and oxalate appeared significantly down-regulated.

After 24h (short-term) low-nitrate supply (T1), several amino acids, such as glutamate, alanine, asparagine, serine, valine, phenylalanine and tryptophane significantly decreased in roots of AM222 compared to AM22. By contrast, among carbohydrates and TCA, glucose together with malate and citrate, respectively appeared to be up-regulated. In shoots, a short-term low nitrate treatment caused an up-regulation of glutamate, glutamine and proline amino acids and of citrate in the TCA cycle, while a down regulation of glucose and sucrose was evidenced in AM222 compared to AM22.

A long-term nitrate limitation (16 days) caused a higher expression level of almost all amino acids among which glutamate, glutamine and proline in N-use efficient genotype, AM222. Further, sucrose was down-regulated while an increased level of both glucose and fructose was observed. Among organic acids involved in TCA cycle, citrate was down-regulated. Similarly, in shoots, a decrease in sucrose and an increase in glucose and fructose level was observed in AM222. Among amino acids and organic acids, glutamate and glutamine and citrate and succinate were up-regulated. The observations on metabolic profile changes, in both roots and shoots, indicated that specific responses were adopted to face nitrate limitation.

The metaboanalyst module “MetPa” allowed us to perform a detailed analysis of plant metabolism pathways and networks affected by N-limitation in both roots and shoots of NUE-contrasting genotypes. In particular, the pathways affected was that related to the amino acids metabolism, mainly alanine, aspartate and glutamate metabolism (Table 19; Figure 28).

However, it was noteworthy that, in both roots and shoots at T1, the glycine, serine and threonine metabolism was also highly impacted.

This result was not surprising because Mauceri et al. (*JIPB* submitted) demonstrated that NUtE more than NUpE component was important in determining high NUE efficiency in eggplants at low N inputs, as already reported in tomato (Abenavoli et al., 2016) and in maize (Bertin and Gallais, 2000).

Furthermore, they also demonstrated that glutamine synthetase GS as a key enzyme influencing NUtE, and consequently NUE, conferring high efficiency to AM222.

So, the understanding N-partitioning process within root/shoot, the amino acids up- or down regulated in contrasting NUE genotypes were already reported as important factors influencing NUE (Masclaux-Daubresse et al., 2010; Xu et al., 2010; Mu et al., 2015), and our results confirmed the key role of amino acids also in eggplant, previously poorly characterized.

Recently, amino acids transport processes in both root and shoot were reported to strongly influence N uptake and metabolism, most probably through a feedback regulatory mechanism and shoot-to-root signaling (Sanders et al., 2009; Tan et al., 2010; Zhang et al., 2010, 2015; Forde, 2014; Santiago and Tegeder, 2016). Furthermore, the internal pools of amino acids within plants may indicate nitrogen status by providing a signal that can regulate nitrate uptake by the plant (Miller et al., 2008).

Why alanine, aspartate and glutamate pathway could play an important role to confer NUE efficiency to AM222 ? Glutamine, asparagine, glutamate, and aspartate are the predominant amino acids transported in plants for utilization and storage (Okamoto and Pilot, 2011). Indeed, as nitrate was taken up by roots, it can be stored in vacuoles or assimilated via conversion to ammonium, or, alternatively, it was loaded into xylem vessels and transported to the shoots where it was assimilated. In eggplant, there is scarce information where the assimilation process occurred. However, in tomato, nitrate, acquired by roots, was transported to shoot and both these processes were regulated by the long-distance nitrate transporter LeNRT2.3, allowing tomato to utilize nitrate more efficiently (Fu et al., 2015; Abenavoli et al., 2016). Ammonium, originating by nitrate reduction, photorespiration or amino acids recycling, is assimilated through the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Lea and Mifflin, 1974; Lea and Ford, 1994). In addition, cytosolic asparagine synthetase (AS) transfers glutamine to a molecule of aspartate to generate glutamate and asparagine (Lam et al., 2003). The C skeletons required for N assimilation derived from tricarboxylic acid cycle (TCA), highlighting the close relationship between C/N metabolism (Lawlor, 2002). Finally, all these processes are controlled by many factors such as soil N availability, plant N status, C status and plant hormones

(Mokhele et al., 2012). So, increasing the N assimilation, utilization and recycling mechanisms could improve NUE in plants, reducing both the environmental and production costs (Good et al., 2007).

The comparison between AM222 and AM22, the efficient and inefficient genotypes, respectively, after a short-term N treatment, highlighted that, under low N supply, L-alanine and L-aspartic acid were significantly more expressed in AM222 roots, while L-glutamic was higher in AM22 roots. In shoots, AM222 showed a significant higher level of L-glutamine, L-asparagine and L-aspartic acid, while AM22 exhibited higher glutamic and succinic acids level. After a long-term N supply, AM222 roots showed a higher level of L-alanine, and L-glutamic and succinic acids, while AM22, the inefficient genotype exhibited a higher level of L-aspartic acid. In shoots, AM222 maintained higher level of L-glutamine, while AM22 displayed a higher level of L-alanine and L-fumaric and succinic acids.

Interestingly, at both short- and long-term N-stress experimental times, L-alanine was the amino acid more expressed in the roots of the efficient eggplant genotype in roots. Alanine together with 2-oxoglutarate were formed by transferring of an amino group from glutamate to pyruvate glutamate via the enzyme alanine amino transferase (AlaAT). AlaAT activity was higher in roots than shoots in rice (Shrawat et al., 2008; Beatty et al., 2009) and this evidence could sustain our results. Recently, Beatty et al. (2013) demonstrated that alaAT overexpression of alaAT from mediated by a root specific promoter affected improved the N-use efficiency of rice. More recently, the increase of alanine together with arginine in both leaf and root of N-tolerant maize it has been were demonstrated observed, that this amino acid together with arginine increased in both leaf and root of N-tolerant maize genotype suggesting genotypes ability to store N source reserves under low N (Ganie et al., 2018).

The amino acid profile in shoots of the efficient AM222 at both T1 and T2 evidenced also, at both experimental time-treatments, a higher level of glutamine. The enzyme involved in its formation is the glutamine synthetase (GS), which has been proposed as a candidate for improving NUE in wheat (Habash et al., 2007; Gadaleta et al., 2011, 2014; Thomsen et al., 2014). GS is present in most species, with three to five isoforms localized in the cytosol (GS1) and a single isoform (GS2) in plastids (Swarbreck et al., 2011). Overexpression of the cytosolic enzyme glutamine synthetase 1 (GS1) has been investigated in numerous cases with the goal of improving crop nitrogen use efficiency (Thomsen et al., 2014). The higher level of glutamine in the efficient genotype was consistent with the results of Mauceri et al. (*JIPB* submitted) whose observed a higher GS activity in AM222. So, these results indicated that GS would be an excellent marker for improvement NUE also in eggplants.

Further analysis including the results obtained in less NUE contrasting genotypes could give more information on NUE eggplant phenotype.

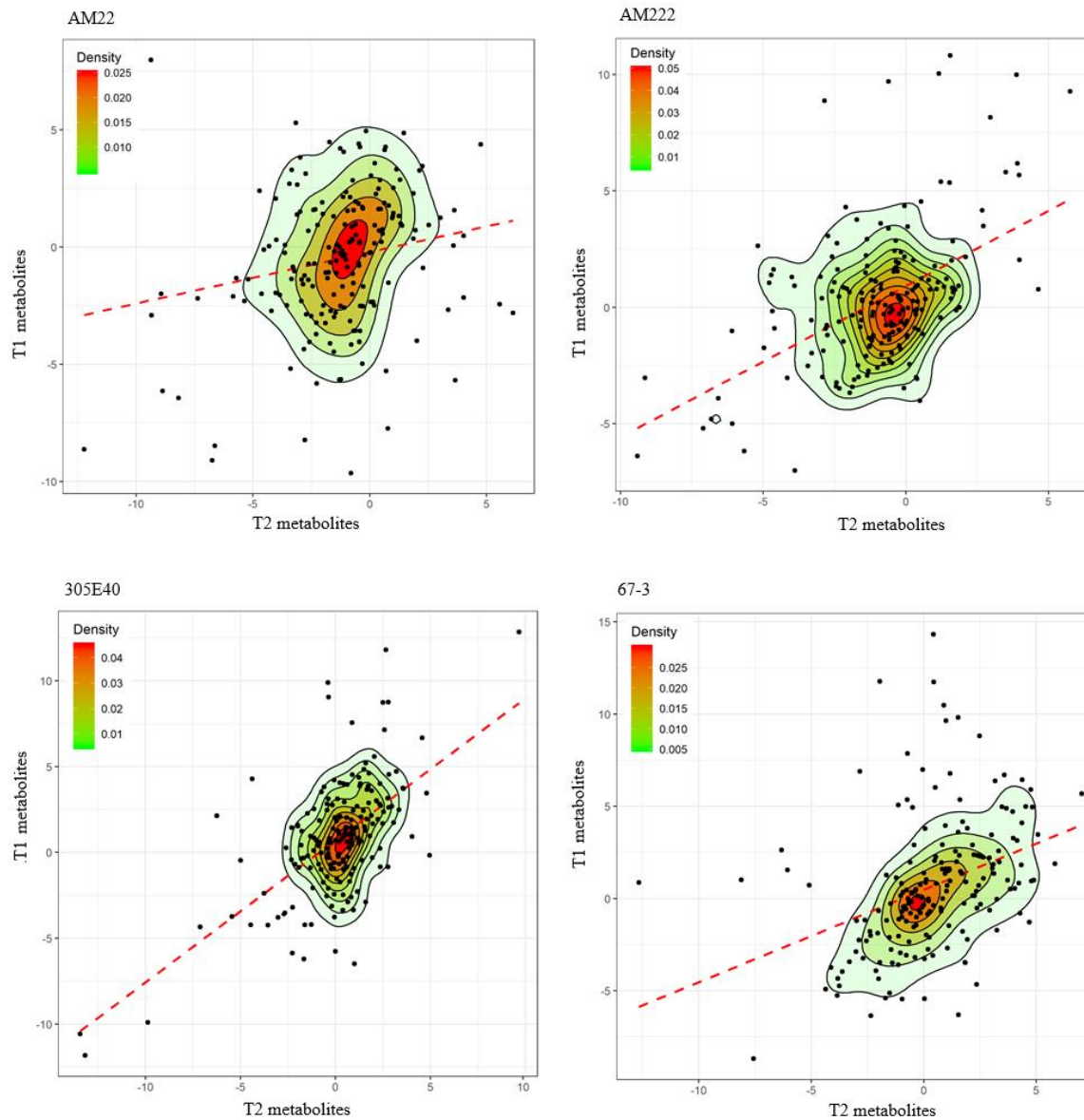


**Figure 24.** Scatter plot distribution of all metabolite groups revealed that there was different trend among genotypes moreover a linear positive correlation between the expression levels was showed only in 305E40.

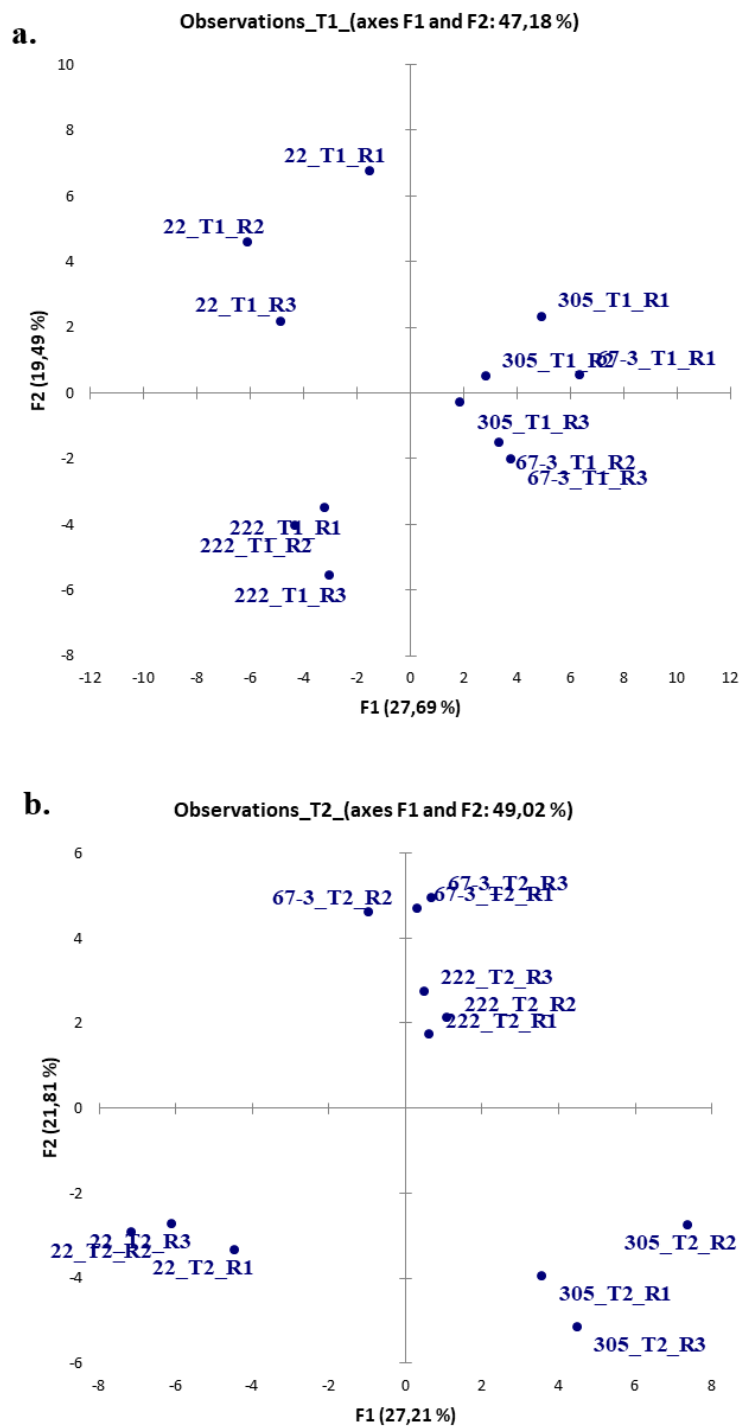


**Figure 25.** Partial least squares - discriminant analysis model scores plot of metabolite profile of four eggplant accessions after t1 (24 h) and t2 (16 days) of low nitrate treatment. principal component 1 (f1) vs. f2 (the explained variances are shown in brackets).

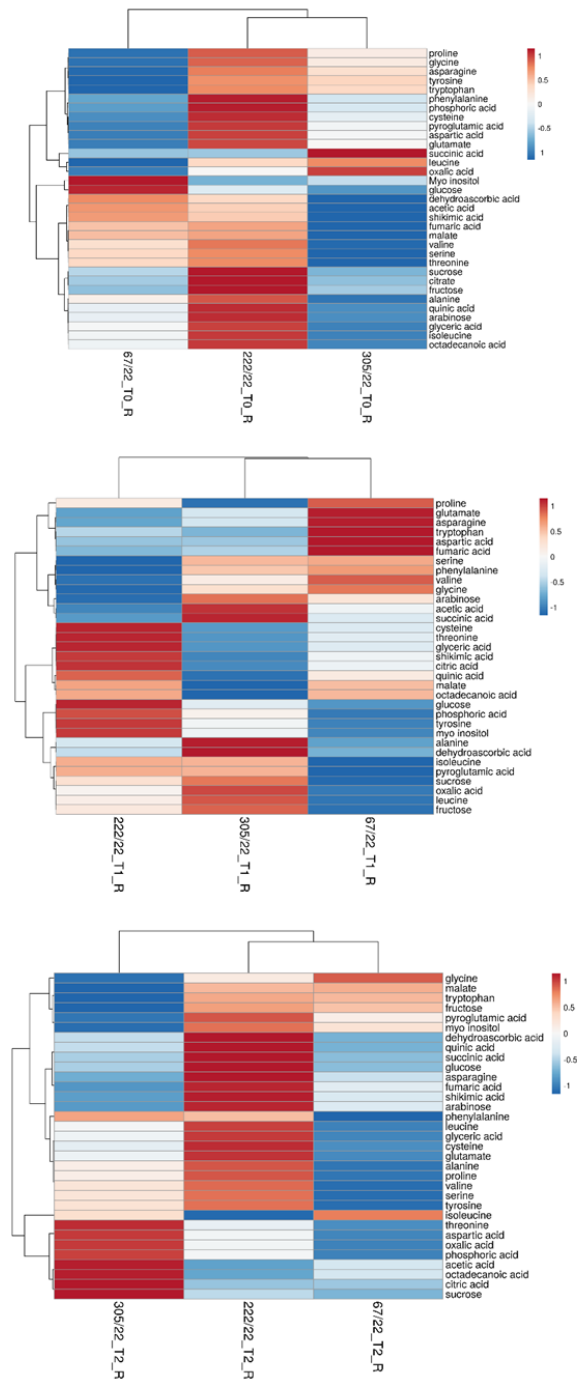


**Figure 26.** Overlay heat map of the root metabolite profiles in AM 22, 67-3 and 305 eggplant accessions, exposed for 0 (A), short-term (24 h) (B) and long-term (16 days) (C) to low nitrate (LN) in comparison with AM 22, the inefficient one. Each square represents the effect of treatment on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively.

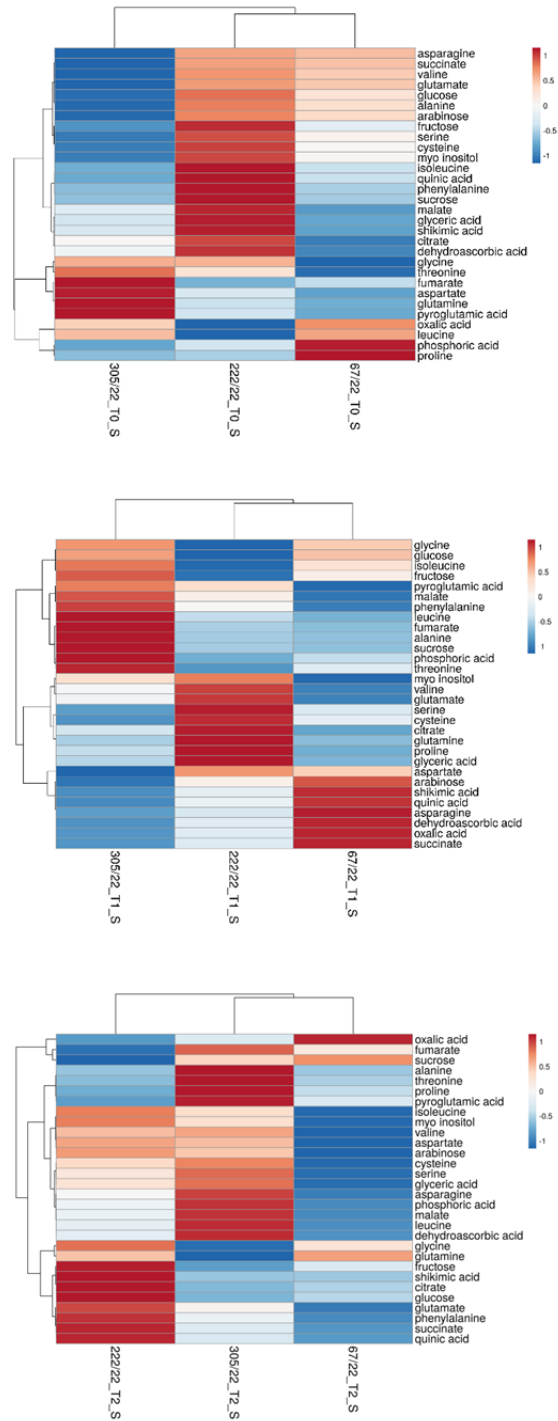


**Figure 27.** Overlay heat map of the shoot metabolite profiles in eggplant accessions (AM 222, 67-3 and 305) exposed for 0 (T0) (A), short-term (T0, 24 h) (B) and long-term (T1, 16 days) (C) to low nitrate (LN) in comparison with the inefficient one (AM 22). Each square represents the effect of treatment on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively.

| Sample             | Pathway                                     | Total Cmpd | Hits | Raw p     | Impact  |
|--------------------|---------------------------------------------|------------|------|-----------|---------|
| Root_T1_222_vs_22  | Alanine, aspartate and glutamate metabolism | 22         | 6    | 0.030331  | 0.54023 |
|                    | Glycine, serine and threonine metabolism    | 30         | 5    | 0.0008007 | 0.53413 |
|                    | Phenylalanine metabolism                    | 8          | 1    | 0.0020281 | 0.5     |
|                    | Isoquinoline alkaloid biosynthesis          | 6          | 1    | 0.5799    | 0.5     |
|                    | Tyrosine metabolism                         | 18         | 3    | 0.82608   | 0.27273 |
| Root_T2_222_vs_22  | Alanine, aspartate and glutamate metabolism | 22         | 6    | 0.0084355 | 0.54023 |
|                    | Glycine, serine and threonine metabolism    | 30         | 5    | 0.12641   | 0.53413 |
|                    | Phenylalanine metabolism                    | 8          | 1    | 0.046027  | 0.5     |
|                    | Isoquinoline alkaloid biosynthesis          | 6          | 1    | 0.065029  | 0.5     |
|                    | Tyrosine metabolism                         | 18         | 3    | 0.0080601 | 0.27273 |
| Shoot_T1_222_vs_22 | Alanine, aspartate and glutamate metabolism | 22         | 7    | 0.0001998 | 0.78161 |
|                    | Glycine, serine and threonine metabolism    | 30         | 4    | 0.0068453 | 0.53413 |
|                    | Phenylalanine metabolism                    | 8          | 1    | 0.44292   | 0.5     |
|                    | Inositol phosphate metabolism               | 24         | 1    | 0.88239   | 0.25131 |
|                    | Arginine and proline metabolism             | 38         | 5    | 0.0047576 | 0.22673 |
| Shoot_T2_222_vs_22 | Alanine, aspartate and glutamate metabolism | 22         | 7    | 0.044228  | 0.78161 |
|                    | Glycine, serine and threonine metabolism    | 30         | 4    | 0.60352   | 0.53413 |
|                    | Phenylalanine metabolism                    | 8          | 1    | 0.37171   | 0.5     |
|                    | Inositol phosphate metabolism               | 24         | 1    | 0.89498   | 0.25131 |
|                    | Arginine and proline metabolism             | 38         | 5    | 0.043706  | 0.22673 |

**Table 19.** Analysis of plant metabolism pathways affected by N-limitation in both roots and shoots of NUE-contrasting genotypes. the pathways affected was that related to the amino acids metabolism, mainly alanine, aspartate and glutamate metabolism.

Alanine, aspartate and glutamate metabolism

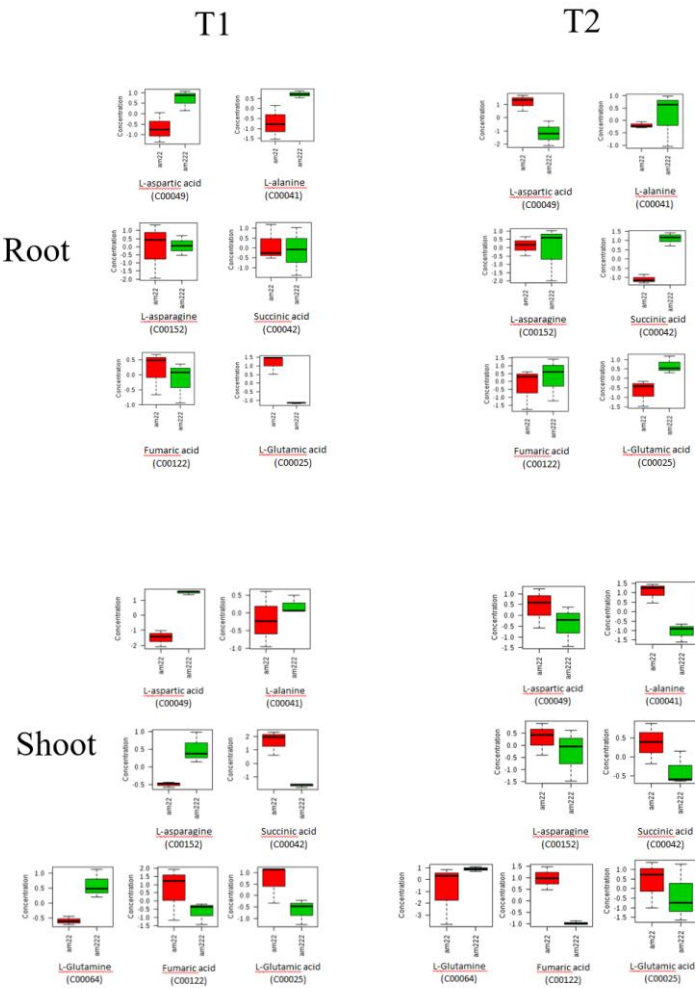
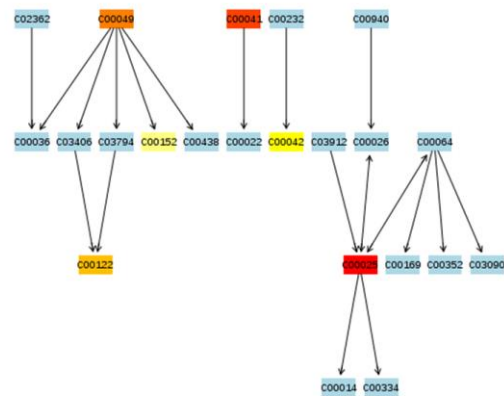


**Figure 28.** Alanine, aspartate and glutamate metabolism pathway, boxplot of relative concentrations for some significantly altered metabolites in AM22 (red) and AM222 (green).

## **CHAPTER 8. TRANSCRIPTOMICS ANALYSIS REVEALED GENES RELATED TO LOW N TOLERANCE IN CONTRASTING N USE EFFICIENT EGGPLANTS**

### **8.1. Introduction**

In intensive agricultural systems, chemicals such as fertilizers and pesticides, together with capital investments in new technical and mechanical tools, the innovative approaches in global agricultural production (based on new selected plant varieties), were the basis of the "green revolution" among the '40 and '70 of last century.

In the 1990s, the traditional breeding strategies supported by Marker-Assisted-Selection (MAS), to improve nutrient management, have been a useful approach to identify the key role specific genes involved in simple and complex traits. Among these, nitrogen use efficiency (NUE) is currently a pivotal challenge for sustainable agriculture and expanding knowledge on this trait is becoming necessary.

NUE, defined as the total biomass or yield produced per unit of N supplied, is a complex quantitative trait, being the results of a large number of genes expression (involved in N-uptake, assimilation and remobilization) and also dependent on environmental factors such as N-availability in soil. Genetic, biochemical and physiological mechanisms on how plants use nitrogen, were the subject of scientific interest over the last 20 years, allowing us to highlight firstly the role of nitrate transporters NRT1.1, NRT1.2, NRT2.1 and NRT2.2 involved in nitrate uptake from the soil (Ho et al., 2009; Miller et al., 2007). Then, also the steps of N-assimilation into amino acids through the glutamine synthase (GS)/glutamate synthase (GOGAT) were intensively studied (Lam et al., 1996; Xu et al., 2012).

More recently, many transcription factors (TF) and protein kinases (PK) were identified as key genes able to orchestrate N-uptake, assimilation, translocation and remobilization (Kant et al., 2011; Masclaux-Daubresse et al., 2010; Simons et al., 2014; et al., 2002; Xu et al., 2012).

Today, Next-Generation Sequencing (NGS) technology, allow us to characterize plants responses to environmental changes and understand the complexity of plant nitrate regulation network at the transcriptional level. Many studies based on transcriptome sequencing in crops, grown under different N conditions, were carried out to identify the expression patterns of N-reactive genes (Amiour et al., 2012; Humbert et al., 2013; Simons et al., 2014). To better understand plant responses to N-low availability, the comparison of differential transcriptional responses of genotypes pair with contrasting NUE could be decisive. In recent studies, NUE-genotypic differences were addressed on different plant species (Hao et al., 2011; Vijayalakshmi

et al., 2015; Todeschini et al., 2016), but till now there are not many reports on differential transcriptomic profiling.

Hao et al. (2011) showed contrasting sensitivity to low N supply in two soybean varieties, identifying differentially expressed genes. Gelli et al. (2011) carried out a study on sorghum root tissue comparing four tolerant and three sensitive genotypes to low N-condition. Furthermore, a more recent microarray experiment on maize roots of different NUE inbred lines, grown under various N levels, allowed identifying nitrate induction triggers in several transcriptional changes (Zamboni et al., 2014). Overall, the results showed that the transcriptional responses were very complex depending on genotype (G) and N availability (N) and their interaction (G\*N) (Bi et al., 2014; Zamboni et al., 2014).

Eggplant (*Solanum melongena* L.) is one of the oldest known domesticated crop, but there are no reports about its physiological and molecular responses to N-uptake, transport and assimilation efficiency. By contrast, the eggplant genome was already drafted by two different Consortium from Italy and Japan (Rotino et al., 2014; Hirakawa et al., 2014) allowing us the possibility to implement genomics and bioinformatics strategies to isolate N-metabolism key related genes via transcriptomic approaches. In addition, the large number of candidate QTL/genes involved in NUE, characterized and mapped in other species, could aided supplemental baits to understand eggplant responses to low N conditions in NUE contrasting genotypes (Mauceri et al., *JIPB* submitted).

In this study, a transcriptome sequencing, using RNA-seq technology, of four NUE-contrasting eggplant genotypes, selected among a core collection by a previous survey in both hydroponic system and pot experiments (see chapter 4) was assessed under sub-optimal nitrate conditions at short and long-term. The aims were: i) to compare the eggplants transcriptomic profiles; ii) to evaluate the enrichment of transcription factors and genes among the significant differentially expressed; iii) to identify novel genes related to the main N pathway. Putative genes/TF related to the most important pathways involved in NUE will be characterized to understand their physiological regulation role and the potential impact for NUE improvement.

## **8.2. Materials and methods**

### **8.2.1. Plant materials**

AM222, 67-3, 305E40 and AM22 seeds, surface sterilized with 5% (v/v) NaClO for 15 minutes and rinsed with deionized water, were then germinated in Petri dishes (Ø 90 mm) on filter paper with 0.1 mM CaSO<sub>4</sub>. After 10 days, seedlings with fully expanded cotyledons, were selected by

uniform size and transferred in hydroponic tanks (4 L, ten seedlings for tank) containing a modified Hoagland's solution. Nitrate was added to solution as  $\text{Ca}(\text{NO}_3)_2$  at 0.5 mM (Low Nitrate, LN) concentration. Furthermore, to balance  $\text{Ca}^{2+}$ , 4.75 mM  $\text{CaSO}_4$  were added in the solution. The growing units were transferred to a growth chamber at 24°C, 65% relative humidity and 14 h photoperiod with  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity for 18 days. The nutrient solution was renewed every three days and pH was adjusted to 5.8 with 1 N KOH.

The sampling for RNA-seq experiment were collected at T0 (control) from plants (12 days old) previously nitrate deprived (0 mM  $\text{NO}_3^-$ ) for 48 hours. The second sampling (T1) was carried out on plants (13 days old) exposed to 0.5 mM  $\text{NO}_3^-$  for 24 hours, while the T2 sampling was done on plants (28 days old) grown at the same low-N condition for 15 days. For each genotype, three biological replicates consisting in eight plants for each were sampled.

### 8.2.2. RNA sequencing and data analysis

Total RNA was isolated and purified using a Mini RNeasy Plant kit (QIAGEN) and a Kit Precision DNase (Primer Design). RNA degradation and contamination were monitored on 1% agarose gel, and quantified using the Nanodrop. Approximately 400 ng of total RNA were used to construct cDNA libraries, following a 3-based end-based sequencing approach (libraries were prepared from the RNA using Transeq as reported in Tzfadia et al., 2018). Samples were sequenced on six lanes of Illumina HiSeq 2500 System, using the SR60 protocol. The output was ~3 million reads per sample.

### 8.2.3. Bioinformatics

The “Mantoux Bioinformatics Institute of the Nancy and Stephen Grand Israel National Center for Personalized Medicine”, Weizmann Institute of Science provided the bioinformatics service for all the upstream analysis.

Reads were trimmed using cutadapt (Martin, 2011) and mapped to eggplant genome SMEL\_V3.2016\_11\_01 from Italian Eggplant Genome Consortium (Rotino et al., 2014) using STAR vers. 2.4.2a (with EndToEnd option and outFilterMismatchNoverLmax was set to 0.04) (Dobin et al., 2013). Counting proceeded over the TES (Transcript End Site) region of each gene (TES region is defined as 200 bp downstream from the end of a gene, and at least 500 bases upstream to the end of the gene), annotated in SMEL\_V3.2016\_11\_01, using HTSeq-count (intersection-strict mode) (Anders et al., 2014).

Principal component analysis (PCA) was carried out to create a low dimensional global map of gene expression data set. The analysis was based on the log2 count reads matrix normalized data and function "prcomp" from the stats package and visualization function for PCA was ggbiplot package in R (GitHub).

#### 8.2.4. *Differential expression analysis*

Differential expression analysis among sampling times and genotypes was conducted using the DESeq2 R package (1.10.1) (Love et al., 2010; Robinson and Oshlack, 2010). The threshold levels for DEG detection were set at FDR-corrected p-value < 0,05 and Log2FC >|1|.

#### 8.2.5. *Metabolism pathway overview*

Pathway analysis was based on the MapMan ontology (Thimm et al., 2004) and figures were defined by a first running of the mercator tool (<http://mapman.gabipd.org/web/guest/mercator>) with the default parameters assigned by the MapMan bin to the eggplant transcripts. Log2 fold changes obtained from the DESeq2 output were used for inputting the software to depict expression changes between two N-use efficient genotypes (AM222 and 67-3) against two inefficient ones (AM22 and 305E40).

#### 8.2.6. *Gene Ontology (GO) and enrichment analysis*

Gene Ontology (GO) enrichment analysis was conducted with the topGO bioconductor package Release (3.8). GO is an annotation to unify the representation of genes and their product attributes across all the species. GO project defined three different vocabulary types of unified biological terms:

BIOLOGICAL PROCESSES (BP): operation or set of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms often involving a chemical or physics transformation.

MOLECULAR FUNCTIONS (MF): refer to the biochemical activity or elemental activities of a gene product at the molecular level, such as binding or catalysis.

CELLULAR COMPONENTS (CC): identify a specific area of the cell or its extracellular environment where the gene product is active. Each GO controlled vocabularies is described by a structure called Directed Acyclic Graph (DAG) that links the above-mentioned terms according to a parent-child relationship. A gene product (represented by an identification code) is associated with a GO code that describes its property. However, a gene product can often hold

more properties, so it can be associated with several different GO terms, and *viceversa* each GO term could be associated with one or more gene products. Indeed, a gene product annotated to a GO term, results also connected to all its parent terms. Mainly two types of relations link the GO terms: “is\_a” and/or “part\_of”; the nodes closest to the graph root are linked to more general concepts, while the more distant ones represent more specific concepts.

#### 8.2.7. Comparative Co-Expression Networks Construction and Visualization

CoExpNetViz tool was used to construct a co-expression network, using a set of queries or "bait" genes as input (chosen by the user) and a minimum set of gene expression data (Tzfadia et al., 2015). CoExpNetViz algorithm proceeds in three main steps; (i) for each chosen bait gene, cohesive values are calculated using mutual Pearson correlation and the information coefficients, (ii) the non-bait (=target) genes are grouped according to the cross-species orthology and (iii) output files are generated and the results can be viewed as network graphs in Cytoscape (Shannon et al., 2003).

CoExpNetViz inputs a set of "bait genes" from one or more species and a set of pre-processed data by species. Usually, genes known to be involved in the same biological process are chosen as baits, the software determines which gene families show members co-expressed with bait genes. The user specifies both positive and negative Pearson correlation coefficients, which are used to determine if two or more genes are co-expressed.

There are many algorithms for network genes co-expression analysis, here we used the Pearson correlation coefficient (PCC), which is the most popular and with a custom implementation of mutual information as a measure for similarity between expression profiles (Song et al., 2012). Thus, co-expressed genes were considered if the correlation falls below 1° or above 99° percentile of expression correlations distribution based on the expression profiles similarity of 4000 random genes. After calculating correlation matrices, positive and negative cut-off values (Vandepoele et al., 2009) were used to translate *r* values into a graph in which nodes and edges represent genes and co-expression relationships, respectively. Family nodes were displayed as circles, each of these representing a partition by a specific color, while bait genes were displayed in white.

### 8.3. Results

#### 8.3.1. Principal Component Analysis (PCA)

Principal component analysis (PCA) is a technique adopted to analyze high-dimensional biological data for their organization in two or three eigenvectors, but it is not valuable to highlight consideration about the biological mechanisms for which the samples clustered. Here, a complex gene expression profiles were organized in simple vector values by combinations of variables with significant variation between samples, and the normalized data of reads count were transformed into Log2. PCA was performed using the R software "prcomp" function, comparing genotypes by times sampling and tissues. The figures showed the biological replicates grouped for the best data interpretation (Figure 29).

Overall, PCA charts provided a clear picture on the significant differences between genotypes mainly in shoot. AM222 and 67-3 genotypes (previously classified as high NUE) appeared the closest among four genotypes in both root and shoot after short-time of exposure (T1).

#### 8.3.2. Differential expressed genes identification

The identification Differentially Expressed Genes (DEGs) between genotypes at short (24h, T1) and long term (15 days, T2) in responses to low N supply were considered, and each comparison was an independent analysis taking into account separately root and shoot. Two comparisons were performed: all the genotypes (AM222, 67-3 and 305E40) against the most N-use inefficient (AM22), and the parental lines of RILs between them (67-3 vs. 305E40). In Figure 30, Differential Expressed Genes (DEGs) of all the comparisons were showed.

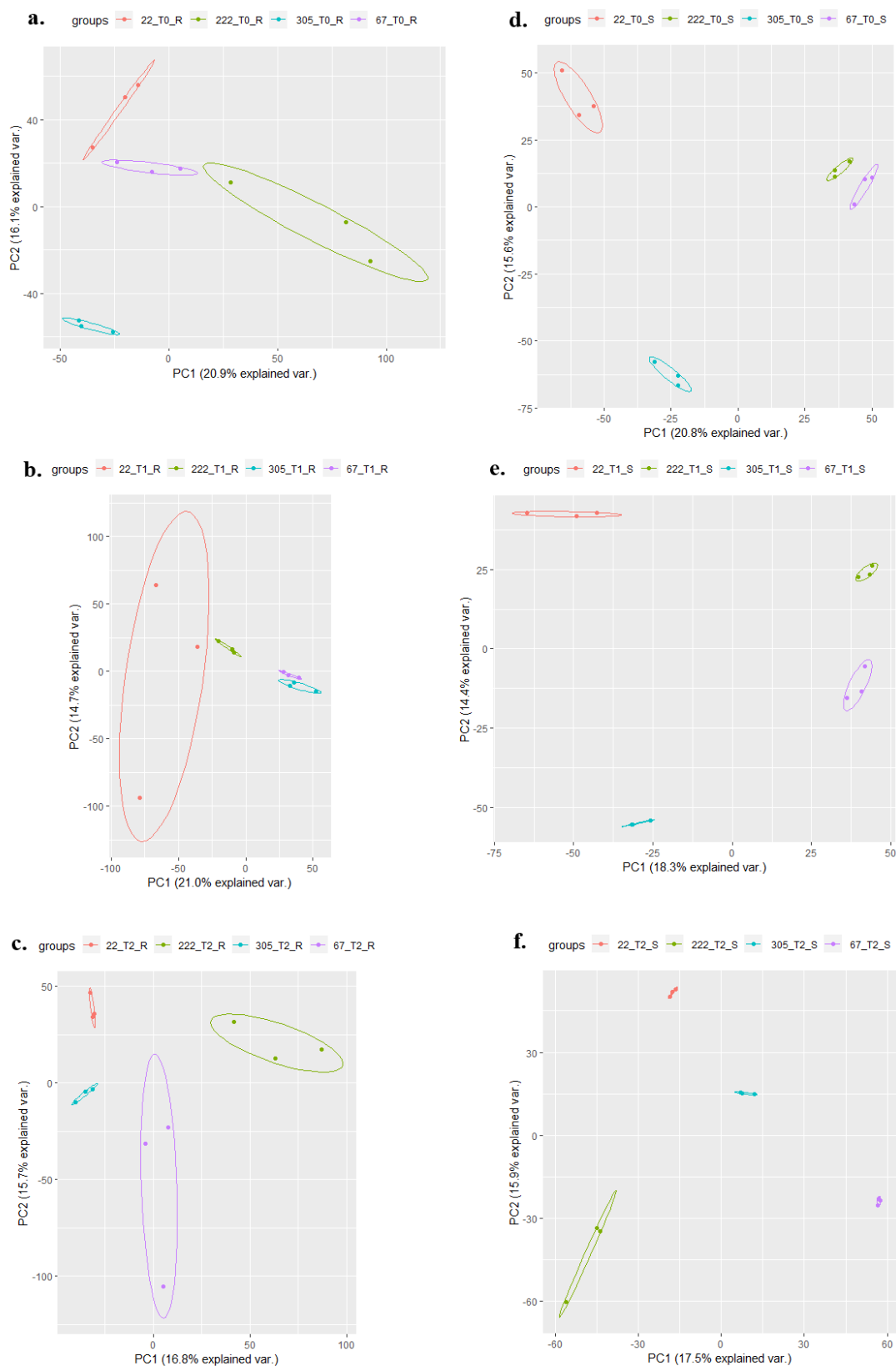


**Figure 29.** Six different subsets of gene expression dataset in eggplants were utilized for PCA analyses: root at T0 (a); T1 (b) and T2 (c); shoot at T0 (d); T1 (e) and T2 (f).

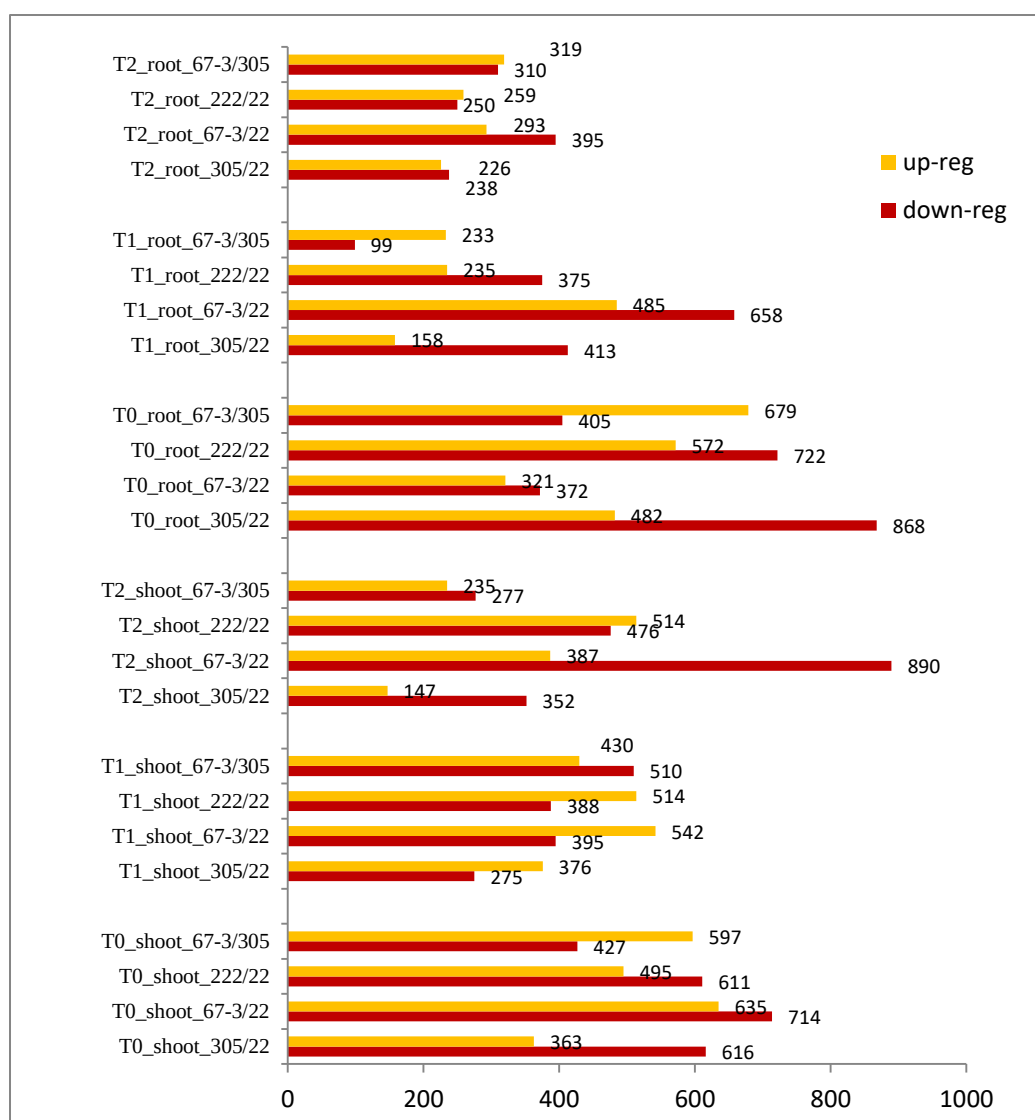


**Figure 30.** Histograms of identified DEGs; all genotypes were compared to AM22, while 67-3 against 305E40.

### 8.3.3. Pathways analysis MAPMAN

Different profiling data sets from root and shoot were visualized in MapMan to highlight which metabolism processes were altered. Firstly, DEGs from AM222, 67-3 and 305E40 together against AM22 were considered, and then two pair of comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) were carried out. Since the results did not show any significant changes in DEGs between the analyses, thus both pair genotypes comparisons were taken into account to better managed the large amount of data, reducing redundant results.

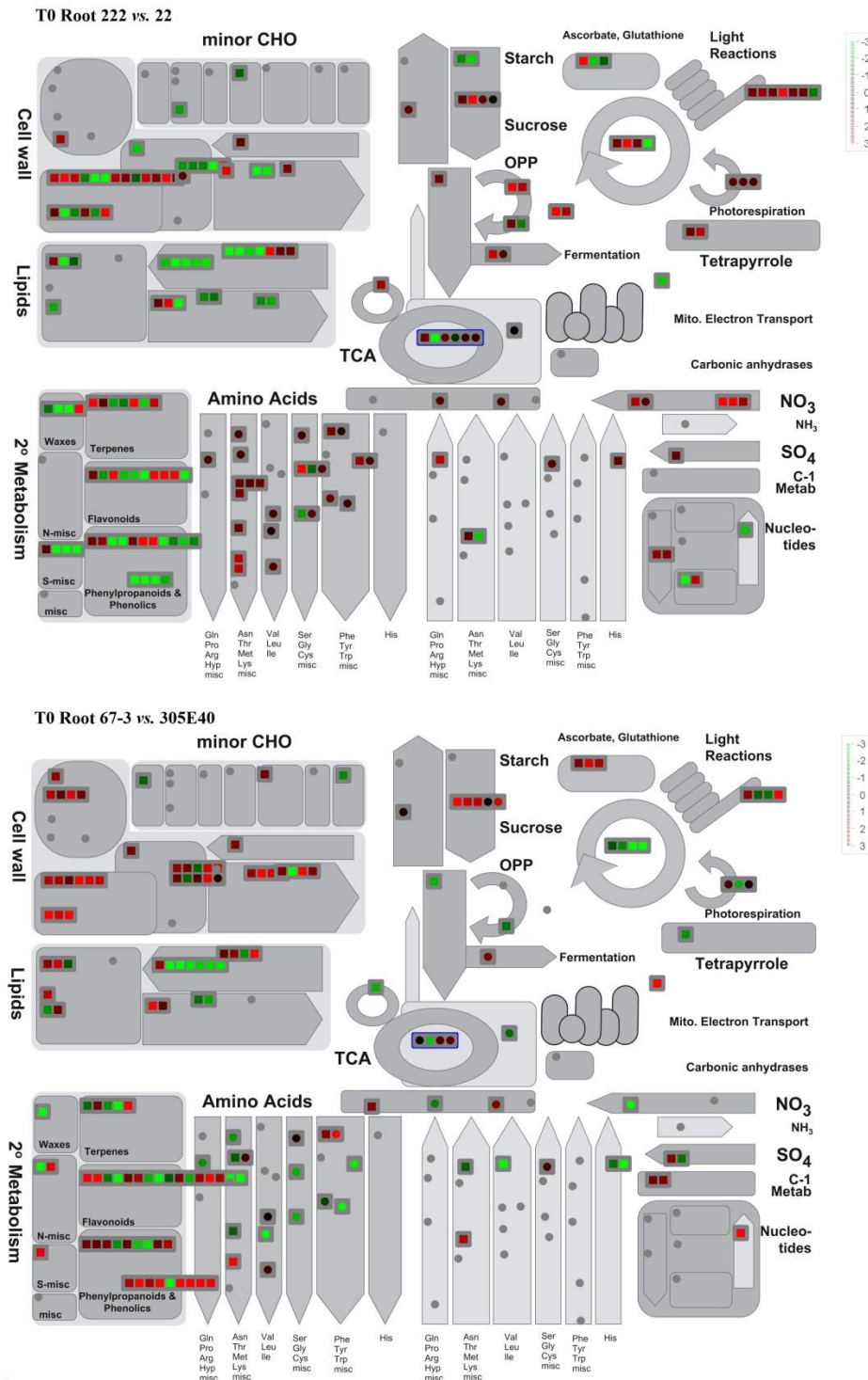


**Figure 31.** An overview of transcript ratios in major metabolic pathways in root at T0 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

In root, the profiling data sets comparisons displayed mainly changes in cell wall, secondary and lipid metabolism transcripts at T0 in both genotype pair comparisons (Figure 31). In shoot, in addition to the pathway changes observed in root, a very significant up-regulation of light reactions and tetra-pyrrole pathways were showed. More interestingly, NiR and NR appeared up-regulated in the N-use efficient genotypes (AM222 and 67-3) (Figure 32). After 24 h, under low N supply (T1), the described changes at T0 in root were confirmed although with a DEGs decrease, while an increase of lipid metabolism related genes transcripts was shown in N-use efficient genotypes (Figure 33). In shoot (at T1), an up-regulation of genes involved in flavonoid and cell wall pathway were observed in AM222 and 67-3, respectively; while both genotypes showed once again a robust activation in light reactions and tetra-pyrrole pathway (Figure 34). Finally, after long-term low N exposure (T2), significant differences were observed in root for phenols in AM222 and flavonoids in 67-3, respectively (Figure 35). Instead of few DEGs were underlined in the light reaction map in shoot (two up-regulated genes in 67-3 and one in AM222 compared to 305E40 and AM22, respectively), while AM222 confirmed DEGs in phenols metabolism compared to AM22 (Figure 36). Once all the maps were generated, the most significant DEGs shared by N-use efficient genotypes AM222 and 67-3 were analyzed.

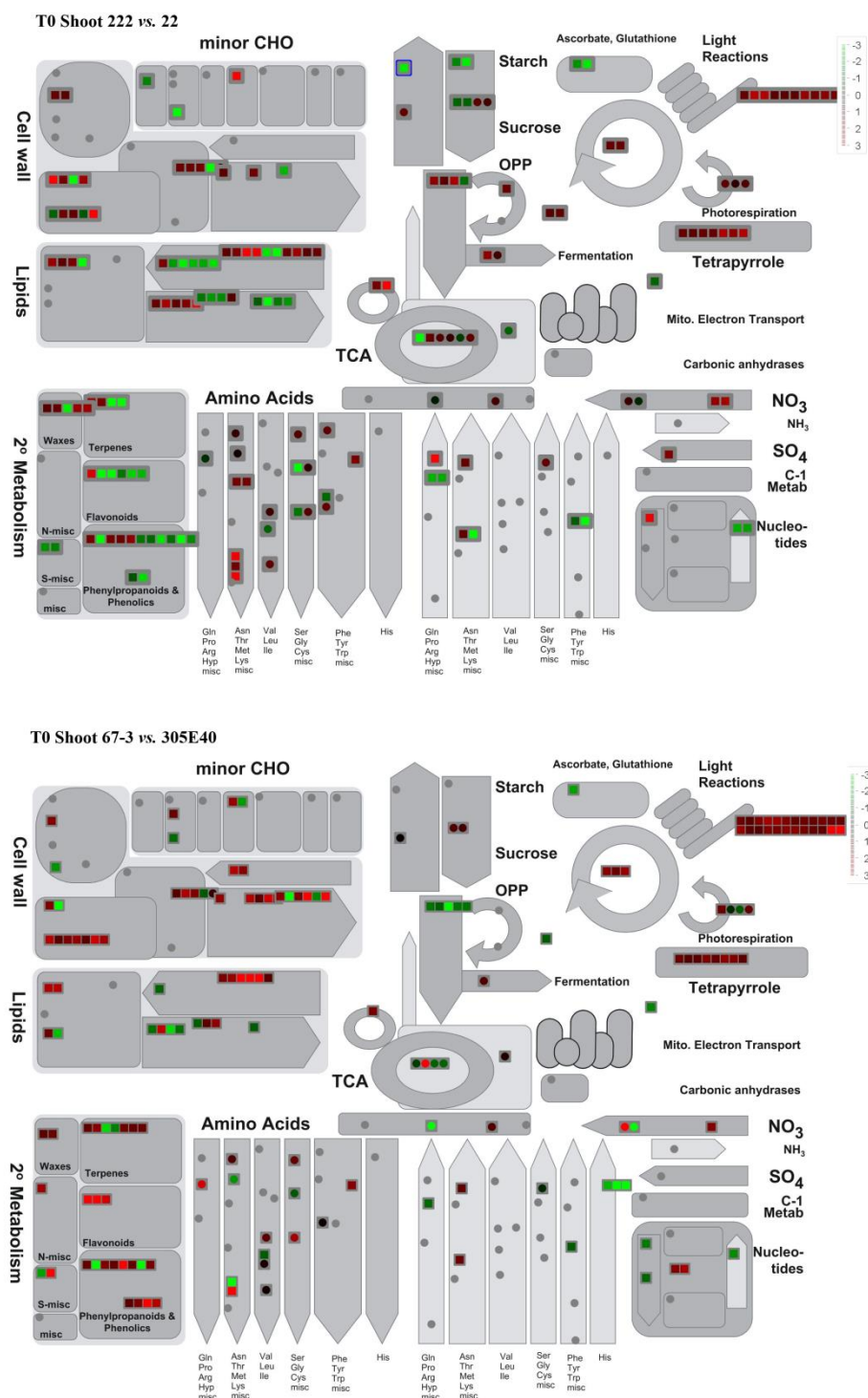


**Figure 32.** An overview of transcript ratios in major metabolic pathways in shoot at T0 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

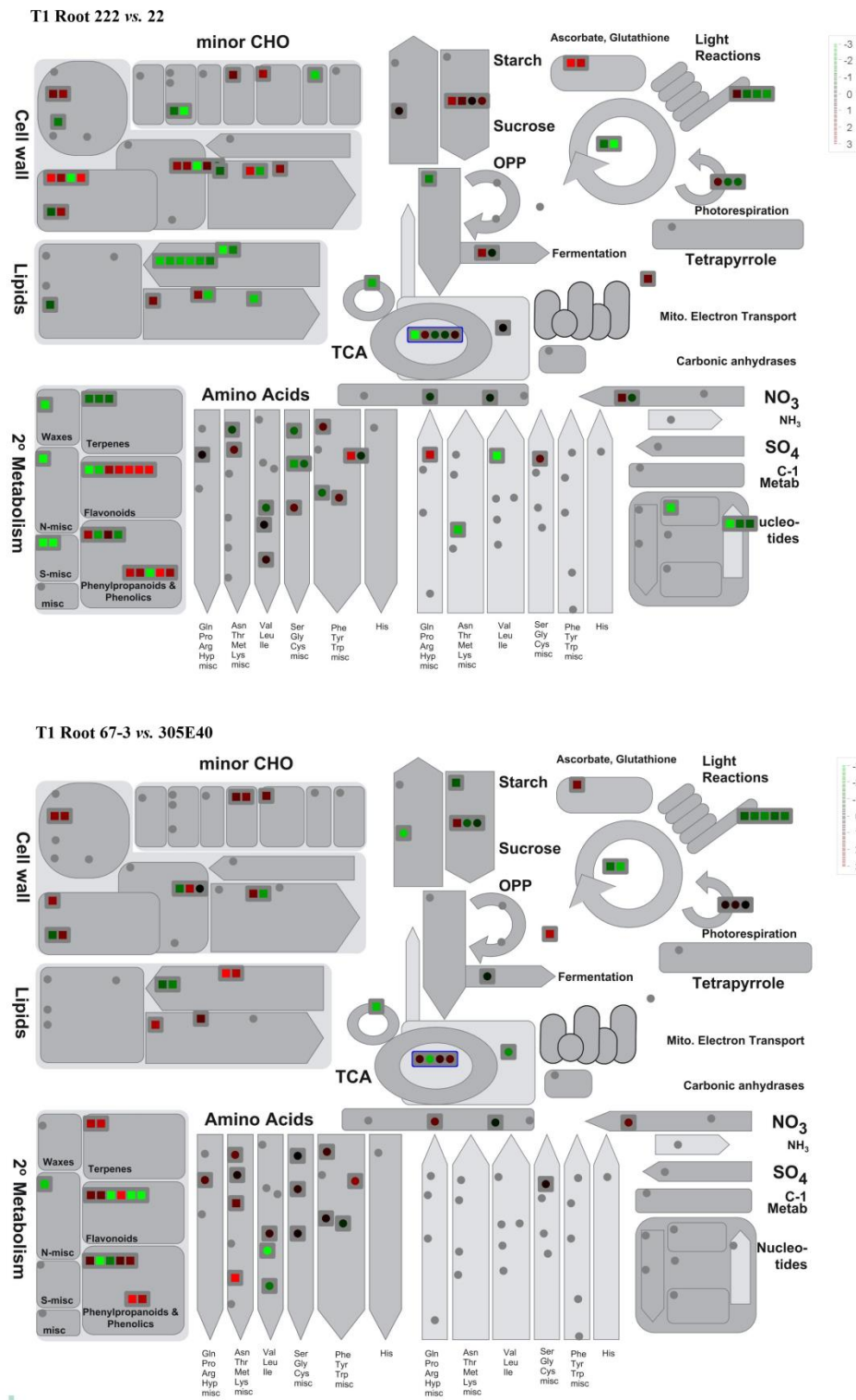


**Figure 33.** An overview of transcript ratios in major metabolic pathways in root at T1 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

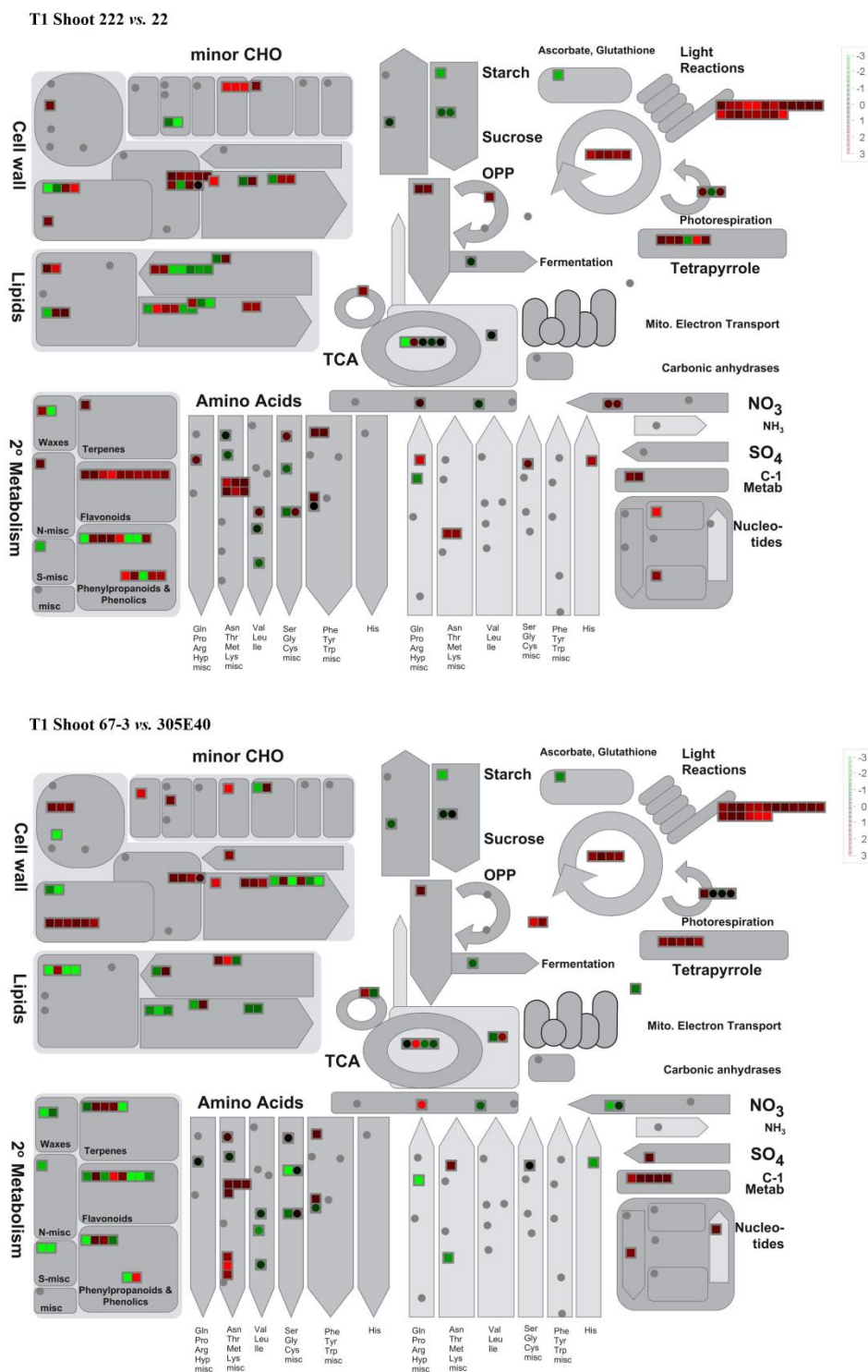
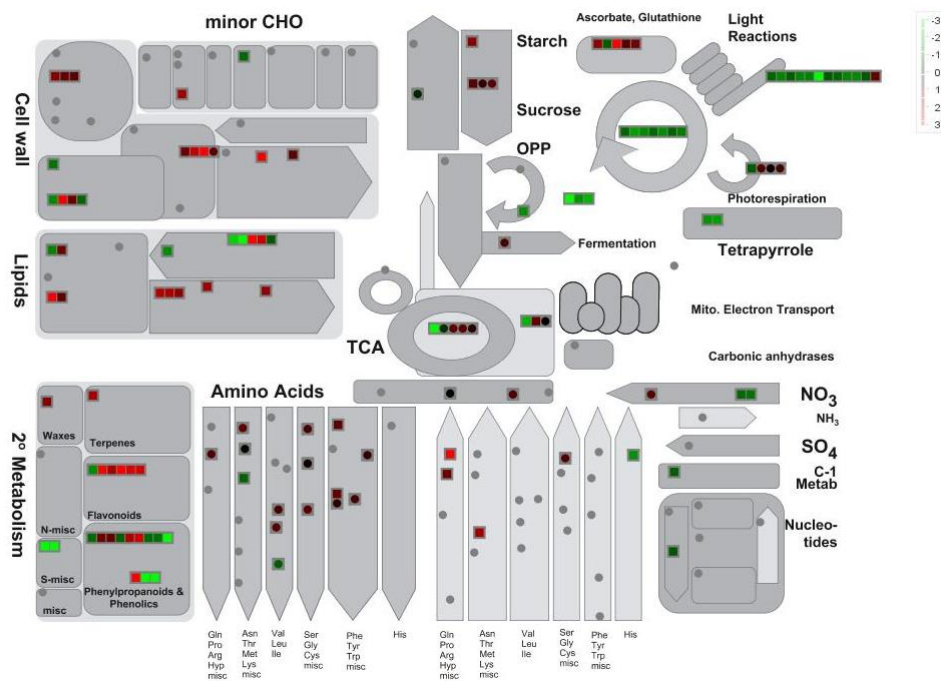


**Figure 34.** An overview of transcript ratios in major metabolic pathways in shoot at T1 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

T2 Root 222 vs. 22



T2 Root 67-3 vs. 305E40

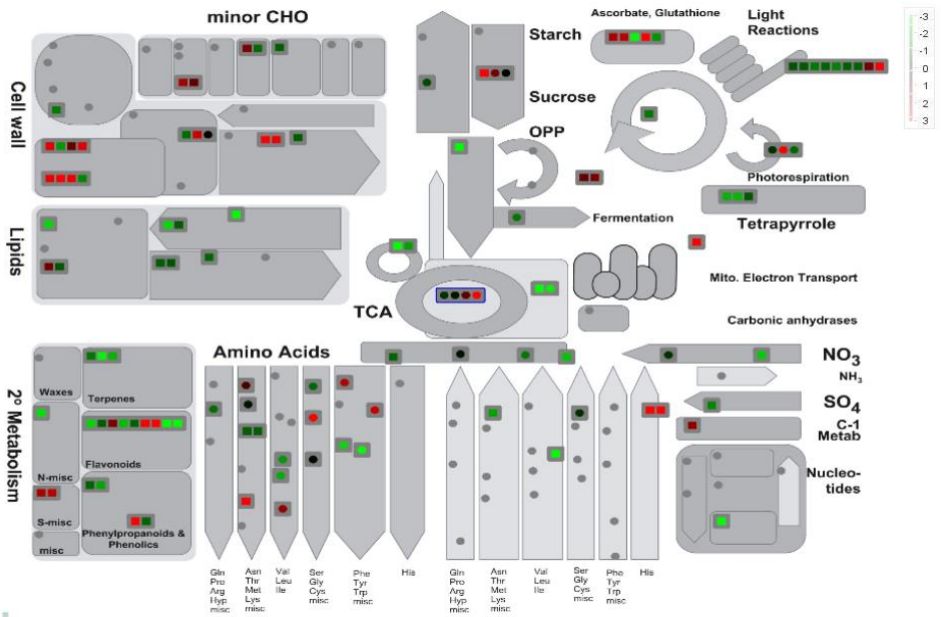


**Figure 35.** An overview of transcript ratios in major metabolic pathways in root at T2 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

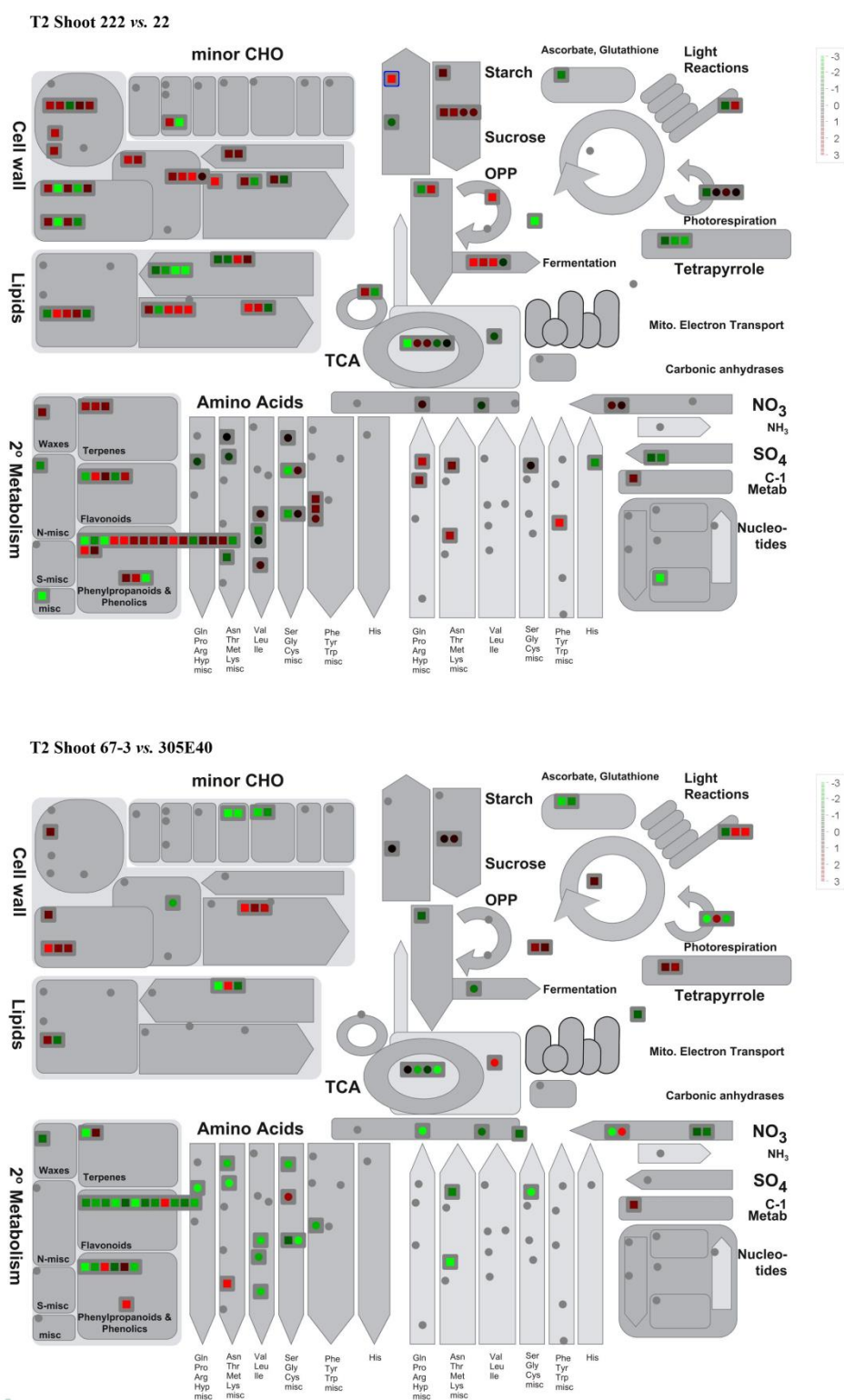


**Figure 36.** An overview of transcript ratios in major metabolic pathways in shoot at T2 in genotype pair comparisons (AM222 vs. AM22 and 67-3 vs. 305E40) is visualized using MapMan. Transcripts significantly up- and down-regulated are indicated in red and green, respectively. The scale bars display fold changes. CHO, Carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

### 8.3.3.1. DEGs shared by N-use efficient genotypes at short- and long-term low N-treatment

All the comparisons between genotype pairs performed through MapMan metabolism overview were able to highlight up-regulated genes in N-use efficient genotypes. In particular, in shoot of both AM222 and 67-3, five genes showed the highest log2FC values of which four genes at T0 and T1, and one at T2. Interestingly, many of these genes were in the light-harvesting complex (LHC) and light receptor (at T0 and T1), while an isoform of oxidoreductase activity ferredoxin-NADP leaf chloroplastic (FNR), which the putative protein plays a key role in regulating the relative amount of cyclic and non-cyclic electron flow, was strongly expressed at T2 in the N-use efficient genotypes. By contrast, the same comparisons were not able to point out any shared DEGs in root in the mapped transcripts of function "metabolism overview". The analysis of significant differential expressed genes in shoot were then described. Light-harvesting complex (LHC) genes showed higher expression levels at all the sampling times (from T0 to T2) in both AM222 and 67-3 compared to the N-use inefficient genotypes, except at T2 where the inefficient ones showed an increased trend in gene expression (Figure 37). Ferredoxin-NADP leaf chloroplastic gene (FNR) showed a higher expression level in AM222 compared to AM22, mainly at T2, while 67-3 showed higher level of transcripts at all the sampling times (Figure 38).

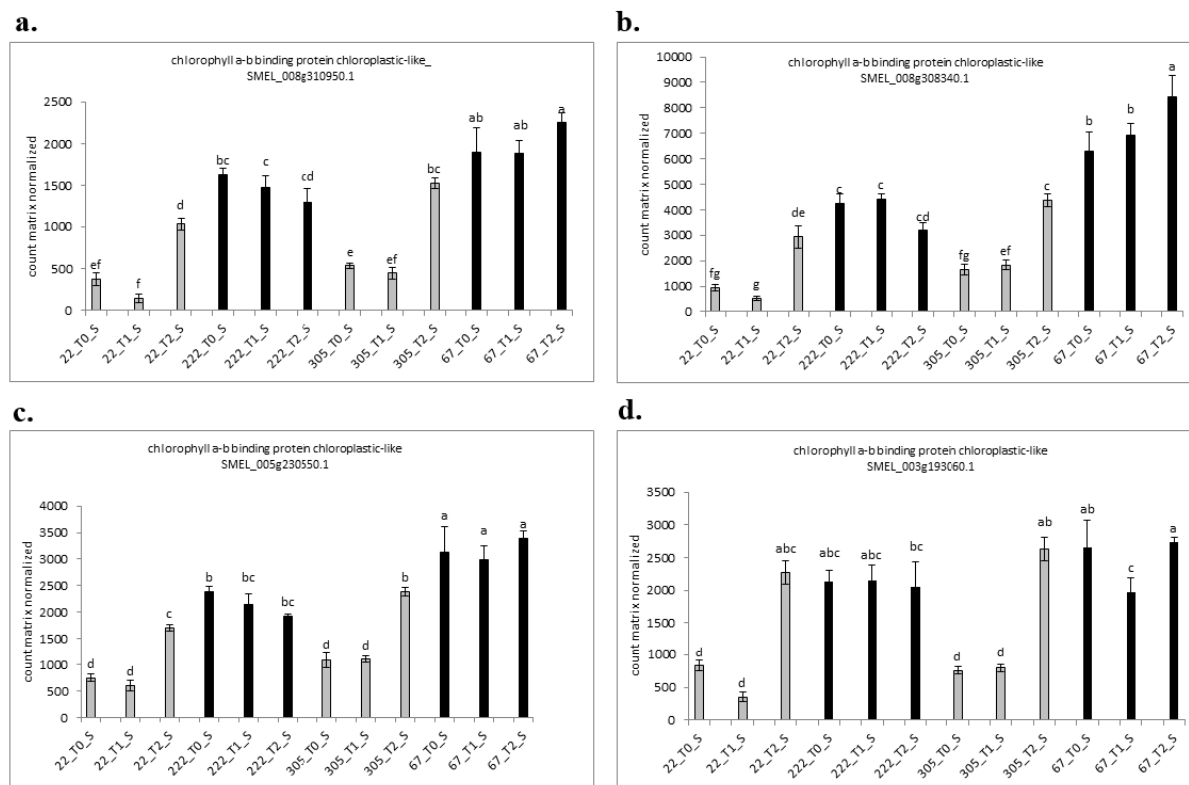


**Figure 37.** Gene expression trends of putative light-harvesting chlorophyll a/b-binding (LHCB) in N-use efficient (black) and inefficient (grey) genotypes; a. SMEL\_008g310950.1; b. SMEL\_008g308340.1; c. SMEL\_005g230550.1; d. SMEL\_003g193060.1.

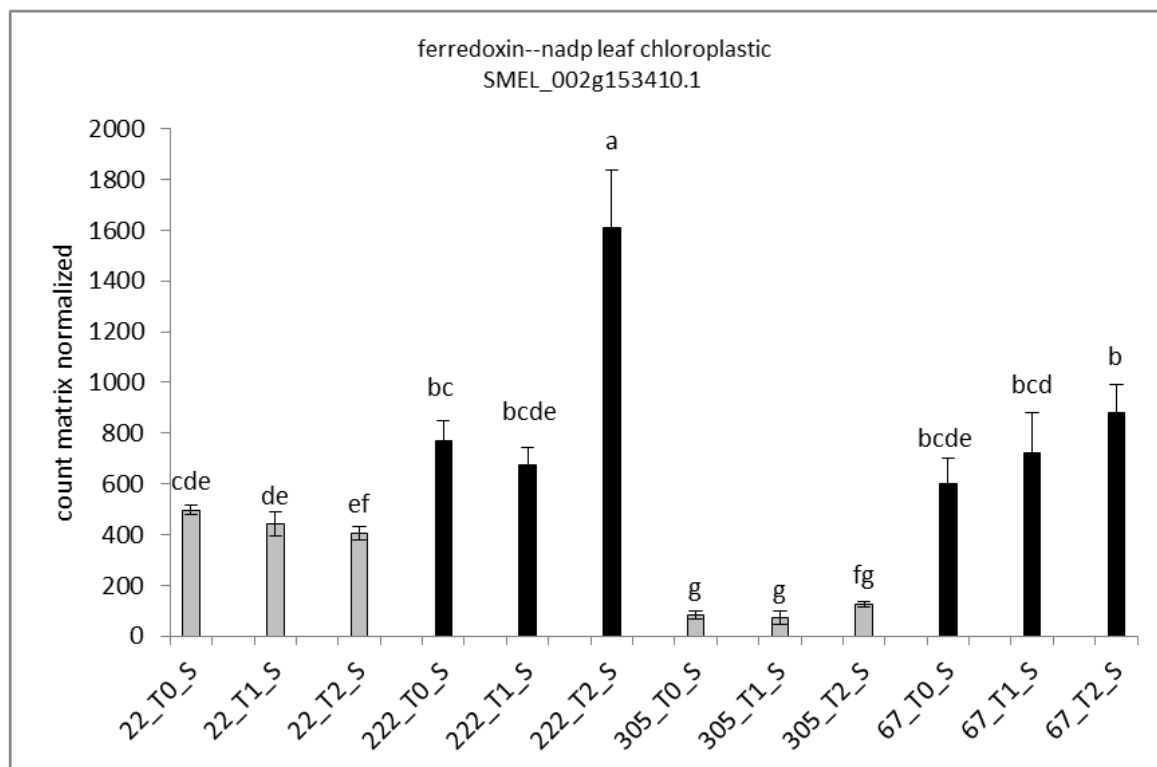


**Figure 38.** Gene expression trend of putative SMEL\_002g153410.1 ferredoxin-NADP reductase, leaf isozyme chloroplast (FNR) in N-use efficient (black) and inefficient (grey) genotypes.

#### 8.3.4. Ontology classification of N-deficiency-responsive genes

A GO Enrichment analysis was also performed to highlight the informative genes not already mapped in the previous analyses. The terms Gene Ontology (GO) define and classify genes and their products, according to the corresponding biological processes, cellular components and molecular functions.

Among the comparisons, more attention was paid to the analysis of DEGs enrichment between all the other genotypes against AM22 (considered the most N-use inefficient genotype), focusing on the main GO categories such as *cellular components*, *molecular functions* and *biological processes*. In the *cellular components*, significant changes in transcript levels of genes involved in carbon metabolism as well as cell wall were observed in many comparisons between genotypes. In *molecular functions*, the most interesting variations were observed in post-translational modifications of farnesyltransferase.

Thus, we focused on the category *biological process* (Figure 39), mainly in the enriched genes involved in the subcategories “GO:0010035 response to inorganic substances”, “GO:0009628 response to abiotic stimulus”, “GO: 0009765 photosynthesis light harvesting” and “GO:

0006995 cellular response to nitrogen starvation”. All the possible relationships (Venn diagram, Figure 40) between differentially expressed genes in eggplant involved in responses to inorganic substances were shown to classify the shared up-regulated genes among three genotypes (AM222, 67-3, 305E40), and between N-use efficient genotypes (AM222 and 67-3).

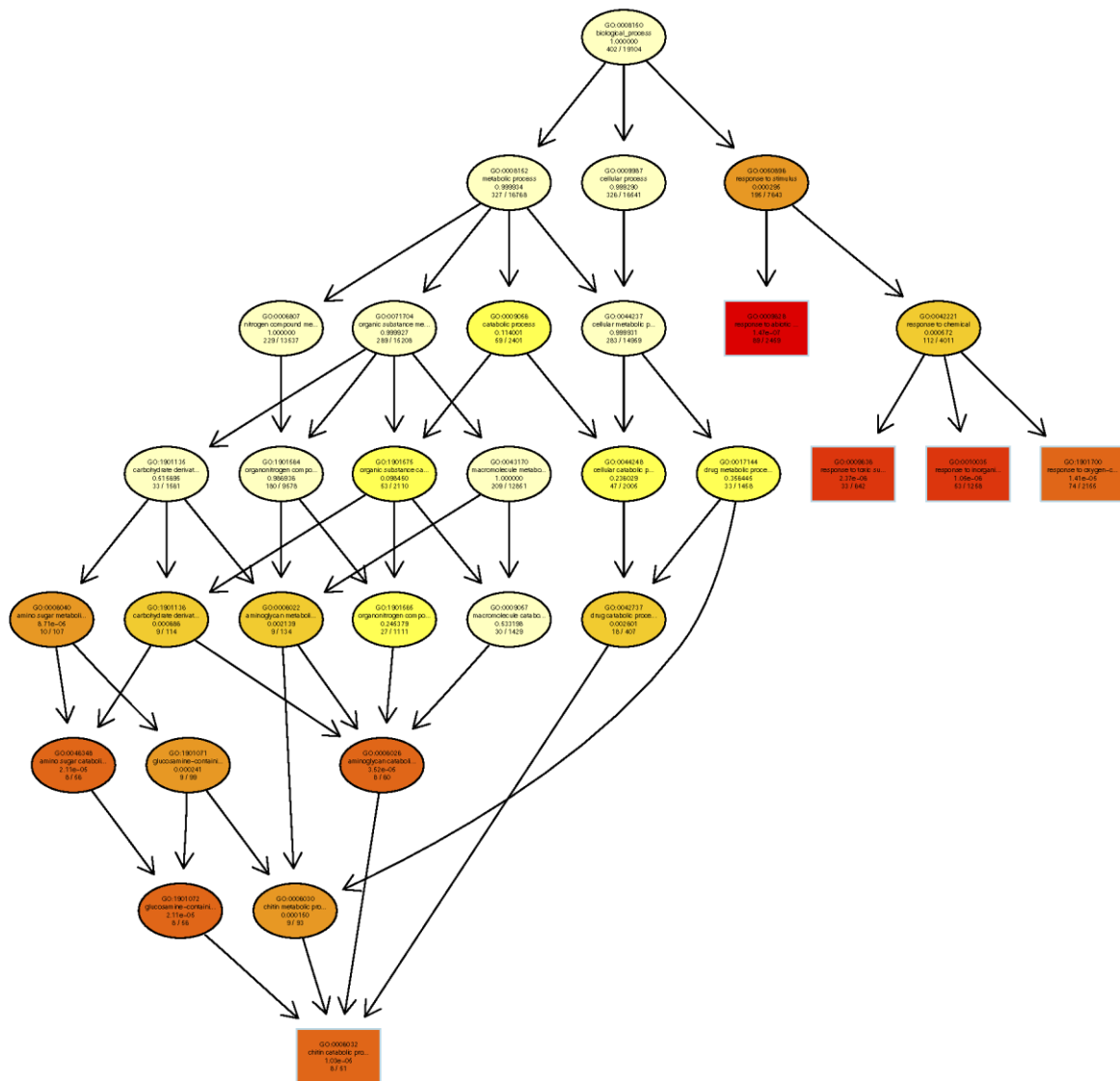


**Figure 39.** One of eighteen directed acyclic graph (DAG) generated from topGO. Taken from the gene ontology (GO) of AM222 vs. AM22 in T1 root. Significant GO terms ( $p \leq 0.05$ ) are marked with color and non-significant GO terms are shown in white boxes. The degree of color saturation of a box is positively correlated to the enrichment level of the term. Solid, dashed, and dotted lines represent two, one and zero enriched terms at both ends connected by the line, respectively.

#### *8.2.4.1. Identification of inorganic substances responsive genes in root*

At N-starvation (T0), in “GO:0010035 response to inorganic substances” all three genotypes (AM222, 67-3 and 305E40) shared 18 genes highly expressed of which 9 appeared involved in N-uptake and metabolism (up-regulated compared to N-use inefficient genotype AM22). Fourteen out of 18 genes were shared by AM222 and 67-3, including orthologue stress-response genes such as chaperonins (heat shock protein) (Figure 40.a).

After 24h of low N exposure (T1), 14 shared genes between AM222 and 67-3 were up-regulated compared to AM22, nine of which were related to multiple stress response (chaperonine heat shock protein and plastid-lipid-associated chloroplastic-like) and a catalase (SMEL\_005g238230.1) in the subcategory “GO:0010035 response to inorganic substances” (Figure 40.b).

Interestingly, among 20 exclusive genes in AM222 compared to AM22 an aquaporin pip2-7-like, sucrose synthase, chitinase-like protein 1-like, cellulose synthase DNA binding isoform 1, NADH-dependent glutamate synthase family protein was included, while among 48 up-regulated genes only in 67-3, twenty-four appeared related to plant stress responses involved in protein folding, dehydration or cell division processes.

Finally, at long-term exposure to low N (T2), in “GO:0010035 response to inorganic substances” the GO enrichment analysis showed a reduced number of shared up-regulated genes among three genotypes (4), while interestingly appeared higher between the N-use efficient genotypes AM222 and 67-3 (21) compared to AM22 (Figure 40.c). Among these last genes, a zinc finger protein zat10-like, a putative 33-like isoform WRKY transcription factor (TF), as well as several calcium binding and responsive to drought and dehydration genes were included.

#### *8.3.4.2. Identification of different GO subcategories responsive genes in shoot*

GO-terms enrichment analysis in shoot highlighted an enrichment for "GO: 0010035 response to inorganic substance" in N-use efficient genotypes (AM222 and 67-3) at T0, while 305E40 did not show enrichment in this GO term and was excluded from the comparison. Nine shared genes between AM222 and 67-3 were identified among which orthologues of nitrite reductase and heat shock 70 kda protein (HSC) (Figure 40.d). GO enrichment analysis showed also the term “GO: 0019755 one-carbon compound transport” altered only in 305E40 compared to AM22, which included putative aquaporins, ammonium and urea transporters genes (data not shown).

At short-term N-stress (T1), interestingly all three genotypes shared a significant alteration in biological process subcategory "GO:0009628 response to abiotic stimulus” compared to AM22

as early response. Among these genes, ortholog genes belonging to light harvesting chlorophyll a/b binding proteins (LHCB) were found (Figure 40.e). Finally, long-term exposure to low N (T2) caused a sharp variation in GO terms in all the genotypes, showing a very interesting enrichment for “GO:0006995 cellular response to nitrogen starvation” including up-regulated shared genes (2) putative involved in N nutrition, of which the catalase SMEL\_005g238230.1 was identified (Figure 40.f).

#### *8.3.4.3. Flag genes expression analysis predicting high NUE in eggplant*

This analysis allowed us to isolate the high expressed predictive genes involved in high N-use efficiency and shared between AM222 and 67-3, previously characterized for high NUE.

Interestingly, the analysis indicated that both genes SMEL\_005g238230.1 (catalase) and SMEL\_006g257490.1 (WRKY, transcription factor 33-like) showed a significant increased expression during time course (from T0 to T2) in N-use efficient genotype pair and an opposite trend in the inefficient pair (AM22 and 305E40) (Figures 41 and 42). BLAST (Basic Local Alignment Search Tool) analysis designed to identify sequence local regions similarity for query SMEL\_005g238230.1 and SMEL\_006g257490.1, showing a strong similarity respectively to a catalase and a WRKY33 transcription factor.

A catalase generally provides a cells protection from the toxic effects of hydrogen peroxide, while WRKY transcription factor (TF) family are reported to be transcriptionally regulated by signaling networks with modulating activity of complex plant processes. Indeed a single TF regulates several different processes being suppressor and activator (Hsieh et al., 2002; Jiang et al., 2009).

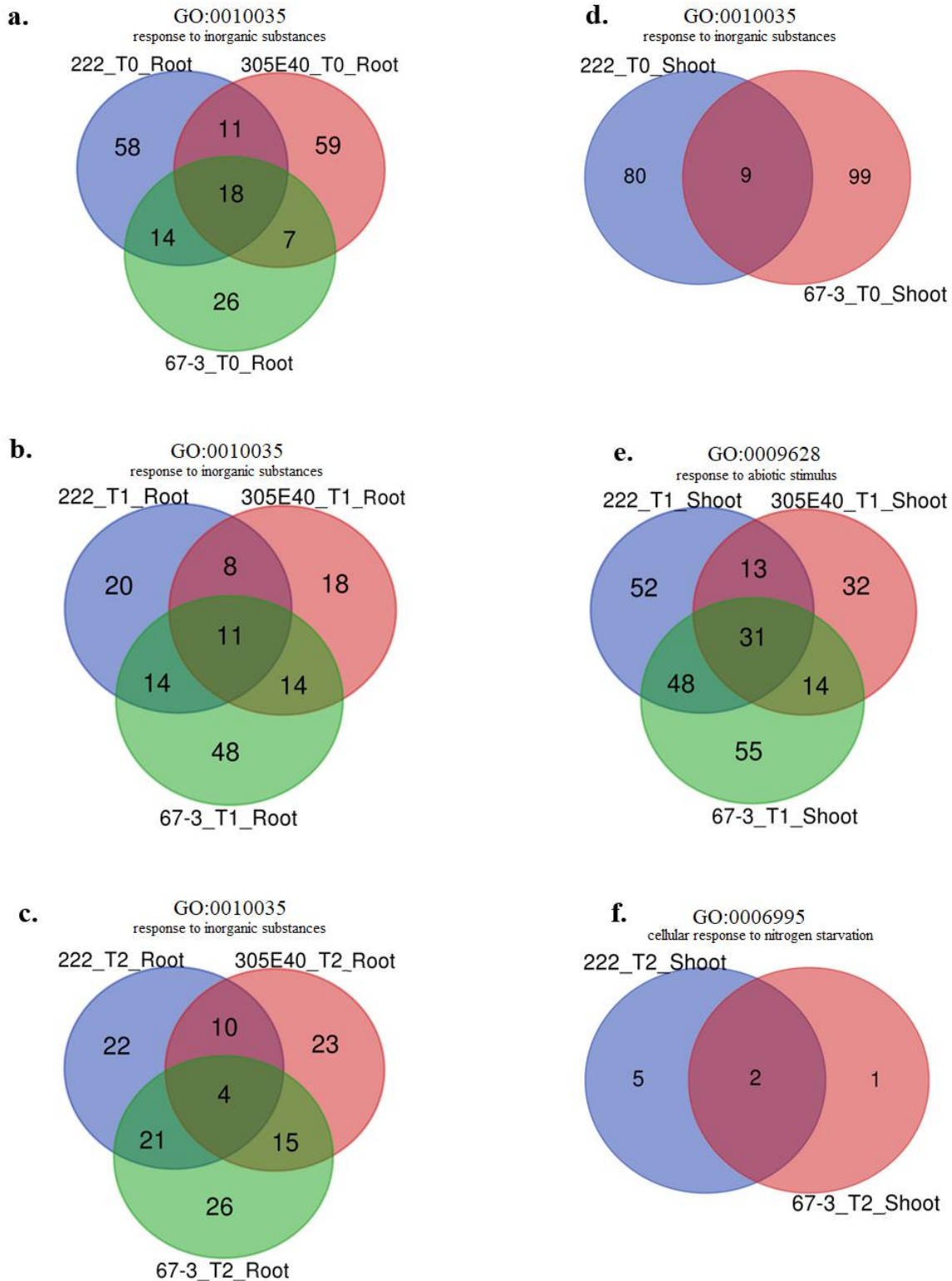


**Figure 40.** Venn diagram that shows all possible logical relationships between and among genotypes in GO terms. A larger number of DEGs is shared between pair “AM222 and 67-3” than “AM222 and 305E40”.

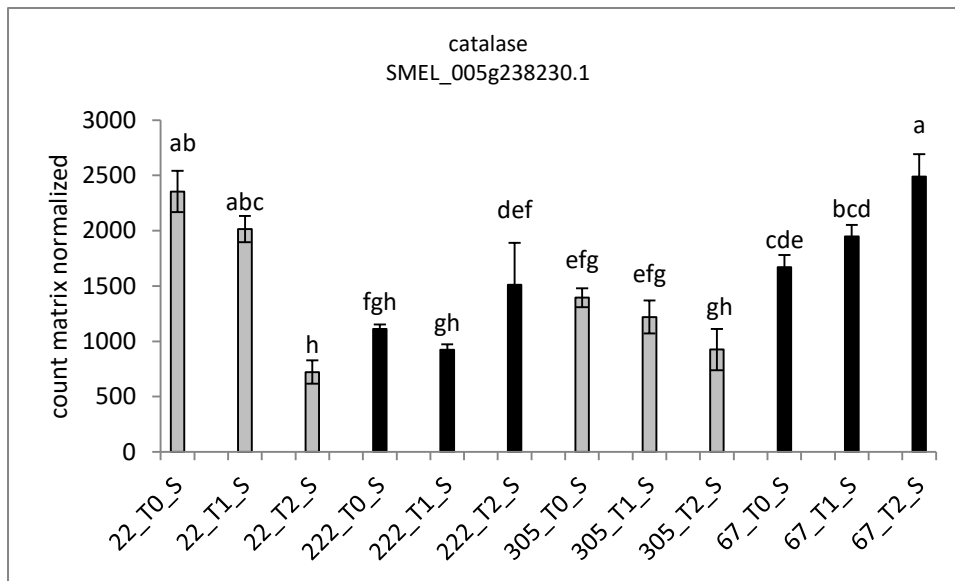


**Figure 41.** Putative catalase gene expression showed an increasing expression time course in N-use efficient genotype pair (black) and an opposite trend in the inefficient pair (grey).

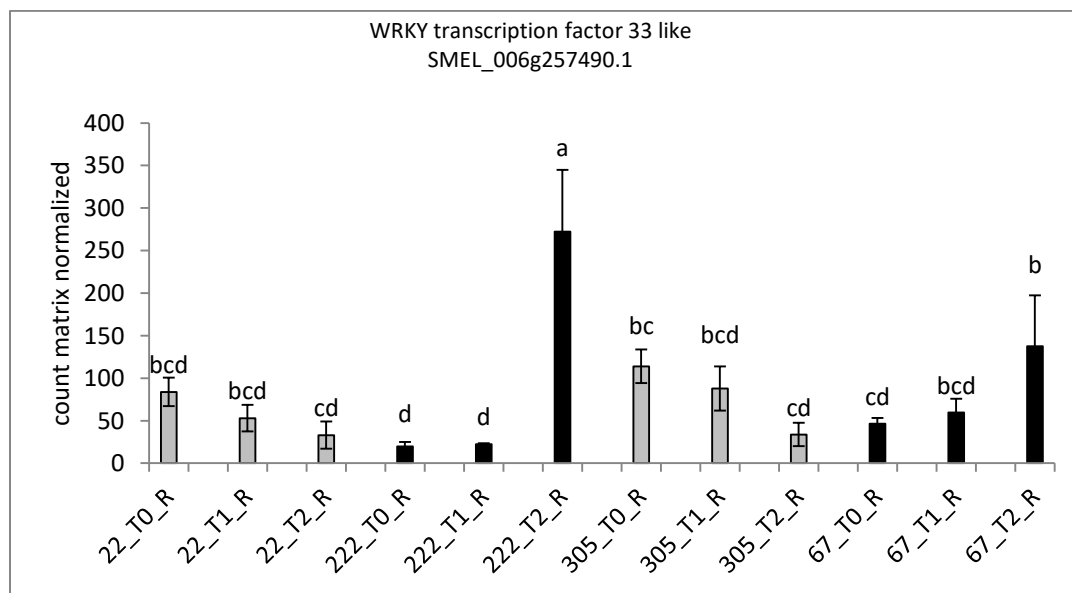


**Figure 42.** Gene expression of putative WRKY33 transcription factor showed an increasing time course expression in N-use efficient genotype pair (black) and an opposite trend in the inefficient pair (grey).

#### 8.3.4.4. Gene co-expression network

CoExpNetViz was adopted to define four independent gene co-expression networks (GCN). The putative light-harvesting chlorophyll a/b-binding (LHCB) (SMEL\_008g310950.1, SMEL\_008g308340.1, SMEL\_005g230550.1, SMEL\_003g193060.1); a ferredoxin -NADP reductase leaf isozyme chloroplastic (SMEL\_002g153410.1); a putative catalase

(SMEL\_005g238230.1) and a putative ortholog of WRKY33 transcription factor (SMEL\_006g257490.1) as input “bait genes”, at T1 were selected.

Alternatively, as non-bait (or target) genes, a matrix count reads normalization for all the genotypes and times sampling were adopted. Gene co-expression networks are able to identify correlations between or among genes resulting active/up-regulated, at the same time and in the same biological process, in one or more genotypes. Based on Pearson's correlation, the relationships among genes were calculated and co-expressed genes were identified.

Four independent genes co-expression networks (GCN) starting from the count reads matrices are shown (Figure 43). A bait node represents a gene whose node is white and diamond-shaped, while the familiar nodes are correlated or anti-correlated to the bait by an edge, a green border and a red margin indicates a positive and negative correlation, respectively. A darker color represents a stronger correlation. The network derived from the four baits genes included in the LHCB previously selected. A block of 53 genes related (Table 20) to the four baits was identified (dark green nodes), other genes were related to three, two or be exclusive to the used baits (Figure 43a). The network (GCN) obtained by using SMEL\_002g153410.1 ferredoxin - NADP reductase, leaf isozyme 1 chloroplastic as bait gene is presented in Table 21 and Figure 43b. Genes co-expression network by using as bait gene the catalase SMEL\_005g238230.1, showed 13 anti-related genes (not reported because of PCC values were lower than -0.80) (Figure 43c). Correlation network for the putative WRKY33 transcription factor SMEL\_006g257490.1 showed 62 co-expressed genes, 21 of which with a PCC score greater than 0.80 (Table 22 and Figure 43d).

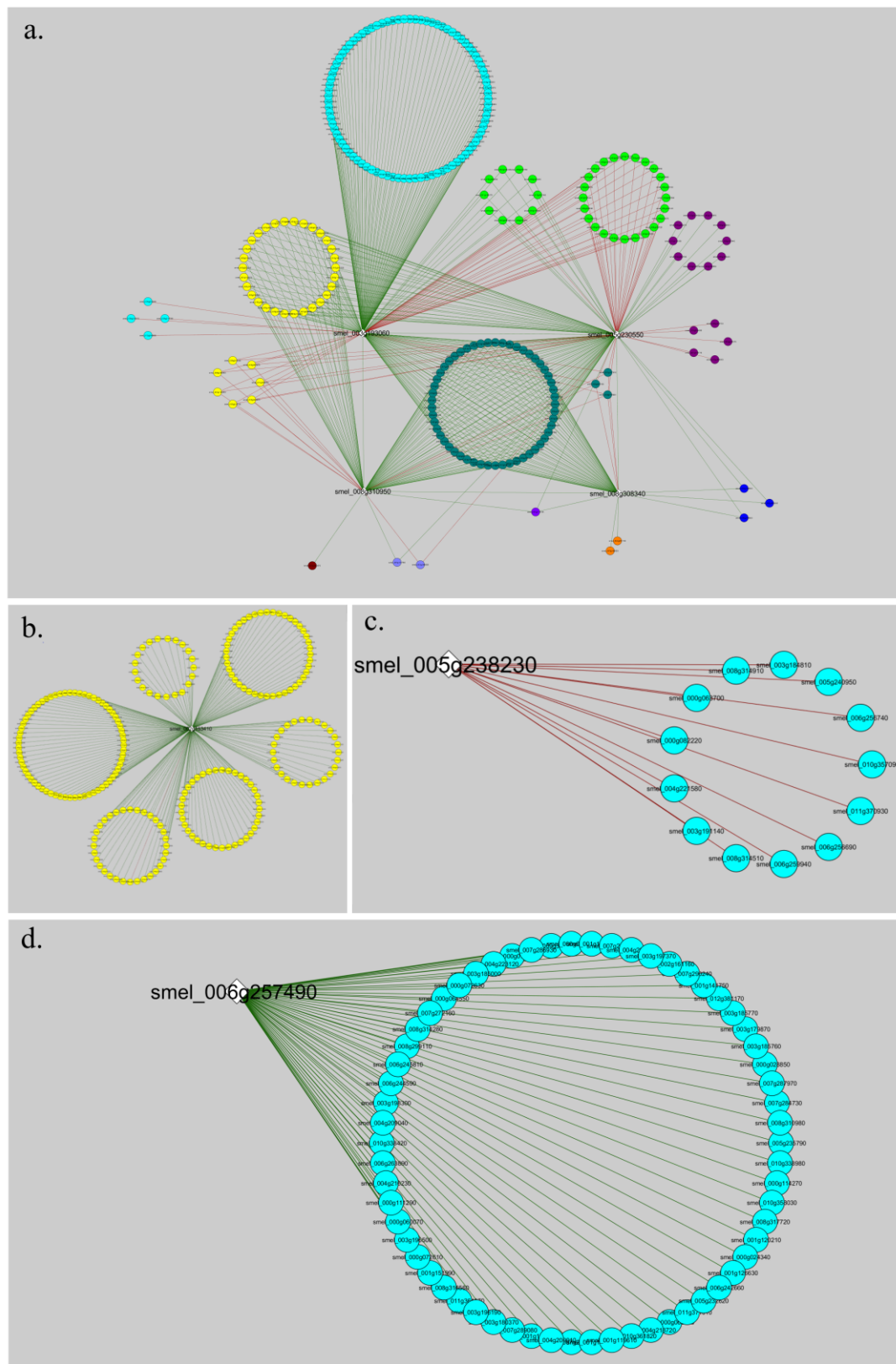


**Figure 43.** Four independent genes co-expression networks (GCN). **a.** Network derived from the four baits genes included in the LHCb; **b.** Network obtained by using as bait gene *SMEI\_002g153410.1* ferredoxin - NADP reductase, leaf isozyme chloroplastic; **c.** network by using as bait gene *SMEI\_005g238230.1*, the catalase; **d.** Network for putative WRKY transcription factor.

| putative gene co-related LHCs                                  | ID                | avg PCC  |
|----------------------------------------------------------------|-------------------|----------|
| photosystem i reaction center subunit iv chloroplastic-like    | smel_006g270050.1 | 0.981971 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_008g308330.1 | 0.978437 |
| photosystem i reaction center subunit chloroplastic-like       | smel_000g071220.1 | 0.976983 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_006g258550.1 | 0.976231 |
| protein curvature thylakoid chloroplastic-like                 | smel_001g129340.1 | 0.969649 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_010g340540.1 | 0.965855 |
| chlorophyll a-b binding protein cp24 chloroplastic-like        | smel_001g121030.1 | 0.96303  |
| chlorophyll a-b binding protein chloroplastic-like             | smel_009g324990.1 | 0.961686 |
| photosystem i reaction center subunit chloroplastic-like       | smel_006g253990.1 | 0.960537 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_004g201560.1 | 0.958704 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_007g287190.1 | 0.953222 |
| photosystem i subunit o                                        | smel_006g266230.1 | 0.950863 |
| protochlorophyllide chloroplastic-like                         | smel_007g290030.1 | 0.950418 |
| coproporphyrinogen-iii chloroplastic-like                      | smel_004g200770.1 | 0.949712 |
| o2 evolving complex 33kd family protein                        | smel_002g166640.1 | 0.947504 |
| uncharacterized loc101214681                                   | smel_003g190650.1 | 0.945128 |
| photosystem i reaction center subunit chloroplastic-like       | smel_006g269390.1 | 0.943611 |
| lipid transfer protein                                         | smel_000g110030.1 | 0.940852 |
| thylakoid lumenal protein chloroplastic-like                   | smel_007g290130.1 | 0.940591 |
| photosystem i reaction center subunit vi- chloroplastic-like   | smel_000g072550.1 | 0.939912 |
| glyceraldehyde-3-phosphate dehydrogenase chloroplastic-like    | smel_004g223250.1 | 0.939286 |
| magnesium-protoporphyrin ix monomethyl ester                   | smel_010g358430.1 | 0.937351 |
| oxygen-evolving enhancer protein chloroplastic-like            | smel_007g285660.1 | 0.937038 |
| membrane family protein                                        | smel_012g381900.1 | 0.935837 |
| photosystem ii 22 kda chloroplastic-like                       | smel_006g256240.1 | 0.931902 |
| photosystem i reaction center subunit iv chloroplastic-like    | smel_000g088030.1 | 0.929767 |
| photosystem i reaction center subunit vi- chloroplastic-like   | smel_003g199020.1 | 0.927532 |
| membrane family protein                                        | smel_012g381920.1 | 0.925608 |
| auxin-responsive family protein                                | smel_009g334990.1 | 0.925408 |
| non-specific lipid-transfer protein 2-like                     | smel_010g354490.1 | 0.924375 |
| ribulose- -bisphosphate carboxylase oxygenase small subunit    | smel_002g159750.1 | 0.923442 |
| magnesium-chelatase subunit chloroplastic-like                 | smel_000g096960.1 | 0.922283 |
| light harvesting-like protein 3                                | smel_008g315800.1 | 0.922052 |
| protoporphyrinogen chloroplastic-like                          | smel_001g136230.1 | 0.921364 |
| o2 evolving complex 33kd family protein                        | smel_002g162100.1 | 0.91907  |
| lysine-rich arabinogalactan protein 19 (LOC107028961), mRNA    | smel_008g315690.1 | 0.918471 |
| glutamyl-trna reductase chloroplastic-like                     | smel_004g217700.1 | 0.917481 |
| chlorophyll a-b binding protein chloroplastic-like             | smel_004g202910.1 | 0.914762 |
| magnesium-protoporphyrin o-methyltransferase-like              | smel_003g195130.1 | 0.912696 |
| thylakoid membrane phosphoprotein 14 chloroplastic-like        | smel_004g200700.1 | 0.912394 |
| dicarboxylate transporter chloroplastic-like                   | smel_012g392360.1 | 0.908269 |
| xyloglucan endotransglucosylase hydrolase                      | smel_000g019860.1 | 0.908252 |
| uncharacterized loc101220016                                   | smel_004g200880.1 | 0.905914 |
| rhodanese cell cycle control phosphatase superfamily isoform 1 | smel_010g346230.1 | 0.905545 |
| 1,4-dihydroxy-2-naphthoyl-CoA synthase, peroxisomal            | smel_003g176670.1 | 0.903715 |
| zeaxanthin epoxidase                                           | smel_002g167380.1 | 0.903485 |
| 2-methyl-6-phytyl- -hydroquinone chloroplastic-like            | smel_003g169560.1 | 0.897481 |
| peptidyl-prolyl cis-trans chloroplastic-like                   | smel_002g164030.1 | 0.895428 |
| gdsI esterase lipase apg-like                                  | smel_003g198480.1 | 0.894764 |
| sodium pyruvate cotransporter chloroplastic-like               | smel_000g053710.1 | 0.890054 |
| low quality protein: uncharacterized partial                   | smel_001g138480.1 | 0.889379 |
| pyrophosphate-energized vacuolar membrane proton pump-like     | smel_007g274000.1 | 0.886776 |
| thioredoxin-like protein chloroplastic-like                    | smel_004g222600.1 | 0.881091 |

**Table 20.** Fifty-three positively related genes to the four baits included in the LHCB.

| putative gene co-related FNR                               | ID                | PCC      |
|------------------------------------------------------------|-------------------|----------|
| nuclear transcription factor y subunit c-                  | smel_001g121580.1 | 0.853605 |
| dna-binding protein smubp-2-like                           | smel_003g186140.1 | 0.825509 |
| probable fructose-bisphosphate aldolase chloroplastic-like | smel_002g158890.1 | 0.821478 |
| auxin-responsive family protein                            | smel_009g334990.1 | 0.80202  |

**Table 21.** Co-expression network using baits ID SMEL\_005g238230.1 for the catalase showed 13 anti-related genes. Because the PCC values were lower than -0.80, they were not taken in to account.

| putative genes co-relate WRKY SMEL_006g257490.1             | ID                | PCC      |
|-------------------------------------------------------------|-------------------|----------|
| af211538_1avr9 cf-9 rapidly elicited protein 180            | smel_000g111290.1 | 0.884535 |
| zinc-finger dna binding protein                             | smel_001g119610.1 | 0.878969 |
| cdp-diacylglycerol--glycerol-3-phosphate 3- partial         | smel_003g180370.1 | 0.860109 |
| mitogen-activated protein kinase 3 (MAPK3)                  | smel_006g245810.1 | 0.855724 |
| gem-like protein 5-like                                     | smel_008g316560.1 | 0.852973 |
| uncharacterized LOC102592249                                | smel_012g381170.1 | 0.851898 |
| uncharacterized LOC102586786                                | smel_011g371510.1 | 0.846697 |
| Yellow-leaf-specific gene 9 (YLS9)                          | smel_010g358030.1 | 0.845609 |
| zinc finger protein zat10-like                              | smel_004g218720.1 | 0.838667 |
| PREDICTED: uncharacterized protein LOC101261845             | smel_001g148360.1 | 0.836447 |
| af211540_1 avr9 cf-9 rapidly elicited protein 75            | smel_011g364570.1 | 0.832105 |
| stress induced protein                                      | smel_007g273570.1 | 0.831041 |
| probable serine threonine-protein kinase chloroplastic-like | smel_004g223120.1 | 0.823134 |
| auxin-responsive family protein                             | smel_007g286930.1 | 0.822581 |
| ethylene-responsive transcription factor 4-like (ERF4)      | smel_007g290540.1 | 0.813443 |
| soybean gene regulated by cold- (SRC2)                      | smel_001g126630.1 | 0.81271  |
| flavonoid 3 -monooxygenase-like                             | smel_003g197370.1 | 0.811116 |
| calcium-binding allergen ole e 8-like                       | smel_003g196190.1 | 0.807826 |
| ethylene-responsive transcription factor 5-like (ERF5)      | smel_003g179870.1 | 0.806611 |
| nudix hydrolase mitochondrial-like (NUDT18)                 | smel_003g196500.1 | 0.803088 |
| respiratory burst oxidase c family protein (RBOHF)          | smel_000g078480.1 | 0.803071 |

**Table 22.** Correlation Network for SMEL\_006g257490.1, a putative WRKY33 transcription factor showed 62 co-expressed genes of which 21 with a PCC score greater than 0.80.

#### 8.4. Discussion

The excess of N-fertilizer applications is already recognized as expensive and environment damaging practice. For this purpose, the selection of high nitrogen use efficiency (NUE) is considered a challenge for maximizing crop yield together with a drastic reduction of N-rate. Understanding mechanisms through which NUE improvement could be reached is nowadays an important plant nutrition research topic.

Many strategies and technological applications have been utilized to dissect the mechanisms underlying a complex trait like NUE. Among the omics technologies, transcriptomics is able to describe a broad account of which cellular processes are up-and down regulated. A major challenge in molecular biology throughout transcriptomics lies in understanding how the same genome can give rise to different cell types and how gene expression is regulated. Thus, this approach could be essential for understanding the complexity of N-responses at the molecular level and predicting the physiological crops responses.

Until now, transcriptomic profiling for dissecting significant NUE differences among genotypes of the same species was carried out in few crops, contributing to understand the complexity of crop N-response. Therefore, many novel transcripts identified from these studies appeared to play an important role in improving plant NUE. A first genome-wide analysis in soybean varieties was capable to identify differentially expressed genes (DEG) at low N supply (Hao et al., 2011). Two maize inbred lines differing in NUE were analyzed for transcriptional responses to different nitrate treatments using a microarray approach (Zamboni et al., 2014). Two other transcriptional profiling studies has been conducted in root tissues of different sorghum and barley genotypes under low N-stress (Gelli et al., 2014; Quan et al., 2016). All these studies laid a basis for clarifying the complexity of plant nitrate regulatory network at the transcriptional level, and the software development able to analyze large biological data in a comprehensive manner through network analysis has become a key approach (Mukhtar et al., 2011).

The present comparative study was aimed at understanding the short- and long-term responses to low N of four contrasting NUE eggplants to identify differential transcripts shared by N-use efficient genotypes compared to inefficient ones.

A distinct transcriptional profile was showed in both N-use efficient genotypes (AM222 and 67-3) compared to the inefficient ones (AM22 and 305E40) along time sampling. In particular, light-harvesting complexes (LHC, localized generally in chloroplast thylakoid membrane) appeared constitutionally higher expressed in AM222 and 67-3, and this trend was confirmed at short- and long-term N-stress (T1 and T2, respectively). These complexes were involved in the light capture, the energy transduction (for photosynthetic reactions) and the reactive oxygen

species (ROS) production, thus we hypothesized a key role in different photosynthetic capacity rate among genotypes (Tanaka et al., 2001; Klimmek et al., 2006). Interestingly, after long-term N-stress (15 days of low N treatment after starvation, T2), a significant up-regulation of ferredoxin-NADP oxidoreductase (FNR) was also observed in the N-use efficient genotypes. The FNR, a thylakoid membrane protein, reduces by oxidation the final product of the photosynthetic electron transport (PET) chain, playing a role in cyclic transport of electrons, positively related to  $\text{NO}_3^-$  assimilation. Indeed, nitrite generated in the cytosol is imported into the chloroplasts and reduced by Fd-dependent NiR to ammonium to sustain high nitrogen use efficiency, which in turn is fixed by the GOGAT Fd-dependent (Hoff, 1994; Sugiura et al., 2007). Herein, we assumed that FNR would contribute in N-use efficient genotypes to both lower energy cost in nitrate assimilation and oxidative stress response (Hanke et al., 2005; Lintala et al., 2007). Plant cells produced hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), mainly during photosynthesis, photorespiration, recognized both as a reactive, and toxic molecular species capable of causing damage to various cellular structures and as a signalling molecule in transduction networks for abiotic stimuli (Willekens et al., 1997; Xing et al., 2007, 2008; Li et al., 2015). Similarly, oxidative stress due to N starvation in plant chloroplast cells results in  $\text{H}_2\text{O}_2$  production from different pathways, by the reduction of  $\text{O}_2$  throughout the Photosynthetic Electron Transport chain (PET) (Pal-Nath et al., 2017; Wang et al., 2017; Chokshi et al., 2017). Thus, we supposed a correlation between higher expressed FNR and nitrate assimilation showed in the eggplant N-use efficient genotypes due to a high NUtE component (Mauceri et al., *JIPB* submitted).

The higher shoot catalase expression, enzyme involved in cell protection against toxic forms of ROS, in the N-use efficient genotypes compared to the inefficient ones confirmed our hypothesis. The results suggested the higher ability of the efficient genotypes to detoxify plant from ROS production, essential for abiotic stress protection in N-stress condition, as previously reported in salt tolerant rice genotypes (Vighi et al., 2016).

Finally, a high significant up-regulation of a transcription factor (TF) belong to the WRKY family gene was also identified in root of the N-use efficient genotypes, at T2. WRKY transcription factor family plays a multitude of roles in plant response to stress, regulating normal growth and development and response to stimuli in plants (Jiang et al., 2017), conferring tolerance to some abiotic stresses (also to  $\text{H}_2\text{O}_2$ ) as recently widely reported (Zheng et al., 2006; Song et al., 2009; Jiang and Yu, 2009; Zou et al., 2010). Furthermore, the analysis of cohesion network revealed the concurrent expression of the WRKY TF with several other genes that could interact in the same specific pathway.

The co-expression network analysis using WRKY as bait allowed us to identify other up-regulated TFs and genes correlated to plant stress responses as previously presented and discussed. Among them, we singled out transcript factors, kinase proteins and other genes that mimicked WRKY expression trend (fig. 42).

Notably, several co-expressed up-regulated transcripts belong to the Mitogen-Activated Protein Kinase (MAPK) family, together with a Yellow-leaf-specific gene 9 (YLS9), a Zinc finger protein ZAT10-like, two ethylene-responsive TFs like (ERFs), a Soybean Gene Regulated by Cold (SRC2), a Nudix hydrolase mitochondrial-like (NUDT18) and a Respiratory Burst Oxidase c family protein (RBOHF).

In plants, MAPK cascades are functional modules encoded by multigenic families responsible for modulating biological processes such as stress response, cellular differentiation and organ development. MAPKs play a role in several signaling networks and could have an integrative function for plant responses to environment; in particular, they are up-regulated in response to nutritional supporting and signaling as recently highlighted (Chardin et al., 2017). Indeed, MAPK6 was able to phosphorylate nitrate reductase NADH2 (NR2) in *Arabidopsis*, highlighting also NR2 role in nitric oxide (NO) biosynthesis and it also could directly regulate nitrate assimilation (Wang et al., 2010).

The results indicated that YLS9 was up-regulated in N-use efficient genotype (AM222) compared to the inefficient ones (AM22). Remarkably, YLS9 was reported to play a role in N remobilization and recycling, important to plants adaptation and survival in unfavourable environmental conditions (Yoshida et al., 2001), suggesting a higher N remobilization ability in the efficient genotypes (Mauceri et al., *JIPB* submitted). This higher ability could be related to leaf senescence, during which macromolecules such as chlorophyll, protein, lipid and nucleic acids are degraded in products that may be translocated to younger leaves or reproductive organs for *de novo* synthesis of cellular components (Gan and Amasino, 1997).

Plant stress responses and related signal transduction pathways appear also under the control of ERF transcription factor family. In particular, ERF5 and ERF6 seem to activate other stress-related TF like WRKY33 and MYB51 under water-limiting condition in *Arabidopsis* (Dubois et al., 2013). The same role is played by zinc finger protein ZAT10 modulating plant abiotic stress responses and the reactive oxygen-defence (Mittler et al., 2006; Sewelam et al., 2013). Consistently, an ERF4 and ERF5 together with a zat10-like protein are co-upregulated in AM222 induced by the eggplant WRKY33.

Finally, Absciscic Acid (ABA) controls cellular processes like regulation of seed dormancy, seed maturation and vegetative growth during plant development, but also a protective role in

response to abiotic stresses including drought, salinity and cold (Finkelstein et al., 2002). Interestingly, the up-regulated SRC2 gene in AM222 was reported as  $\text{Ca}^{++}$ -dependent activator of NADPH oxidase catalytic genes, RBOHF, which in turn function in ABA-induced ROS production in guard cells, demonstrating that ROS production is rate-limiting for ABA signal transduction (Kwak et al., 2010).

In conclusion, transcriptomics analysis in NUE-contrasting eggplant genotypes highlighted the central role of some TFs family, which are up-regulated in AM222 and 67-3 N-use efficient genotypes compared to the inefficient ones (AM22). One transcription factor belonging to WRKY family and involved in plant stress responses, showed a significant up-regulation in AM222 compared to AM22 (*8-fold* higher expression). Interestingly, WRKY33 triggered a correlated higher expression of 21 genes and other TF families, many of which closed related to N-assimilation and remobilization, plant abiotic stress stimuli and ROS rate-limiting production.

## CHAPTER 9. SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

### 9.1. Genetic variation for N-use efficiency in eggplant

The identification of genotypes with improved Nitrogen-Use-Efficiency (NUE), i.e. more able to uptake, utilize and remobilize N available in soils, could be an important challenge to maintain high crop yields in a sustainable agriculture. Eggplant yield is highly dependent to N-fertilizers, and until today, its adaptive responses to N-limited fertilization are limited, and the genetic mechanisms underlying a complex trait such as N-use efficiency are still unknown.

In the PhD thesis, for the first time, genetic variation in response to nitrate supply was evaluated in eggplant (*Solanum melongena* L.) to select NUE-contrasting genotypes and to identify physiological and molecular flag traits which confer high NUE value.

For this aim, nineteen eggplant accessions, representing varied geographic origins and morphological traits a large germplasm collection of 250 accessions, were firstly screened in a hydroponic system at low and high nitrate levels (LN and HN). Two eggplants, AM222 and AM22, were identified as N-efficient and inefficient genotypes, respectively. The selected genotypes were then grown in the greenhouse, in pot experiments, together with 67-3 and 305E40 accessions from parents of a RIL segregant population recently developed from a F<sub>2</sub> population, and crop yield was evaluated. The results confirmed also in pot experiments AM222 as the most efficient genotype, based on N fruit content, NUE and NUtE component. Interestingly, similar performance was observed in 67-3 that can be considered as N-use efficient genotype, while AM22 and 305E40 were considered as N-use inefficient one. The difference in fruit production may be determined by different N translocation and remobilization, which strongly affected NUtE. Interestingly, AM222 exhibited higher GS activity and genes related expression, which might be responsible for high NUtE.

Overall, our results indicated the key role of N-utilization component (NUtE) more than the N-uptake efficiency (NUpE) to confer high NUE in eggplant. The remobilization of N from leaves to fruits may be a strategy to enhance NUtE, suggesting GS as a key enzyme. Thus, NUtE, fruit dry weight and GS could be considered the best flag traits to identify rapidly high NUE eggplant genotypes.

Furthermore, a high heritability ( $h^2_B$ ) and G×E interaction of some target traits, such as LRL, RF and NUtE, could suggest these traits useful to high selection gain.

To deeply understanding the genetic mechanism underlying this complex trait, the four selected eggplant genotypes (AM222, AM22, 67-3 and 305E40) were employed for both transcriptomics

and metabolomics approach. In particular, taking into account both short- and long-term N-stress, the same experimental design for -omics techniques was adopted, focusing on N metabolism networks in order to identify differential gene transcripts and metabolites able to explain the NUE difference in both N-contrasting genotype pairs. These results are the object of a manuscript already submitted to Journal of Integrative Plant Biology.

## **9.2. Molecular cloning, functional assay and allelic variant analysis of N-metabolism related genes**

In this study, the assessment of gene expression of the main high affinity nitrate transporter *SmNRT2.1*, together with *SmNRT3.1* and *SmNRT3.2*, encoding for its protein partner in NUE-contrasting eggplants was carried out. A first functional analysis in eggplant root, showed an up- and down-regulation of these genes at low and high N (LN and HN). A specific assay was also set up to demonstrate the proteins interaction between *SmNRT2.1* and both members of NRT3 family. *SmNRT2.1*, *SmNRT3.1* and *SmNRT3.2* transcript levels showed different pattern of expression induced by low and high N among genotypes. As expected for their involvement in HATS functionality, at LN, *SmNRT2.1* showed a higher transcript level compared to HN in all genotypes, the same trend was observed for *SmNRT3.1*, except in 67-3 genotype, where the expression level resulted rather undetectable at both N levels. Interestingly, the opposite expression pattern was detected for *SmNRT3.2* which appeared higher expressed at HN, again except 67-3 genotype that showed a significant higher gene expression at LN. Thus, 67-3 genotype seemed impaired in *SmNRT3.1* compared to other genotypes and, as previously reported in other species.

Thus, a functional assay as Bimolecular Fluorescence Complementation (BiFC) was set up to visualize the protein-protein interaction in living cells (*Arabidopsis* mesophyll protoplasts) showing similar pattern of YFP fluorescence for both *SmNRT2.1*-*SmNRT3.1* and *SmNRT2.1*-*SmNRT3.2* pair protein complexes which suggest that *SmNRT2.1* protein is able to interact in vivo with both *SmNRT3.1* and *SmNRT3.2*. Interestingly, the fluorescence appeared located in different areas of the plant cell suggesting that protein complexes localization should be deepened understood.

Here, we demonstrated, for the first time, in eggplant the protein-protein interaction *SmNRT2.1*-*SmNRT3.1*, as firstly reported in *Arabidopsis* (Okamoto et al., 2006). The protein-protein interaction *SmNRT2.1*-*SmNRT3.2* never described before opened a new scenario since *NRT3.2* was previously described as responsive to biotic stress so that interesting questions could be addressed about crosstalk between abiotic/biotic stress

responses in eggplant, as already reported in tomato. To analyze the function and the interaction of the main N-transporters in eggplant, *NRT2.1*, *NRT3.1* and *NRT3.2* were cloned to construct cassettes of expression for *Agrobacterium*-mediated eggplant genetic transformation to over-express or silencing genes of interested. Stable genetic transformation experiments, for both overexpressing and silencing gene targets, are actually underway.

### 9.3. Allelic variant analysis of CLCa gene

A natural genetic variation in another N-metabolism related gene, the chloride channel protein (CLCa) gene (carrier protein involved in the storage of nitrate in the vacuole), in position of the CDS 245A in 245G which determines a missense mutation for the change of the TAT/tyrosine codon in TGT/cysteine was identified. The consequence of this variation in the protein is under evaluation, paying attention to characterize and clarify a possible functional role of identified SNP on the function and performance of the transporter. A computer-generated model as a basis for determining the secondary structure of the CLCa vacuolar nitrate transporter. The TMHMM software generated a model in which both N- and C-terminal ends of CLCa protein were inside and outside alternatively in 67-3, while in 305E40 genotype both CLCa N- and C-terminal ends appeared outside protein. Differing numbers of TM domains and orientations were deduced for CLCa by computational analysis and structural analysis of the predicted protein sequence. Two eggplants, 67-3 and 305E40, characterized by a variation in a single amino acid in the CLCa deduced protein sequence allowing us to predict a topology of 9 and 10 TM for 67-3 and 305E40 genotypes, respectively.

Taken together, we could speculate that CLCa protein variants due to a substitution of a tyrosine with a cytosine may have generated some structural changes. To validate these conclusions *in vivo* determination of regions orientation of the hypothetical loop should be necessary in further experiments.

### 9.4. Metabolomics profiling to highlight differential N-related compounds

To get more insight into the effects of N-limited supply on the primary metabolism, root and shoot of NUE contrasting eggplants were analyzed at short- (24h of N-limited supply after N starvation, T1) and long-term (16 days of N-limited supply, T2) treatment through GC-MS untargeted metabolomic to identify differential metabolites among genotypes into the pathways

N-metabolism related. A detailed analysis of pathways and networks affected by N-limitation in both roots and shoots was carried out through the metaboanalyst module “MetPa”.

In particular, the amino acid metabolism, mainly alanine, aspartate and glutamate metabolism appeared the more affected by treatments and noteworthy glycine, serine and threonine metabolism was also highly impacted. These results were in accordance with the previous PhD results (Mauceri et al., *JIPB* submitted) where the most important role of NUtE component in determining high N-use efficiency at low N inputs was demonstrated. Thus, why alanine, aspartate and glutamate pathway could play an important role to confer N-use efficiency to AM222? Glutamine, asparagine, glutamate, and aspartate are the predominant amino acids transported in plants for N-utilization and storage. It is well known that  $\text{NH}_4^+$  primarily originated by nitrate reduction is assimilated through GS/GOGAT cycle. In addition, cytosolic asparagine synthetase (AS) transfers glutamine to a molecule of aspartate to generate glutamate and asparagine. The comparison between AM222 and AM22, contrasting for N-use efficiency at short- and long-term N limited supply, highlighted a significantly different amino acids profiling both in roots and shoots.

Interestingly, L-alanine was the amino acid more expressed in the roots of N-use efficient genotype, that together with 2-oxoglutarate were formed by transferring an amino group from glutamate to pyruvate glutamate *via* the enzyme alanine amino transferase (AlaAT). Recently, *alaAT* overexpression mediated by a tissue specific root promoter was demonstrated to improve N-use efficiency in rice. More recently, the increase of alanine together with arginine in both leaf and root of N-tolerant maize were observed, suggesting genotype ability to store N source under low N.

The amino acid profile in shoots of the efficient AM222 at both T1 and T2 evidenced also a higher level of glutamine. The enzyme involved in its formation is the glutamine synthetase (GS), which has been proposed as a candidate for improving NUE in wheat. The higher level of glutamine in the efficient genotype was consistent with the results of Mauceri et al. (*JIPB* submitted), which observed a higher GS activity in AM222. So that, these results indicated that GS would be an excellent marker for improvement NUE also in eggplants.

## **9.5. Transcriptomics analysis and differential expressed N-related genes**

Based on the evidence discussed above a whole transcriptome analysis was performed on the NUE-contrasting genotypes adopting the same experimental design of metabolomics. The main aim was the detection of differential expressed genes (DEGs) between the efficient and

inefficient genotypes at short- (T1) and long-term (T2) N-limited supply. The main up-regulated genes predictive for eggplant high N-use efficiency was the target of the wide transcriptomics analysis. To this end, we focused on isolating the most interesting shared genes between AM222 and 67-3 previously characterized for high NUE compared to the inefficient one (AM22 and 305E40) along time sampling.

MAPMAN pathway metabolism overview and GO-terms enrichment analysis highlighted changes in “response to inorganic substances”, “light reactions” and “cellular responses to nitrogen starvation”.

Among these categories, genes belonging to the Light Harvesting Complex (LHC), the ferredoxin-NADP<sup>+</sup> reductase (FNR) and catalase in shoot, while in root the putative WRKY transcription factor (TF) were detected.

In particular, LHC (localized generally chloroplast thylakoid membrane) appeared constitutionally higher expressed in AM222 and 67-3, and this behaviour was confirmed at short- and long-term N-stress (T1 and T2, respectively). These complexes were reported for their role in light capture, energy transduction (for photosynthetic reactions) and the production of reactive oxygen species (ROS), thus we hypothesized a key role in different photosynthetic efficiency rate among genotypes.

Interestingly, after long-term N-stress (15 days of low N treatment after starvation, T2), a significant up-regulation of ferredoxin-NADP oxidoreductase (FNR) were also observed in the N-use efficient genotypes. FNR, a thylakoid membrane protein, reduces by oxidation the final product of the photosynthetic electron transport (PET) chain, playing a role in cyclic transport of electrons positively related to NO<sub>3</sub><sup>-</sup> assimilation. Herein, we assumed that FNR would contributed in N-use efficient genotypes to both lower energies cost in nitrate assimilation and oxidative stress response.

Catalases are also involved in cell protection against ROS toxic forms, and the isoform detected in eggplant appeared significantly higher expressed in N-use efficient genotypes compared to the inefficient one. These results provided another confirmation that a higher shoot catalase expression on the efficient genotypes seemed an indicator of detoxifying ability from ROS, essential for abiotic stress protection in those N-stress tolerance genotypes, as previously reported in salt tolerant rice genotypes.

Finally, we also identified in the root of N-use efficient genotypes a high significant up-regulation of a transcription factor (TF) belong to the WRKY family gene. WRKY transcription factor family was reported to play a multitude of roles in the plant response to stress, regulating normal growth and development and response to stimuli in plants (Jiang et al., 2017). The

analysis of cohesion network revealed the concurrent expression of this eggplant WRKY TFs with several other genes that could interact in the same specific pathway. The co-expression network analysis using WRKY as bait allowed us to identify other up-regulated TFs and genes correlated to plant stress responses as previously presented and discussed. Among them, we singled out transcript factors, kinase proteins and other genes that mimicked WRKY expression trend. Notably, several co-expressed up-regulated transcripts belong Mitogen-Activated Protein Kinase (MAPK) family, together with a Yellow-leaf-specific gene 9 (YLS9), a Zinc finger protein ZAT10-like, two ethylene-responsive TFs like (ERFs), a Soybean Gene Regulated by Cold (SRC2), a Nudix hydrolase mitochondrial-like (NUDT18) and a Respiratory Burst Oxidase c family protein (RBOHF).

In particular, MAPK cascades are functional modules encoded by multigenic families responsible for modulating biological processes such as stress response, cellular differentiation and organ development. MAPKs play a role in several signalling networks and could have an integrative function for plant responses to environment, in particular, MAPKs up-regulation in response to nutrients supporting and their involvement in nutritional signalling were recently highlighted (Chardin et al., 2017). Remarkably, YLS9 was recently reported to play a role in N remobilization and recycling, important to plants adaptation and survival in unfavourable environmental conditions. In our system, YLS9 up-regulation could be related to a higher N remobilization ability of N-use efficient genotype (AM222) compared to the inefficient one (AM22) previously observed in greenhouse experiment (Mauceri et al., *JIPB submitted*).

The ERF transcription factor family regulates plant stress responses and related signal transduction pathways. In particular, ERF5 and ERF6 seem to activate other stress-related TF like WRKY33 and MYB51 under water-limiting condition in *Arabidopsis*. The same role is played by zinc finger protein zat10 modulating plant abiotic stress responses and the reactive oxygen-defence. Consistently, an ERF4 and ERF5 together with a zat10-like protein are co-upregulated in AM222 induced by the eggplant WRKY.

Interestingly, the up-regulated SRC2 gene in AM222 was reported as  $\text{Ca}^{++}$ -dependent activator of NADPH oxidase catalytic genes, RBOHF, which in turn function in ABA-induced ROS production in guard cells, demonstrating that ROS production is rate-limiting for ABA signal transduction.

In conclusion, transcriptomics analysis in NUE-contrasting eggplant genotypes highlighted the central role of some TFs family up-regulation in AM222 and 67-3 N-use efficient genotypes compared to the inefficient ones (AM22). One transcription factor WRKY33, belongs to WRKY family, reported to have a multitude of roles in plant stress responses, showed a significant up-

regulation in AM222 compared to AM22 (*8-fold* higher expression). In addition, WRKY33 interestingly triggered a correlated higher expression of 21 genes and other TF families, many of which closed related to N-assimilation and remobilization, plant abiotic stress stimuli and ROS rate-limiting production.

## 9.6. Conclusion and future perspectives

Overall, based on morphological and physiological traits, differential metabolites and genes between two pairs of NUE-contrasting genotypes the main results of this PhD thesis can be summarized:

- 1) Four eggplant genotypes contrasting for NUE were identified;
- 2) The main role of NUtE component in eggplant N-use efficiency was demonstrated;
- 3) The knowledge on morpho-physiological and genetic flag traits in NUE-contrasting eggplants were significantly improved;
- 4) Some key metabolites and genes able to enhance N-metabolism cycle efficiency were identified through an integrated metabolomics and transcriptomics approach;
- 5) Cloning, functional assay and allelic variant analysis of genes involved in N-uptake, transport and assimilation furnished new perspective for analyzing the mechanism underlied NUE-contrasting phenotypes.

For the future perspectives, we believe that these genotypes can be considered the first *breeding lines* useful for the following objectives:

- 1) The analysis of mutants overexpressing N-related genes aiding to improve the knowledge of NUE in eggplant;
- 2) The genotyping and phenotyping of segregant populations for associations/QTL related to low N responses focusing mainly on eQTL;
- 3) The setting up of genomic selection based on whole genome information;
- 4) The setting up genomic prediction models for fruit production and quality, based on the information of the populations evaluated and tested taking into account the impact of low N stress to foster the selection of novel high NUE varieties.

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## List of papers

- 1) **Mauceri A., Bassolino L., Lupini A., Badeck F., Rizza F., Schiavi M., Toppino L., Abenavoli M.R., Rotino G.L., Sunseri F. (2018)** Genetic variation in eggplant (*Solanum melongena* L.) for Nitrogen Use Efficiency (NUE) under contrasting  $\text{NO}_3^-$  supply. *Journal Integrated Plant Biology* (submitted).