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TESI DI LAUREA

The maize *Zmccd8* mutant, impaired in strigolactones synthesis, displays different physiological and molecular responses to nitrogen in field

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ABSTRACT

Never as nowadays, due to the increasing global population and climate changes, which led to the frequent occurrence of extreme events; agriculture need to face urgent challenges to limit the consequences of environmental stress. The trend that's been used lately is to increase the chemicals treatments both to counter biotic stress deriving from the increase in Phyto-parasites or to improve tolerance to abiotic stress, due for example to the lack of nutrients. However, this cannot be the resolution to these adversities because the unrestrained use of chemicals already led to problems such as the rise of residues levels in agriculture products and the water eutrophication; that are not no longer sustainable. The improvement of crops tolerance to low nutrient levels is crucial to reduce the use of chemical fertilizers. One approach could be studying how the major crops sense, uptake and use the essential nutrients. Nitrogen (N) is and one of the most important macronutrients for plants, this explains both why it is even the most used as fertilizer and why it is so important to maximize the Nitrogen Use Efficiency (NUE).

Among the most productive crops we can for sure find maize (*Zea mays* L.) which provides a large amount of food as well as feed for the livestock; so it is very interesting to understand how it absorbs and assimilates nitrogen. It is known that the main forms absorbed by maize are nitrate (NO_3^-) or ammonium (NH_4^+), these molecules are not just used as nutrients but also function as -signals. In recent studies it is been underlined how the perception of nitrate is mostly achieved through the transition zone (TZ) of maize root and seems to involve nitric oxide (NO), reactive oxygen species (ROS) homeostasis, and hormonal (auxin and strigolactones) signalling. The role of Strigolactones (SLs), should be investigated further, since it is still not very clear how these carotenoid-derived phytohormones work, despite being heavily involved especially in abiotic stress response, acting both as endogenous and exogenous signalling molecules.

To understand the role played by SLs in the response to N starvation, we assessed the phenotypical, physiological and transcriptional responses of the wild type *Zea mays* L. inbred line B73 and compared them with those observed for plants of the *ZmCCD8*

knockout mutant. Both genotypes have been grown in field and both were tested in condition of urea fertilization and without urea supply. *ZmCCD8* is characterized by a knockout mutation of the *CCD8* gene which encodes the CAROTENOID CLEAVAGE DIOXYGENASE8 that is necessary for the biosynthesis of SLs. For the physiological essay the DUALEX SCIENTIFIC+™ was used weekly to assess levels of chlorophyll (CHL), anthocyanins (ANTH), flavonoids (FLAV) and NBI value (Nitrogen Balance Index). The plant growth has also been assessed in correspondence of the same time-points by measuring the heights, internodes length, stems circumferences, leaves number and length.

Regarding the transcriptional responses, at selected time-points total RNA was extracted from leaves of both genotypes and both treatments and the expression of previously selected genes was assessed. Our results confirmed the crucial involvement of SLs in the adaptation of maize to low nitrogen and allowed to identify few key molecular components underlying their action and potentially suitable to be used to improve the maize tolerance to nitrogen shortage.

Riassunto

Mai come oggi, a causa dell'aumento della popolazione mondiale e dei cambiamenti climatici, che hanno provocato il frequente verificarsi di eventi estremi; l'agricoltura deve affrontare sfide urgenti per contenere le conseguenze degli stress ambientali. La strategia adottata ultimamente è quella di aumentare i trattamenti chimici sia per poter contrastare lo stress biotico derivato dall'aumento dei fitoparassiti sia per migliorare la tolleranza agli stress abiotici, conseguenti, per esempio, alla carenza di nutrienti. Tuttavia, questa non può essere la soluzione a queste avversità, poiché l'uso eccessivo di sostanze chimiche ha già causato problemi come l'aumento dei livelli di residui nei prodotti agroalimentari e l'eutrofizzazione delle acque; che non sono più sostenibili. Per ridurre l'uso di fertilizzanti chimici è fondamentale migliorare la tolleranza delle colture alla scarsità dei nutrienti. Per questo scopo, un approccio potrebbe essere quello di studiare come le piante coltivate percepiscono, assorbono e utilizzano i nutrienti essenziali. L'azoto (N) è uno dei macronutrienti più importanti per le piante, questo spiega sia perché è il più utilizzato come fertilizzante, sia perché è così importante massimizzare l'efficienza d'uso dell'azoto (NUE).

Tra le colture più produttive troviamo sicuramente il mais (*Zea mays* L.) che fornisce una grande quantità di cibo oltre che di mangime per il bestiame; è quindi molto interessante capire come assorba ed assimili l'azoto. Nitrato (NO_3^-) ed ammonio (NH_4^+) sono le principali forme azotate assorbite dal mais, queste molecole non vengono utilizzate dalla pianta solo come nutrienti, ma svolgono anche la funzione di molecole segnale. In studi recenti è stato evidenziato come la percezione del nitrato in mais avvenga principalmente attraverso la zona di transizione (TZ) della radice e che coinvolga il monossido di azoto (NO), la regolazione dell'omeostasi delle specie reattive dell'ossigeno (ROS), e la via di segnalazione di ormoni (auxine e strigolattoni) che infine attivano una via di trasduzione nel resto della radice. Il ruolo degli Strigolattoni (SL), in particolare, dovrebbe essere ulteriormente approfondito, poiché non è ancora molto chiaro come funzionino questi fitormoni derivati dai carotenoidi, nonostante siano largamente coinvolti soprattutto nella risposta allo stress abiotico, agendo sia come molecole segnale endogene che esogene.

Per comprendere il ruolo svolto dagli SLs nella risposta alla carenza di N, abbiamo valutato le risposte fenotipiche, fisiologiche e trascrizionali della linea wild type di *Zea mays* L. B73 e le abbiamo confrontate con quelle delle piante del mutante knockout *ZmCCD8*. Entrambi i genotipi sono stati coltivati in campo ed entrambi sono stati testati in condizioni di fertilizzazione con urea e senza somministrazione di urea. Il mutante *ZmCCD8* è cateterizzato da una mutazione knockout del gene *CCD8* che codifica per la CAROTENOID CLEAVAGE DIOXYGENASE8 necessaria per la biosintesi degli SLs. Per il saggio fisiologico è stato utilizzato settimanalmente il DUALEX SCIENTIFIC+™ per valutare i livelli di clorofilla (CHL), antociani (ANTH), flavonoidi (FLAV) ed il valore NBI (Nitrogen Balance Index). Contemporaneamente abbiamo controllato la crescita delle piante misurandone le altezze, la lunghezza degli internodi, le circonferenze dei fusti, il numero e la lunghezza delle foglie.

Per quanto riguarda le risposte trascrizionali, l'RNA totale è stato estratto dalle foglie di entrambi i genotipi e trattamenti ed è stata valutata l'espressione di geni precedentemente selezionati in corrispondenza di specifici time-points. I nostri risultati hanno confermato il coinvolgimento cruciale degli SLs nell'adattamento del mais a basse quantità di azoto e hanno anche permesso di identificare alcuni componenti molecolari che sembrano svolgere un ruolo chiave in questa regolazione e potenzialmente e che potrebbero quindi costituire dei buoni candidati per il miglioramento la tolleranza del mais alla carenza di azoto.

1. INTRODUCTION

1.1. Maize (*Zea mays L.*)

1.1.1. Domestication

Maize (*Zea mays* L.), originated by a short and bushy plant called Teosinte native of lowland Mexico, is now an annual herbaceous tall plant with a complicated root system belonging to the family of Poaceae, formally known as Graminaceae, subfamily Maydeae and class monocots (John F. Doebley et al. 2019). Maize was domesticated from Teosinte, by 6300 years ago in Mexico. After initial domestication, early farmers continued to select for advantageous morphological and biochemical traits in this important crop (Viviane Jaenicke-Després et al. 2003), it is known that few QTL genes are responsible for the most of maize domestication (John F. Doebley et al. 2016). Beginning from the tillering trait which is largely controlled by the gene *Teosinte branched 1* (*tb1*), which acts as a repressor of organ growth and contributes to apical dominance by repressing lateral branch outgrowth (Sarah Hake et al. 2002), as showed in **figure 1.1**. In maize a retrotransposon insertion in this gene results in higher expression of this allele and thus stronger repression of tillering (Anthony Studer et al. 2011).

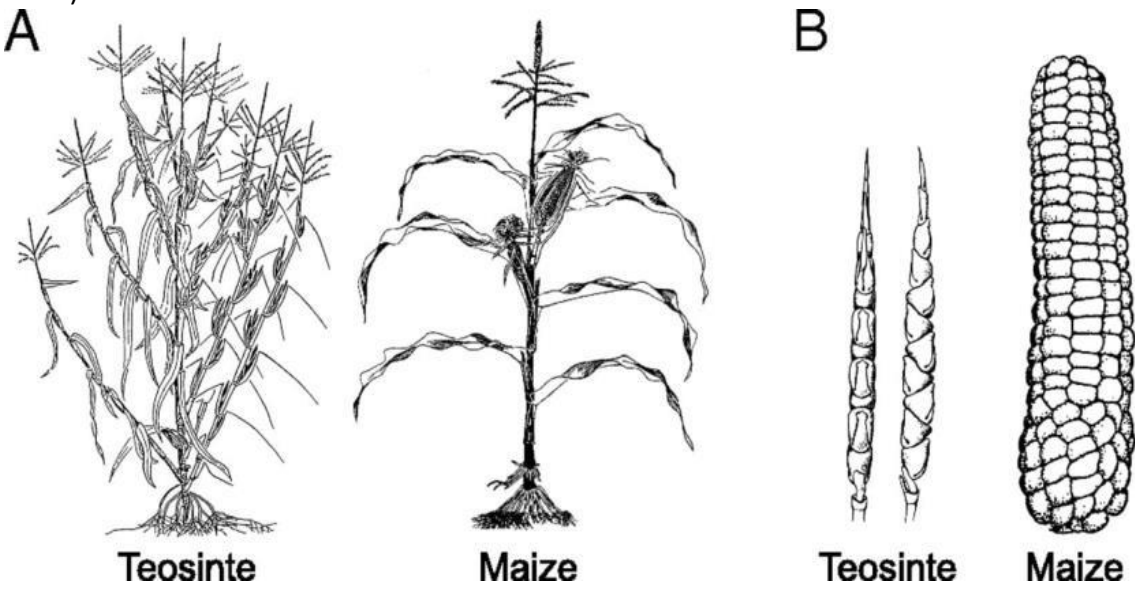


Figure 1.1. Transition from the bushy form of Teosinte (left) to the single-stemmed (right) form of the varieties cultivate (John F. Doebley et al. 2019).

Another domestication QTL that has been domesticated is *Teosinte glume architecture 1* (*tga1*), Teosinte seeds are covered by a hardened fruit case composed of lignified glumes, whereas maize kernels are "naked" on the ear. Although the components of the fruit case are present in maize, their development is disrupted so that the kernels are not encased as in Teosinte (Jane Dorweiler et al. 1993). Another important trait for the domestication has been the reduction in number and an increase in size of inflorescence. Teosinte has long lateral branches that bear multiple small ears at their nodes and tassels at their tips. In maize, thanks to the *prol1.1* allele there are much shorter lateral branches that are tipped by a single large ear with no additional ears at the branch nodes (John F. Doebley et al. 2013). At last, in order to maximise the productivity corn cob always bigger have been selected through the time as it can be seen in **figure 1.2.**

Figure 1.2. Maize cobs uncovered by archaeologists show the evolution of modern maize over thousands of years of selective breeding. Photo © Robert S. Peabody Museum of Archaeology, Phillips Academy, Andover, Massachusetts.

1.1.2. Relevance

Maize has never been as relevant as nowadays, it is one of the world's major crop since it has been estimated that the corn has become the main food supply for the diet of the population of 94 developing countries, including Africa, some regions of Asia and South America (D. P. Chaudhary et al.2014), nevertheless it has been valued that just by the

combined production of 10 countries accounts for 87%, 78% and 68% of global total production of maize, rice and wheat, respectively (Guoyong Leng et al 2019). Since the past 50 years the world's cereal production has increased from about 876 Mt to 2848 Mt (FAOSTAT 2018), and the corn is by far the most cultivated cereal, even higher than wheat and rice, with a total of 1,151 million tons harvested in the 2022/2023 (Shahbandeh, 2023). Maize relevance is not only related to the food supply, but it is even the principal resource for animal feed, industrial production such as bioethanol (39% of world's bioethanol production is obtained with maize) and corn syrup and other maize products (Foley, 2013). Corn cultivation is so extensive that it has a main role in world's Water Footprint, which is used as a measure of the amount of fresh water utilized in the production or supply of goods. It has been calculated that between all the agricultural productions cereals are the ones that requires the most water, but maize in particular has the biggest Water Footprint of them all (Daniela Lovarelli et al. 2016), as shown in the **figure 1.3**.

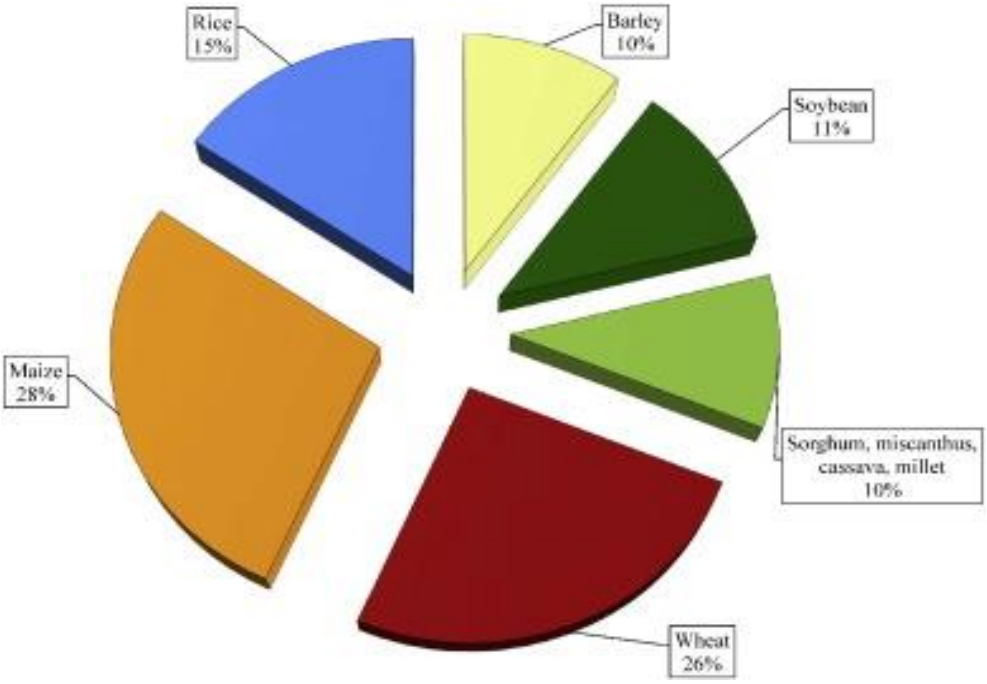


Figure 1.3. Subdivision of the Water Footprint of major crops (Daniela Lovarelli et al. 2016)

1.1.3. Root system

Considering the relevance of maize as seen in the previous paragraph can be understood the need to improve its stress tolerance and so the productivity as well, to do that it is necessary to know the complex root system of maize. First of all, the roots can be divided into an embryonic, which come directly from the seed, and a post-embryonic system that comes from the shoot instead. The embryonic root system comprehends primary root (PR) and seminal roots (SR); while the post-embryonic root system includes the crown roots (CR) under the soil and brace roots (BR) out of the ground (Feldman et al. 1994). PR is the first root to emerge, and it is necessary for the anchoring, mineral absorption and perception of external stimuli (Ive De Smet et al. 2012). Both PR and SR, are essential for the seedling stability after germination, generally their function terminates with the complete development of the post-embryonic system (Feldman et al. 1994). All these root classes can develop lateral roots (LR) which are part of the post-embryonic system and are fundamental for the exploration of the soil, water, nutrient uptake and to establish positive mutualistic symbiosis (Hochholdinger et al. 2004).

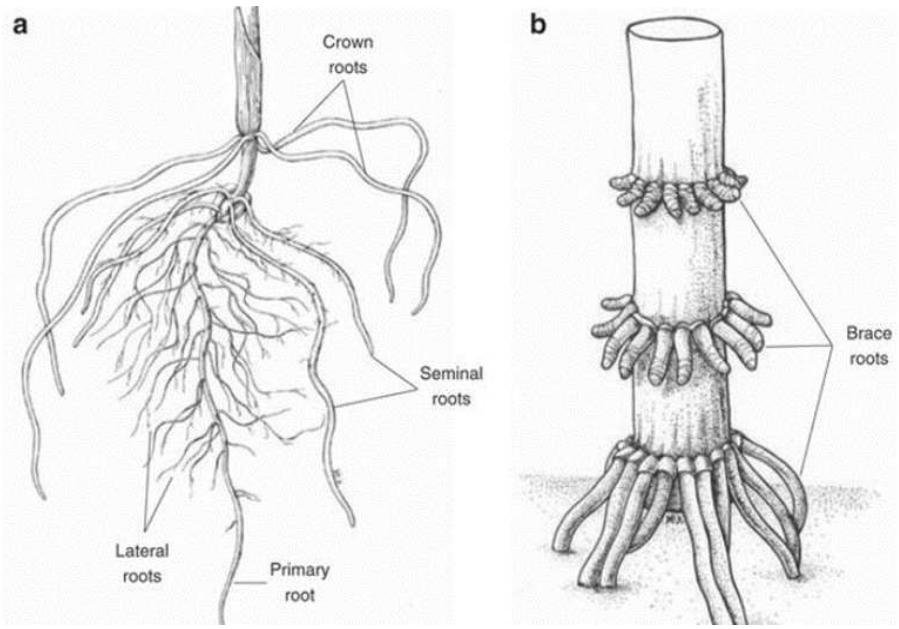


Figure 1.4. Maize root system representation (Hochholdinger et al. 2009).

Roots can be divided longitudinally into four consecutive zones, starting from the apical meristem located on the root tip, composed by undifferentiated meristematic cells in continuous division. Continuing towards the plant can be found the transition zone that is involved in various endogenous and exogenous signals perception and translates them into adaptative responses (Baluška et al. 2013; Trevisan *et al*. 2014). Then there is the elongation zone, a zone of rapid cell elongation, and at last there is the maturation zone composed of cells highly differentiated and specializated that absorb water and mineral nutrients. Root hairs and lateral root primordia develop in the maturation zone (Baluška et al. 2013; Trevisan et al. 2014).

1.2. Climate changes

1.2.1. Trend overview

It is now well known that climate change has acquired an important place among the major contemporary problems together with a growing and more demanding world population. The aim of this paragraph is to examine their actual entity and the effect they have already had and will have on agriculture, and so put at risk many countries food security. Changes to Earth's climate driven by increased human emissions of heat-trapping greenhouse gases are already having widespread effects on the environment, such as droughts, wildfires, and extreme rainfall, which are happening faster than scientists previously assessed (www.nasa.gov/). The past nine years have been the warmest years since modern recordkeeping began in 1880, specially 2022, which tied for the fifth warmest year on record (www.earthobservatory.nasa.gov/).

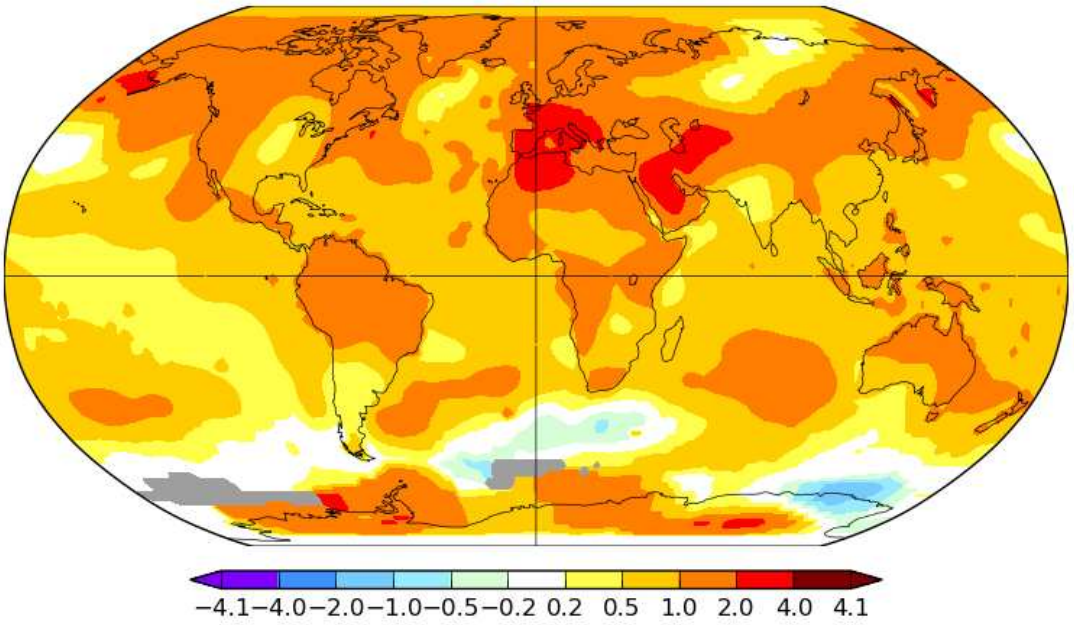


Figure 1.5. The map shows the average surface air temperatures differences in 2013–23 compared to 1963–73, (www.nasa.gov/).

Climate extremes, such as droughts or heat waves, lead to harvest failures threatening the food security of communities worldwide. That's why improving our understanding of their impact on crop yields is crucial to enhance the resilience of the global food

system (Elisabeth Vogel et al. 2019). It is in fact estimated that globally during the past four decades a loss of 1820 million Mg of cereals (maize, rice and wheat) has been caused by droughts (Guoyong Leng et al. 2019), global wheat production in particular is estimated to fall by 6% for each °C of further temperature increase and become more variable over space and time (S. Asseng et al. 2014).

One of the most sensitive areas to climate change due to human activities are drylands home to more than 38% of the total global population (Jianping Huang et al. 2015). Predicting the areal change in drylands is therefore essential for taking early action to prevent the aggravation of global desertification. The increasing aridity, enhanced warming and rapidly growing human population will exacerbate the risk of land degradation and desertification in the near future as in **figure 1.6**. This acceleration in dryland expansion rate could result in half of the global land surface covered by drylands at the end of this century (Jianping Huang et al. 2015).

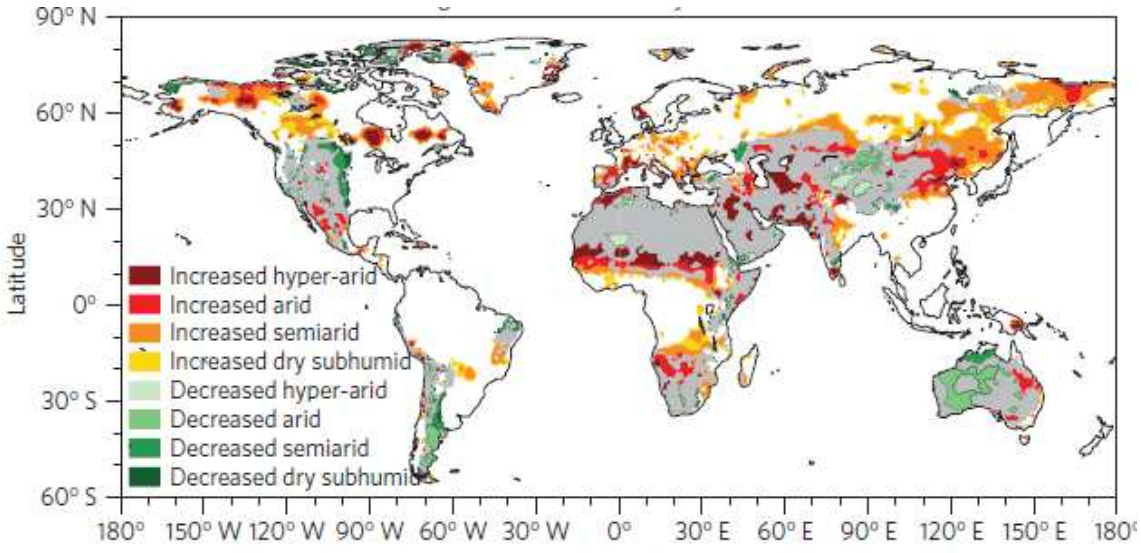


Figure 1.6. In the image are represented the previous expectation of dryland expansion (in grey) together with the latest estimated variations (colored). It shows how latest prediction cover a wider area with even increased entities (Jianping Huang et al. 2015).

1.2.2. Impact on agriculture

Over agricultural areas, disasters arising from extreme weather cause marked damage to crops and food system infrastructure, with the potential to destabilize food systems and threaten local to global food security, such disasters expected to become more common in the future. Since wheat, rice, maize, and soybean provide two-thirds of human caloric intake, it is critical to assess the impact of global temperature increase on production of these crops to maintain stable the global food supply (Bing Liu et al. 2017). In fact, at present, without CO_2 fertilization, effective adaptation and genetic improvement, it is estimated that each Celsius degree increase in global mean temperature, on average, would reduce global yields of wheat by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1% (Bing Liu et al. 2017).

A study has quantified that climate changes are responsible for the variability more than 60% of the yield variability in maize, rice, wheat and soybean in many regions (Graham K. MacDonald et al. 2015). Many of these regions were in the most productive global areas such as Midwestern U.S. and the Chinese Corn Belt for maize, and Western Europe and Australia for wheat. Over a total area of 94 million hectares, 39% of the maize yield variability caused by variations in temperature, precipitation, or their interaction, explained by climate translates into an annual fluctuation of 22 million tons in global maize production. Similar climate variability has affected rice, wheat and soybean annual production variability of 3, 9 and 2 million tons, respectively (Graham K. MacDonald et al. 2015).

These data are rather alarming when compared to the estimated losses caused by extreme weather disaster of the whole period from 1964 to 2007: a loss of 1,820 million tons due to droughts (approximately equal to the global maize and wheat production in 2013), and 1,190 million tons due to extreme heat disasters (more than the global 2013 maize harvest) (Navin Ramankutty et al. 2016).

Many studies classify maize as the major crop subjected to losses due to climate changes, this can be explained by the fact that maize is generally grown during summer months, which have the highest probabilities of extreme heat. Wang Lixin et al. 2016 compared maize with wheat and maize resulted more sensitive to drought, because

under comparable water reduction (approximately 40%), wheat had only 20% yield reduction, while maize experienced approximately 39% yield reduction.

Another consequence of climate changes is the eutrophication of aquatic ecosystems caused by the excessive of fertilization to try to contain the loss of production due to the stressful environment. It has been calculated that the addition of phosphorus and nitrogen to watersheds is almost entirely from fertilizers. The current global rate of application of phosphorus and nitrogen in fertilizers to croplands is respectively 14.2 Tg and 62 Tg a year. Few agricultural regions have very high phosphorus and nitrogen application rates, resulting as the main contributors of these excesses. Will Steffen et al. 2015 suggest that a redistribution of phosphorus and nitrogen from areas where it is currently in excess to areas where the soil is naturally poor may simultaneously boost global crop production and reduce the eutrophication of aquatic ecosystems.

1.3. Nitrogen

1.3.1. Nitrogen in agriculture

Nitrogen (N) is an essential mineral nutrient for the survival of plant species, it is one of the essential macronutrients and together with Sulphur (S), it forms covalent bonds with Carbon (C), constituting organic compounds. It is used for the formation of amino acids, proteins, nucleic acids, coenzymes and other important metabolic components for the plants, such as ATP and chlorophyll. It is in fact the fourth most abundant element in plants, after C, hydrogen (H) and oxygen (O). The molecular forms most used by plants are the inorganic forms, nitrate (NO_3^-) and ammonium (NH_4^+) and the organic components included in amino acids, peptides and proteins (Nacry et al 2013). Although NO_3^- is the most abundant ionic form, it is also the most soluble and therefore the most mobile along the soil profile due to water action; NH_4^+ instead has less leaching since it is adsorbed by the soil particles. Due to this different the plant induces the formation of a phenotypically different root system. In preponderance of the anion NO_3^-, more subjected to leaching phenomena, the elongation of the primary (PR) and lateral (LR) roots downwards are stimulated, in order to reach deeper soil profiles. On the other hand, for the cation NH_4^+, less mobile, an increase in root density occurs, inducing the proliferation of lateral root primordia (LRP) and the elongation of LRs (Hong Liao et al. 2015). However the 99% of the total Nitrogen is found in its inactive molecular gaseous form N_2 that is the most abundant component of the atmosphere, representing 78% of the air (Wang G. et al. 2020), but it can be used by the plants only due to the presence of mutualistic symbioses with nitrogen-fixing bacteria, which are capable of capturing N_2 and converting it into NH_4^+ (Nicolai Lehnert et al. 2018). Most of the nitrogen therefore comes from the fixation of atmospheric N mostly operated by bacteria and some algae, defined as diazotrophs which through the activity of nitrogenases, they can fix N_2 and convert it into ammonia (NH_3), in atmospheric conditions (Wang G. et al. 2020). In agriculture, the absence of these usable forms is a problem since it leads to a reduction in soil fertility, erosion and desertification (Wang G. et al. 2020), and even because the nitrogen in particular unlike the other elements (phosphorus, potassium and calcium), does not have a mineral reserve pool (Robertson and Vitousek, 2009).

Therefore, the strong demand for nitrogen in agriculture has led to the development of industrialized nitrogen fixation by recreating this natural reaction through the Haber-Bosch process, with the aim of producing nitrogen fertilizers. In this way the Ammonia is produced by making hydrogen and N_2 react under very high temperature and pressure conditions (Wang G. et al., 2020). In agriculture, more than 120 million tons of N/year are applied in the form of synthetic fertilizers. In 2005, it was estimated that total industrial N fixation was 121 Tg. Anthropic activity, every year, fixes and introduces into the terrestrial biosphere more N_2 than all natural processes combined (Jan Willem Erisman et al. 2008). However, of the total fertilizers applied, only the 30-50% is absorbed by plants, the rest is lost or metabolized by soil microorganisms with a deleterious environmental impact (Nicolai Lehnert et al. 2018). These massive fertilizer inputs lead so to alterations in microbial communities and their efficiency, causing a series of cascading problems for the environment and the health of living beings (Robertson and Vitousek, 2009). Under aerobic conditions, NH_3 is oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) by soil bacterial. The nitrification reaction in an oxidized environment occurs spontaneously, resulting in pollution from NO_3^- which leads to the eutrophication of water (Wang G. et al. 2020). In the absence of oxygen, instead, the NO_3^- and the NO_2^- are reduced to N_2 through the denitrification process by anaerobic heterotrophic bacteria and fungi. The issue in this case is due to the denitrification, since the nitrous oxide (N_2O) is obtained as final product, although a good part is readily reduced by bacteria to restore the N_2 in gaseous form, part of the N_2O is released into the atmospheric environment (Wang G. et al. 2020). It is estimated that approximately 75% of N_2O in the atmosphere is converted into nitrogen monoxide (NO), a powerful greenhouse gas that impacts the ozone layer, promoting global warming. NO is estimated to have a 100-year warming potential, which is 300 times higher than that of CO_2 (G. Wang et al. 2020). It is therefore important to seek solutions to limit losses of N in the environment and reduce the quantity of chemical fertilizers used, for this purpose varieties of plants that are highly efficient in the uptake and use of N are sought. A fundamental parameter for this purpose is the NUE (Nitrogen Use Efficiency), a parameter that defines the capacity to use N, determined by the weight of the total biomass or the yield of a given crop in relation to the quantity of N distributed

in the soil (Nacry et al. 2013). NUE can be further distinguished into the Nitrogen Uptake Efficiency (NUpE) defined as the ratio between N absorbed and that available in the soil and the Nitrogen Utilization Efficiency (NUtE) which is the biomass production per unit of N acquired by the plant (Nacry et al. 2013). Approaches for NUE optimization are mainly focused on finding genotypes with more efficient N uptake together with a better NO_3^- allocation and metabolism. However, there are also agronomic techniques for the optimization such as rotations, in order to improve the ability of plant communities to use the available N, and the application of precision agriculture, intervening with fertilizations in an adequate and localized way at the exact times and with the adequate formulations. Enhanced Efficiency Fertilizers (EEF) have also been developed, these fertilizers have a greater efficiency thanks to the use of substances capable of slowing the release of the nutrient, delaying so the nitrification processes and preventing a large loss of the mobile forms of N (Robertson and Vitousek, 2009).

1.3.2. Nitrate Sensing

Nitrate itself acts as signal molecule in many processes, it induces a response called Primary Nitrate Response (PNR) (Y.Y. Wang et al. 2018), which causes the up regulation of nitrate transporters and nitrate/nitrite reductase genes (Crawford and Glass, 1998). The dual-affinity NRT1.1/NPF6.3 and the high-affinity NRT2.1, which are membrane transporters for the uptake of NO_3^-, seem to be directly involved in the nitrate sensing, for this reason they are called 'transceptor' (Bouguyon et al. 2015; Munos et al. 2004). In particular when there is a low external concentration of nitrate the NRT1.1/NPF6.3 is phosphorylated so providing to this receptor a high affinity for nitrate, whereupon is induced the expression of NRT2.1 a HATS (high affinity transporter). They both act in the LR regulating their development in response to the nitrate (Krouk et al. 2010). The uptake of NO_3^- by the transceptor NRT1.1 induces a phospholipase C (PLC), that increases cytosolic levels of IP3 (inositol trisphosphate). The high concentration of IP3 induces the opening of channels for Ca_2^+ and so an accumulation of cytosolic Ca_2^+ occurs (Riveras et al. 2015). This in turn determines a phosphorylation cascade which activates the proteins kinases CPK10/30/32, promoting the phosphorylation of transcription

factor NLP7 (NIN-LIKE PROTEIN 7), which finally in the nucleus regulates the target transcription genes inducing the response to nitrate (Alvarez et al. 2020).

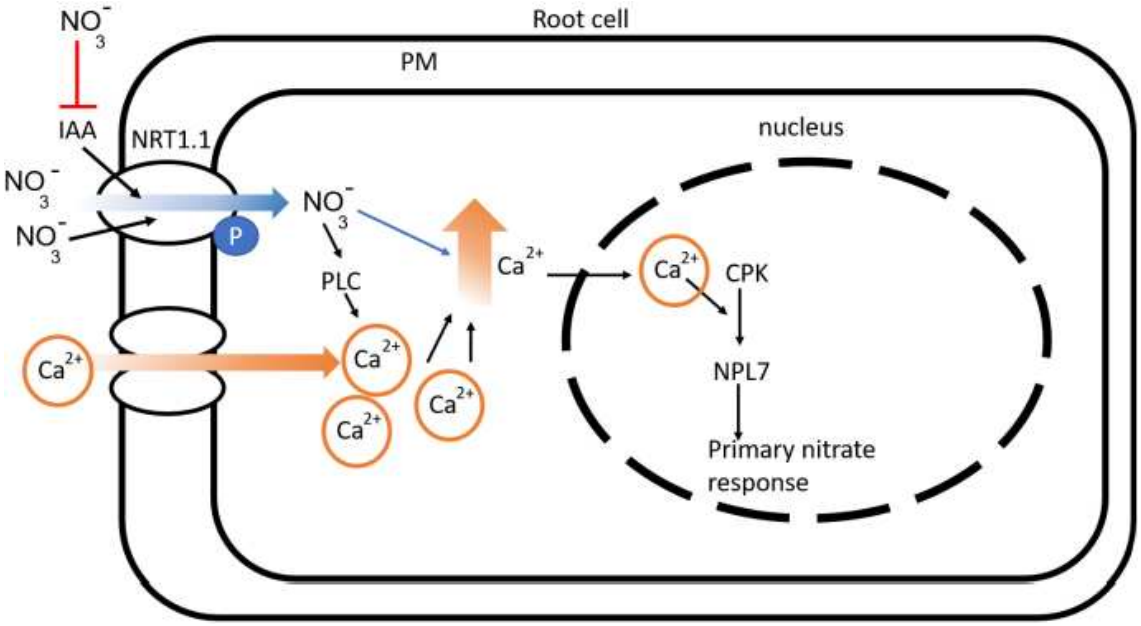


Figure 1.7. Nitrate sensing in the *Arabidopsis* root cell (Ravazzolo et al. 2021a).

1.3.3. Nitrogen uptake

As already mentioned, the nitrogen forms that can be assimilated from plants are molecular nitrogen (N_2), nitrate (NO_3^-) and ammonium (NH_4^+). However, molecular nitrogen (N_2) is assimilated only in the case of association with nitrogen-fixing bacteria that converts it into NH_4^+ (Tegeder and Masclaux-Daubresse, 2018). For the nitrate (NO_3^-) uptake it seems that at first there are some transporters called cHATS, which are High Affinity Transporter System constitutively expressed and therefore present even in the absence of the nitrate ion (Nacry et al. 2013). This ready-to-use systems is necessary in emergency situations since as the ion appears, in a concentration range of 6-20 μ, they are immediately active, so they are associated with the function of receptors for exogenous signals. The nitrate that enters through the cHATS seems to have no nutritional value, but instead it allows the early activation of other more efficient absorption systems. (Nacry et al. 2013)

Four different families of nitrate transporters have been identified, but only for two of these their role in the uptake of NO_3^- has been confirmed (Y.Y. Wang et al. 2018). The

first of these is the NRT1/PTR, known as Nitrate Transporter1/Peptide Transporter Family mainly made up of LATS, or Low Affinity Transporter System (Tsay Y. Et al. 2012). NRT1.2 is in fact a strictly low affinity transporter, while NRT1.1 possesses both high and low affinities depending on the phosphorylation (Nacry et al. 2013). This family of transporters is not saturable, characterized by a linear activity that increases as the external concentration increases, for this reason in a high concentration range (>1 mM), they are more effective than HATS (High Affinity Transporter System) (Nacry et al. 2013). The second family involved in the NO_3^- uptake is the NRT2 represented by HATS. These transporters, unlike LATS, are saturable and able to absorb especially at low concentrations up to 1 µM (Nacry et al. 2013). In particular NRT2.1 which gives the greater contribution to absorption, representing 75% of the total activity of HATS (Miguel Cerezo et al. 2001).

The last two families are the CLC (*chloride channels*) and the SLOWLY ACTIVATING ANION CHANNEL which seem to be involved in the efflux, transport and allocation of nitrate inside the plant (Wang et al. 2018).

Regarding the ammonium uptake it changes according to the concentrations of NH_4^+, in fact at low concentrations an active transport operated by AMT (Ammonium Transporters1) saturable HATS is required, in *Arabidopsis* the AMT1 is responsible for the 95% of the NH_4^+ uptake. When the NH4+ concentration gets higher a passive non-saturable low-affinity uptake system through aquaporins and cation channels occurs (Chiasson et al. 2014).

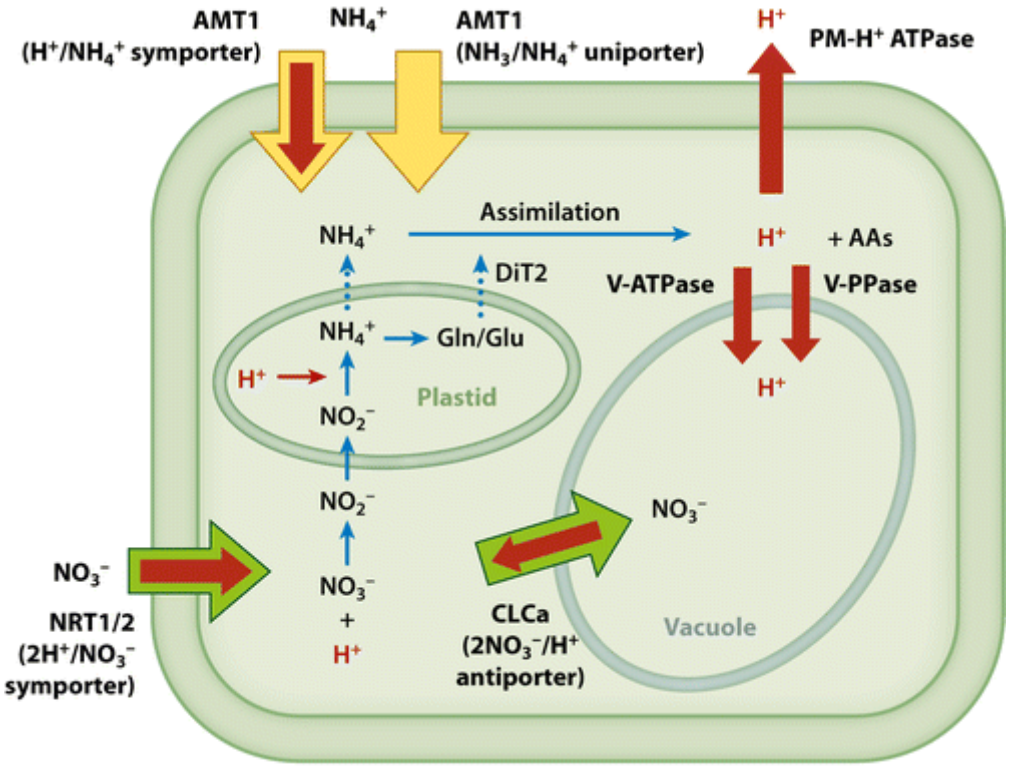


Figure 1.8. Relationship between ammonium and nitrate uptake (Guohua Xu et al. 2012).

Since the urea represents the form used globally for more than half of all nitrogen fertilizer applied to crops, it is necessary to deeply understand how its uptake in plants works (Witte, 2011). Urea nitrogen can enter in the plant either directly, or through the form of ammonium or nitrate after its degradation by the soil microbes. A passive transport takes place at high concentrations of environmental urea, mediated by aquaporins, in particular by the major intrinsic proteins (MIPs) which are further divided in 4 subclasses (TIPs, SIPs, PIPs and NIPs) (Witte, 2011). But at low concentrations an active transport by a high affinity urea transporter is needed, this transporter is the DUR3, identified in *A. thaliana*. This protein is expressed in the plasma membrane of root epidermal cells, especially in nitrogen starved plants, and seems to be involved both in the uptake of environmental urea and in its internal transport (Liu et al. 2003).

1.3.4. Assimilation

In order to be assimilated the NO_3^- has to be reduced to NH_4^+ and then linked to a carbon chain, becoming glutamate and/or glutamine. This reaction needs two enzymes: the nitrate reductase (NR), a cytosolic enzyme, which converts NO_3^- to NO_2^- with NADH as electron donor (Andrews et al. 2013) and nitrite reductase (NiR) that transfers electrons to NO_2^- using the ferredoxin (Fd), reducing it to NH_4^+ (Sakakibara et al. 2012). Now as NH4+ the ions can be incorporated into organic molecules as amino acid thanks to the action of glutamine synthetase (GS), followed by the glutamate synthase (GOGAT) (Miflin and Habash 2002). GS has two main isoforms: the cytosolic one (GS1) and the plastid one (GS2) (Cren and Hirel, 1999). While GS2 is responsible for the primary N assimilation of the leaves photorespiratory ammonium, the GS1 is mainly involved in the primary N assimilation in roots (Tegeder and Masclaux-Daubresse, 2018).

The pathway of N assimilation is showed in **figure 1.9**.

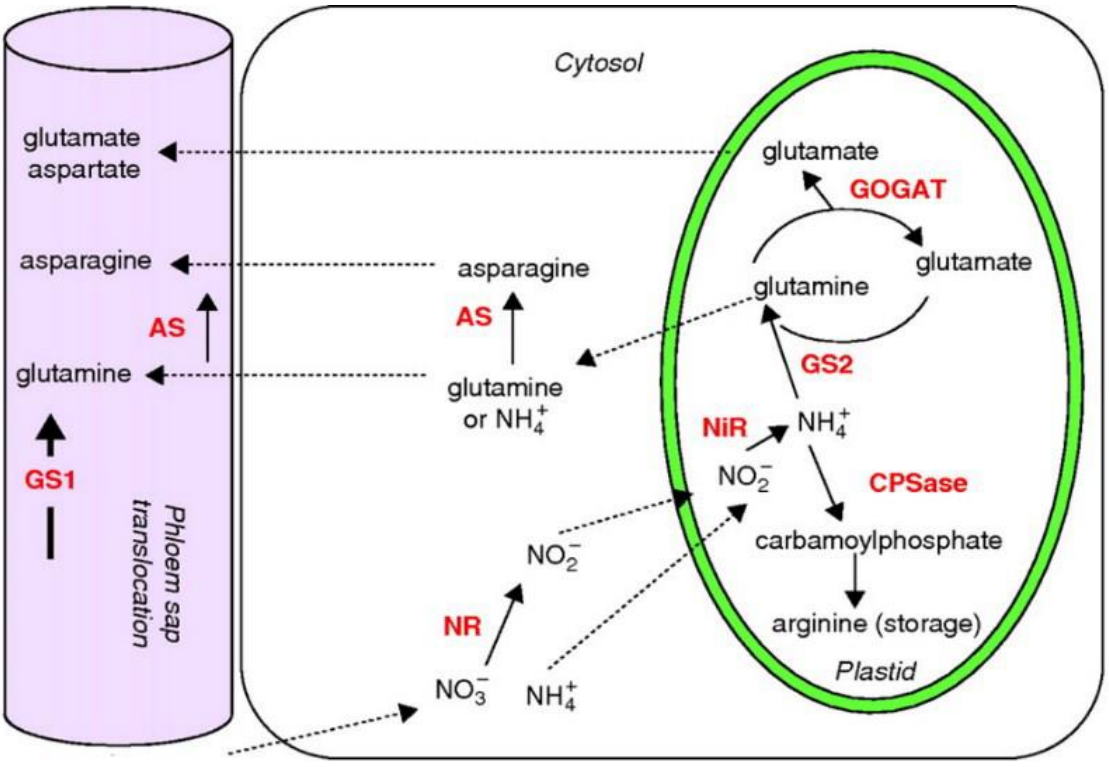


Figure 1.9. Representation of the enzymes involved in the nitrogen management in plants (AS=asparagine synthetase; GOGAT=glutamate synthase; GS1/2=glutamine synthetase; NiR=nitrite reductase; 1/2NR=nitrate reductase) (Masclaux-Daubress et al. 2010).

1.4. Strigolactones (SLs)

1.4.1. Strigolactones identification and chemical structure

Strigolactones (SLs) are phytohormones derived from carotenoids that were initially discovered as compounds exudates from the roots capable of inducing the germination of many parasitic species (Cook et al. 1966). Strigol was the first identified natural SL, a stimulant for the germination of the parasitic plant *Striga* from cotton exudates (Cook et al. 1972), later the orobanchol was identified because of its role in the germination induction in the *Orobanche* species (Yokota et al. 1998). It was even found that these same compounds are crucial for establishing mutualistic symbiosis with arbuscular mycorrhizal fungi (AMF) and are capable of inducing branching of fungal hyphae for the colonization of host roots helping with the uptake of inorganic nutrients (Akiyama et al. 2005). Only in 2008 SLs were identified as a new class of phytohormones, acting as endogenous and exogenous signal molecules in response to multiple environmental stimuli (Umehara et al. 2008). They are mostly involved in the regulation of broad aspects related to plant growth and development (Yoneyama and Brewer, 2021). Between the main roles of SLs as endogenous signals can be found the regulation of the root system architecture (Koltai, 2011) aiming to mediate adaptive responses to both biotic and abiotic stress, in particular the lack of nutrients (Decker et al. 2017). They are even involved in the architecture of the shoot system by inducing the internodes elongation (de Saint Germain et al. 2013), the thickening of the stem (Augusti et al. 2011) and inhibiting the axillary bud growth to prevent branching (Barbier et al. 2019). The chemical structure that distinguishes these molecules is the butenolide ring (D-ring), essential for the bioactivity and linked through a stereochemical R-configuration to the variable part of the molecule, conserved in all the natural SLs. Based on the variable part SLs can be divided in two macro groups: the canonical ones, which contain a tricyclic lactone (ABC-ring), and the non-canonical, in which the ABC-ring is absent. Canonical ones can be further distinguished due to their C-ring junction stereochemistry: the strigol-type SLs which have the C-ring in β-orientation (8bS-configuration) or the orobanchol-type SLs if the C-ring is in α-orientation (8bR-configuration) (Jia et al. 2018). However, SLs are not the only molecules that own a D-ring, also the Karrikins, which are

simpler molecules identified in the smoke of burning plants (Nelson et al. 2009), own D-ring. Since their structure is similar, they also share some signal transduction with SLs, but without inducing parasitic plants germination (Waters et al. 2013). SLs can now be synthesized and between the synthesized ones the rac-GR24 is probably the most used.

1.4.2. SLs biosynthesis and signalling

The first step for the SLs synthesis is the conversion of the *all-trans*-β-*carotene* to carlactone (CL), the SLs precursor, this process takes place in the plastid and it is operated by three enzymes. The first one is the carotenoid isomerase DWARF27 (D27) followed by two carotenoid cleavage dioxygenases CCD7 and CCD8 (Wu F. et al. 2022). At this point in *Arabidopsis* the gene *MORE AXILLARY GROWTH1 (MAX1),* which encodes a cytochrome P450 monooxygenase (CYP711A1), converts CL in carlactonoic acid (CLA) in the cytoplasm, where an unknown methyltransferase adds a methyl group to the CLA converting it into methyl carlactonoate (MeCLA). In the end the lateral branching oxidoreductase (LBO) oxidizes the MeCLA (Brewer et al. 2016). In rice instead Os900 (CYP711A2), a MAX1 homologue, converts the CL into 4-deoxyorobanchol (4DO), which is converted into orobanchol by Os1400 (CYP711A3) (Zhang et al. 2014).

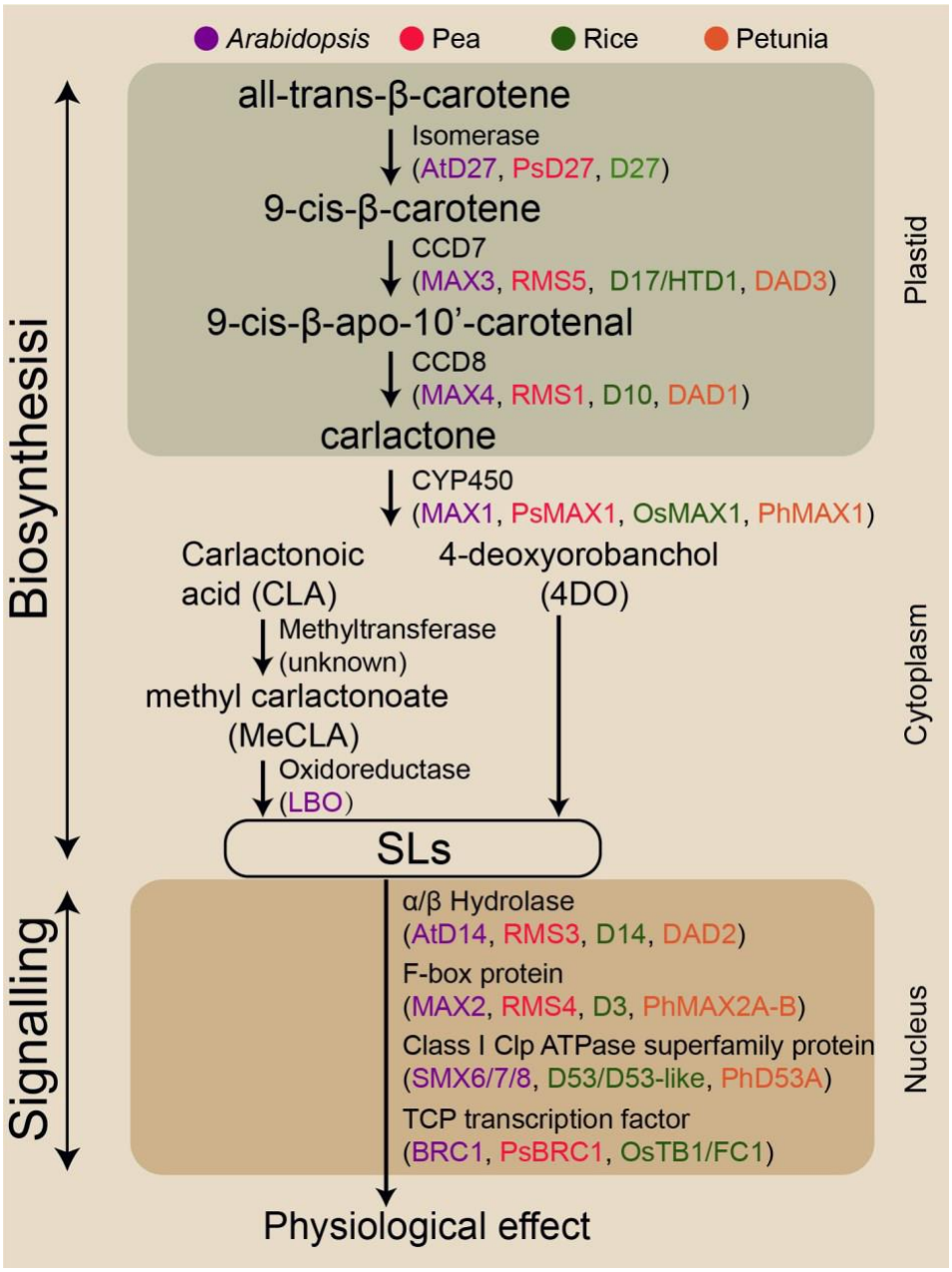


Figure 1.10. Schematic representation of SLs with different nomenclature for different plants (Wu F. et al. 2022).

In case of lack of nutrients, mainly the phosphate and nitrogen, probably due to the need of attracting AMF improving the uptake, both SLs biosynthesis and exudation are enhanced (Gutjahr, 2014), as a result of increased transcript levels of SLs biosynthesis genes, such as *MAX3-MAX4* in Arabidopsis (Ito et al. 2016) and D27-D10 in rice (Umehara et al. 2015). To finely control their homeostasis a negative feedback

mechanism takes place, indeed reductions of MAX3 and MAX4 transcript levels are observed afterward a GR24 supply in Arabidopsis (Mashiguchi et al. 2009).

In the absence of SLs, D53 suppresses SLs signal transduction but in the presence of SLs, the SLs receptor α/β hydrolase DWARF14 (D14) can bind the SLs and thanks to its conserved catalytic triad (Ser-His-Asp) with hydrolase activity, it divides the SLs in two parts. At this point a change of conformation occurs and so it can interact with the F-BOX proteins MAX2/D3 (Zhao et al. 2015). The SCF complex, of which MAX2/D3 is a part, will now be able to ubiquitinate D53 and so to redirect it to the 26S proteasome for its degradation, resulting in SLs signal transduction (Mashiguchi K. et al. 2020).

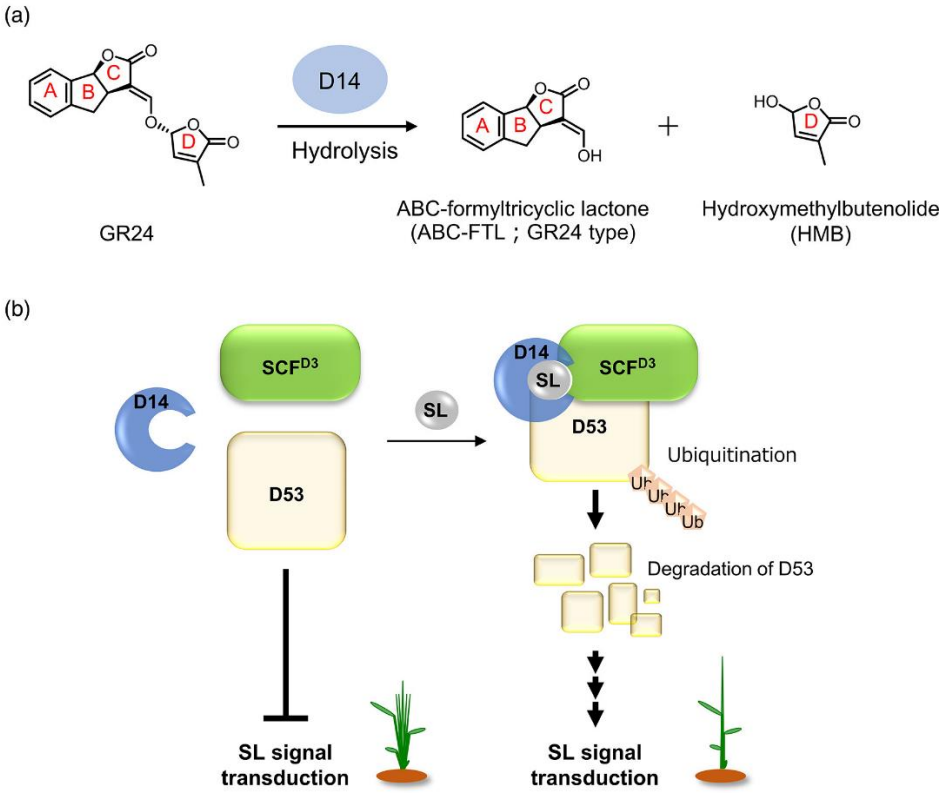


Figure 1.11. SLs mechanism of perception by D14 and its partner proteins. (Mashiguchi K. et al. 2020).

1.4.3. Stricolactones transport

Due to SLs recent interest as phytohormones and its low concentration, SLs transporters have been only partially identified and characterized. PDR1 (*PLEIOTROPIC DRUG RESISTANCE* 1) has been the first SLs exporter ever characterized from *Petunia hybrida,* thanks to the *pdr1* mutant that had decreased levels of orobanchol in root exudates and so displayed a lower interaction with AMF (Kretzschmar et al. 2012). It is a G-type ABC transporter strongly expressed in root tip cells (Sasse *et al*. 2015), particularly in specialized root cortex cells called Hypodermal Passage cells (HPCs) which are the entry point for the AMF colonization (Sharda and Koide, 2008). Therefore, it is believed that PDR1 is mainly involved in the SLs exudation, but a recent study proved that PDR1 is even involved in the loading of SLs into dormant buds (Shiratake et al. 2019).

In maize two genes have been investigated for their role as SLs transporter: maize homolog of PDR1 and *ZmWBC33* (a WBC transporter) (Trevisan et al. 2016). ZmWBC33 is a WBC subfamily ABCG transporters in maize, which showed a marked induction of its transcription in case of nitrogen and phosphate deficiency and a downregulation in case of NO_3^- and NH_4^+ supply. Regarding *ZmPDR1* instead just a slight regulation of its expression was found in response to the N supply or deprivation. Significant levels of *ZmWBC33* transcripts were even observed in root vascular tissues and in the apical meristem of lateral root primordia (LRP) at different stages (Ravazzolo et al. 2019). Another interesting feature about the *ZmWBC33* transport is that its transcripts co-localize together with those of *ZmCCD8* in stem, in cortex and in epidermis. The fact that *ZmCCD8* and ZmWBC33 are co-localized even in shoots vascular tissues, suggests that SLs synthesis takes place also in the aerial part (Lopez-Obando et al. 2015) and supports the hypothesis that ZmWBC33 could be involved in the cell-to-cell flux of SLs in maize root (Ravazzolo et al. 2019).

1.4.4. Strigolactones and abiotic stress

SLs are widely involved in the plant response to many abiotic stresses, in many studies it has been shown how plants SLs biosynthesis or signalling deficient mutants are more sensitive to drought, salt, osmotic stress and lack of nutrients. It has been proved how SLs positively regulates drought and high salinity responses in *Arabidopsis* since plants SLs-deficient and SLs-response mutants showed a hypersensitivity to drought and salt stress (Chien Van Ha et al. 2014). Furthermore, the expression profile of transcripts involved in the SLs biosynthetic and signaling pathways confirms the involvement of SLs in the response to salt and alkaline stresses (Yanhua Qiao et al. 2020). In addition, some specific responsive transcription factors controlling the biosynthesis of SLs in *Arabidopsis* resulted regulated by drought and salt stresses (Marzec and Muszynska, 2015). High levels of SLs have also been found in roots of *Oryza sativa L.* plants subjected to water shortage (Haider et al. 2018). These levels can be explained by the fact that in order to prevent water loss the SLs, together with H_2O_2/NO production, play a prominent role in inducing an ABA-independent stomatal closure, which results in plant tolerance to environmental stress (Guodong Wang et al. 2018). Another important cause of abiotic stress is represented by the nutrient starvation and also in this case the SLs are crucial to regulate plant root architecture to improve the adaptation to nutritional stress. The expression of SLs biosynthesis genes in rice roots is stimulated by N- or P-limiting conditions (Sun et al. 2014). In tomato *Solanum lycopersicum* a reduced root growth was observed in case of in SLs-depleted plants that were maintained under continuous P deprivation. In particular the root hair length, lateral root number and root tip anatomy were impaired, only in plants grown under low P, while it was not affected significantly when plants were supplemented with adequate P (Santoro V. et al. 2020). SLs inhibits also the shoot branching to promote the root system growth, which is a key response of adaptation for plants under nutrient limited conditions and severe environments (K. Yoneyama et al. 2011). Besides being involved in the rearrangement of the plant architecture, SLs can even be exudated in the soil, thanks to the increase of transcripts encoding SL transporters, during P starvation, to attract AMF in the rhizosphere helping with the nutrients supply (Lanfranco et al. 2018). The exudation of

SLs by rice is higher under mineral-deficient conditions, whereas increasing N and P doses reduce the amount of strigolactones in the exudates (H.J. Bouwmeester et al. 2011). Unfortunately, SLs also induce the germination of many obligate parasite species, such as witchweeds (Striga, Alectra spp.) and broomrapes (Orobanche, Phelipanche spp.) (David C Nelson, 2021). However, in maize it has been recently showed that by inhibiting, with knockout mutations, the activity of the cytochrome P450 ZmCYP706C37, together with ZmMAX1b and ZmCLAMT1, which are all involved in the SLs biosynthesis, a change of the exudated SLs composition occurs. In particular promoting the biosynthesis of two SLs, the zealactol and zealactonoic acid, instead of the major maize SLs, the zealactone results in a reduced induction of *Striga* germination (Guan et al. 2023).

Furthermore, SLs have a dual function in plant nutrition: a role in the direct and indirect acquisition of nutrients and a role in optimizing resource allocation (Rameau et al. 2019). In rice it has been proven that SLs are required for the proper distribution and reallocation of N into different plant tissues in cases of N starvation (Luo et al. 2019).

All the useful functions of SLs in case of abiotic stresses reported earlier in this paragraph, suggest that these phytohormones are an optimal target for the design of plant tolerance enhancer chemicals for a sustainable and modern agriculture. The supply of synthetic SLs could therefore have a crucial role in reducing erosion, desertification and soil degradation by enhancing the resistance systems already present in plants allowing them to growth in these adverse conditions (Akash Tariq et al. 2023). It has been shown in various experiments (Santoro. et al. 2020, Ruyter-Spira et al. 2011, H. Koltai et al. 2011, López -Bucio et al. 2003) how an exogenous SLs supply is able to stimulate the primary root and lateral root number under low P (Santoro et al. 2020), or even to contrast the reactive oxygen species induced in apple seedlings under KCl stress by enhancing the enzyme activities of peroxidase and catalase and maintaining the osmotic balance. In Arabidopsis a treatment with exogenous rac-GR24 increased the number of cells in the meristem (M) and in the transition zone (TZ) leading to the primary root growth, thus reaching deeper into the ground and helping with the nutrient's uptake (Ruyter-Spira et al. 2011). Lastly in Arabidopsis (**figure 1.12)** and tomato it has been shown how rac-GR24 inhibits the LR development while on the other

hand it induces longer RH (H. Koltai et al. 2011), which are essential to anchor the root to the ground and to extend its nutrient uptake area (López -Bucio et al. 2003)

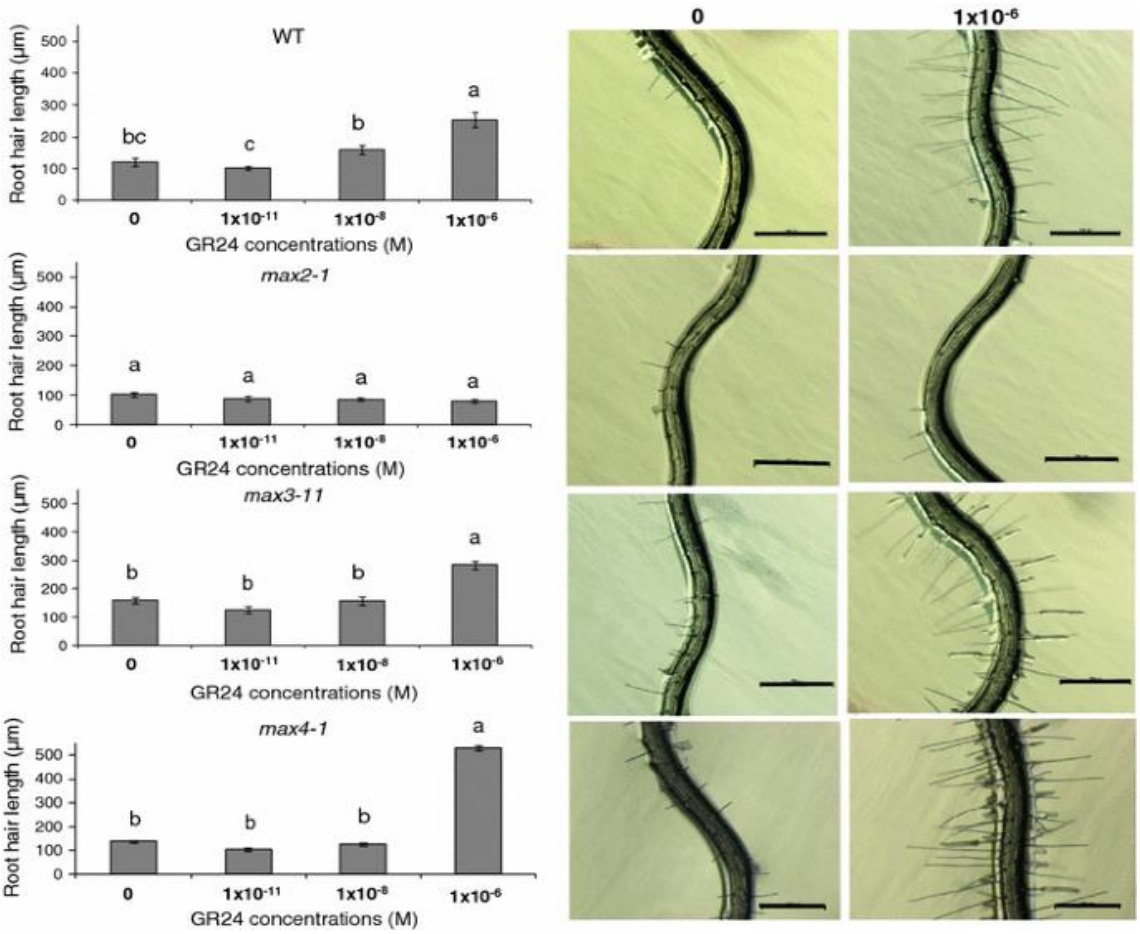


Figure 1.12. Effects of exogenous GR24 exposure on the root hair length of Arabidopsis seedlings (H. Koltai et al. 2011).

1.4.5. Strigolactones role in nitrogen response

An improvement in cops NUE enhancement could be achieved by further understanding the mechanisms of the NO_3^- and NH_4^+ response, leading so to an important reduction in the use of N fertilizers (Ravazzolo et al. 2020). This response obviously takes place in whole root, but the transition zone (TZ) seems to be the most important portion for nitrogen sensing and transduction because of its massive transcriptomic and proteomic reprogramming caused by the presence of nitrogen (Trevisan et al. 2015).

So the TZ has been demonstrated to function as the sensory part of the root, able to elaborate a developmental response from the perception of the external environment, thanks to the involvement of endogenous signals (Baluška et al. 2010). Among those, auxin, SLs and nitric oxide (NO) are involved in the early NO_3^- signalling in maize TZ (Trevisan et al. 2015).

Regarding the SLs, their biosynthesis and exudation is strongly enhanced by N deficiency, in particular zealactone and carlactonoic acid are produced in N starving roots (**Figure 1.13.)** (Ravazzolo et al. 2019).

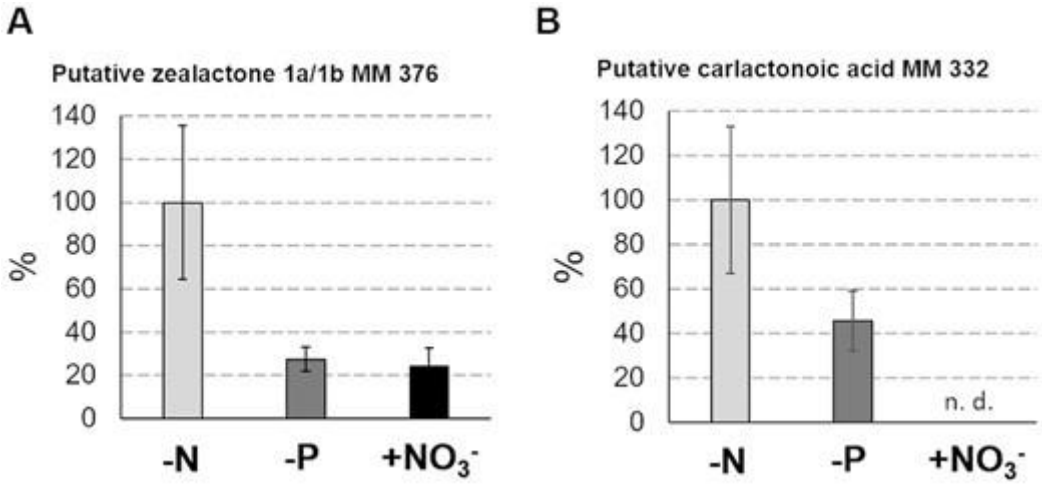


Figure 1.13. SLs main forms (zealactone (A) and carlactonoic acid (B)) quantification in maize root of seedlings after 24 hours of incubation under N-deficient conditions and exposed for 24 h to NO_3^-. Phosphate-starved seedlings (-P) quantification in root tissues is included as positive control (expressed in percentage, normalized to average amount per fresh weight) (Ravazzolo et al. 2021).

It has been seen that NO_3^-, drastically inhibits SLs synthesis and exudation: just after 2 hours from NO_3^- supply a strongly inhibition in expression of *ZmCCD7* and *ZmCCD8* occurs in cells of the TZ, while 24 hours are enough to totally switch down SL exudation (Ravazzolo et al. 2019). NH_4^+ instead results less effective as it needs more time for the inhibition of SLs (Ravazzolo et al. 2019). The inhibition in the transcription of genes involved in SLs biosynthesis, signalling and transport after NO_3^- supply, is actually detectable in all the four zones of the PR, but it is mainly evident in the TZ. (Manoli et al. 2016)

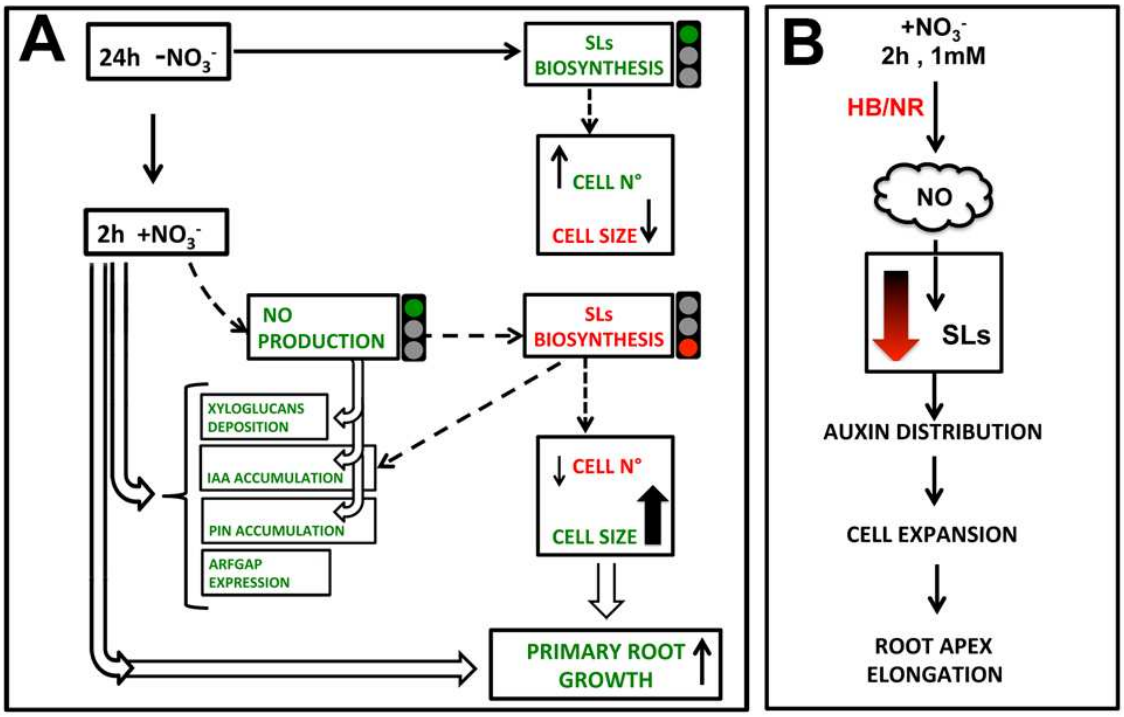


Figure 1.14. A hypothesis of how the interplay that occurs in the NO_3^- signalling controls the PR growth, acting on the TZ enlargement, proposed by Manoli et al. 2016.

1.4.6. SLs crosstalk with the other phytohormones

The most important interaction of the SLs is with the auxin since together this two phytohormones can affect synergistically the root development and shoot branching (Zhang et al. 2020). Regarding the shoot branching it seems that the axillary buds remain dormant as long as the auxin is accumulated in the shoot tip thus determining apical dominance. Therefore, the buds are not directly regulated by the auxin that does not enter the buds, so the dormancy signal needs to be mediated by SLs (Kebrom, 2017). Auxin does in fact up-regulate the SLs biosynthesis by increasing the *MAX4/D10* expression (Arite et al. 2007). SLs in turn seem to act negatively on the transcription of *PINs* genes, which are necessary for the polar transport of the AUX, and even altering the localization of their relative proteins in the plasma membrane, modifying the auxin transport and canalization in both shoots and roots (Shinohara et al. 2013). Due to the high levels of auxin in the SLs biosynthesis mutants, recently it has also been hypothesized, that the SLs may directly regulate the auxin biosynthesis (Ligerot et al. 2017). These two hormones are particularly important even for the development of the root system in case of abiotic stresses (**figure 1.15**), mainly affecting the roots elongation (Mayzlish-Gati et al. 2012) and regulating the LR emergence and development (Olatunji et al. 2017). The effect that these 2 hormones have on the root's architecture is mainly caused by the negative regulation of the SLs on polar auxin transport thus creating different gradients of auxin in the roots, in particular for the M, TZ and LR (Koltai et al. 2010). SLs, modulating the auxin efflux, seems to be involved even in the regulation of the RH by increasing the auxin levels of the epidermal cells and so promoting the RH elongation, mostly in case of P starvation (Y. Kapulnik et al. 2011).

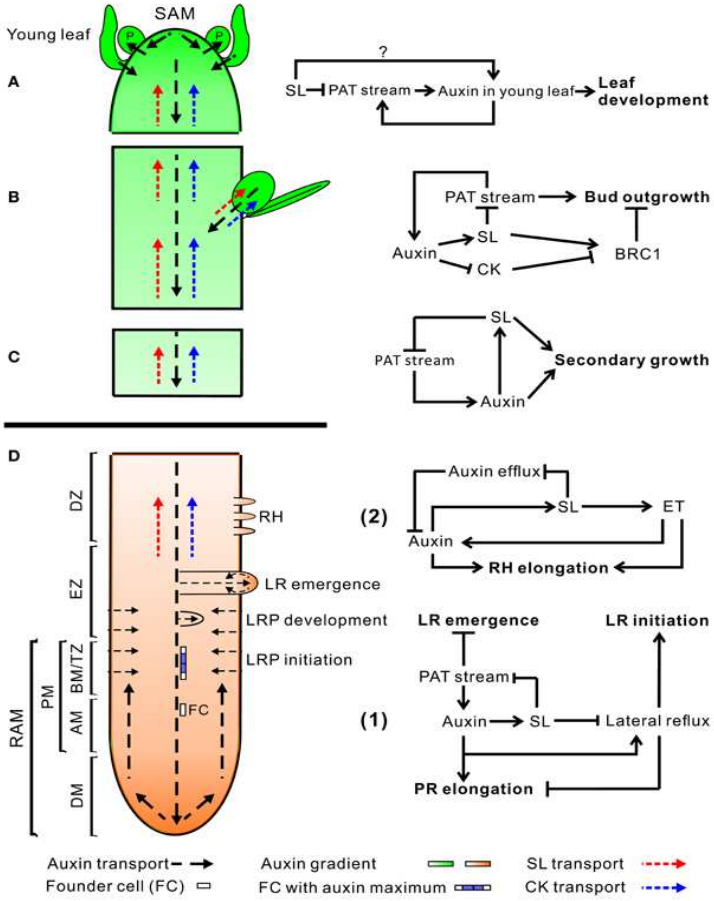


Figure 1.15. Representation of SLs and auxin interaction in the regulation of shoot and root development (Ruyter-Spira et al. 2013)

Regarding the interaction between SLs and cytokinins (CKs), they act antagonistically in shoot system, since CKs represses the expression of *BRC1* to induce the bud outgrowth, while usually it is enhanced by SLs to keep the bud dormant (Dun *et al*. 2012). Instead, SLs and CKs can act synergistically in the root system where they regulate the LR and PR development acting on the expression of the gene SHORT HYPOCOTYL 2 (Koren et al. 2013).

Abscisic acid (ABA) and SLs are closely related, it seems that ABA acts upstream in the regulation of SL biosynthesis and at the same time SLs are required for many responses correlated with ABA. Low expression of SL biosynthesis genes and thus a lower content in SLs have been found in ABA-deficient plants (López- Ráez et al. 2010). The relationship between SLs and ABA is not surprising, in light of the above described importance of SLs for the plant response to abiotic stresses. Mutants for SLs signaling or biosynthesis are

less sensitive to treatments with exogenous ABA in the presence of drought (Visentin et al. 2016), because of a reduced stomatal sensibility to ABA. At last ABA interact antagonistically with the SLs by reducing their biosynthesis and signalling, in the induction of the tillering which is usually inhibited from the SLs (G. Wang et al., 2018).
Concerning the interaction of SLs and ethylene it is known that they are both needed for the elongation of the hypocotyl and RH (Omoarelojie *et al*. 2019). It has been proved that ethylene acts upstream in this regulation, in fact RH elongation in *Arabidopsis* was enhanced when treated with GR24 if the plants were not treated even with aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis (Lee and Yoon, 2020).
As far as the crosstalk between SLs and Gibberellins (GAs), it is known that in rice plants treated with synthetic GAs levels of transcripts of genes for the SLs biosynthesis and transport tends to drop resulting in decreased SLs exudation (Ito et al. 2017). It is also well known that these two phytohormones are responsible for the stature of plants by inducing the internodes elongation, but it seems that SLs act independently from GAs to stimulate internode elongation. SLs in particular affect the stem elongation by stimulating cell division and not the cell length while GAs can stimulate both (A. de Saint Germain et al. 2013).

1.5. ZmCCD8

1.5.1. *ZmCCD8* overview

As stated above *Zea mays L. CAROTENOID CLEAVAGE DIOXYGENASE8 (ZmCCD8)* is an essential gene for the SLs biosynthesis. *CCD8* is a single copy *CCD8* gene in maize, located in the long arm of chromosome 3. It has an open reading frame of 1719 bp, which is divided in four exons, that encodes for a 572 residues protein. The sequence of the *ZmCCD8* has an 84.9%, 59.7%, and 58.9% amino acid identity respectively with rice D10, D10-like, and *Arabidopsis* MAX4 and it is localized in plastid (Auldridge et al. 2006; Guan et al. 2012).

1.5.2. Characteristics of the mutant *ZmCCD8* (zmccd8::Ds)

To understand how the SLs control maize branching Guan et al. in 2012 used a knock-out mutant for the *ZmCCD8* gene, since this protein is essential for their biosynthesis. The knock-out was obtained thanks to the insertion of a Ds transposon (*zmccd8*::Ds), reported in this line from Vollbrecht et al. (2010), the *Ds6-like* element was inserted into a junction between the second intron and third exon of the *ZmCCD8 gene* (**figure 1.16 A**). To check the eventual expression of *ZmCCD8,* primers flanking the insertion site (primers F2 and R2) have been used for a quantitative RT-PCR, resulting in efficiently amplified *ZmCCD8* transcripts for the wild-type (WT) seedlings (*ZmCCD8*-F), while no expression was found in the mutant seedlings suggesting that the *zmccd8* mutant could not produce an intact transcript (**figure 1.16 B**). A truncated mRNA was than expected in the *zmccd8* mutant, so this time a second pair of primers were used (primers F1 and R1), these primers bind a sequence located upstream of the insertion site. The results showed a very high expression of a truncated, nonfunctional *ZmCCD8* transcripts (*ZmCCD8*-U) in the shoots of *zmccd8* seedlings, caused by the loss of feedback repression. In roots samples instead the level of the truncated *ZmCCD8* transcript was quite law suggesting that the amount of feedback repression could be tissue specific (**figure 1.16 C**). To confirm the negatively regulation hypothesis seedlings grown in hydroponic solution were treated with a synthetic SLs (GR24) showed, resulting in decreased levels of *ZmCCD8*-U mRNA both for the *zmccd8* mutant and for the WT (**figure 1.16 D**) (Guan et al. 2012).

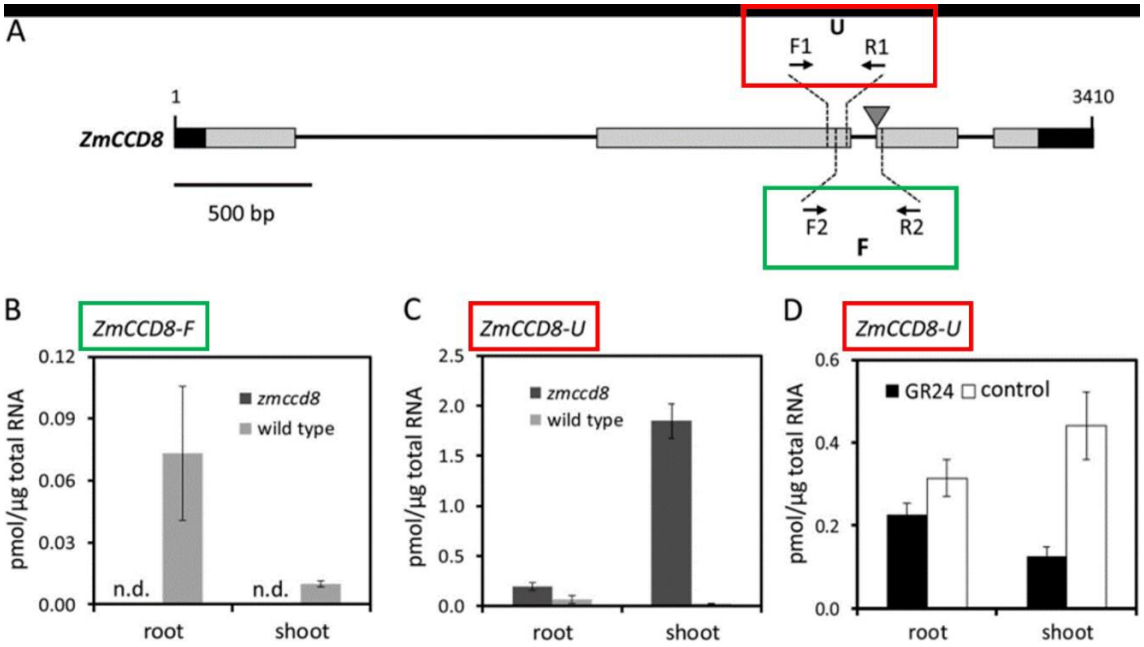


Figure 1.16. Structure and expression of the zmccd8::Ds insertion mutant (Guan et al. 2012)**.**

After 14 days of growth of *zmccd8* mutant and WT seedlings showed differences in their phenotype. While buds in the WT seedlings were not visible in the axil of the first leaf, they were evident on the majority of *zmccd8* seedlings. After 14 days of growth of *zmccd8* mutant and WT seedlings showed differences in their phenotype. While buds in the WT seedlings were not visible in the axil of the first leaf, they were evident on the majority of *zmccd8* seedlings. Furthermore, when all the buds became visible there still was a difference in their length, *zmccd8* buds were twice as long of the WT ones, but a suppression of buds' outgrowth in *zmccd8* seedlings was assessed when treated with GR24, confirming that this phenotype trait depends on the lack of SLs signaling (**figure 1.17 A, B, C**). The growth of axial buds was visible in 6-week-old *zmccd* plants grown in the field, while no buds were visible in the WT plants (**figure 1.17 D**). In the end, after 60 days, the *zmccd8* plants had three times the number of axillary branches compared to the WT plants (**figure 1.17 E**). The mild branching phenotype of *zmccd8* plants is probably responsible for the shorter stature, ear length and thin stems when compared to the WT, in fact, as shown in **figure 1.17 F**.

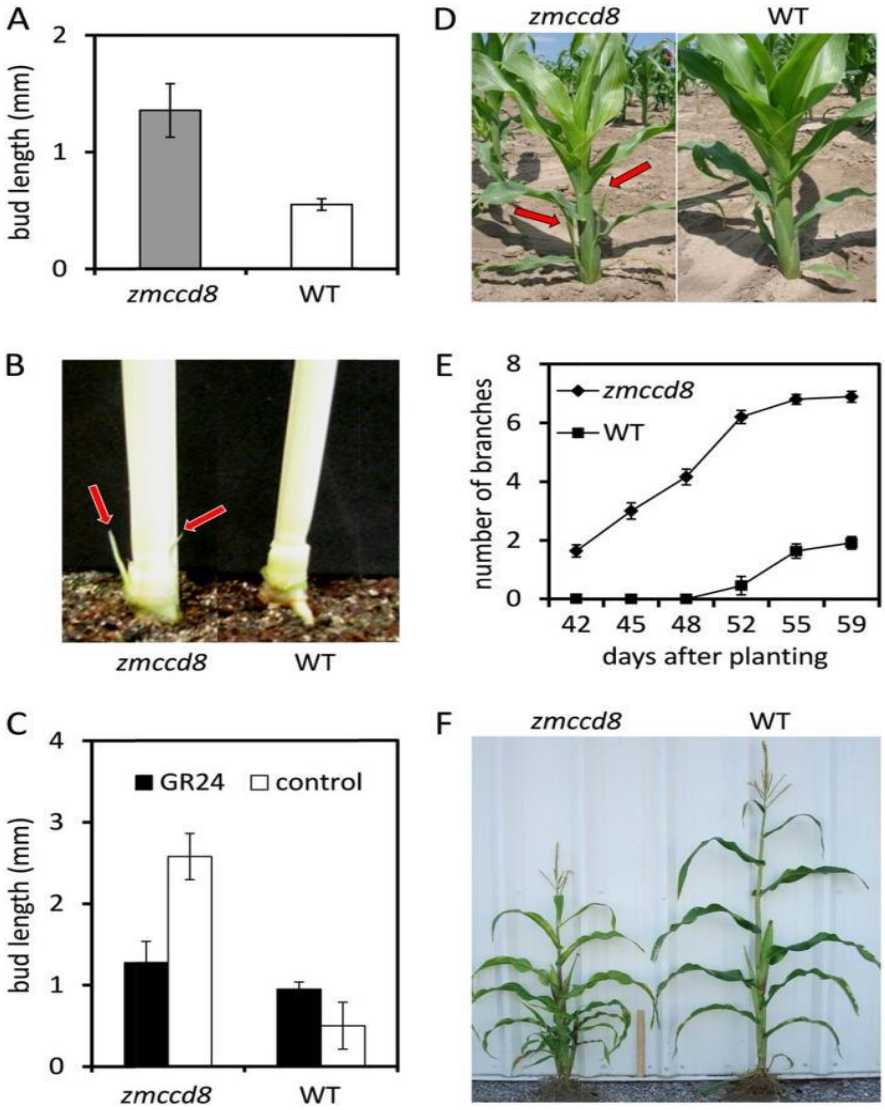


Figure 1.17. Zmccd8::Ds mutant branching phenotypes compared to WT, and its rescue using GR24 (Guan et al. 2012).

All the internodes of *zmccd8* stems node were shorter than WT internodes, in particular the internodes between positions 5 and 14 (**figure 1.18 A**). Ear length, ear diameter and shank diameter of the *zmccd8* mutant were 28%, 18% and 41% less, respectively, than those of the WT plants (**figure 1.18 B**). Another difference in the phenotype is in the tassels dimension, since tassels of *zmccd8* were longer than those of WT plants, furthermore *zmccd8* tassels tended to drop probably because of the longer length together with the narrower stems (**figure 1.18 C**). Regarding the root system there are significant differences, in the **figure 1.18 D** can be seen how smaller and less developed the root system of *zmccd8* mutants was compared to the WT. The primary root of the WT was 24% longer than the primary root of *zmccd8* and the length of nodal roots in

WT seedlings was 13.25 cm while it was of just 2.3 cm for the mutant (**figure 1.18 E)**. The last difference in the root system was a delay in nodal roots emerging in the *zmccd8* mutant, since after 10 days of growth all the WT seedlings had two nodal roots emerging between coleoptile and mesocotyl while no detectable nodal roots were found in the mutant seedlings (**figure 1.18 F)** (Guan et al. 2012).

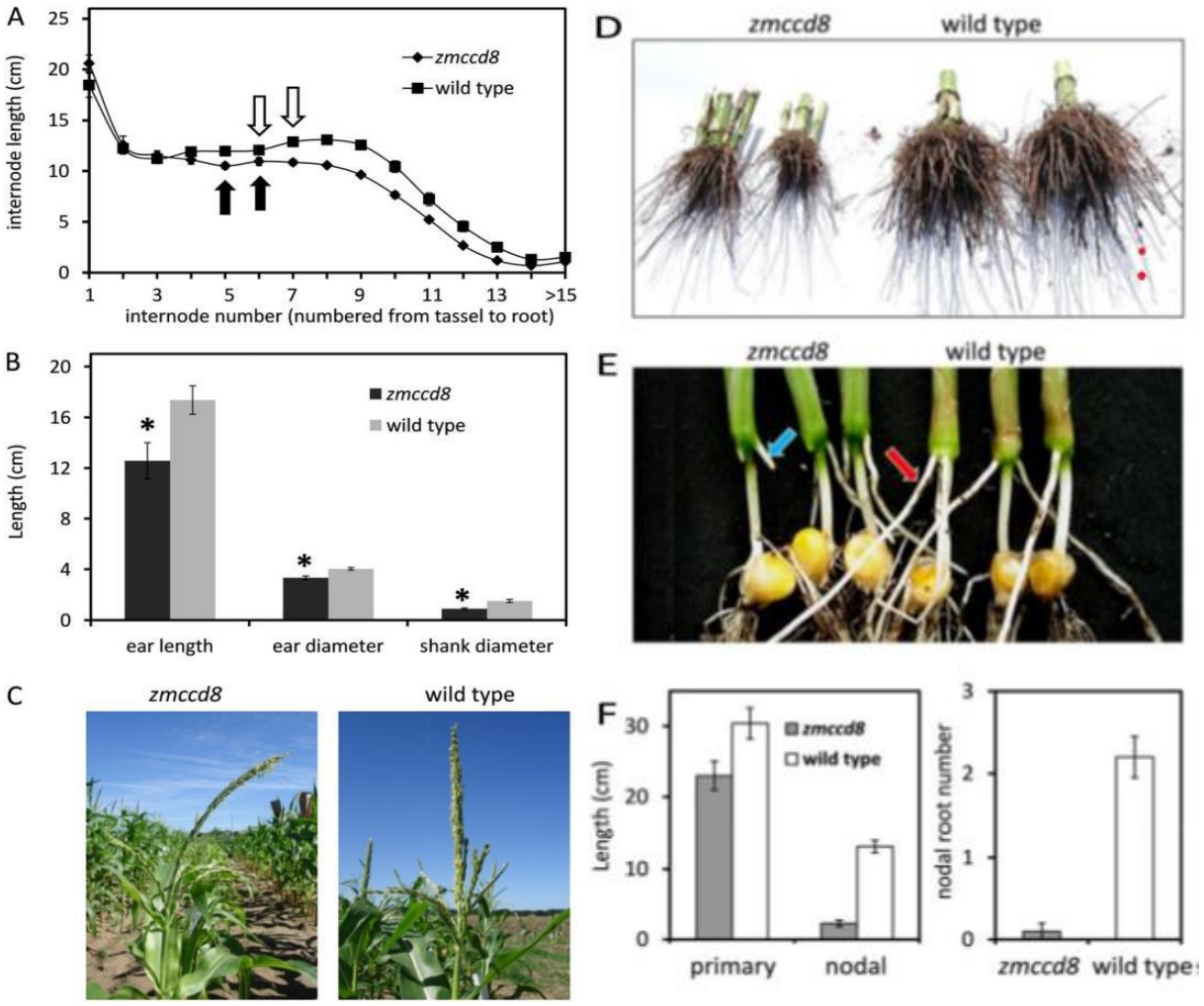


Figure 1.18. Internodes, ear, and tassel phenotypes (A, B, C) and root phenotype of *zmccd8* mutants (D,E,F) (Guan et al. 2012).

2. AIM OF WORK

One of the nowadays critical issues in agriculture is the lack of nutrients, which combined with the climate changes and all the problems correlated, are leading to a really stressful environment for plants growth. Therefore the aim of this study is to deeply characterize the role of SLs in the response of maize plants to the N availability, with the final purpose of better understanding the molecular basis of NUE in this important crop.

In order to do this, we grew the wild type *Zea mays L.* B73 inbred together with the *ZmCCD8* knockout mutant in field and, at 58 days after transplant, we fertilized half the plants of both lines with an urea treatment, leaving the other half without any supply. To check the phenotypical differences between the plants during the growth we measured every week the heights, internodes length, stems circumferences, leaves number and length. We even assessed physiological responses with the DUALEX SCIENTIFIC+™ to assess levels of: chlorophyll (CHL), anthocyanins (ANTH), flavonoids (FLAV) and NBI value (Nitrogen Balance Index) essential to check the global status of the plant. Lastly, we performed molecular essays to assess the level of transcript abundance for key genes involved in the biosynthesis, transport and perception of strigolactones, the transport and assimilation of nitrogen, sulphur and iron transport and compartmentation, at four time points after the fertilization (10, 23, 31 and 43 days after urea fertilization).

3. MATERIALS AND METHODS

3.1. In field maize growth conditions and experiment setup

For this study we planted 50 plants of the wild type (WT) of maize inbred line B73 and 50 plants of the *ZmCCD8* knockout mutant for the *ZmCCD8* gene, obtained with an insertion of a Ds transposon (*zmccd8*::Ds) into a junction between the second intron and third exon resulting so in a truncated nonfunctional protein preventing this genotype from producing SLs; both lines were kindly provided by Dr. Jiahn-Chou Guan, University of Florida, USA. Each plant had its own pot and was kept for 3 weeks in the greenhouse under controlled conditions. After this period the plants had grown enough to proceed with the transplant in field (Azienda Agraria Sperimentale L. Toniolo, Legnaro, PD), where we realized 2 straight lines of plants, one for each genotype. Each line was separated in half so that we could easily fertilize with urea (46% of N content, Cauvin Agricoltura, Genoa) just one half for each genotype, thus creating two different levels of nitrogen concentration in the soil. The fertilization with urea for half of the plants took place after 61 days after transplant (DAT) from the greenhouse to the open field, and 3 days after it was also necessary a treatment (Coragen® Mais by FMC Agro Italia) to contain the damages caused by the corn borer. All treatments and analyses are reported in the **Table 1**.

Hence, we obtained 4 different theses (**figure 3.1**): wild type of maize inbred line B73 grew with urea (WT +N) and *ZmCCD8* knockout mutant grew with urea (*zmccd8* +N); wild type of maize inbred line B73 grew without N source (WT -N) and *ZmCCD8* knockout mutant grew without N source (*zmccd8* -N). When we started with the analyses, we found out that some of the transplanted plants died in the process, thus remaining 24 plants for both the urea treated genotypes and 23 plants for both the untreated genotypes.

Date	DAT	DAU	Description
5/4/2023	/	/	sowed maize seeds in the greenhouse
21/4/2023	0	/	transplanted maize seeds in field
22/5/2023	31	/	phenotypical analysis and physiological analysis (only DUALEX SCIENTIFIC+™)
30/5/2023	38	/	phenotypical analysis and physiological analysis
6/6/2023	45	/	phenotypical analysis and physiological analysis
14/6/2023	53	/	phenotypical analysis and physiological analysis
19/6/2023	58	/	urea fertilization for wild type B73 +N and *ZmCCD8* +N plants
21/6/2023	60	2	phenotypical analysis and physiological analysis
22/6/2023	61	3	treatment to contain the damages caused by corn borer
29/6/2023	68	10	phenotypical analysis, physiological analysis and first sampling for RNA extraction and gene expression analysis
12/7/202	81	23	phenotypical analysis, physiological analysis and second sampling for RNA extraction and gene expression analysis
21/7/2023	89	31	phenotypical analysis, physiological analysis and third sampling for RNA extraction and gene expression analysis
2/8/2023	101	43	fourth sampling for RNA extraction and gene expression analysis

Table 1. Experiment setup (DAT = days after transplant, DAU = days after urea).

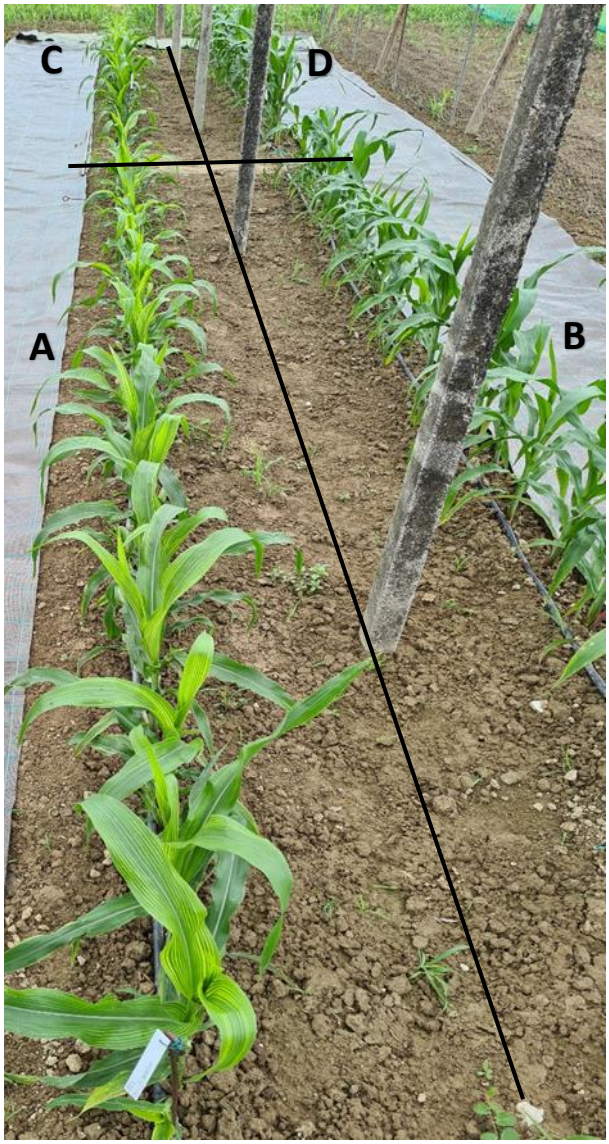


Figure 3.1 The result of the transplanted plant and the disposition of the 4 theses are shown in the image above: **A**) *zmccd8* +N, **B**) WT +N, **C**) *zmccd8* -N, **D**) WT -N.

3.2. Phenotypical analysis

To check the phenotypical differences between the plants of the two different genotypes, we decided to measure the height, internode length, stem circumference, leaf number and length of all the plants. The height was measured from the soil to the last fully developed node of the plants, internodes length was calculated by dividing the height for the number of the plant's leaves, the stem circumference was measured at the bottom of the plant where it reaches its maximum, for the leaves number we excluded the cotyledon and the newest leaves that were not enough developed, finally leaf length was measured only for the third leaf from the top, for the entirety of its length. All the measures have been checked through ANOVA statistical analysis with R-Studio (Posit team, 2023).

3.3. Physiological analysis

For the physiological analysis we were able to measure leaf values of chlorophyll, anthocyanins, flavonoids and NBI value using the portable and non-destructive DUALEX SCIENTIFIC+™ instrument (Force-A, Orsay, France in **Figure 3.2**). It is a device that can determine levels of chlorophyll, flavanols and anthocyanins contents in leaves, and it also calculates the NBI (Nitrogen Balanced Index), which is an important indicator of plant nitrogen status. The measures of the chlorophyll content in the leaf is accomplished thanks to the transmittance ratio of two different wavelengths: the far-red which gets absorbed by chlorophyll while the near infrared is used as reference. The flavanols and anthocyanins content is calculated thanks to a differential ratio of chlorophyll fluorescence. The principle of chlorophyll' measurement is called the screening effect of polyphenols on chlorophyll fluorescence (DUALEX SCIENTIFIC+™ manual, www.force-a.com) and it consists in a comparison between a first reference excitation near-infrared light which is not absorbed by the polyphenols and a second specific light that gets absorbed by polyphenols. Only the fraction of the light that reaches the chlorophyll in the mesophyll generates a near-infrared chlorophyll fluorescence. As recommended, in order not to affect the measurements, the measures

were taken at the half of all the leaves and away from the midrib. All the measures have been checked through ANOVA statistical analysis with R-Studio (Posit team, 2023).

Figure 3.2 The DUALEX SCIENTIFIC+™ (www.force-a.com)

3.4. RNA extraction and cDNA synthesis

For the total RNA extraction, we sampled 100 mg of all the leaves from 3 selected plants for each of the 4 theses and grinded them in a mortar using liquid nitrogen to help us maintaining the cold chain and avoiding RNA degradation. Then we used the Spectrum™ Plant Total RNA Kit (Sigma, St Luis; MO, USA) following the manufacturer's protocol including a DNase step. To quantify the RNA, it has been used the Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) together with an agarose gel electrophoresis for a qualitative check of the RNA to make sure it was not degraded. In order to check the transcription levels of the RNA, we operated a reverse transcription PCR, according to the internal laboratory protocol, using the M-MLV reverse transcriptase by Promega to obtain the cDNA as template for the quantitative PCR.

3.5. Quantitative reverse transcription PCR (qRT-PCR)

A quantitative reverse transcription PCR was than performed on the obtained cDNA to investigate the gene expression of selected genes at 4 different time points (**Table 1**). The qRT-PCR was accomplished using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) and SYBR Green reagent (Applied Biosystems, Monza, Italy), according to the manufacturer's instructions. All the genes were tested with 2 biological replicates and 3 technical replicates. The gene relative expression was determined according to the Livak and Schmittgen (2001) method, using *MEP* (*membrane protein PB1A10.07c*, Zm00001d018359) as housekeeping gene, according to Manoli *et al.* (2012). A melting curve analysis was realized to evaluate the specificity and investigate for possible off targets and primer dimers. Every result has been checked through ANOVA statistical analysis with R-Studio (Posit team, 2023).

In the **Table 2** are reported al the target genes and the sequences of the relative primers used in qRT-PCR. Primers were designed using Primer3 web tool, version 4.0.0 (http://bioinfo.ut.ee/primer3/; Rozen and Skaletsky, 2000), together with their functions.

Gene name	Maize GDB accession id	Forward	Reverse
CCD7	Zm00001eb074640	TCCGGCTCGCGCAGATTC	CTGCCCAGAACCCATGGA
Carotenoid cleavage dioxygenase 7, involved in SL biosynthesis			
CCD8	Zm00001eb153000	AGAAAGGTGTCTCTGCTGCT	CTATGGGCTCGCTCACATGA
Carotenoid cleavage dioxygenase 8, involved in SL biosynthesis			
WBC33	Zm00001eb305190	CGCTAACACGGTCTCATCAA	ATCATCATCAGCCCTTCGAC
ABC transporter G family member 11, putative involved in SL transport			
MAX2	Zm00001eb376660	GAACAAGACCGGCATCCAAC	TTAACTCGTCAGGCCTCCAG
Encoding F-box protein MAX2 involved in SL signaling			
D53	Zm00001eb404740	ACCCTGAGACTGGTTTCCTG	CTACAGTACGGTGGGTGGTG
Dwarf ortholog53, repressor of SL signaling			
NR	Zm00001eb176470	ATGATCCAGTTCGCCATCTC	GTCCGTGGTACGTCGTAGGT
Nitrate reductase(NADH)1, involved in nitrate assimilation			
NRT1.1	Zm00001eb023600	GCCATCAACCTCGTCCTCTT	CCACCGATTTCAGGCCAAAA
Encoding the protein NRT1, a dual-affinity nitrate-specific transport protein, also a nitrate sensor			
ASN3	Zm00001eb013430	ACTGGAGGATAAGAACGACTGG	GCGGCAGTACAACACGTAAC
Asparagine synthetase 3, involved in nitrogen storage and remobilization			
ASN4	Zm00001eb396990	GGACTCAAAGCCTTCACCGA	TCATCTCATCCGTCACCTGG
Asparagine synthetase 4, involved in nitrogen storage and remobilization			
GS1	Zm00001eb253820	ATGATCGCCGAGACCACCAT	GGAACGGAAGGAACAATGG
Glutamine synthetase 4, involved in NH4+ assimilation and translocation			
GS2	Zm00001eb432590	TTCGCCGTGACAGAGAAAGG	AACCGTACAAGTGTCCAGCA
Glutamine synthetase 1, role in the assimilation of NH4+ derived from nitrite reduction in the plastids			
LBD7	GRMZM2G017319_T01	GTCTTCGTCGCCAAGTTCTT	AGTTGCCCGTCCACATGAG
Lateral Organ Boundaries 7, negative regulator of anthocyanin biosynthesis and of some N-response genes			
AAAP10	Zm00001eb080600	ACACTCACATGGTTCCAGCA	ATCGTCCTCTGCATTTCGGC
Amino acid/auxin permease 10, involved in amino acid transport			
AAAP22	Zm00001eb145880	GGTCTCTTCCAACCCTTTAAA	ACCTGACTACATTCTTCTTCGA
Amino acid/auxin permease 22, involved in amino acid transport			
PTR2	Zm00001eb251550	TGTTGTGTCGGTGCCCAAG	CTGGAGACGGGTATGGGTTG
Homolog of A. thaliana Peptide Transporter 2 (PTR2), transports a wide range of N-containing substrates			
PTR5.6	Zm00001eb062900	TGTTGTTGCTCTCACACAAGG	AGCATGTGATGAACTAGAACGA
Encoding a NRT1/ PTR FAMILY 5.6 protein involved in di-tripeptide transmembrane transport			
SULTR4	Zm00001eb004550	GTCACCGAAACGCAGCAAC	CTTCTTCTTCCTCGCTCGCT
Sulfate Transporter 4, mediates the uptake and translocation of sulfate			
SULTR6	Zm00001eb154590	TAGGCGTCTTCAGGTTAGGG	GAGGTCTGTCTTTGGCGTGA
Sulfate Transporter 6, mediates the uptake and translocation of sulfate			
NAS2	Zm00001eb014700	AAGAGGGAGGAGATGGCAAC	ACACGAGAGATTGAAACAGCG
Nicotianamine Synthase 2, involved in the biosynthesis of nicotianamine, a metal ion chelator			
NAS4	Zm00001eb218430	CTTTGGAGTGGGCGAGGTAT	CGTTGCGTGTGTGGAGAGAA
Nicotianamine Synthase 4, involved in the biosynthesis of nicotianamine, a metal ion chelator			
NAS6	Zm00001eb396110	GGAATTGTGTGTGGTAACCGT	ACTTCACAGTGCATGACATCA
Nicotianamine Synthase 6, involved in the biosynthesis of nicotianamine, a metal ion chelator			
VIT1	Zm00001eb312010	GGCTACGGTCACTGCTTCTT	CAGCGGGTCACAGTTTGGAA
Vacuolar Iron Transporter 1.2-like, an iron transporter required for iron sequestration into vacuoles			
VIT2	Zm00001eb427520	TCCACATACAAGTCTCGTCGT	CCGATGTCAACAAGAGCAAGC
Vacuolar Iron Transporter 2, an iron transporter required for iron sequestration into vacuoles			
MEP	Zm00001eb257640	TGTACTCGGCAATGCTCTTG	TTTGATGCTCCAGGCTTACC
Housekeeping gene, encoding the membrane protein PB1A10.07c (MEP)			

Table 2. In the table above are listed the accession id and the primer sequences for each gene.

4. RESULTS

4.1. Phenotypical analysis

In order to examine the plants growth and to check for phenotypical significant differences the plants have been analysed almost weekly (we tried to maintain the weekly cadence by adapting to the weather conditions). All the plants have been tested for the phenotypical analysis, for a total of 24 plants for both the WT+ and *Zmccd8*+ and 23 plants for both the WT- and *Zmccd8*-.

4.1.1. Leaves number

We started by counting all fully formed leaves, excluding the cotyledon leaf, and the averaged results are reported in **figure 4.1**. No significant differences were detected at 31 and 38 DAT, while from the 45 DAT to 60 DAT, a slightly but significant difference occurred between the four theses in particular for the WT- which resulted having the most leaves followed by the WT+ and in the end by both the *Zmccd8*+ and *Zmccd8*- which shared similar values. The differences increased particularly from the second time point after the urea supply (68, 81 and 89 DAT), where the values align for the same genotypes, with both the WTs having 1/2 leaves more than both the mutants which never even reached the average of 11 leaves.

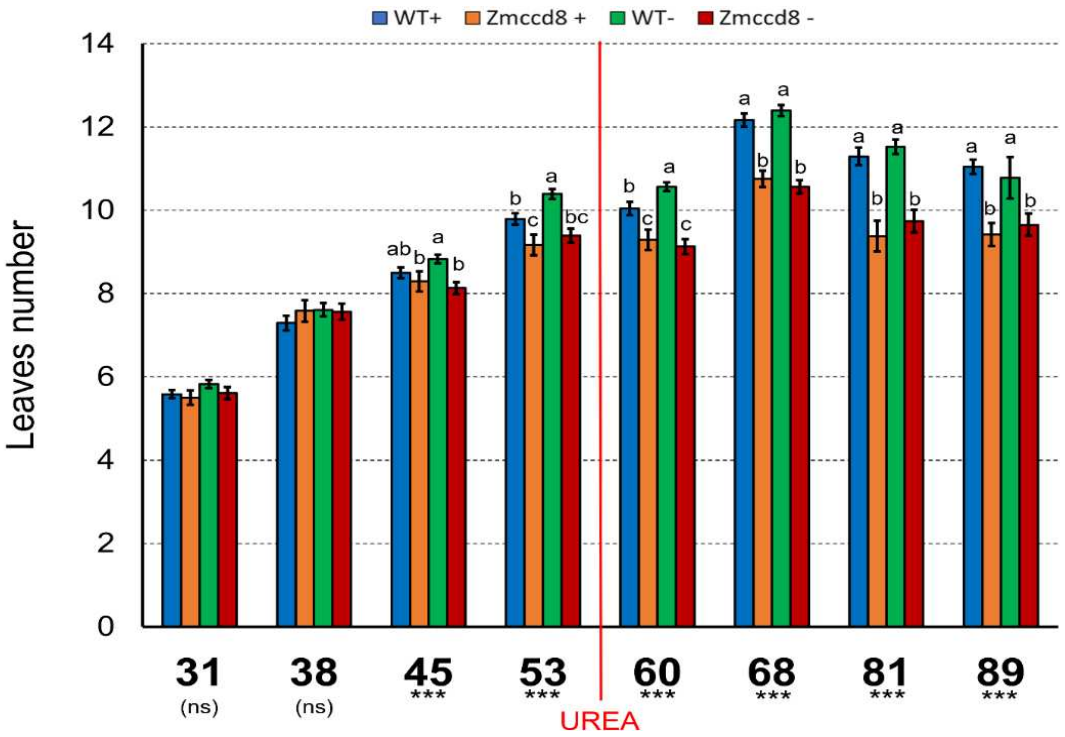


Figure 4.1. Average leaves number in WT maize plants supplied with urea (WT+, blue) or without urea (WT-, green), and in *zmccd8* mutant maize plants supplied with urea (*zmccd8+*, orange) or without urea (*zmccd8-*, red). On the horizontal axis the DAT (days after transplant) are reported, namely the days in which the analyses were carried out. The vertical red line represents the moment when the urea was supplied. Significance ANOVA p-value codes are reported under each DAT: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p> 0.05 'ns' not significant.

4.1.2. Plants heights

For the plant heights, we measured the height of each plant starting from the soil to its last fully developed node, and from the average of the values we obtained the **figure 4.2**. The results are similar to the ones we obtained for the leaves number (**figue. 4.1**), as there were no significant differences at 31 and 38 DAT. At 45 DAT both the WTs got slightly but significantly higher of both the mutants. The differences start getting wider at 53 and 60 DAT, in particular for the WT- which displayed the highest value followed by the WT+ and at last by both the *Zmccd8+* and *Zmccd8-* which shared lowest similar values. The greatest difference was detected at 68 DAT, where the same genotypes grouped together, and both WT+ and WT- resulted around 40 cm higher than *zmccd8-* and *zmccd8+*. This clustering of the two genotypes is also maintained in the last 2 time points (81 and 89 DAT), but the difference is lightly reduced to approximately 30 cm between the WTs and the mutants.

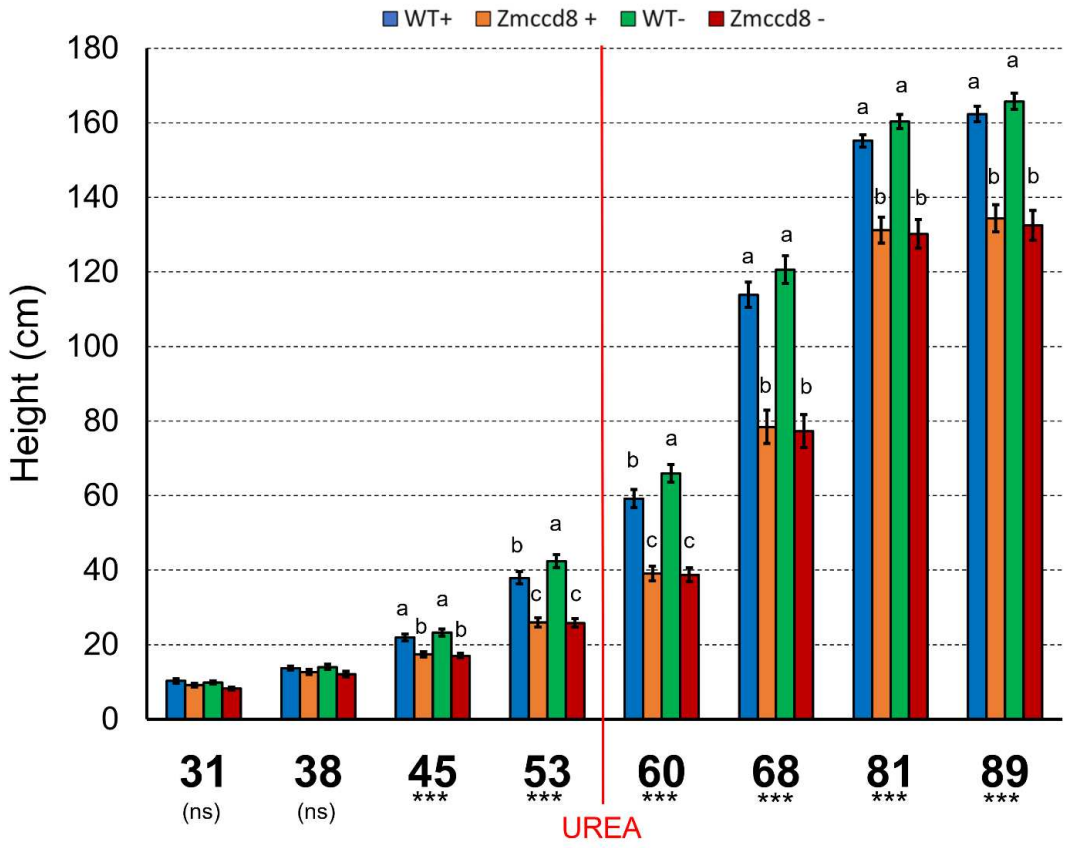


Figure 4.2 Average plant heights in WT maize plants supplied with urea (WT+, blue) or without urea (WT-, green), and in *zmccd8* mutant maize plants supplied with urea (*zmccd8*+, orange) or without urea (*zmccd8*-, red). On the horizontal axis the DAT (days after transplant) are reported, namely the days in which the analyses were carried out. The vertical red line represents the moment when the urea was supplied. Significance ANOVA p-value codes are reported under each DAT: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p> 0.05 'ns' not significant.

4.1.3. Internodes lengths

In the **figure 4.3** are reported the averages of the internode lengths, which have been obtained by dividing the heights values for the leaves number of each plant. Differently from what has been seen for the leaf number and plant height, in this case there were differences starting from the first time point (31 DAT) with the WT+ which resulted to have slightly but significantly longer internodes than by the *Zmccd8*+ and WT-, which shared similar lengths, and finally *Zmccd8*- which had the shortest internodes. Starting from the 38 DAT and for the 4 following time points (45, 53, 60 and 68 DATs), the genotypes once again started clustering together, with the WTs internodes that became progressively longer as the DATs increased reaching a maximum difference of around 2

cm between the WTs and both the mutants in the 2 time points right after the urea fertilization (60 and 68 DAT). However, the differences became non-significant between all the four theses at 81 DAT and turned to be back slightly but still significantly different at 89 DAT, with the WTs having the longest internodes followed by the *Zmccd8+* and at the end by the *Zmccd8-*.

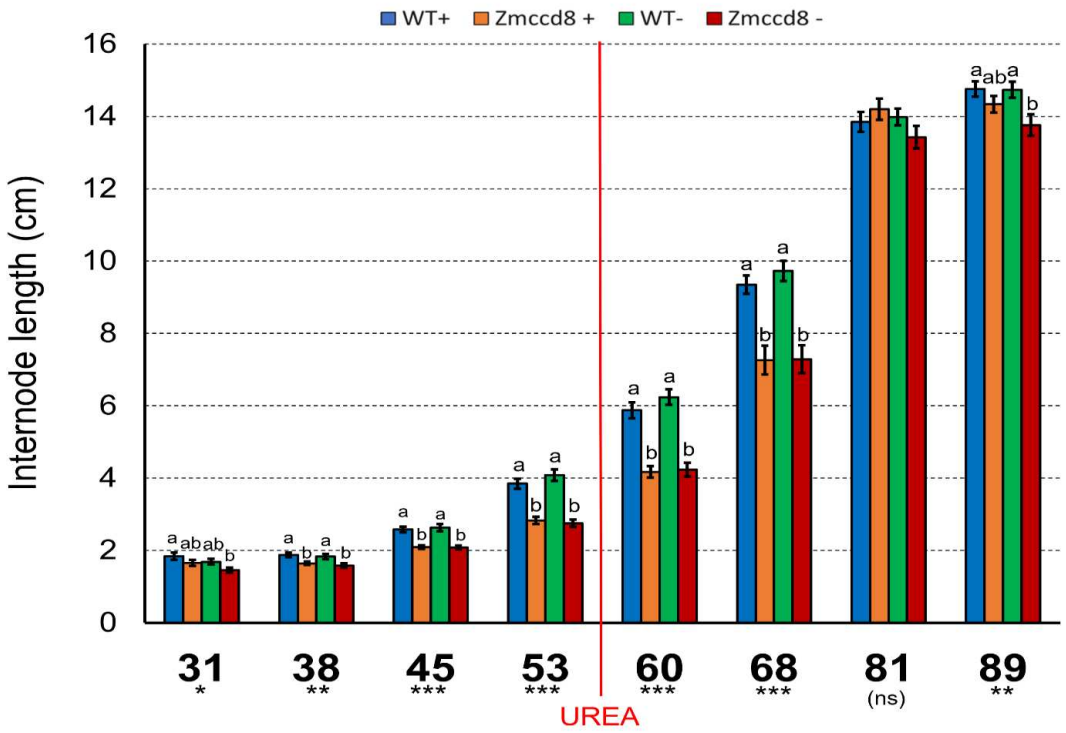


Figure 4.3 Average plant internode lengths in WT maize plants supplied with urea (WT+, blue) or without urea (WT-, green), and in *zmccd8* mutant maize plants supplied with urea (*zmccd8+*, orange) or without urea (*zmccd8-*, red). On the horizontal axis the DAT (days after transplant) are reported, namely the days in which the analyses were carried out. The vertical red line represents the moment when the urea was supplied. Significance ANOVA p-value codes are reported under each DAT: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p> 0.05 'ns' not significant.

4.1.4. Stems circumferences

Regarding the stems circumfernces, the avereges of the measures were taken at the bottom of the plant where it reaches its maximum are reported in the **figure 4.4.** No significant differences could be detected until the second time point (38 DAT) when the WT- had the widest circumference while all the other 3 theses shared similare values. For all the following DATs the trend was similar, in particular with the circumferences of the WT- remain the widest, togheter with the WT+ (even if at 53, 60 and 68 DAT, result to be statistically smaller but still very similar) reaching 2 cm more than both the mutants which always clustered togheter.

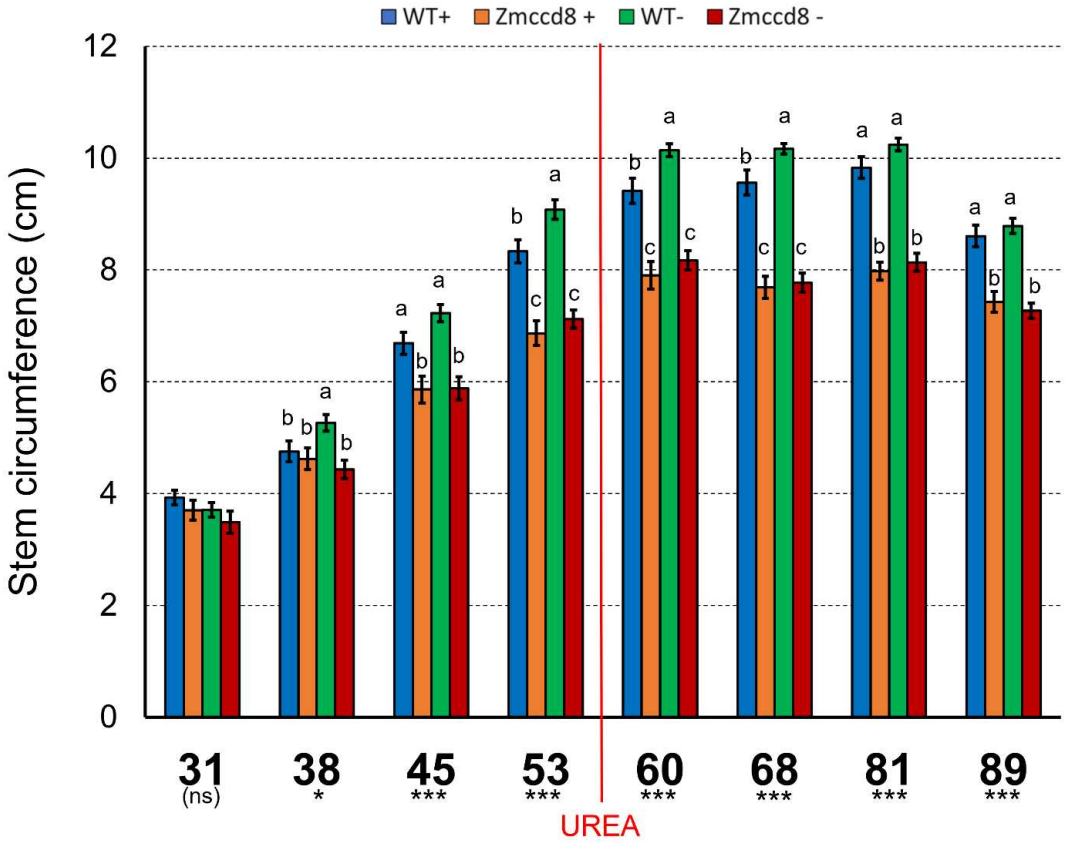


Figure 4.4. Average stem circumference in WT maize plants supplied with urea (WT+, blue) or without urea (WT-, green), and in *zmccd8* mutant maize plants supplied with urea (*zmccd8+*, orange) or without urea (*zmccd8-*, red). On the horizontal axis the DAT (days after transplant) are reported, namely the days in which the analyses were carried out. The vertical red line represents the moment when the urea was supplied. Significance ANOVA p-value codes are reported under each DAT: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p> 0.05 'ns' not significant.

4.1.5. Third leaf length

At last, we measured the entire length of the third leaf from the top of each plant (**figure 4.5).** Starting from the first time point until the fifth one (31, 38, 45, 53 and 60 DAT), the trend was always similar: the same genotypes grouped together, with the WTs leaves reaching the largest difference of 15 cm longer compared to both the treatments in the mutant. A shift occurs at 68 DAT, so after more than a week of fertilization, showing the *Zmccd8*+ treatment grouped together with the WTs. Then, the trend changed again at 81 DAT when the WT- had the longest leaves followed by the WT+ and at last by both the mutants. At the end (89 DAT), all the leaves lengths were reduced, reaching non-significantly different values.

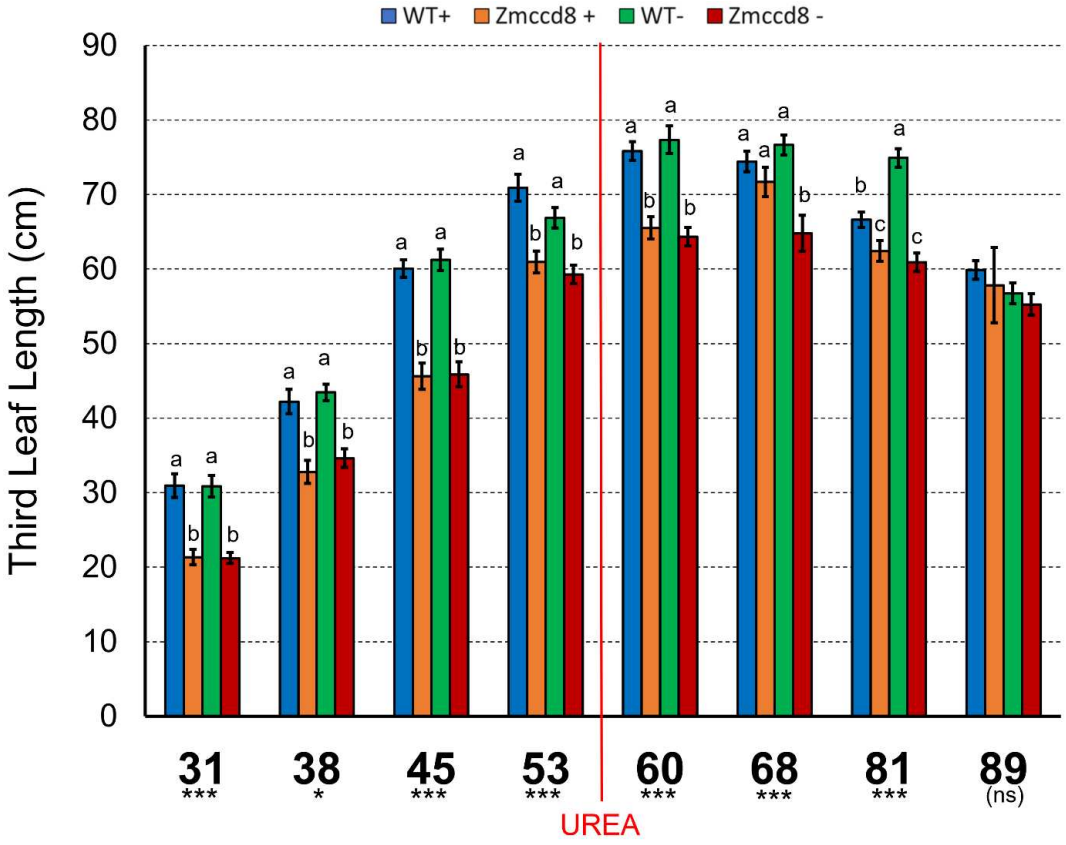


Figure 4.5. Average third leaf length in WT maize plants supplied with urea (WT+, blue) or without urea (WT-, green), and in *zmccd8* mutant maize plants supplied with urea (*zmccd8*+, orange) or without urea (*zmccd8*-, red). On the horizontal axis the DAT (days after transplant) are reported, namely the days in which the analyses were carried out. The vertical red line represents the moment when the urea was supplied. Significance ANOVA p-value codes are reported under each DAT: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p> 0.05 'ns' not significant.

4.2. Physiological analysis

The physiological analysis had been carried out, simultaneously with the phenotypical analysis, with the optical sensor DUALEX SCIENTIFIC+TM (Force-A), in particular we measured the levels of chlorophyll, anthocyanins, flavonoids and NBI value of each leaves of all the plants. Given the large amount of data, we grouped the leaves with the same behaviour into 5 groups to facilitate understanding: group 1 (leaves 1-2), group 2 (leaves 3-4-5), group 3 (leaves 6-7-8), group 4 (9-10-11), group 5 (leaves 12-13). The groups were organized to represent the arrangement of the leaves in the plant, so in group 1 there were the oldest and closest leaves to the ground, while in group 5 there were the newest and tallest.

4.2.1. Chlorophyll

In the **figure 4.6**, the averages of the increasing chlorophyll levels of all the 5 groups are reported. Starting from the first group, for all the four theses the values overall kept growing until the 81 DAT and then the values of WT+ and both mutants decrease, with the exception of the WT- which does not change at 89 DAT. Furthermore, an inversion in the trend was observed between the 31 DAT, when both mutants have higher values than the WTs, and the 38 DAT, when no significant differences were measured with the 45 DAT when the mutant chlorophyll content got definitively lower than the WTs. These differences started to get even bigger from 60 DAT until the end of the experiment.

In the second group, the trend in the values growth was conserved until the 81 DAT, then the values rest the same as 89 DAT. In this case the chlorophyll content of both the WTs was immediately higher than the mutants starting from 31 DAT. In particular, statistically higher values can be found in the WT- in most of the time points, followed by the WT+ and at last from both the mutants which share similar values.

The same trend was observed also in the third group, with a growth until 81 DAT and a stasis until 89 DAT. In this case the difference in the chlorophyll content was more marked between the WT- and the *Zmccd8*- reaching peaks of 15 DUALEX units compared to the difference of 11 DUALEX units between the WT+ and the *Zmccd8*+.

In the fourth group, even though these leaves grew up first in the mutants at 38 DAT, as soon as they grew up in the WTs too (45 DAT) their values were already higher than the mutants' ones. The growth in this group occurred faster than the other groups until the

81 DAT and even in this case there was a stasis until 89 DAT. Also, in this group the differences were more marked in the treatments without the urea supply, especially until 68 DAT.

In Group 5, an exponential growth occurs between 68 and 81 DAT in all the plants with the exception for the *Zmccd8*- in which these final leaves grew up only at 81 DAT. In both the 81 and 89 DAT the same genotypes clustered together, with the WTs showing statistically higher chlorophyll content than both the mutants.

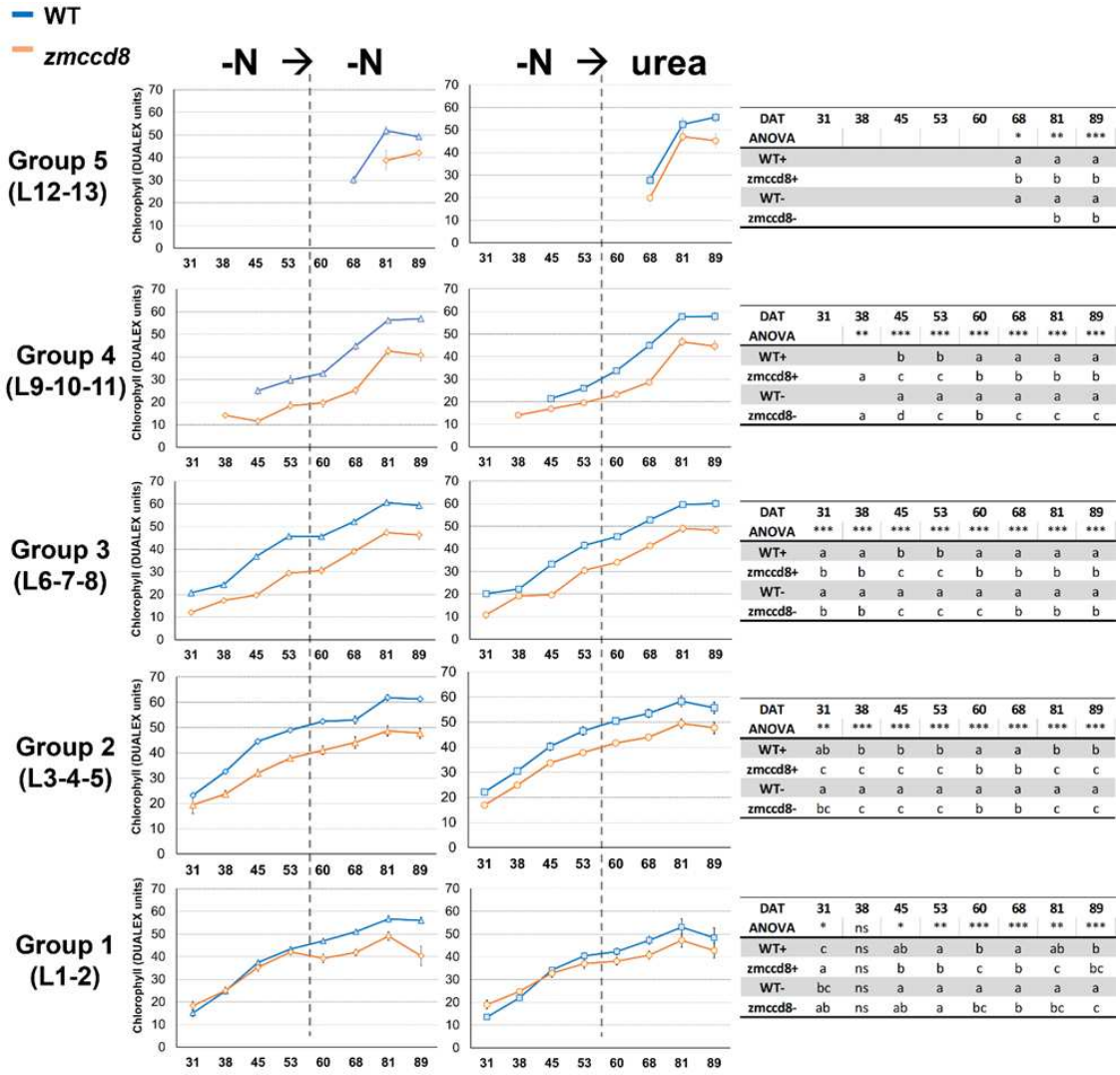


Figure 4.6 The average chlorophyll content (expressed as DUEALEX units) of each group of leaves in the vertical axis and the number of the DAT (days after transplant) in which the analyses were carried out on the horizontal axis. Next to the graphs there are tables with the statistical results obtained by each group: similar letters at corresponding DAT within treatments are not significantly different by an ANOVA test ($p < 0.05$). In blue the WTs and in orange the mutants, the grey dotted line showed when the urea was supplied. Significance ANOVA p-value codes are reported as follow: $p<0.001$ '***'; $p<0.01$ '**'; $p<0.05$ '*'; $p>0.05$ 'ns' not significant.

4.2.2. Flavonoids

Looking at the **figure 4.7** it can be seen how the flavonoids levels tend to decrease contrary to what it has been seen for the chlorophyll in the previous paragraph.

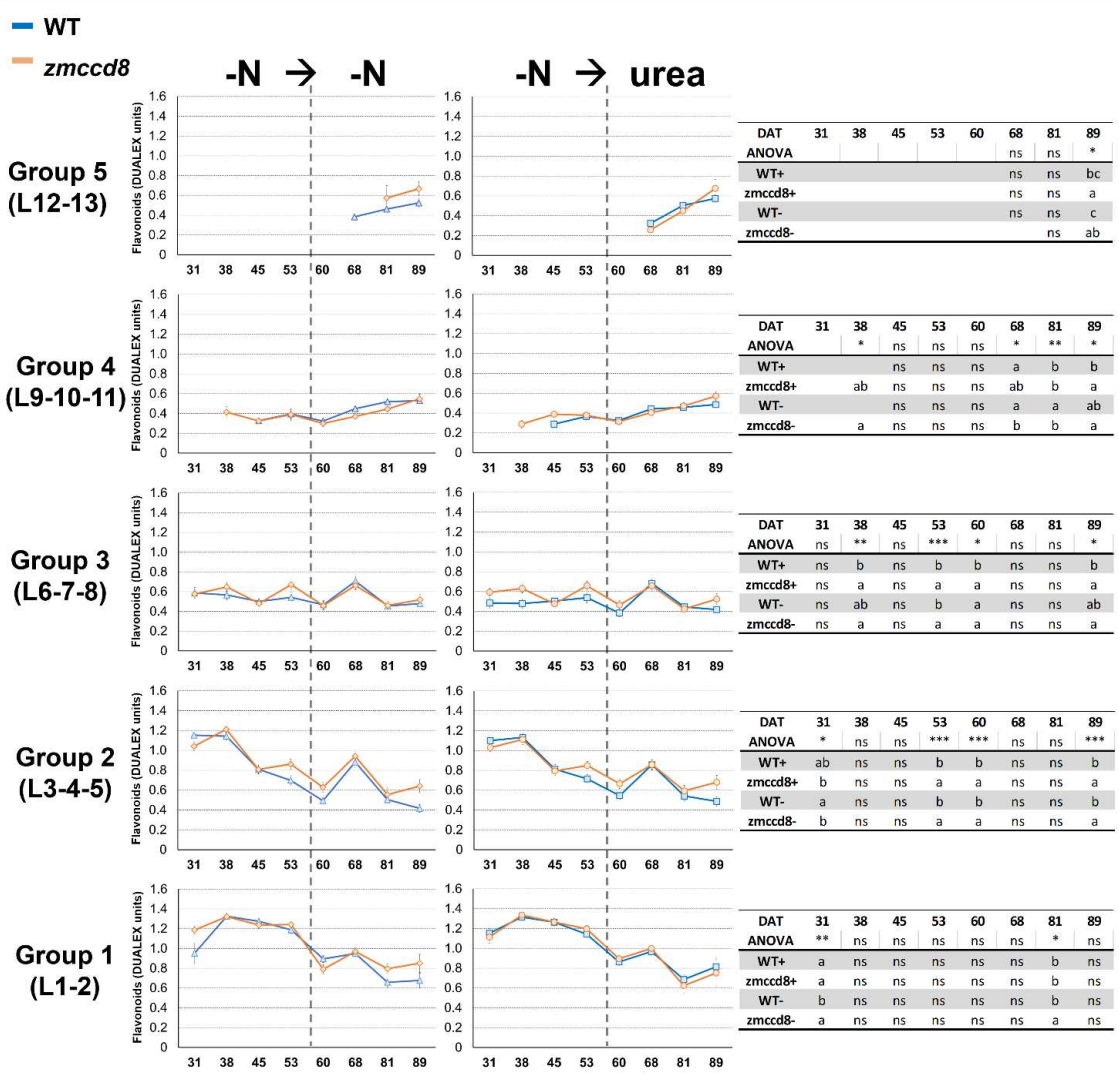
In the first group, the flavonoids levels globally decreased although there are some exceptions at 38, 68 and 89 DAT. There were no significant differences in the values between the four theses apart from the 81 DAT in which *Zmccd8*- had the lowest ones.

An overall decrease was also visible in the second group, with the exception for a significant growth in flavonoids content at 68 DAT. The differences were often non-significant, but at 53, 60 and 89 DAT both mutants have similar values which were higher than WTs. Furthermore, all the four theses at 89 DAT had lower flavonoids content than the group 1 ones.

In the third group the values did not change much during the experiment, reaching similar values from 31 to 89 DAT. Therefore, at most of the DAT the values are not significantly different, despite that there are some higher peaks especially in the mutants at 38, 53 and 68DAT, even in the end, at 89 DAT, the mutants have slightly higher values than the WTs.

In the fourth group instead a small growth in the flavonoids content could be found. The differences were not significant until 68 DAT, when together with 81 DAT the WT- had statistically higher values than the other 3 thesis, while at 89 DAT the mutants reached the highest values followed by WT- and at last by WT+.

The growth gets more marked in the fifth group, where there were not significant differences until 89 DAT, when the *Zmccd8*+ has the highest flavonoids content followed respectively by *Zmccd8*-, WT+ and WT-.



DAT	31	38	45	53	60	68	81	89
ANOVA						ns	ns	*
WT+						ns	ns	bc
zmccd8+						ns	ns	a
WT-						ns	ns	c
zmccd8-							ns	ab

DAT	31	38	45	53	60	68	81	89
ANOVA		*	ns	ns	ns	*	**	*
WT+			ns	ns	ns	a	b	b
zmccd8+		ab	ns	ns	ns	ab	b	a
WT-			ns	ns	ns	a	a	ab
zmccd8-		a	ns	ns	ns	b	b	a

DAT	31	38	45	53	60	68	81	89
ANOVA	ns	**	ns	***	*	ns	ns	*
WT+	ns	b	ns	b	b	ns	ns	b
zmccd8+	ns	a	ns	a	a	ns	ns	a
WT-	ns	ab	ns	b	a	ns	ns	ab
zmccd8-	ns	a	ns	a	a	ns	ns	a

DAT	31	38	45	53	60	68	81	89
ANOVA	*	ns	ns	***	***	ns	ns	***
WT+	ab	ns	ns	b	b	ns	ns	b
zmccd8+	b	ns	ns	a	a	ns	ns	a
WT-	a	ns	ns	b	b	ns	ns	b
zmccd8-	b	ns	ns	a	a	ns	ns	a

DAT	31	38	45	53	60	68	81	89
ANOVA	**	ns	ns	ns	ns	ns	*	ns
WT+	a	ns	ns	ns	ns	ns	b	ns
zmccd8+	a	ns	ns	ns	ns	ns	b	ns
WT-	b	ns	ns	ns	ns	ns	b	ns
zmccd8-	a	ns	ns	ns	ns	ns	a	ns

Figure 4.7. The average flavonoids content (expressed as DUEALEX units) of each group of leaves in the vertical axis and the number of the DAT (days after transplant) in which the analyses were carried out on the horizontal axis. Next to the graphs there are tables with the statistical results obtained by each group: similar letters at corresponding DAT within treatments are not significantly different by an ANOVA test ($p < 0.05$). In blue the WTs and in orange the mutants, the grey dotted line showed when the urea was supplied. Significance ANOVA p-value codes are reported as follow: $p<0.001$ '***'; $p<0.01$ '**'; $p<0.05$ '*'; $p>0.05$ 'ns' not significant.

4.2.3. Anthocyanins

Anthocyanins displayed a similar trend to the Flavonoids (**figure 4.8**), showing a reduction in their content through the time occurs in all the groups.

A decrease was particularly evident in the first group until the 45 DAT, with the WT+ having the highest values at 31 and 38 DAT. Non-significant differences were reported for the 45, 53, 60 and 81 DAT. At 89 DAT the *Zmccd8*- reached highest values in comparison with the other 3 thesis.

The second group shared a similar trend with the first one, but had statistically greater differences between the two genotypes, in which the mutants had higher anthocyanins content than the WTs.

The differences between the two genotypes got even wider in the third group, in particular between the *Zmccd8*- and the WT- at 45, 53 and 60 DAT. Differently, at 89 DAT the difference was greater between the urea supplied treatments.

The group 4 showed a constantly decrease until the 81 DAT when the mutants had a great fall in anthocyanins content but still keeping their values higher than both the WTs. This gap got even bigger between the 81 and 89 DAT, since a stasis in the WTs content occurred, while both the mutants increased their anthocyanins.

Even in the last group a decrease in the values was observed until the 81 DAT, while they did not really change between 81 and 89 DAT, with the mutants always having higher anthocyanins content than the WTs.

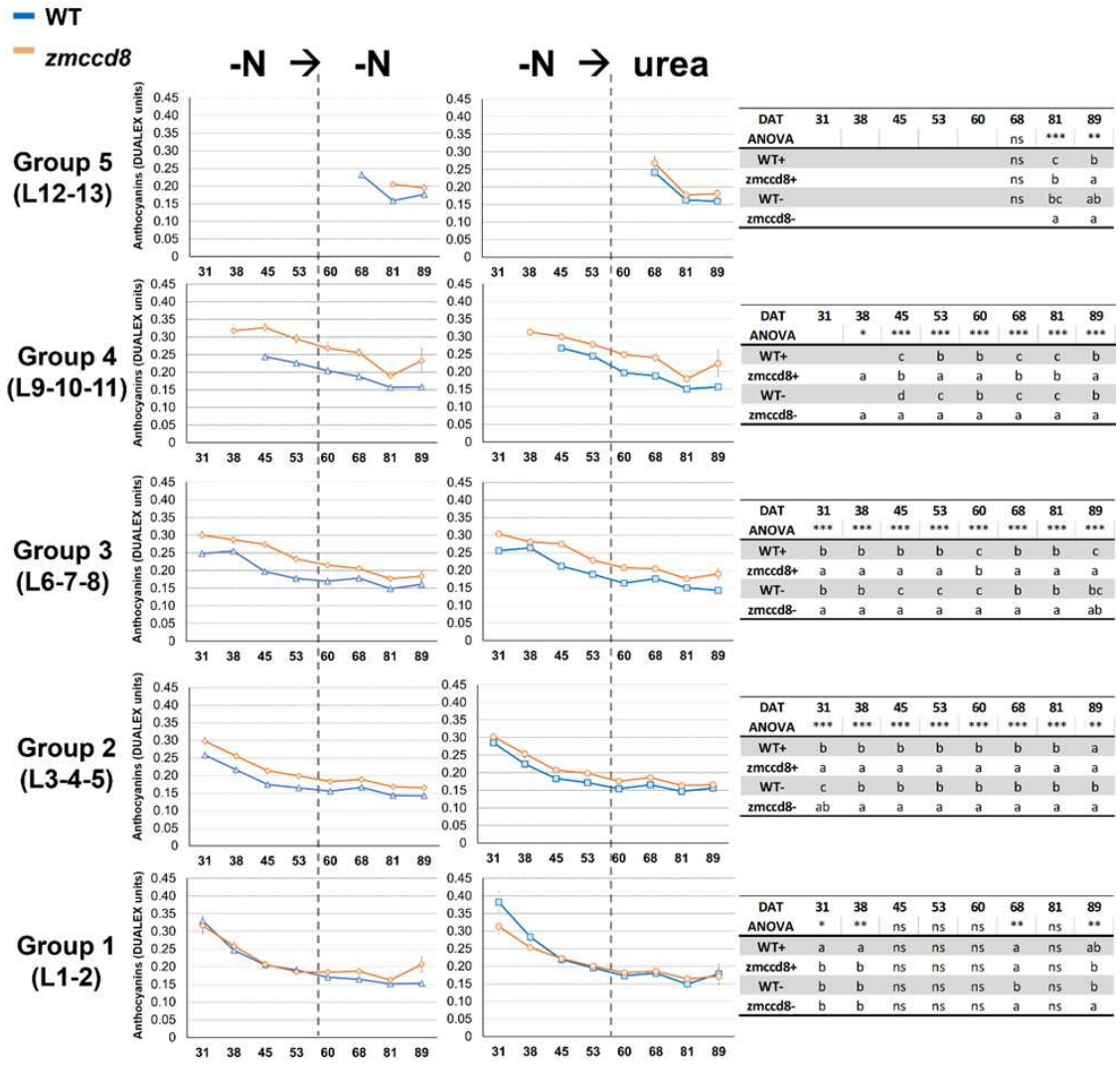


Figure 4.8. The average anthocyanins content (expressed as DUEALEX units) of each group of leaves in the vertical axis and the number of the DAT (days after transplant) in which the analyses were carried out on the horizontal axis. Next to the graphs there are tables with the statistical results obtained by each group: similar letters at corresponding DAT within treatments are not significantly different by an ANOVA test ($p < 0.05$). In blue the WTs and in orange the mutants, the grey dotted line showed when the urea was supplied. Significance ANOVA p-value codes are reported as follow: $p<0.001$ '***'; $p<0.01$ '**'; $p<0.05$ '*'; $p>0.05$ 'ns' not significant.

4.2.4. Nitrogen Balance Index

In the **figure 4.9** it can be noticed how the NBI increase, through the time, was similar to the one seen in **figure 4.6** for the chlorophyll content. Actually, the Nitrogen Balance Index (NBI) was obtained by the instrument as the ratio between CHL and FLAV content. In the first group, the NBI kept growing, except for the stasis between 60/68 DAT and 81/89 DAT. The WT- reached the highest values at 68 and 81 DAT, while no significant differences were found at 89 DAT.

NBI values were still growing in the second group with a significant peak at 60 DAT for the WT+, followed by remarkable fall in all the four theses at 68 DAT. In the following time point (81 DAT) a rise in the NBI values already occurred, reaching similar and non-significant values for all the four theses. However, a great gap was found at 89 DAT between the two genotypes, since the NBI increased in both the WTs unlike the mutants. The exact same trend was also observed in the third group, but the differences between genotypes got even bigger. Actually, group 3 displayed the maximum difference in the NBI levels, about 60 DUALEX units. It occurred both between the WT- and *Zmccd8*- at 53 DAT, and at 60 DAT between the WT+ and *Zmccd8*+.

Nevertheless, in the fourth group these leaves grew up first in the mutants at 38 DAT, as soon as they grew up even in the WTs at 45 DAT their NBI values were already around 40 DUALEX units higher than both the mutants. The overall values displayed and increased, without the fall at 68 DAT but just a transient pause in the growth. The WTs NBI values were always higher than the mutants, as exception for the 81 DAT, when the WT- had lower values similar to the mutants. In the end we found the highest values in the WT+, followed by the WT- and at the end by both the mutants.

In the last group (group 5), no statistical differences could be found at 68 DAT, almost the same happened at 81 DAT, with the exception for the *Zmccd8*- which developed its leaves in this time point, having the lowest NBI values. In summary, the same genotypes still clustered together, with the WTs having higher values than both the mutants.

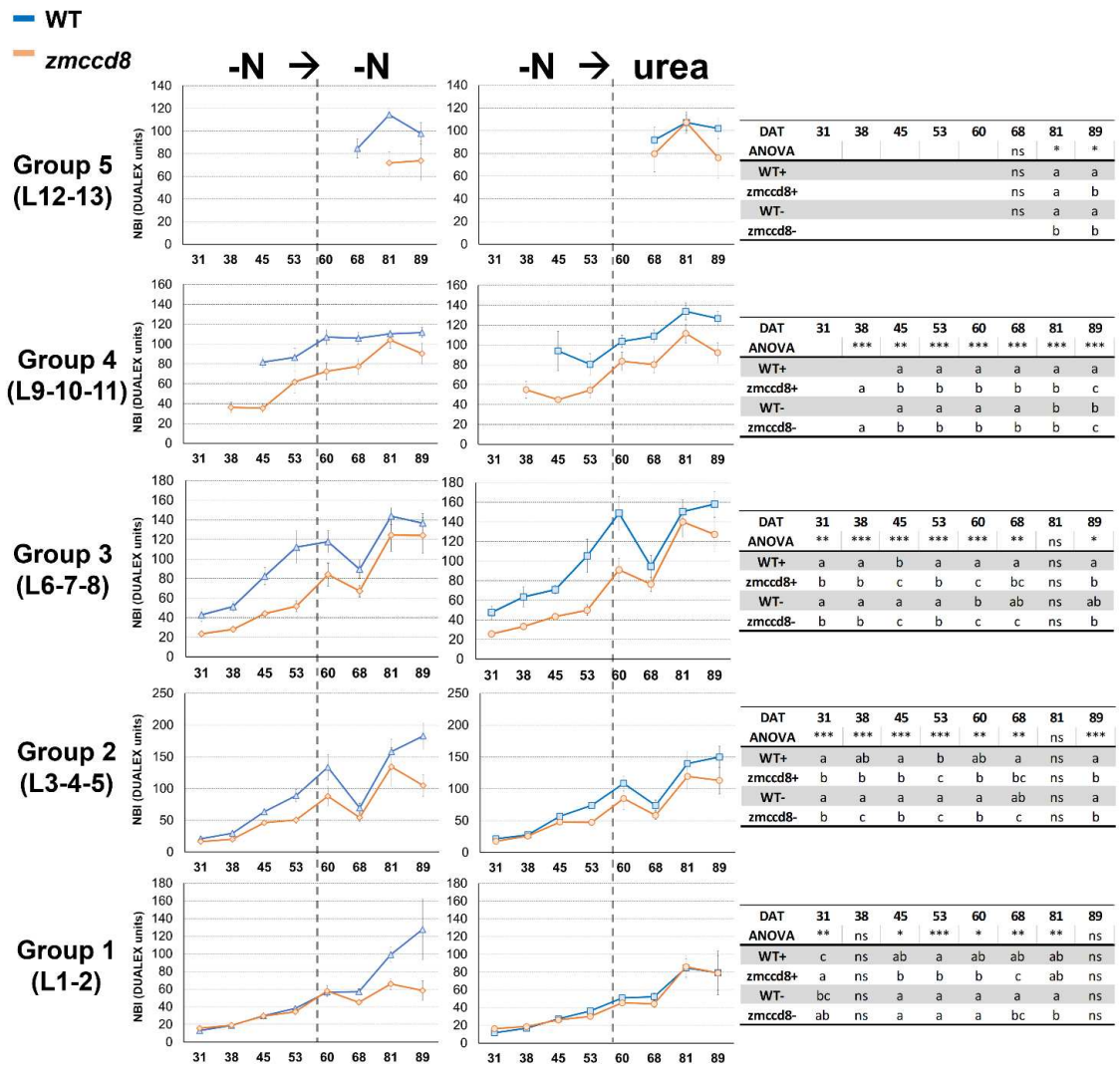


Figure 4.9. The average Nitrogen Balance Index (NB) values (expressed as DUEALEX units) of each group of leaves in the vertical axis and the number of the DAT (days after transplant) in which the analyses were carried out on the horizontal axis. Next to the graphs there are tables with the statistical results obtained by each group: similar letters at corresponding DAT within treatments are not significantly different by an ANOVA test ($p < 0.05$). In blue the WTs and in orange the mutants, the grey dotted line showed when the urea was supplied. Significance ANOVA p-value codes are reported as follow: $p<0.001$ '***'; $p<0.01$ '**'; $p<0.05$ '*'; $p>0.05$ 'ns' not significant.

4.3. Molecular analysis

In order to deeply and better understand the molecular roles operated by strigolactones in response to nitrogen, we organized the graphs, obtained by the averages of our qPCR results, into 4 groups:

I. genes for the biosynthesis, the transport and the perception of SLs
II. genes for the nitrogen uptake, transport and assimilation
III. genes for the sulphur transport and for the amino-acid transport
IV. genes for the iron chelation and compartmentation.

Each gene of the groups was checked at four different time points, all operated after the urea supply (**Table 1**). *AAAP10* and *PTR2* were analysed too but the results were not included because they were too poorly expressed.

4.3.1. *CCD8* and Group I

As expected, *CCD8* was not expressed at all in both the mutants, while it was more expressed in the WT- than the WT+ at 23 and 31 DAU, even if the opposite happened at 43 DAU **(figure 4.10)**.

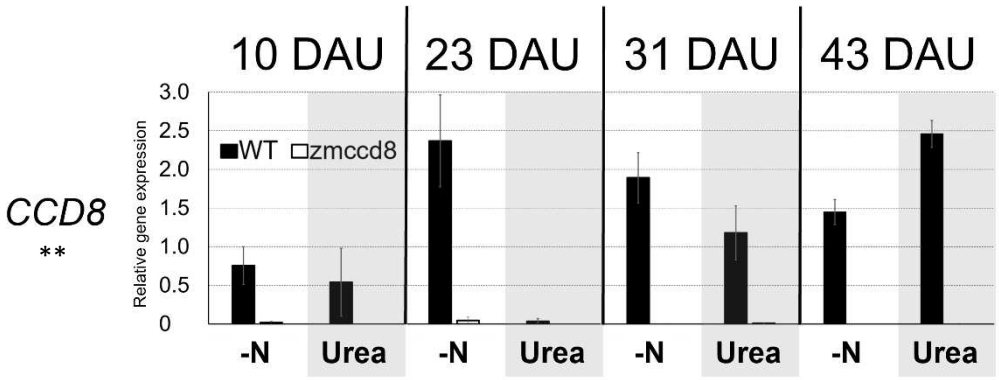


Figure 4.10 Relative gene expression of *CCD8* at four different DAU (days after urea). In black the WTs and in white the *zmccd8* mutants, with "-N" stands for the plants grew without N supply and "Urea" stands for plants supplied with urea as N source. Data are means ± SE for three biological replicates. Significance ANOVA p-value codes are reported as follow: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p>0.05 'ns' not significant.

Group I (**figure 4.11**) includes genes involved in SL biosynthesis, transport and the perception. *CCD7* is a gene involved in SL biosynthesis, it displayed a significant difference in its expression at 23 DAU (days after urea), in particular it was mostly expressed in both the non-supplied genotypes while it is definitely less expressed in the WT+ and not expressed in the *Zmccd8*+. No significant difference can be found for the other time points.

Concerning *WBC33,* a gene encoding a putative SL transporter, its transcription was higher at 10 DAU, to later gradually decrease in the following time points. The only significant difference could be found at 23 DAU where the expression in *Zmccd8*+ was about twice than the other 3 thesis.

For the genes involved in the SL perception, the transcription of both *MAX2* and *D53* was assessed. Regarding *MAX2*, all the four theses had about the same expression at 10 DAU, it remained the same 23 DAU for both the non-supplied, while it was less expressed in the WT+ and almost not expressed at all in *Zmccd8*+ (a similar behaviour to the expression of *CCD7* at the same DAU). An inversion of the trend occurs at 31 DAU when for both genotypes this gene was more abundantly transcribed in response to urea provision. At the end of the experiment all the four theses had similar values of the first time point at 43 DAU.

In the case of *D53* instead a difference in the expression could be found already at 10 DAU when it was more expressed in WT compared to the mutant in all conditions. Differently, at 23 DAU the gene expression in WTs decreased to the level of the mutants with no significant differences, a slightly increase in the expression levels occurred in all the cases but in particular for WT+. As for *MAX2,* even in this case the expression levels of all the four theses in the last time point were similar to the first one.

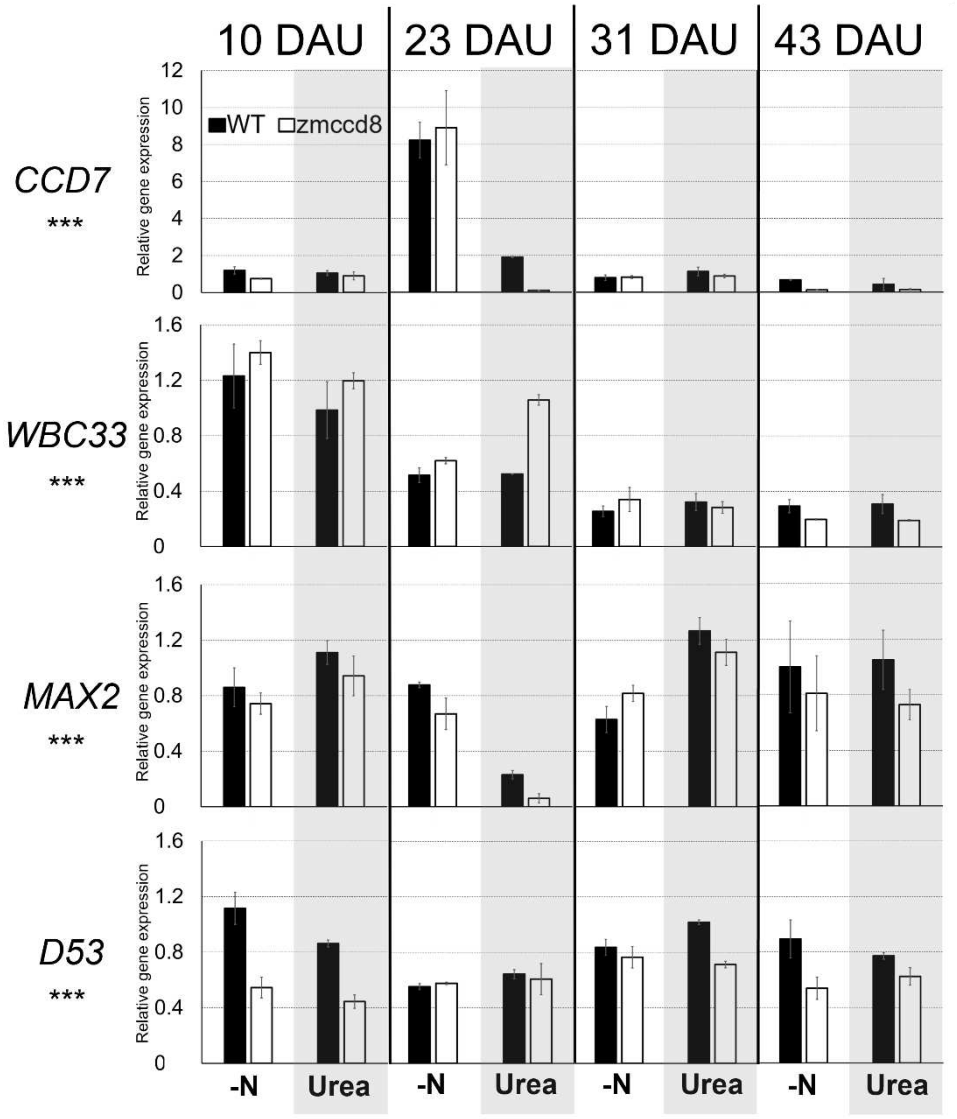


Figure 4.11. Relative gene expression of Group I genes I (biosynthesis, transport and perception of SLs) at four different DAU (days after urea). In black the WTs and in white the *zmccd8* mutants, with "-N" stands for the plants grew without N supply and "Urea" stands for plants supplied with urea as N source. Data are means ± SE for three biological replicates. Significance ANOVA p-value codes are reported as follow: $p<0.001$ '***'; $p<0.01$ '**'; $p<0.05$ '*'; $p>0.05$ 'ns' not significant.

4.3.2. Group II

The group II includes genes for N uptake, transport and assimilation (**figure 4.12**). The gene encoding the nitrate transceptor NRT1.1 showed a doubled expression in both the WTs if compared to the mutants at 10 DAU. In all the other 3 time points, instead, its expression was strongly downregulated for all the four theses, with only a slightly increase in WT- at 23 DAU and *Zmccd8*- at 31 DAU.

With regard to the nitrate reduction, the *NR* expression was evaluated and resulted similar in all the four theses at 10, 31 and 43DAU, while it got higher at 23 DAU showing the highest transcription in the *Zmccd8.*

No significant differences were observed in the case of *GS1* transcription. *GS2* expression was no significantly altered for the first 2 time points (10 and 23 DAU), but it increased reaching the highest expression in WT- followed by the *Zmccd8*- and then from both the urea-supplied genotypes from the 31 DAU. At 43 DAU, its expression in the non-supplied genotypes decreased back, even though the WT- remained slightly higher than the others.

As far as *ASN3* was concerned, it showed a very low expression in the WT+ at 10 DAU which was approximately 5 to 6 times lower than what observed for the other treatments. The exact opposite occurred instead in the following time point, since the expression in the WT+ was higher than all the others. The expression slightly increased at 31 DAU, in particular for *Zmccd8*+ while at 43 DAU, it slightly decreased, with the exception of *Zmccd8*+ which showed a *ASN3* transcription higher in comparison to all the others. Moving to *ASN4*, in the first time point (10 DAU) the two treatments had similar behaviour with values very similar among genotypes. In particular the mutant showed generally higher levels of expression, with the exception of *Zmccd8*+ at 23 DAU. Even in the last 2 time points *Zmccd8*+ showed an ASN4 transcription higher compared to *Zmccd8*-.

At last, the transcription of *LDB7*, a gene previously identified as a marker of nitrogen response in maize, was also assessed. In the first time point, *LBD7* showed a higher expression in WT+ than the mutants, which in turn displayed higher LBD7 expression values in comparison to the WT-. In WT+ its expression decreased, so no significant

differences were detected at 23 DAU. However, at 31 DAU the values of expression of this gene decreased for mutant and WT-, while it greatly increased in the WT+ All the expression levels decreased in the last time point, in particular for the WT+ resulting in a significant difference only between the non-supplied genotypes, since in the WT- it was definitely more expressed than in *Zmccd8*-.

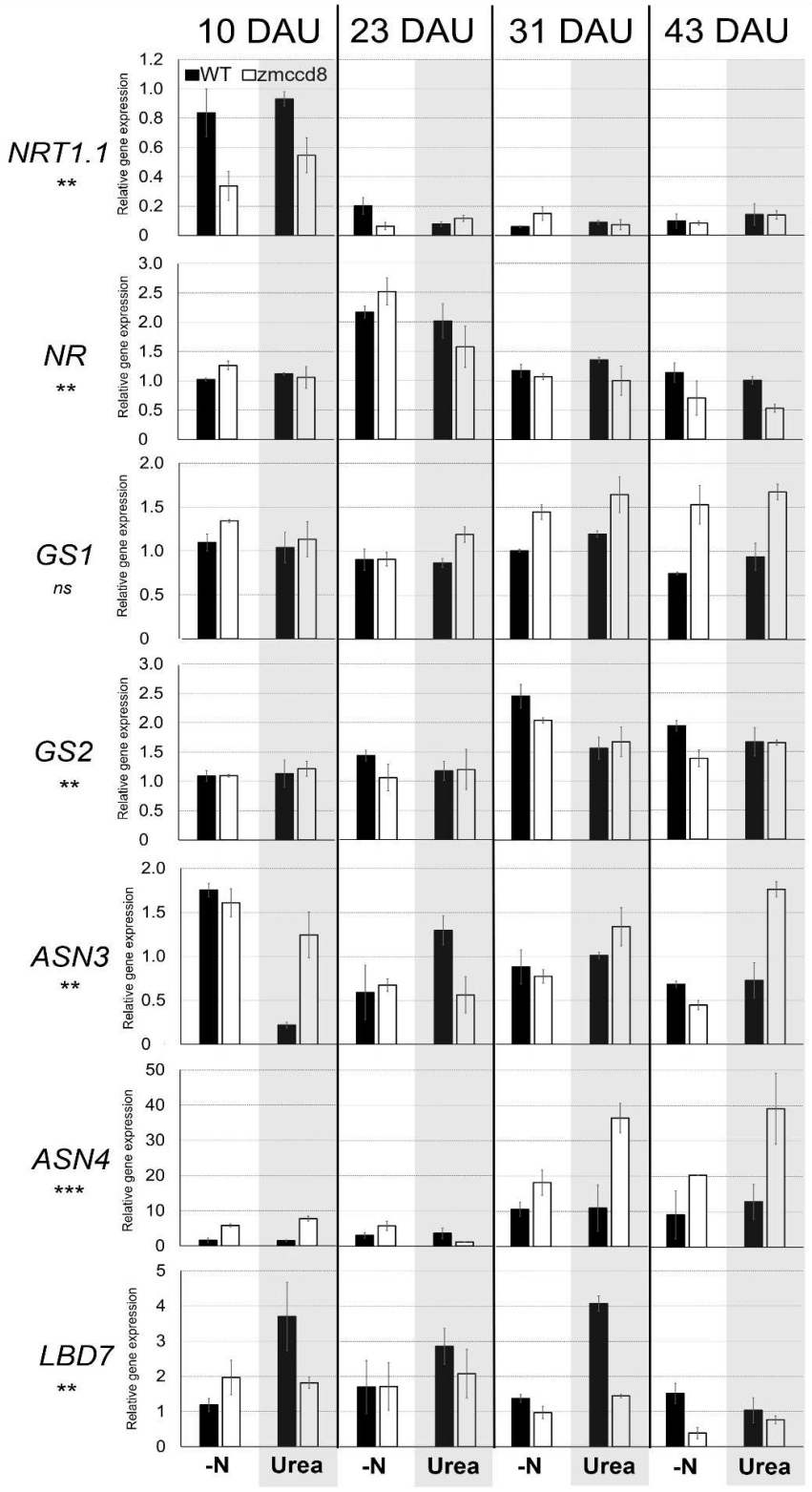


Figure 4.12 Relative gene expression of Group II genes I (N uptake, transport and assimilation) at four different DAU (days after urea). In black the WTs and in white the *zmccd8* mutants, with "-N" stands for the plants grew without N supply and "Urea" stands for plants supplied with urea as N source. Data are means ± SE for three biological replicates. Significance ANOVA p-value codes are reported as follow: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p>0.05 'ns' not significant.

4.3.3. Group III

In the group III **(figure 4.13),** the results of the genes involved in the transport of the sulphur (*SULTR4*, *SULTR6*) and in the amino acid transport (*AAAP22*, *PTR5.6*) are reported. Starting from the sulphur transporters, at 10 DAU an higher expression of *SULTR4* was found in the supplied genotypes. The greatest difference was observed at 23 DAU with an expression in WT- which was 4 times the expression observed for *Zmccd8*- and WT+, while no expression resulted in *Zmccd8*+. All the values measured for the four theses dropped significantly in the next time point, with no significant differences. At the last time point the values measured in both the WTs showed a further decrease resulting so in both the mutants having higher expression. *SULTR6* expression in the WTs seemed always more expressed in the first time point, where WT- and WT+ had twice the expression levels of both mutants. Once again at 23 DAU, as seen also for *SULTR4*, the WT+ had a *SULTR6* expression way higher than all the others, which shared similar values. In the last two time points the expression of both WTs decreased while it did not really change in the mutants, resulting so in a higher expression in the WTs at 31 DAU and comparable levels at 43 DAU.

Moving to the amino acid transporter, at 10 DAU only a slightly higher expression of *AAAP22* was found in both the WTs. The gene expression remained the same in almost all the treatments at 23 DAU, with the exception for a peak in the expression of WT- which then resulted the most expressed. At 31 DAU the highest expression was found in the WT+, since the expression levels decreased only in the WT-, while in all the other treatments they lightly increased. There were no differences at 43 DAU in both WTs and *Zmccd8*-, while a great increase occurred in *Zmccd8*+, reaching twice the expression of all the others. Regarding *PTR5.6*, once we operated the statistical analysis on its results, it turned out to be non-significant.

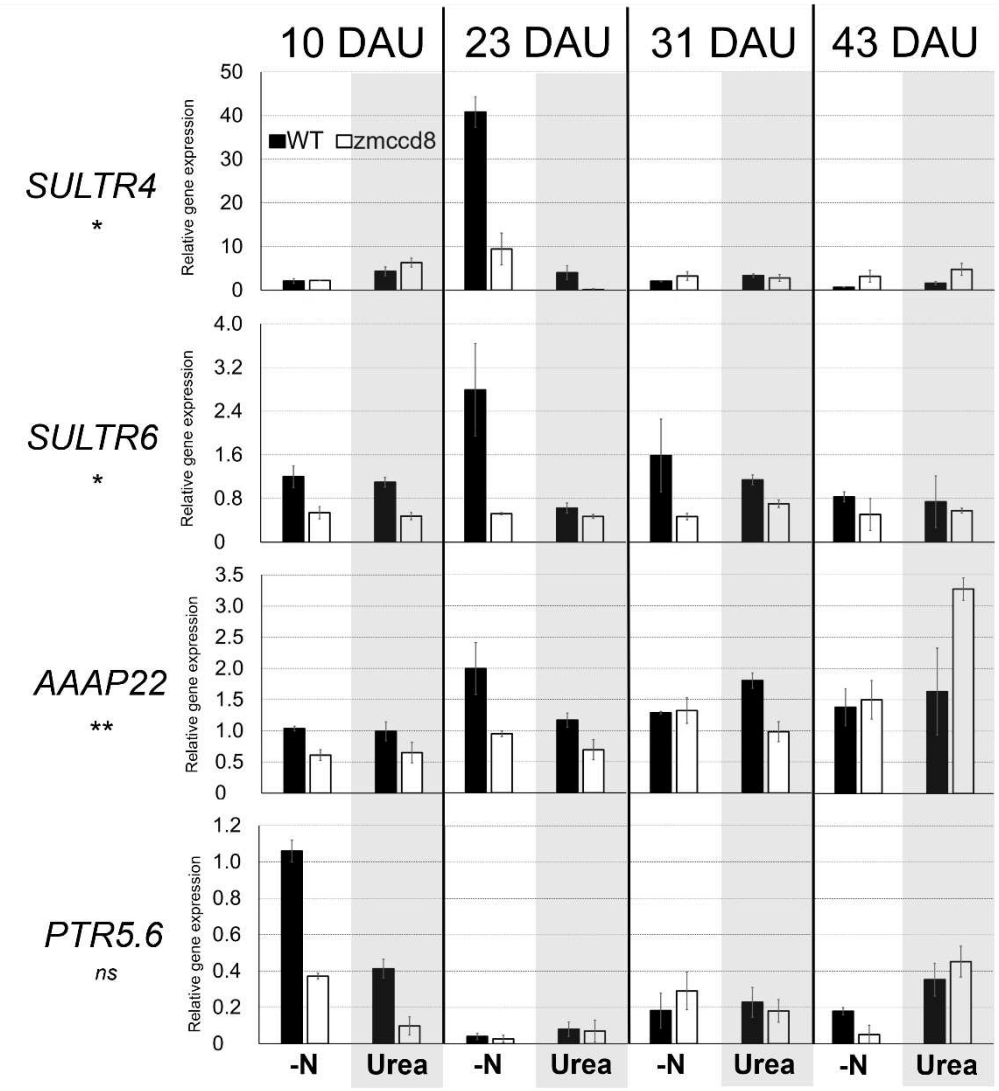


Figure 4.13. Relative gene expression of Group III genes I (sulphur and amino acid transporters) at four different DAU (days after urea). In black the WTs and in white the *zmccd8* mutants, with "-N" stands for the plants grew without N supply and "Urea" stands for plants supplied with urea as N source. Data are means ± SE for three biological replicates. Significance ANOVA p-value codes are reported as follow: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p>0.05 'ns' not significant.

4.3.4. Group IV

The first three genes reported in group IV (*NAS2*, *NAS4* and *NAS6*, **figure 4.14**) are involved in the nicotianamine biosynthesis and so in the iron chelation. A clear difference in the expression pattern of *NAS2* was visible already at the first time point, since the expression in WT- was significantly more than *Zmccd8-*, while no expression at all was found in the urea supplied genotypes. A lack in the expression occurred at 23 and 31 DAT. A low expression was found at 43 DAU in all the thesis, with the exception of the WT- in which it was not expressed at all. Moving to *NAS4* expression, just a slightly difference was found at 10 DAU, with both the WT+ having values a few higher than the mutants. A comparable increment occurred then at 23 DAU, with the exception of the WT- which increased significantly more than the others, even its expression level stayed the same in the next time point, while the others kept growing. So as result at 31 DAU the WT+ showed the greatest expression followed by the WT- which shared similar levels with both the mutants. At the final time-point *NAS4* expression slightly decreased to comparable levels in all the four theses. Comparable expression levels could be found in the first time point of *NAS6* for both the WTs and *Zmccd8-*, while in the *Zmccd8+* it was almost not expressed. A great difference later occurs at 23 DAU when a lower level of expression was found in the *Zmccd8-* and WR+, while no significant expression levels were detected in all the four theses in the last 2 time points.

VIT 1 and VIT 2 encode vacuolar iron transporters, *VIT1* resulted to be the most expressed in the WT- at 10 DAU, since the other 3 theses had about the half of its expression levels. No significant differences were found in the following 2 time points (23 and 31 DAU), while at 43 DAU it was more abundantly transcribed in *Zmccd8+*. According to statistical analysis *VIT2* results were not significant.

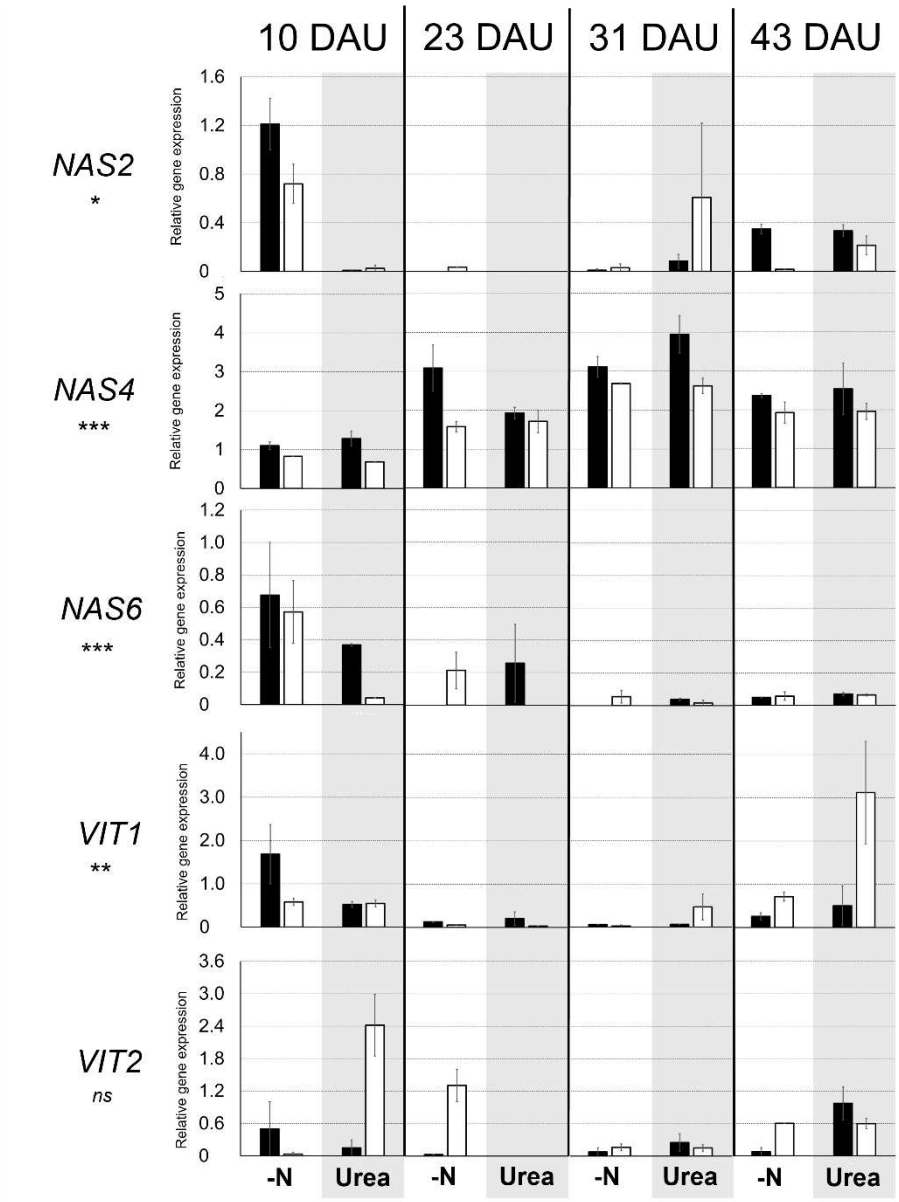


Figure 4.14. Relative gene expression of Group IV genes I (nicotianamine biosynthesis and iron chelation) at four different DAU (days after urea). In black the WTs and in white the *zmccd8* mutants, with "-N" stands for the plants grew without N supply and "Urea" stands for plants supplied with urea as N source. Data are means ± SE for three biological replicates. Significance ANOVA p-value codes are reported as follow: p<0.001 '***'; p<0.01 '**'; p<0.05 '*'; p>0.05 'ns' not significant.

5. DISCUSSION

Nutritional deficiencies are among the most widespread abiotic stresses, in particular the stress induced by nitrogen (N) deficiency, which causes severe conditions such as reduced growth, leaf chlorosis in plants and global crop yield reduction (Zhang et al. 2020; Tollenaar and Lee 2002). To face these adverse conditions, plants have developed a compensatory mechanism that includes various elements, among which the strigolactones (SLs) result involved. In fact, an increase in their production and exudation occurs in N deprived plants (Ravazzolo et al. 2019). Therefore, to better characterize the SL role in the N-response, together with their mechanisms of action, we monitored the phenotypical, physiological and transcriptional responses in the field of the wild type *Zea mays* B73 (WT) and *ZmCCD8* knockout mutant (*Zmccd8*) grew in N-shortage conditions or supplied with urea as N source after 60 days in open field.

Since this mutant does not produce SLs, we analysed how it is impaired in the application of the compensation mechanism in response to N deficiency. Starting from the phenotypic results it was immediately clear that, due to the altered adaptive response, both the *Zmccd8* plants grew in N-deficiency (*Zmccd8*-) and those supplied with urea (*Zmccd8*+) suffered more than the WT plants, underlining the importance of SLs in the adaptation to stressful conditions. Actually, the mutants' plants had values significantly lower than the WTs for all the phenotypical parameters we checked. Only two exceptions can be found in the internodes (**figure 4.3**) and leaves lengths (**figure 4.5**), in which these differences were erased respectively at 81 and 89 DAT, but these results could be mostly attributable to the senescence. This suffering condition can be explained by a reduced photosynthetic activity, a reduction in the assimilative capacity of CO_2, an incorrect accumulation of metabolites in the leaves caused by the lower accumulation of chlorophyll (a nitrogen-containing compound) (Muchow 1989) in the leaves of the *Zmccd8* mutants. As seen also in **figure 4.6**, the chlorophyll content was almost always higher in the WT leaves, in particular the difference was gradually more evident in the upper leaves. Since it is known that, especially in case of N deficiency, plants transport the N to the young developing tissues from the older leaves (Aguera et al. 2010), and this trend was not found for *Zmccd8* mutants in our data, this could

confirm that SLs are involved in leaf senescence and N reallocation (Ueda and Kusaba, 2015). Furthermore, it seems that SLs could also be involved in inhibiting the Chl degrading enzymes activity, maintaining the chloroplast thylakoid membrane stability and regulating the Chl binding to the membrane proteins (Zhou et al. 2012), explaining so the lower chlorophyll content in both the mutants.

Regarding the flavonoid values, as reported in the paragraph 4.2.2, a decrease both over time and age of leaves was detected, but no significant differences were found across all the four theses, except for the 53 DAT in which they were higher in the mutants. Since anthocyanins are a subgroup of the flavonoids, they share with these ones the trend of decrease in their values over time. However, in the **figure 4.8** a clear difference in the content between the different genotypes can be found. Although the values are comparable in group 1, the difference in their content gradually increased as the groups go by. This difference in the anthocyanins content was especially relevant since the biosynthesis of these violet water-soluble pigment occurs in response to stresses, N deficiency included (Liang et al. 2018). Actually, in case of a stressful condition the concentration of reactive oxygen species (ROS) rises, so plants in order to face this toxic increase of ROS, activate different enzymatic and non-enzymatic antioxidant agents, and the anthocyanins are among the main of the non-enzymatic ones (Kovinich et al. 2015). A lower content of anthocyanins therefore indicates a less suffering condition (Francini et al. 2019), as we observed in the WT treatments.

Finally, as regard to the physiological analyses, considering that the NBI is calculated by the ratio between chlorophyll and flavonoids contents and the chlorophyll contents were higher in the WTs, while the flavonoids were slightly higher in the mutants, it can be understood why the trend described for the NBI in paragraph 4.2.4 was similar to the Chl trend and more evident. In fact, there were no significant differences in the first group of leaves, while starting from the second group, the amount of the difference increased as we moved through the groups, reaching a gap between the two genotypes even bigger than what has been seen for the chlorophyll content. In the study realized by De Souza et al. in 2022, the NBI resulted as one of the best performing indicators for the N plants status, directly correlated with nitrogen content per biomass. Therefore,

once again we can tell how *Zmccd8*, due to the lack of SLs, results more suffering and exposed to abiotic stresses than the WT.

However, based on the results obtained from the phenotypical analyses, no significant differences emerged between the two N-treatments, but only between the two genotypes. On the contrary, analyses of Chl content and N-index seemed to indicate a slightly more evident susceptibility of *Zmccd8* to N-deprivation. The mutant seemed in fact to be less adapted to N-starvation, being less able to set up the above described compensation mechanisms.

Moving to the molecular results, the first thing that we could appreciate in **figure 4.10** is that *CCD8* was not expressed at all in the mutants, while it was expressed in WT, as expected. Furthermore, another important deduction can be made from these first data: probably the fact that CCD8 resulted more expressed in the WT- than the WT+ at 23 and 31 DAU, may depend on the absence of the urea supply in the WT- that resulted in a more stressful condition, which so needed a higher biosynthesis of SLs to face it. In support to this hypothesis, it is known how CCD8 represents a reliable marker for the SLs biosynthesis levels and so even and indirect marker for the N response (Ravazzolo et al. 2019; Ravazzolo et al. 2021). However, this response was clearly appreciable only at 23 DAU which seems therefore to represent the time point at which the plant better perceives the nitrogen status.

A similar trend of expression was also found for the *CCD7* expression in **figure 4.11**, which it is also involved in the SLs biosynthesis. In fact, no significant different expressions were found in any time points except for the 23 DAU, when *CCD7* reached its maximum expression only in the non-supplied genotypes, once again showing how the SLs are necessary in case of stressful conditions. Despite *Zmccd8* was not able to produce SLs, CCD7 resulted highly expressed anyway also in this genotype (in *Zmccd8*- at 23 DAU), probably because of a compensation mechanism since it works upstream of the CCD8 (Guan et al. 2012). Regarding the SL transport, the expression of *WBC33* was higher at 10 DAU for all the four theses, and its expression then gradually decreased in all the following time points. A hypothesis to explain this behaviour is that the expression of this putative SLs transporter, could be necessary for a rearrangement of the SLs already basally present to optimize their use before that they get well induced.

When the biosynthesis is finally induced and the SLs reach an optimal concentration across the plant the expression of the transporter can be reduced, explaining its decrease through time. At last, regarding the SLs perception, a difference in the *MAX2* expression occurred at 23 DAU, when no expression was found in the urea supplied genotypes, probably because at 23 DAU most of the supplied nitrogen had been absorbed and so the SLs adaptive response was not necessary. While the expression of the SLs response suppressor *D53* did not really change in mutant through time since it gets modulated/degraded by the presence of SLs which are not produced in this genotype. Its expressions in fact resulted averagely higher in the WT since an efficient suppression of the SL response is required in this case. Furthermore, the expression levels of D53 in the WT were lower at 23 DAU when a significant SLs biosynthesis occurred.

In **figure 4.12** the first significant difference can be immediately found in the N uptake, in fact in *NRT1.1* at 10 DAU the expression was higher in WT, in both the nutritional regimes, suggesting that the absence of the SLs in the mutants invalidates the correct regulation for the N uptake. Another interesting conclusion can be made from the results for the expression of both *ASN3* and *ASN4*, since they were always expressed significantly more in the *Zmccd8*+ in both the last two time points, as if the lack of SLs compromises the ability to correctly modulate the metabolism in response to the environmental N content in this genotype. Lastly it is interesting to notice how *LBD7*, which is a gene involved in the N response (Zhang et al. 2014), resulted more expressed in the WT+ in the presence of N (for the first 3 time points, since probably the levels of the suppled N at 43 DAU were really low), underlining so how a correct response takes place in the WT in presence of N. Furthermore, the fact that *LBD7* expression was significantly lower in the *Zmccd8*+ once again confirm the role of the SLs in the response to N.

an interesting behaviour was found in terms of expression of genes involved in sulphur transport/uptake, as described in the paragraph 4.3.4. No significant differences were found across the four theses in most of the time points, as exception of the expressions for both the tested genes in the WT- at 23 DAU. In this time point in fact the WT- reached significantly higher values than the other 3 thesis, exactly as it was seen in its expression

of *CCD7* and *MAX2* at the same time point. This similarity in the results for both the synthesis of SLs and for the sulphur transport can be explained since the SLs are induced in the response to many abiotic stresses, including sulphur starvation stress (Umehara et al. 2021). This close correlation demonstrates so how the SLs strongly up-regulate genes for the sulphur uptake to face its eventual deficiency. Umehara et al. 2018 in fact underlined how the symptoms of rice plants under sulphur deficiency were similar to the SLs biosynthesis/signalling deficient mutants' phenotypes.

As far as *AAAP22* expression was concerned, a less marked than what was seen for *ASN3* and *ASN4,* but still significant overexpression in WT- at 23 occurred. Even in this case, as seen for *ASN3* and *ASN4*, in the WT- at 43 DAU a strong induction expression of *AAAP22* occurs. This trend of expression of the genes involved in N assimilation seems to suggest that this genotype, due to the absence of SLs, is not able to modulate its metabolism in response to the environmental N content.

Finally, as regard to the genes involved in iron transport and chelation in **figure 4.14**, a similar trend was found in all genes except for *NAS4*. In particular, at 10 DAU higher expression levels can be found for *NAS2*, *NAS6* and *VIT1* in both the N non-supplied genotypes. The increase in their gene expression could be an attempt of the non-supplied plants to compensate for the lack of iron, since it has been reported that low levels of N interfere with iron absorption by maize plants, resulting so in significantly lower iron content in maize plants under N deficiency than the plants of control (Mekonnen et al. 2023). Regarding *NAS4*, once again at 23 DAU its expression results higher in WT-, as already seen in many genes before, suggesting that its overexpression is part of the correct response to N starvation that occurs in WT-.

These results clearly confirmed how the SLs are essential for a correct response to N.

6. CONCLUSIONS

From the results that we obtained a greater broad-spectrum view of the action of Strigolactones in *Zea mays* L. can be appreciated, although further analyses to finely understand their action in the response to maize deficiency need to be carried out in the future.

Many significant differences were highlighted between the two genotypes during their growth. Starting from the physiological results of the leaves pigments, in which the reduced contents of chlorophyll and NBI are probably the cause of the greater suffering in the *Zmccd8* plants. This worse health condition in the mutant plants was also confirmed by the higher levels of anthocyanins present in their leaves, thus confirming the fundamental role of strigolactones. The physiological differences observed then resulted in significant differences in the phenotype of the plants since the WTs under the same growth conditions always showed greater dimensions compared to the mutant plants, underlining the crucial importance of strigolactones for the achievement of a correct growth in maize.

Furthermore, significant differences were found also at the molecular level, probably due to an impaired regulation of the response to N deficiency in the mutant that does not produce SLs.

One last conclusion can be given by the absence of significant differences, attributable to the different nitrogen supply in the treatments, in the phenotypical results, despite the molecular differences observed. This disagreement suggests that the difference in nitrogen concentration that we obtained in the field was not so restricting and therefore compensated by the molecular responses of the plants. Furthermore, since in the mutant plants the molecular response was impaired due to the lack of strigolactones, differences in the physiological analyses between the two mutants were still detectable. In particular the *Zmccd8*- plants, which were non nitrogen supplied, showed worse health conditions than the supplied *Zmccd8*+ plants.

Future experiments are needed to set up new protocols to better study the exact response to *Zmccd8* to nitrogen deprivation and to widen the ongoing research also to further abiotic stresses.

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