University of Naples "Federico II"
Ph.D. in *Agrobiology and Agrochemistry*
XXVIII cycle (2013-2016)

GENOMICS OF OSMOTIC STRESS RESPONSES IN *ARABIDOPSIS* AND CROPS

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Tutor: Dr. Maria Stefania Grillo

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To my family

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1. Introduction

1.1 Abiotic stresses and crop production: the osmotic component

By 2050 the global population will reach 9.1 billion people, 34 percent higher than today. In an era where the global yield growth rate of the major crops has been steadily declining, food security in the near future is becoming a major concern (FAO, 2009). Abiotic stresses, such as salinity and drought are primary environmental constraints to crop production. Current prediction models anticipate an increase in frequency and intensity of episodes of drought, flood, and heat waves due to climate change phenomena (Ahuja et al., 2010; Mittler and Blumwald, 2010). Warmer temperatures will also result in extensive soil salinization, as sea levels rise, with a decrease in land suitable for agriculture (Morison et al., 2008).

Today, soil salinity affects more than 40 million hectares of irrigated land, with deleterious effects on crops. High concentrations of NaCl in soil challenge plants with both an osmotic and an ionic stress component: on the one hand, a decrease in soil water potential reduces water uptake and, on the other hand, Na^+ ions negatively influence metabolic processes, mainly due to a competition in uptake with K^+, an essential cofactor for many enzymes (Zhu, 2001). This ion imbalance and the hyperosmotic condition lead to molecular damage resulting in slower crop growth rates, reduced tillering and delayed reproductive development (Zhu, 2001; Roy et al., 2014). Water deficit stress is even more pervasive and economically damaging, causing an average yield loss of more than 50% (Hu and Xiong, 2014). Several studies have reported the sensitivity to drought of the major crop species, such as most modern varieties of maize (*Zea mayze*) (Lobell et al., 2014), rice (*Oryza sativa*) (Siringam et al., 2013), tomato (*Solanum lycopersicum*) (Foolad, 2007) and potato (*Solanum tuberosum*) (Jaarsma et al., 2013). Drought directly arrests plant growth as a result of increased soil water potential and photosynthesis inhibition by stomatal closure. Water stress is often accompanied by high temperatures that induce photo-oxidation and, consequently, production of reactive oxygen species and oxidative damages (Li et al., 2009). Because water deficit and soil salinity share the osmotic stress component, plant responses to drought and salt are closely related and the mechanisms overlap (Zhu, 2002; Osakabe et al., 2014). Breeders have made important progress in improving and developing stress-tolerant crops, but these still cannot meet the global food demands. Therefore, there is immediate need to discover still unknown processes involved in osmotic stress response for future breeding applications.

1.2 Physiological aspects of plant response to osmotic stress

1.2.1 Morphological and anatomical modifications

Roots are the organ through which plants explore the surrounding soil, searching for water and nutrients. Therefore, they are the first organs exposed and responding to osmotic stress. In order to maximize water uptake, plants modify root architecture in stress condition, increasing primary root elongation to explore deeper layers of soil (Malamy, 2005; Mittal et al., 2015). At the whole plant level, the root to shoot ratio is increased, with a reduction in leaf number and leaf area (Blum, 1996).

On leaf epidermis, stomata and trichome density are affected. Stomata play an important role in the photosynthesis and transpiration rates, regulating the gas exchange. Similarly, trichomes increase solar radiation reflection, reducing leaf temperature and, therefore, the transpiration process. Accordingly, reduction of stomata and trichome density are important traits to osmotic stress tolerance (Gianoli and Gonzalez-Teuber, 2005), that can be interpreted as an adaptation to minimize water loss (Skirycz et al., 2010).

A general reduction of cell proliferation and expansion is, indeed, an important stress adaptive response to reduce transpiration rate and energy requirements (Skirycz et al., 2010). This, however, also translates in a decreased photosynthetic activity and biomass production, resulting in a reduction in plant growth.

1.2.2 Photosynthetic responses and gas exchanges

The negative modulation of photosynthetic activity is the major cause of reduction in plant development under osmotic stress. Carbon fixation through photosynthesis is a complicated process involving different components, such as CO_2 assimilation and reduction pathways, photosynthetic pigments, and electron transport chain. Damages at any level caused by stress reduce the overall photosynthetic capacity (Ashraf and Harris, 2013). During osmotic stress, the stomata closure determines decreased CO_2 assimilation, which in turn causes deactivation of Rubisco enzyme, responsible for CO_2 fixation (Chaves et al., 2009). Similarly, the action of other important enzymes involved in the regulation of the Calvin cycle is altered by stress condition, including the Fructose-1,6- bisphosphatase (FBP). In fact, FBP is considered as one of the potential enzymes that cause the reduction of photosynthetic activity under stress conditions (Ashraf and Harris, 2013).

During salt stress, also the increased levels of Na^+ and Cl^- ions perturb the photosynthetic processes. The toxic Na^+ cation causes the reduction in photosynthetic pigments, such as chlorophylls (Chl). The decreased contents of these pigments under stressful condition is probably

due to impaired biosynthesis or accelerated pigment degradation. This reduces light absorbing efficiency of photosystem I (PSI) and II (PSII) with a high decrease in photosynthetic capacity (Eckardt, 2009). Osmotic stress affect the function of both PSI and PSII also reducing electron transport through them. This results in a decreased production of ATP and NADPH, the energy-storage molecules that are essential for CO_2 fixation.

1.2.3 Reactive oxygen species protection

Reactive oxygen species (ROS) are reactive molecules containing oxygen, such as singlet oxygen (O_2), superoxide anion (O_2^-), hydroxyl radical ($HO^·$) and hydrogen peroxide (H_2O_2). Although they are products of the normal metabolism of oxygen, their accumulation causes oxidative damage to proteins, DNA, and lipids (Gill and Tuteja, 2010).

In plants, only 1% of the absorbed oxygen produces ROS (Bhattacharjee 2005). In control conditions, these species are efficiently removed by non-enzymatic and enzymatic antioxidants.

Under osmotic stress, instead, when CO_2 is limited, the electron transport chain causes the over-production of ROS, which can exceed the ability of the antioxidant systems, causing oxidative stress (Anjum et al., 2010). In this context, the removal of ROS is essential for stress tolerance. This is mainly obtained by oxyreductant enzymes as gluthatione peroxidase (GPx), superoxide dismutase (SOD) and catalase and by antioxidant compounds, such as ascorbic acid, thioredoxin, gluthatione.

Several studies have shown that the overexpression of scavenging antioxidant enzymes enhances stress tolerance. For example, transgenic plants overexpressing *OsMT1α*, coding for a SOD, showed increased levels in antioxidant enzyme activities and improved drought tolerance in rice (Yang et al., 2009). Similarly, the overexpression of Ascorbate peroxidase (*APX*) in Tobacco chloroplasts displayed improved plant tolerance to salt and water deficit (Badawi et al 2004).

1.2.4 Hormonal regulation

Several adaptive mechanisms in plants, are related to increase in endogenous abscisic acid (ABA) levels. ABA mediates numerous processes in growth and development, including seed maturation and germination and stomatal movements.

In Arabidopsis, osmotic stress rapidly induces expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*), encoding the rate-limiting enzyme in ABA biosynthesis (Iuchi et al., 2001) in roots. ABA is then loaded in vascular tissues and transported through passive diffusion to leaves where it is perceived in guard cells. ABA signalling leads to the activation of outward ion

channels and inhibition of inward channels, resulting in an efflux of cations and anions, followed by water, thus reducing the turgor of guard cells resulting in stomatal closure (Schroeder et al., 2001; Kim et al., 2009).

ABA also plays an essential role in the initiation and maintenance of seed dormancy. Genetic studies have been used to isolate several ABA-insensitive (ABI) mutants, that are able to germinate when exposed to concentrations of exogenous ABA that inhibit germination of wild-type seeds (Finkelstein et al., 1994). The best characterized are the transcription factors ABI3 and ABI5, genetic components of the ABA signalling pathway in seed germination. Both regulate transcription of a large number of seed development genes. In particular, ABI3 acts upstream of ABI5 to maintain embryos in quiescent state in order to protect plants from drought (Lopez-Molina et al., 2002).

Other hormones have also been implicated in the regulation of stress-related processes.

Growing evidence suggests the role of auxin, indole-3-acetic acid (IAA) in stress responses. IAA is mainly required for plant morphogenesis, such as tropistic root growth, flower organ development and vascular tissue differentiation (Zhao, 2010). The transcript levels of several auxin-related genes change under drought stress, suggesting an auxin role in drought tolerance. Moreover, recent studies provide evidence about the correlation of auxin homeostasis and ABA synthesis. In rice, the balance of IAA and ABA homeostasis plays a crucial role in plant growth and abiotic stresses response, suggesting an crosstalk between ABA and auxin at the biosynthesis and signalling levels (Du et al., 2012).

1.3 Biochemical mechanisms of plant response to osmotic stress

1.3.1 Protective role of secondary metabolites

Exposure to drought, high salinity and low temperature leads to cellular dehydration. This removal of water from the cytoplasm results in a decrease of cytosolic and vacuolar volumes. In response to stress, plants enhance the production of specific sets of primary and secondary metabolites, that act as osmoprotectants, osmolytes, antioxidants, and secondary messengers to limit cellular damage. The net accumulation of these osmolytes lowers the cellular osmotic potential and draws water into the cell to maintain turgor pressure. These solutes include amines (polyamines and glycinebetaine), amino acids (proline), soluble sugars (glucose, sucrose, trehalose), and polyols (mannitol, sorbitol and inositol) (Singh et al., 2015). Metabolic plasticity in the accumulation of these osmoprotective compounds is a promising indicator of plant acclimation to stress condition. Since some crops have low levels of these compounds, the manipulation of genes involved in osmoprotectant biosynthesis

pathways is one of the strategies to improve stress tolerance in plants (Reguera et al., 2012; Conde et al., 2015).

Polyamines (PAs) are small aliphatic nitrogen compounds, ubiquitous to all organisms. The biological functions of PAs are associated with their cationic nature. In plants, polyamines act as regulatory molecules implicated in fundamental cellular processes, including embryogenesis, floral development and pollen tube growth (Tiburcio et al., 2014). Significant accumulation of the three most common PAs, putrescine (Put), spermidine (Spd), and spermine (Spm), occurs during biotic and abiotic stress (Wen and Moriguchi, 2015). Glycine betaine (GB), a quaternary ammonium compounds derivative of glycine, is considered the major osmolyte involved in cell membrane protection. In response to various abiotic stresses such as drought and salinity, GB is accumulated in chloroplasts and other plastids of many plant species. One of the principal role of GB is that it encourages water flow into cells to maintain the intracellular osmotic equilibrium and regulates the cascade of signal transduction (Ranganayakulu et al., 2013). The overproduction of GB in various plants including maize (Quan et al., 2004) and cotton (*Gossypium hirsutum*) (Lv et al., 2007) by modulation of two key genes involved in GB biosynthesis, *betA* (encoding choline dehydrogenase) and *CMO* (choline monooxygenase), results in improved yield production under stressful field conditions.

Accumulation of proline under stress condition in many plant species has also been correlated with stress tolerance. Proline (Pro) is a versatile amino acid essential both as component of protein and as free amino acid. To avoid cellular dehydration, proline facilitates water uptake and reduces the accumulation of Na^+ and Cl^- (Ashraf and Foolad, 2007). In plant cells, Pro biosynthesis takes place in the cytosol and the plastids. The principal precursor is glutamate, converted to Pro by two consecutive steps catalyzed by PYRROLINE-5-CARBOXYLATESYNTHETASE (P5CS) and P5C REDUCTASE (P5CR). The degradation of Pro occurs in mitochondria by the reverse action of PROLINE DEHYDROGENASE (PDH) and PYRROLINE-5-CARBOXYLATE DEHYDROGENASE (P5CDH) (Kumar et al., 2015). The over-expression of biosynthetic genes significantly enhances endogenous levels of proline and increases drought stress tolerance in wheat (*Triticum aestivum*) (Vendruscolo et al., 2007) as well as in rice (Su et al., 2004) promoting growth, antioxidant defence system and decreasing rate of uptake of Na^+ and Cl^- .

Additional osmoprotectants include reduced forms of sugars, such as glucose, sucrose, fructose and trehalose. Sugars provide carbon for cellular metabolism and regulate growth and development of plants. During salinity and drought stress, sugars and sugar alcohols regulate the osmotic adjustment, protect membranes by interacting with protein complexes and enzymes, and scavenge toxic ROS (Van den Ende and Valluru, 2009). The regulation of trehalase activity by expression of

trehalose synthesis-related genes improves tolerance to abiotic stresses in rice (Garg et al., 2002) and alfalfa (*Medicago sativa*) (Suárez et al., 2009), decreasing aggregation of denatured proteins (Ashraf and Harris, 2004; Koyro et al., 2012). Similarly, sugar alcohols including mannitol, sorbitol and inositol improve stress tolerance of plants. Mannitol is the most common polyol in nature and is synthesized in mature leaves and then translocated through the phloem and oxidized to mannose or stored and used as carbon source. During abiotic stress, mannitol is accumulated in the cytosol to stabilize macromolecules and to act as scavenger of hydroxyl radicals (Conde et al., 2011).

1.4 Molecular response to osmotic stress

Drought stress affects several aspects of plant physiology and metabolism by causing an osmotic imbalance. In addition, salt stress also causes an ionic imbalance mainly due to the presence of toxic Na^+ ions. Many signalling proteins, such as transcription factors, protein kinases and phosphatases, are involved in signal transduction during plant adaptation to osmotic and ionic stress. The role of these regulatory genes ranges from the perception of stress signal to induction of tolerance effector genes. A fine modulation of the activity and abundance of all these components in the different plant organs is necessary to achieve stress tolerance (Zhu, 2002).

1.4.1 Ionic homeostasis regulation

During salt stress, entry of Na^+ and Cl^- ions into the cells causes significant ion imbalance and might cause severe physiological disorders. Since Na^+ inhibits many enzymes, its entry should be minimized. Two major transporters maintain low Na^+ concentrations in cytoplasm: the Na^+/H^+ exchanger 1 (*NHX1*), located in the tonoplast, and SALT OVERLY SENSITIVE 1 (SOS1, also known as NHX7) (Yamaguchi et al., 2013) $Na^{+/}H^+$ antiporter located in the plasma membrane.

In plants, the SOS pathway (Figure 1) is a key mechanism for ion homeostasis control at the cellular level. High concentrations of Na^+ ions initiate a calcium signal that activates the upstream regulator SOS3, a calcium binding protein, which in turn binds and activates the serine/threonine protein kinase SOS2. The SOS3-SOS2 complex controls the activity of SOS1 and regulates the activity of several tonoplast located transporters and enzymes such as the V-ATPase, responsible for generating the proton driving force that allows the compartmentation of ions into the vacuole against gradient. SOS3-SOS2 complex may also regulate the activities of other transporters, such as NHXs (Zhu, 2002).

While the SOS signalling pathway mainly exports Na^+ out of the cell, most NHXs are essential for Na^+ detoxification through compartmentation of ions into vacuole. This compartmentation, in addition to avoid Na^+ toxicity in the cytosol, uses Na^+ ions as osmolyte in the vacuole to reach

osmotic homeostasis. Overexpression of *NHX1* in *Arabidopsis thaliana* and its orthologs in other plant species, such as tomato or rice, appear to improve plant salt tolerance (Zhang et al., 2001). Similarly, constitutive overexpression of SOS1 lowers shoot Na^+ content and improves salt tolerance (Shi et al., 2002). Conversely, mutations in SOS1 cause extreme salt stress sensitivity in plants (Deinlein et al., 2014; Zhu, 2001), both glycophytes and halophytes (Oh et al., 2009).

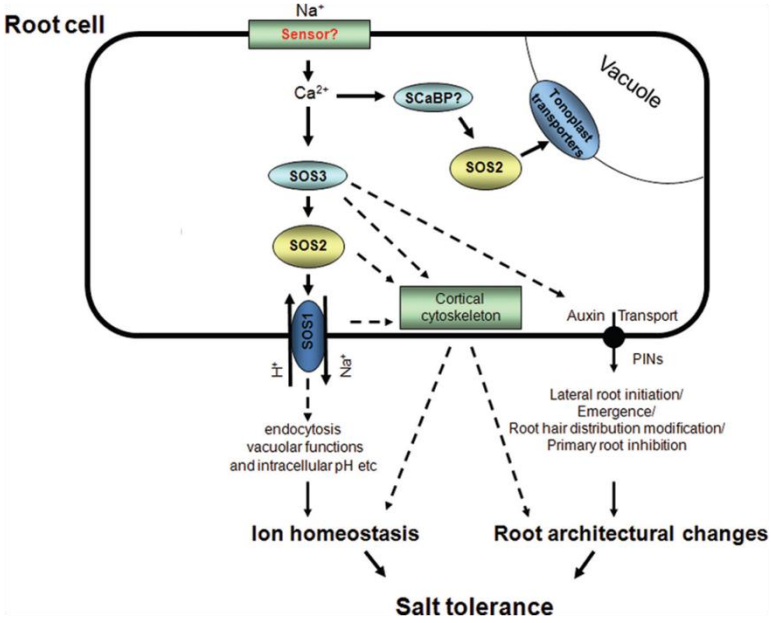

Figure 1. Ionic homeostasis regulation by SOS pathway as reported in Ji et al. (2013). High Na+ stress initiates a calcium signal that activates the SOS3-SOS2 protein kinase complex, which then stimulates the Na+/H+ antiporter SOS1. Solid arrows indicate established, direct regulations, while dashed lines indicate suggested links between the components represented.

1.4.2 Osmotic homeostasis regulation

A key role in the regulation of stress response processes is played by the hormone ABA. An important breakthrough in understanding the osmotic stress responses has been the recent identification of the ABA PYR/PYL/RCAR receptors (PYRABACTIN RESISTANCE; PYR1-LIKE; REGULATORY COMPONENT OF ABA RESPONSE), and the elucidation of their mechanism of action in ABA-mediated signalling cascades (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009).

Osmotic stress induces an increase in ABA concentration, perceived by the PYR/PYL/RCAR receptors. When ABA is ligated, a conformational change is induced in PYR/PYL/RCARs, which therefore become able to interact and inhibit PP2C protein phosphatases. This mechanism prevents the PP2Cs-mediated inactivation of SnRK2 kinases (Sucrose non-fermenting 1-related protein kinase) via dephosphorylation (Klingler et al., 2010).

ABA-activated SnRK2s phosphorylate an important class of transcription factors, AREB/ABF (ABA-RESPONSIVE CIS-ELEMENT BINDING PROTEIN/ABA-RESPONSIVE CIS-

ELEMENT BINDING FACTOR), that induce the expression of several genes whose promoter regions contain an ABRE motif (ABA-responsive cis-element). These genes are principally involved in the ABA signaling under osmotic stress and include RD29B, and several basic leucine zipper (bZIP) proteins (Jakoby et al., 2002; Shinozaki et Yamaguchi-Shinozaki., 2007; Hubbard et al., 2010; Klingler et al., 2010). In addition, the ABA signalling cascades also regulates stomatal movements, by stimulating closure and inhibiting opening. Several transporters located on the plasma membrane of guard cells are under the control of OST1, an SnRK2-type kinase. When released from PP2C inhibition, OST1 phosphorylates and activates stomatal closure promoters such as anion transporter SLAC1 and ROS generator AtRBOHF (Geiger et al., 2009; 2010, Sirichandra et al., 2009). By contrast, OST1-operated phosphorylation results in the inactivation of promoters of stomatal closure. For example, phosphorylation at Thr306 results in reduction of the activity of KAT1 (Sato et al., 2009).

Other important transcription factors, MYCs and MYBs, are synthesized after the accumulation of endogenous ABA, indicating that they are also key components in the ABA-dependent regulatory pathway (Valliyodan and Nguyen, 2006; Abe et al., 2003). MYB44 interacts with the ABA receptor RCAR1/PYL9, reducing its inhibitory activity on ABI1 (Li et al., 2014). In Arabidopsis it was highly expressed in guard cells with other MYB proteins, AtMYB60 and AtMYB61, playing roles in stomatal movements. AtMYB60 is mainly expressed when stomata are open. In contrast, AtMYB61 is specifically expressed in guard cells in the darkness by signals that induce stomatal closing (Cominelli et al., 2005; Liang et al., 2005).

Various stress-related genes do not respond to ABA treatment, suggesting the existence of an ABA-independent pathway in response to osmotic stress. This pathway is regulated by DREB2 proteins (DRE/CRT-BINDING PROTEIN 2), members of the AP2/ERF family of plant-specific transcription factors. Several studies reported the role of *DREB2A* and *DREB2B* in regulatory mechanism of plants during osmotic and heat stress (Sakuma et al., 2002, 2006).They bind to dehydration-responsive cis-element/C-repeat (DRE/CRT), sited in the promoter region of genes showing ABA-independent expression in stress responses (Nakashima et al.,2006; Yoshida et al., 2014)

In recent years, evidence for interaction between the AREB/ABFs and DREB has been reported. DREB1A, DREB2A, and DREB2C proteins interact physically with AREB/ABF proteins (Lee et al., 2010). Analyses of *DREB2A* genes have shown the presence of an ABRE motif in the promoter region, required for the *DREB2A* expression in dehydration response. Chromatin immunoprecipitation (ChIP) analyses have verified that *DREB2A* is regulated by AREB1/ABF2, AREB2/ABF4 and ABF3 under osmotic stress (Kim et al., 2011). These data suggest crosstalk

between elements of the ABA-dependent and ABA-independent signaling pathways (Figure 2).

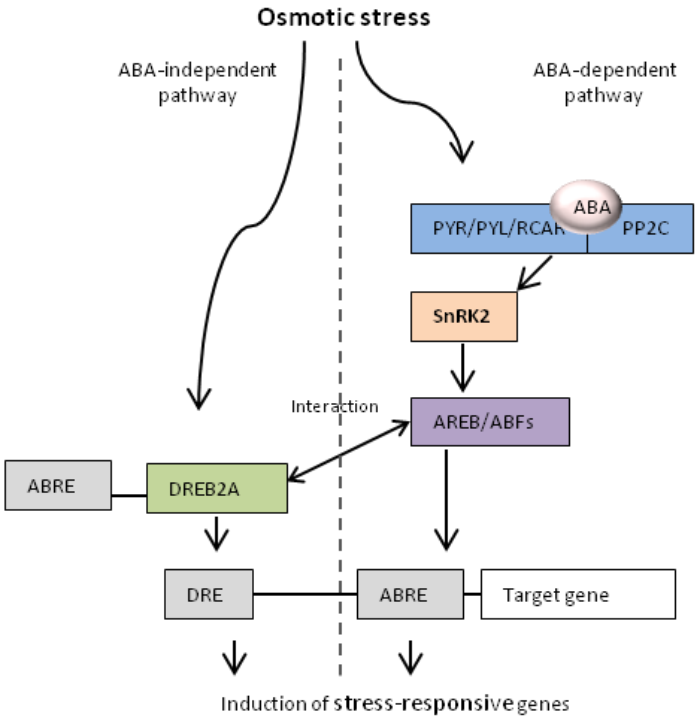

Figure 2. Schematic representation of ABA-dependent pathway and crosstalk with ABA-independent for activation of osmotic stress-dependent gene expression. In presence of ABA, PP2C-PYR/PYL/RCAR receptor complexes activate SnRK2s. In turn, SnRK2s phosphorylate a variety of substrates, including AREB/ABFs, and modulate their activities. Recent studies suggest crosstalk between the AREB/ABF-SnRK2 pathway and ABA-independent signaling due to the presence of ABRE motif in DREB2A promoter (Modified from Yoshida et al., 2014).

1.5 Genomic tools for isolation and characterization of key genes

Several genomic approaches offer valid methods to elucidate the molecular basis of stress response in plants at the transcriptomic, proteomic and metabolomic levels. The neologism "omics" refers to high throughput analyses of genome expression (transcriptomics), proteins and protein-protein interactions (proteomics) and metabolite profiling (metabolomics) (Figure 3).

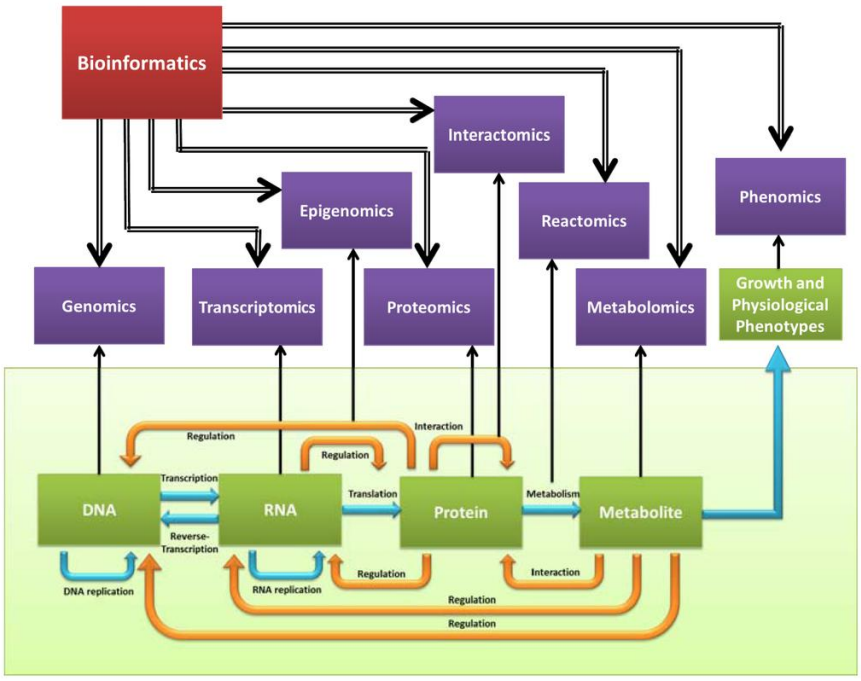

Figure 3. General representation of -omics tools, their crosstalk and connections with cellular metabolism (Li et al., 2013).

1.5.1 Omics sciences: large-scale identification of candidates

Since plant stress response has a complex genetic basis, it is not unexpected that hundreds to thousands of genes or proteins modify their expression under stress conditions. A standard method to study this molecular change is to compare the expression of genes during stress to unstressed condition. In recent times, several studies have demonstrated that the large-scale transcriptome profiling using microarrays and the most recent technology of RNA sequencing (RNA-seq) are powerful tools for identifying genes involved in plant stress tolerance. RNA-seq is an next-generation sequencing (NGS) technologies, that provides a far precise measurement of transcripts levels from very small amounts of cellular materials (Wang et al., 2009). Numerous studies have been carried out in model species as well as crops. By using this technique, 28,335 unique genes were identified from sorghum (*Sorghum bicolor*) shoot and root exposed to polyethylene glycol (PEG) or exogenous ABA. Interestingly, the function of more than 50 differentially expressed genes was not previously described (Dugas et al., 2011). In addition to finding novel transcripts, RNA sequencing allows the identification of allele-specific expression, splicing models and splicing junctions (Malone et al., 2011). In maize, Kakumanu et al. (2012) observed 76,000 novel splice junctions that account for 23% of the total number identified.

Furthermore, to understand the acclimation of plants to soil water deficit, Bogeat-Triboulot et al. (2007) have analyzed the expression profiles of approximately 6,340 genes in *Populus euphratica*, a poplar growing in arid regions, under drought stress. They did not find complete correlation

between transcripts and proteins directly involved in plant stress response. The alteration in gene expression at transcript level does not often correspond with the changes at protein and consequently at metabolic level. Therefore, investigation of changes in plant proteome and metabolome is also highly important (Kosova et al., 2011).

A global analysis of protein expression, using a 2-D gel-based protein separation method followed with protein identification by Mass Spectrometry (MS), revealed several plant proteins responding to drought and salt stress. Numerous studies have been carried out in crops, such as rice and maize using this approach (Salekdeh et al., 2002; Cui et al., 2005) and have identified components of transcription and translation machinery and especially enzymes catalysing changes in metabolite levels.

The approaches used in plant metabolomics studies include metabolic fingerprinting, aimed at identification of metabolic patterns associated with a particular stress condition without identification or quantification of metabolites, and metabolite profiling used to simultaneously measure of all metabolites in a sample. Hence, metabolomics also significantly contributes to the study of stress mechanisms in plants by identifying different compounds, including products of stress metabolism, stress signal transduction molecules as well as molecules involved in acclimation response of plant (Fiehn, 2002; Shulaev et al., 2008)

1.5.2 Finding a needle in a haystack: forward and reverse genetics

Despite the amount of transcriptome and proteome information, the functions of many identified genes still need to be established. Ten years ago, It was estimated that proteins without a previously studied domain or motif represent between 18 and 38% of the eukaryotic proteome (Gollery et al., 2006). Functional genomics is a branch of molecular biology that uses the massive wealth of data produced by genome-wide techniques to describe function and interactions of genes (Cushman et al., 2000). The main approaches adopted are based on the large-scale identification of the genetic basis responsible for an observed phenotype (Forward genetics), or the investigation of the effect of induced mutation or altered expression of a selected gene (Reverse genetics). While forward genetics is based on the phenotypic screening of large mutant populations obtained by chemical (e.g. ethyl methane sulfonate) or physical (X-rays, Ultraviolet) mutagenesis of wild-type seeds, the reverse genetics method is based on the production of gain- and loss-of-function mutants in which the gene of interest is overexpressed and knocked-out, respectively.

Large-scale collections of loss-of-function mutant lines using these types of insertion tags were generated in *Arabidopsis thaliana* (Sessions et al., 2002) and in rice (Jeon et al., 2000; Miyao et al., 2003), but the functional characterization of these knockout mutants, requires a major effort, further

complicated by issues raising from functional redundancy or lethality of the insertions.

Transgenic approaches can be used as an alternative or complementary method to reveal the function of candidate genes. The enhanced expression of a gene from a constitutive promoter can be an extremely useful approach for testing its function in several cell types. The Gateway system for expression of proteins in transgenic plants, uses oligopeptide epitope tags to allow immunoblotting or affinity purification of the analyzed proteins (Fritze and Anderson, 1999). This is an useful method for the identification, isolation and biochemical analysis of single proteins or multi-protein complexes. For example, a gain-of function strategy was adopted by generating *AHK1* overexpressing Arabidopsis plants, to confirm its role as positive regulator of abscisic acid (ABA) signaling during drought and salt stress (Tran et al., 2007). Likewise, Davletova et al. (2005) assign a function for the zinc-finger protein ZAT12 in reactive oxygen and abiotic stress signaling, using *Zat12* gain- and loss-of-function Arabidopsis lines. Constitutive expression of *Zat12* results in enhanced expression of oxidative and light stress-response transcripts.

T-DNA activation tagging has become the most popular method for generating gain-of-function mutants in plant. This technique utilizes a random insertion of a T-DNA vector carrying cauliflower mosaic virus 35S enhancer elements, which effect transcriptional activation of flanking genes. T-DNA insertion mutant population were created in Arabidopsis (Weigel et al., 2000), rice (Jeong et al., 2006), tomato (Carter et al., 2013) and tobacco (Liu et al., 2015).

Armed with such information and these powerful tools, it will be possible to isolate, control and optimize plant tolerance traits for improved crop development and productivity.

1.6 Aims and Contents of the thesis

The aim of the PhD project was to identify and characterize genes involved in the plant response to osmotic stress.

Using microarray analysis, several genes whose expression was differentially regulated in *Solanum tuberosum* culture cells adapted to high concentrations of polyethylene glycol (PEG) were previously identified in the laboratories of CNR-IBBR (Ambrosone et al., 2011). The present research was aimed at verifying the functional role in stress tolerance of fifty of these genes by studying their orthologues in *Arabidopsis thaliana.* Taking advantage of the tools available for the model species a reverse-genetic approach was performed. The large-scale phenotype screening of *knockout* mutants in presence of NaCl or ABA allowed the identification of three genes previously uncharacteryzed for involvement in abiotic stress responses: *DRT111* (At1g30480) a predicted RNA-binding protein, *TIP41-like* (At4g34270) a predicted component of the TOR pathway and *SIN-like* (At5g49530) a putative subunit of RNA polymerase III. In order to characterize their function, different approaches were used. In particular:

- Gene expression analyses during a time-course of 7 days revealed the response of *DRT111, TIP41-like* and *SIN-like* to different exogenous stimuli (hormones, salts and osmotic agents);
- Promoter analyses evaluated the expression of *DRT111, TIP41-like* and *SIN-like* in different plant tissues and developmental stages. Interestingly, *DRT111* is expressed in specific tissues that respond to osmotic stress conditions, such as thricomes and stomata. while *TIP41-like* and *SIN-like* are widely expressed in all the tested developmental stages, with particular high activity in vascular tissue and inner walls of guard cells respectively;
- The sub-cellular localization analysis, using Arabidopsis plant overexpressing the fusion proteins between YFP and each protein, identified DRT111 and SIN-like in the nucleus and TIP41-like in the cytoplasm;
- Gain and loss-of function approaches were finally applied. Phenotype analyses in different stress conditions of plants with altered expression of *DRT111, TIP41-like* and *SIN-like* (knockout mutants and overexpressing lines), suggested an role of *DRT111* in seed germination and an *SIN-like* involvement in root development. While *TIP41-like* appears involved in several ABA-mediated responses to osmotic stress.

Among the differentially expressed genes in the *S.tuberosum* adapted cells, the Arabidopsis ortholog AtRGGA was previously isolated and partially characterized at CNR-IBBR (Ambrosone et

al., 2015). Therefore, our research activity further clarified the molecular mechanisms through which AtRGGA regulates osmotic stress responses. In particular:

- An Electro mobility shift assay (EMSA), using recombinant, His-tagged AtRGGA and total RNA or specific ribosomal RNAs demonstrated that AtRGGA binds rRNA *in vitro*;
- RNA immunoprecipitation, that was carried out during an 3-month stage in Julia Bailey-Serres laboratories at the University of California, Riverside (USA), provided evidence that that AtRGGA is capable to bind rRNA *in vivo*;
- Finally, a yeast two-hybrid screening was performed to identify interacting partners of AtRGGA. Interestingly, several putative AtRGGA partners are mainly involved in RNA processing, transport and ribosome biogenesis, suggesting that AtRGGA might affect RNA stability.

The second part of the work focused on the identification of stress-regulated genes in *Solanum lycopersicum* in response to limiting water input (Iovieno et al., 2016). A careful monitoring of physiological parameters during drought stress progression and recovery indicated that drought leads to a decrease of leaf gas exchanges, such as stomatal conductance and CO_2 assimilation, and to a consistent accumulation of proline and ABA.

- Genome-wide comparisons of stress responses across two cycles of drought and rehydration were carried out using Illumina deep sequencing of RNA populations followed by cluster analysis to identify stress-induced patterns of transcriptome profiles.

  Cluster analysis of the 966 Differentially Expressed Genes (DEGs) indicated that drought stress largely results in down-regulation of gene expression with only a small subset of induced genes. Enrichment analysis of Gene Onthology (GO) categories in DEGs indicated that genes belonging to the photosynthesis, light harvesting and photosystem I and II GO category were over-represented in down-regulated genes, along with histones and cell proliferation and cell cycle GOs, while stress-related GOs were enriched in drought-induced transcripts.

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2. Functional characterization of genes implicated in osmotic stress responses in *Arabidopsis thaliana*

2.1 Introduction

Arabidopsis thaliana is the most studied model and the first plant species whose genome was sequenced (Kaul et al., 2000). Using this information, microarrays and, more recently, RNA sequencing have flourished in a variety of genome-wide expression studies aimed at characterizing developmental steps and plant processes, including responses to abiotic stresses. It is now established that a great percentage of the Arabidopsis gene pool (up to 12 %) is differentially expressed in response to high salt, drought, and cold stress (Ma et al., 2006), with an extensive overlap between the three signalling cascades (Seki et al., 2002). Time course analyses revealed two major groups of genes with different expression kinetics. One group, showing rapid and transient changes in expression, contains genes with regulatory roles, such as transcription factors. By contrast, effector genes such as transcription factor target genes, showed gradual induction after long-term exposure. These results indicate that numerous transcriptional regulatory mechanisms play important roles in stress signalling pathway (Seki et al., 2002; Kreps et al., 2002).

Using these large-scale data sets, Swindell and colleagues (2007) identified genes among 10 Arabidopsis ecotypes that exhibited different patterns under temperature stress. Interestingly, they found correlation with changes in gene expression and ecotype variation strongly related to geographical temperature gradients, suggesting that genes exhibiting a different expression pattern play an essential role in stress response pathways and in particular in temperature acclimation.

Although transcriptomic studies have biological limitations due to the modest information on proteins activity provided by mRNA abundance (Feder and Walser, 2005), they constitute a valuable starting point to identify genes potentially involved in stress response pathways. Such candidates need to be subjected to a functional validation to prove their involvement in adaptation mechanisms, since many responsive genes may constitute markers of stress, whose changes in expression are a sign of cellular injury induced by the treatment.

Even though intensive research has been devoted to the study of transcriptomic changes in response to stress, RNA regulatory mechanisms are only recently emerging as key processes participating in the modulation of cellular responses to stress (Nakaminami et al., 2012).

These mechanisms include both transcriptional and post-transcriptional regulations through the main action of RNA granules, small RNAs and RNA-binding proteins (RBPs) (Figure 4).

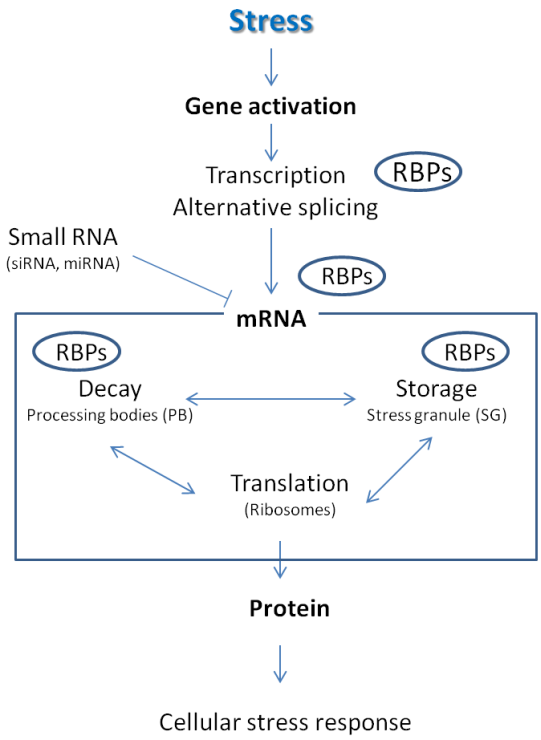

Figure 4. RNA regulation in plant stress response. Non-coding RNAs are implicated in transcriptional and post-transcriptional regulation. mRNAs are stored in stress granules (SGs) and their decay such as decapping, deadenylation and degradation occur within processing bodies (PBs). RNA binding proteins (RBPs) are involved in several phases of RNA regulation metabolism. Modified from Nakaminami and colleagues (2012).

Regulation of RNA metabolism involves directly or indirectly RBPs, distinguished in classes based on the presence and organization of several different functional motifs and domains. In plants, the most common are the RNA Recognition Motif (RRM) and K Homology (KH) domains (Lorkovic, 2009). Other domains and motifs include the Tudor SN domain, arginine repeats (SR), glycine-rich domain (GR), zinc finger domain (ZnF) (Burd and Dreyfuss 1994; Albà and Pagès 1998; Lorkovic and Barta 2002), Arginine/Glycine (RGG/RGX) motif and cold shock domain (CSD) (Nakaminami et al., 2012; Ambrosone et al., 2012).

RBPs are involved in different transcriptional and post-transcriptional processes such as the maturation of mRNA through splicing, polyadenylation, capping and export from the nucleus. Transcriptome analysis shows that 42% of Arabidopsis genes have alternatively spliced forms, resulting in an increased diversity of proteins. Alternative splicing is frequently associated to environmental conditions (Filichkin et al., 2010). Several splicing and splicing-related factors play important roles in the abiotic stress response. SKIP, an Arabidopsis component of the spliceosome confers osmotic stress tolerance by controlling alternative splicing of several stress-related genes, such as *NHX1*, *CBL1*, *P5CS1*, *RCI2A*, and *PAT10* (Feng et al., 2015). Therefore, one splicing factor may regulate several genes with different splicing forms. In wheat, the transcription factor

DREB2 has three splice variants. While under cold stress all forms show transient increases, during drought and salt stress only the level of two transcripts is increased, suggesting a specific alternative splicing control in plant stress response (Egawa et al., 2006).

In addition to mRNA splicing, RBPs are involved in different post-transcriptional processes such as control of mRNA stability and decay (Nakaminami et al., 2012). In response to stress conditions, plant cells rapidly modify processes related to mRNA translation into protein. Specific mRNAs are compartmentalized in stress granules (SGs) and processing bodies (PBs), dynamic ribonucleoprotein structures located in the cytosol. In these structures, mRNAs are stored or degraded to avoid energy costs associated to the translation of dispensable proteins. PBs are present constitutively, however their size and number change rapidly in response to stress. PBs are closely linked to mRNA decay processes such as decapping, deadenylation and degradation (Anderson and Kedersha, 2009). SGs are only formed under stress conditions and are mainly involved in translation and sorting of specific mRNAs for diverse fates (Yang et al., 2014).

An impact of stress on translation efficiency has been shown in the case of hypoxia. The RNA binding protein UBP1 participates in the selective mRNA translation mechanism during hypoxia by sequestering mRNAs in SGs. Upon re-oxygenation, stress granules dissolve and mRNAs return to actively translating polysomes (Sorenson and Bailey-Serres, 2014). Growing evidence suggests that PBs and SGs interact with each other. In Arabidopsis, the RNA-binding protein TSN (Tudor Staphylococcal Nuclease) was identified as component of SGs, involved in stress tolerance through the stabilization of stress-regulated mRNAs and the modulation mRNA levels of key enzyme in gibberellin biosynthesis (Yan et al., 2014). Double mutants *tsn1/tsn2* showed a drastic reduction in germination, growth, survival and fitness under high salinity stress (dit Frey et al., 2010). Recent studies report that TSN is also an integral component of PBs during heat stress playing an important scaffolding role (Gutierrez-Beltran et al., 2015). The heat shock protein HSP90, constitutively expressed in the cytosol of eukaryotic cells, is involved in the formation of both PBs and SGs (Matsumoto et al., 2011), suggesting the cooperation of these two structures in the RNA regulatory processes during stress response.

Finally, RBPs mediate some other important mechanisms, such as the regulation of small RNAs.

In the last years, transcriptome analyses have shown numerous non-coding RNAs (ncRNAs) expressed from unannotated genomic regions. These ncRNAs include long non-coding RNAs and small RNAs, such as micro RNAs (miRNAs) and small interfering RNAs (siRNAs). The small RNAs mainly control the gene expression by post-transcriptional regulation. They target complementary mRNAs for degradation or translation repression (Tang et al., 2003; Chen, 2004). Consequently, their down-regulation causes the accumulation of their target mRNAs (Shukla et al.,

2008). Interestingly, several plant miRNA target genes have been identified as important players in stress response in Arabidopsis (Sunkar and Zhu, 2004, Sunkar et al., 2006) and, more recently, in several crops such as rice and cotton (Mittal et al, 2016; Gao et al., 2016).

The action of RNA-binding proteins in many aspects of RNA metabolism and their involvement in abiotic stress response, make them interesting research targets.

Previously, a transcriptome study was carried out at CNR-IBBR, using an experimental system based on *Solanum tuberosum* culture cells exposed to a gradual increase of polyethylene glycol (PEG) (Adapted) or subjected to short-term PEG-treatment (Shocked) (Leone et al., 1994; Figure 5).

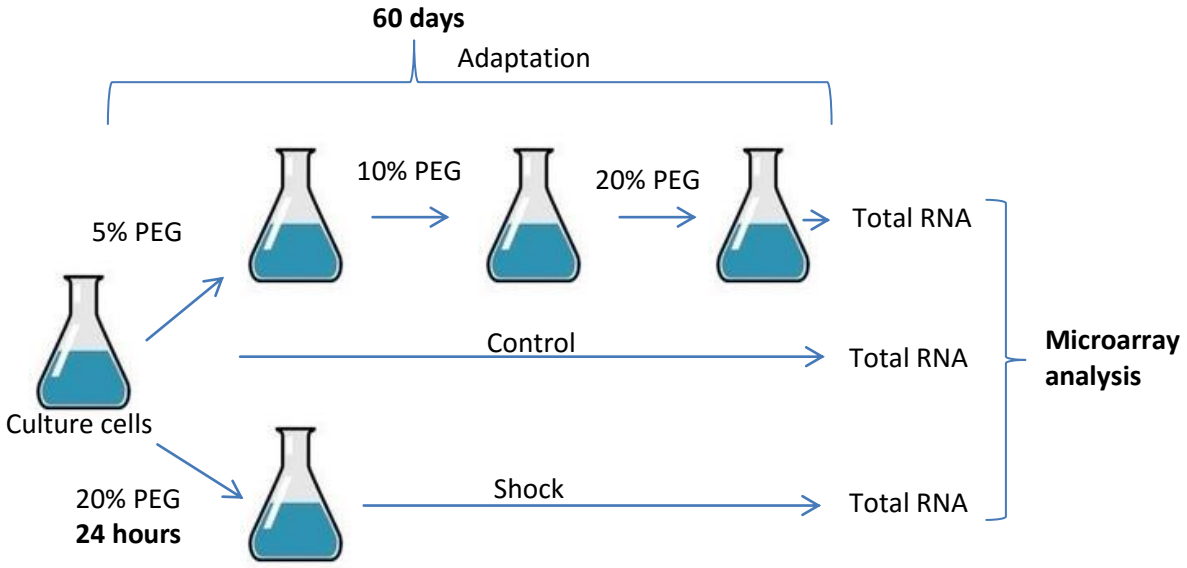

Figure 5. The experimental setup developed to study adaptation mechanisms in a simplified system. Adaptation: *Solanum tuberosum* culture cells exposed over the span of 60 days to a gradual increase in polyethylene glycol (PEG) up to 20% (W/V). Shock: 24h treatment with PEG 20% (W/V). Total RNA was isolated from control, shocked and adapted cells and used for microarray analysis.

Using Potato 10k cDNA slides from TIGR (The Institute for Genomics Research), microarray analyses were carried out comparing gene expression in control, shocked and adapted cells and thus providing a global view of the changes induced by osmotic stress. 129 up-regulated genes and 415 down-regulated genes during PEG adaptation, and 337 up-regulated and 184 down-regulated genes in response to PEG short-treatment were identified (Ambrosone et al., 2011). Several genes were specifically regulated in adapted rather than shocked cells, possibly indicating a functional involvement in adaptation mechanisms (Ambrosone et al., 2011). The present research was aimed at verifying the functional role of a subset of these genes by studying their orthologues in

Arabidopsis and taking advantage of the several tools available for the model species. Interestingly, several of these genes have assigned functions related to RNA metabolism.

Among the differentially expressed genes in adapted cells, the *S.tuberosum* RGGA (*StRGGA*, GenBank acc. no. FM209282) gene was previously partially characterized at CNR-IBBR. *StRGGA* was specifically induced in cells gradually adapted to PEG, while no change in gene expression was observed when shocked cells were analyzed (Ambrosone et al., 2011). The deduced protein sequence of StRGGA shares 63% sequence homology with the protein encoded by the locus *At4g16830* of Arabidopsis, which, therefore, was hypothesized to be the Arabidopsis ortholog (AtRGGA) (Ambrosone et al., 2015). The gene encodes a glycine-rich RNA-binding protein of 355 aa containing a RGG box domain. The second part of this research activity was thus focused on the study of the molecular mechanisms through which AtRGGA regulates osmotic stress responses.

2.2 Materials and Methods

2.2.1 Plant materials, growth conditions and stress treatments

Arabidopsis plants of the Col-0 ecotype were used throughout this study. Wild type Col-0 plants, mutants and transgenic lines were grown as described by Weigel and Glazebrook (2002).

For gene expression studies, 12-d-old seedlings grown on solid GM medium (1X MS Salts, 3% Sucrose, pH 5.7) were transferred to plates containing 35% (W/V) PEG, NaCl (120 mM), or ABA (10 µM) and incubated for 2, 5 and 7 days.

Large scale mutant phenotype screening

For germination analyses, seeds were sown in the presence of NaCl (50mM and 100mM) or ABA (0.5µM and 2µM). Germination was scored in terms of fully expanded cotyledons from 2 to 7 days of incubation. Survival tests were carried out using 18-d-old seedlings germinated on GM plates and transferred after 5 days to media containing NaCl (90mM and 180mM) or ABA (50µM and 100µM). Survival was scored daily in terms of absence of necrotic or bleached leaves. To score root growth in the presence of NaCl or ABA, 4-d-old seedlings grown on GM medium and showing similar primary root length were transferred to GM medium or medium with NaCl (80mM and 120mM) or ABA (20µM and 50µM). Photographs were taken, and root length was scored 14 days after transfer.

Phenotyping of DRT111 knockout mutants and overexpressing lines

For germination analysis of *drt111* knockout mutants, Col-0 and *DRT111*-overexpressing lines, seed's were sown in presence of ABA (5 µM). Germination was scored in terms of radicle emergence 2, 4 and 6 days after stratification. Water loss analysis was performed on detached leaves of 3-week-old plants as described by Mustilli et al. (2002).

Phenotyping of TIP41-like knockout mutants and overexpressing lines

For germination, *tip41-like*, Col-0 and *TIP41-like* overexpressing seeds were sown on ABA-containing medium (0.5µM and 2µM). Germination was scored in terms of fully expanded cotyledons after 7 days of incubation. Survival tests were performed using 5-d-old seedlings grown on GM plates and transferred to ABA-containing medium (50µM). Survival was scored daily in terms of absence of necrotic or bleached leaves from 3 to 6 days after transfer. Water loss analyses were performed on detached leaves of 3-week-old plants as described by Mustilli et al. (2002).
Root growth in the presence of 20µM and 50µM of ABA was tested using 4-d-old seedlings grown on GM medium and transferred to GM medium or medium containing ABA. Root length was scored 10 days after transfer.

SIN-like: knockout mutants and overexpressing lines

Root growth was analyzed for *sin-like*, *SIN-like* overexpressing plants and Col-0 on GM medium. Photographs and root length measurements were performed 14 d after germination.

AtRGGA:knockout mutants and overexpressing lines and stress treatments

rgga (SALK_143514) and *AtRGGA* overexpressing lines (35S::FLAG-RGGA) were already available in CNR-IBBR laboratory (Ambrosone et al., 2015). Survival tests were performed using 18-d-old seedlings germinated on GM plates and transferred to plates containing ABA (50μM). Survival was scored daily in terms of absence of necrotic or bleached leaves. Root growth in the presence of ABA was analyzed: 4-d-old seedlings grown on GM medium and showing equal primary root length were transferred to GM medium or medium with 20 μM ABA. Photographs were taken, and root length was scored 10 d after transfer.

For EMSA analyses, Col-0 10-d-old seedlings grown on GM plates were transferred to GM or GM plus NaCl (180mM) plates for 48 hours prior to RNA extraction. RIP assay was performed using Col-0 and *AtRGGA* overexpressing lines (35S::FLAG-RGGA) grown as described for the EMSA assay

2.2.2 Bioinformatic analyses

Bioinformatic analyses were performed using softwares and databases available online on The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) and the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). In particular, the BLAST algorithm (Altschul et al., 1990) was used to determine homologies between potato and Arabidopsis genes. BLAST also provides identity percentages between nucleotide (BLASTn) or protein (BLASTp) sequences. Putative domains present in protein sequences were identified using several softwares such as InterProScan (www.us.expasy.org) and Pfam (www.pfam.wustl.edu).
The protein alignments were obtained using the multiple alignment tool MUSCLE *(http://www.ebi.ac.uk/Tools/msa/muscle/).* Gene expression levels in different tissues and developmental stages were analysed *in silico* using the Arabidopsis microarray data displayed in the eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

2.2.3 DNA extraction and T-DNA knockout mutant selection

Genomic DNA was extracted from leaf tissues (100mg) using C-TAB buffer (100 mM Tris-HCl pH 7.5, 1.4M NaCl, 20 mM EDTA, 5g C-TAB, 1% β-ME). Samples were then heated for 30’ at 70°C and DNA was extracted adding an equal volume of Chloroform: Isoamil alcohol= 24:1. Samples were then gently shaken and centrifuged for 15’ at 14,000 g. The upper phase was subsequently recovered and DNA was precipitated with 0.7 Volume Isopropanol overnight at –20°C. Samples were subsequently centrifuged for 15’ at 14,000 g and the pellet was washed with 70% EtOH and centrifuged (14,000 x g) for 10 min. DNA was then resuspended in 50 μl of H_2O. DNA quality and quantity was checked by agarose electrophoresis.

The T-DNA insertion mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC), and homozygous plants were selected by PCR. Primers were designed using the T-DNA Express primer design tool (http://signal.salk.edu/tdnaprimers.2.html) and listed in Table 1. PCR on genomic DNA was performed using standard protocols (Sambrook et al., 1989). Two PCR procedures were used to verify zigosity status and select homozygous *knockout* lines for each mutant. One procedure used the gene specific forward and reverse primers (LP and RP) to amplify a product from chromosomes without the insertion. The other used the BP primer, that anneals in the left border of the T-DNA, combined with the gene specific primer RP, to amplify the product from a chromosome carrying the insertion (Figure 6). Both amplification products were identified in heterozygous individuals. Conversely, there was one amplification product, only using the BP-RP primers, in homozygous individuals.

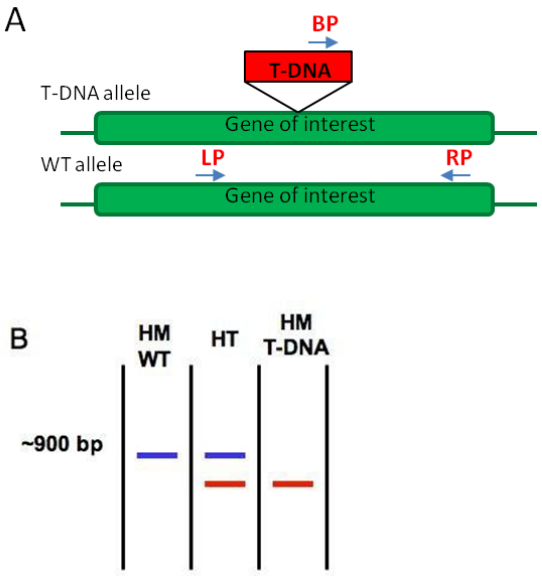

Figure 6. Isolation of homozygous T-DNA insertion lines A) Schematic representation of T-DNA insertion and B) Example of PCR product using T-DNA left border (BP) and gene (LP-RP) specific forward and reverse primers. HM T-DNA: homozygous or HT: heterozygous for the T-DNA. HM WT: wild-type

Table 1. List of primers used for T-DNA insertion confirmation

Name	Sequence 5'-3'
N461572_LP	TGATTTTTCAAATTACAGGGGTG
N461572_RP	GCAAAGCAACAAAGCAAGTTC
N520997_LP	GAGCAACCTGCAACTGATAGG
N520997_RP	CTGAACCGCTGCTCTACAATC
N526159_LP	TTCGACTATTCACCGTTCGAC
N526159_RP	TCCAATTAGGAGGCACAACAC
N528065_LP	CGATGGATTAGCGAACTATGC
N528065_RP	GAGCAAACAATCAACAATGGC
N531836_LP	AAGCATCTACAAGGCCATGG
N531836_RP	AACGGCAAATGATCAGATCAC
N548763_LP	AAGCAATGGAACGAAGAACAG
N548763_RP	TCCTTGTTTGAAACGCATACC
N551573_LP	AGCTTGTTCTGCAGATTCTCG
N551573_RP	TAAATTTGGGTTTGGTTTCCC
N552218_LP	GTTTTTCTCTATTGGGCTCGG
N552218_RP	TAACCCCAGGCACATCACTAG
N555304_LP	TCTTCATTGCATTCGATACCC
N555304_RP	GACGCAGCGTAATCATTAAGG
N555559_LP	GAAGAGTCTGACAGGCCACTG
N555559_RP	AGGGATGTTTTCGTAACCCAG
N590614_LP	TCGTTCTGTCAAATTTGGACC
N590614_RP	AGAGTCCAACAAAACATTGCG
N599137_LP	GAAAAGACACTCAAGGAGGGC
N599137_RP	CTCCCTTGTCTGCGATTACAG
N602739_LP	TCTTATGACACCTCTCCGTCG
N602739_RP	CGTTTCATCCTCTGCTTTCTG
N604980_LP	AAAAATATTCACGCGATGGTG
N604980_RP	CATCTAAACAGCGAGCTTTGC
N610626_LP	TCATCGCAGTTTGAATTTACG
N610626_RP	CTGCATCAGAAAGGAGTGGAG
N623494_LP	ATTGAAATAGTCGATGCACGG
N623494_RP	AGGTTATAAGGGTCACAGGCC
N631268_LP	CAGTCTTGAGATCGTAAGCCG
N631268_RP	TCAAACAAATAACCCCAAAAATG
N639651_LP	TGAGGCTGCTTGGAGATTATC
N639651_RP	TCTCGAAAAGCACAAACATTG
N640822_LP	CGAATCTGATTTTGTGATTCG
N640822_RP	GAATTTGGTAGGTGCATAGCG
N644455_LP	TAATGGGTATTGGTCCAGCTG
N644455_RP	ATGTGGTTTCGGTGACTTGAG
N650032_LP	GAAAAATTGTTTTGTACAGTCTCAGC
N650032_RP	AATATGGCTCAATTGGGGAAC
N651519_LP	GGAGGAGATTGGATTGGATTC
N651519_RP	GTGCAGTGTCCATAGGAAAGC
N652421_LP	CTCAGAAACGCTCTGTGAAGG
N652421_RP	TCATAAGGAAACGGTCACGAG
N653221_LP	ACTTCAATGGTCCAAAATCCC
N653221_RP	TTTTCATTCGGACCTGACTTG

Table 1. Continued

Name	Sequence 5'-3'
N654482_LP	GAACAACGTGAAAAGGGTTTG
N654482_RP	TGACTTTCATGCTCATTGCTG
N656570_LP	TCAAACTGGACAAAATAAAGCG
N656570_RP	TGCAGGCTACAGCAGCTAAAG
N658116_LP	GACCGATCACTGATGAGAAGC
N658116_RP	ACAACCACTGCTTCAACATCC
N658342_LP	TTAGCATTTCGTCAATGGTCC
N658342_RP	AACAATCAATTGGCACAAAGC
N659242_LP	GCATCTTCAGTTGTTTCTCGC
N659242_RP	CATAAGTCCATCTCAGCTCGC
N659880_LP	TGGCTACATTCGGTGAATTATG
N659880_RP	GCTTCATACTGCGAGAGTTGG
N660190_LP	CAATGGAAGCTGCTGATCTTC
N660190_RP	ACACTGCACGGGTTAAGAATG
N663323_LP	CAAGGAGTTAGTGAGGGACTCG
N663323_RP	GCGTGTTGGTTAAGATACCATC
N663425_LP	AAGCCGAATCCGATAAAAGTC
N663425_RP	ATTCGGAAGTGGAAGCTCTTC
N663463_LP	GATGCTGCATATGGTTGTGTG
N663463_RP	GTTGAACGATAAGCTTGCGAG
N664667_LP	GTGCTTGAGAAATTAGGTGCC
N664667_RP	TCAAATAAGCATTTGGAACCG
N665071_LP	TTCGCTTAAGAGCTTAGCACG
N665071_RP	GGTAGAAGTAGATGGCCCAGG
N665483_LP	AGCTCTGCTTCTGTCTTTCCC
N665483_RP	GGAACAGTAGAGCAAGGCATG
N668973_LP	CTGATGCCTTTTACATCGAGG
N668973_RP	ATCCAAACTCGGGAGAGAGAG
N670017_LP	CTTGTGGATTTGGTGGTTGAC
N670017_RP	TTATGGCATTTTACGGCGTAG
N671231_LP	CTCGCCATTGATTACGAAGAG
N671231_RP	TTTGTTTTTGATTGTGGTATCG
N672556_LP	TGGTAAGAAAATAGGAAGGTGCC
N672556_RP	CTGTGAAGGACCTTGTGCTTC
N672654_LP	GCAAAGATTTAGAAGATTTAAATCG
N672654_RP	AACAACACACAGACCGGCTAC
N674601_LP	ATTAGGGAAACGGGAAAGGAG
N674601_RP	TTGCAACCTTGGTTTCAACTC
N681249_LP	ATCCAGTTGGGCCAATCTAAG
N681249_RP	CTCAAACAAACCAAAATTCGC
N686155_LP	TTGATTCACTGTGGAAAAGGC
N686155_RP	AGATGTGTGGGTGAGAACAGG
N840594_LP	TCTGGTAAACATGCCGAAATC
N840594_RP	TTGTGGTAGACTTCCCATTGG
N854650_LP	TTTTGCAGCATCAAAGTGTTG
N854650_RP	ACCTAAGAACTCAAGCAGCCC
N870155_LP	TTTCCTCTTTCTCTTCCGCTC
N870155_RP	TTGCTCAACGGTTAATTCTGG
N871551_LP	GCAATCAGCATGAGAGAGACC
N871551_RP	TCTCCATCAATGCTTTAACGG
N877264_LP	CACTGCATCTCGGAGCTTAAC
N877264_RP	CTGATTTCCTCTTTGGCACAG
LB_T-DNA	TAGCATCTGAATTTCATAACCAATCTCGATACAC
SAIL_T-DNA	TAGCATCTGAATTTCATAACCAATCTC
WISC_T-DNA	AACGTCCGCAATGTGTTATTAAGTTGTC

2.2.4 Isolation of RNA, cDNA synthesis and qRT-PCR

Total RNA was extracted from leaf tissues (100mg) using TRIzol Reagent (Life Technologies) following the manufacturer's instructions. RNA quantity was measured spectrophotometrically by NanoDrop ND-1000 Spectrophotometer (NanoDropTechnologies), and integrity was verified on a denaturing formaldehyde gel. 1 µg of DNase-treated total RNA was reverse transcribed using SuperScript II Reverse Transcriptase™ and oligo (dT_{20}), according to manufacturer's instructions (Life Technologies).

The complementary DNA was diluted 1:20 and 4.5 µL of diluted cDNA were used for each qRT-PCR reaction, performed with 6.25 µL of 1X Platinum SYBR Green qPCR SuperMix (Life Technologies) and 1.75 µL of primer mix (5µM). Primers used are listed in Table 2. Reaction was performed with ABI 7900 HT (Applied Biosystems). Cycling conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Three biological replicates with three technical repetitions were tested. Quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Elongation Factor *EF1α* was used as endogenous reference gene for the normalization of the expression levels of the target genes. RNA extracted from plants grown in control condition served as calibrator sample for relative quantification of gene expression.

Table 2. List of primers used in gene expression studies

Name	Sequence 5'-3'
AtEF-1 for	TGAGCACGCTCTTCTTGCTTTCA
AtEF-1 rev	GGTGGTGGCATCCATCTTGTTACA
AtRGGA for	GAGCGTCGTAGTGGAACTGG
AtRGGA rev	CTGGCTTCTCTCCAACATCC
DRT111 for	CAATCGGTGCTTATTCCGGC
DRT111 rev	AGGTCTCGCTGGATCGTACT
TIP41-like for	GGAAATTCAGGAGCAAGCCG
TIP41-like rev	GCTTTTGCCTCAACCGTTTCT
SIN-like for	CGACGTTGTTGTTCGTGAAA
SIN-like rev	TCGTATGGACGCCAAGATGG

2.2.5 Plasmid Construction

Cloning of target genes into binary vectors

Gateway technology (Life Technologies) was used to obtain binary vectors for promoter and protein localization studies as well as to produce transgenic overexpressing plants. An outline of this technology is reported in Figure 7. The putative promoter o*f DRT111*, *TIP41-like* and *SIN-like* (corresponding to 2 kb upstream of ATG) was amplified from genomic DNA extracted from Col-0 plants. The coding sequences were amplified with or without STOP codon to warrant correct N-terminus and C-terminus fusion with tags.

PCR amplifications were carried out using Phusion DNA polymerase (Thermo scientific). Primers used are listed in Table 3.

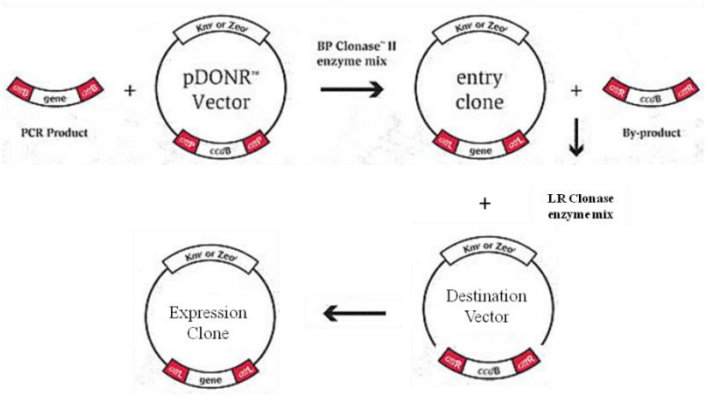

Figure 7. The GATEWAY™ recombinant reaction.

Table 3. List of primers used to clone sequences in pDONR207.

Name	Sequence 5'-3'
DRT111ATGpDONRfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGCTTGGTGGATTATACGGAGAT
DRT111STOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTAATAGCCAGGGATTTCACCTGGAACT
DRT111NOSTOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC ATAGCCAGGGATTTCACCTGGAACT
TIP41-likeATGpDONRfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGGAGACGG TGGTCGATAA
TIP41-likeSTOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTAAACTTTACTAGGGATCTTCAGTTTCTGT
TIP41-likeNOSTOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC AACTTTACTAGGGATCTTCAGTTTCTGT
SIN-likeATGpDONRfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGGACTTTGATGATGATGACAAACCAA
SIN-likeSTOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCATCGAGCTTTCTGTAAAACCCAT
SIN-likeNOSTOPpDONRrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCGAGCTTTCTGTAAAACCCAT
DRT111pDONRpromfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC T CT CAA GTC TGG CAA ACT TTT AAA ACA AAA G
DRT111pDONRpromrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC TTT TTC GCC GCC GGT AAA GAG ACT
TIP41-likepDONRpromfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC CGATGTTAAGCTTATGGATATGCATAGAC
TIP41-likepDONRpromrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTGAATCGCCGGCTAATATTGG
SIN-likepDONRpromfor	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC CTGCAAAGAGAGTGAAGCACGAT
SIN-likepDONRpromrev	GGGGACCACTTTGTACAAGAAAGCTGGGTC ATTTTTACTTCCTGTGACAATGCAAAATTAGGGTTT

The promoter and coding sequences were cloned into pDONR207 (Life Technologies) using BP clonase to obtain entry vectors. LR clonase was used for recombination with destination vectors, which were pMDC164 (Curtis and Grossniklaus, 2003) for promoter studies, pEG101 and pEG104 (Earley et al., 2006) for protein localization studies, and pGWB411 and pGWB412 (Nakagawa et al., 2007) to produce FLAG-tagged overexpressing plants. The destination vectors used are listed in Table 4.

Table 4. List of binary GATEWAY™ destination vectors used in this study

Vector	Reference	Use
pMDC164	Curtis and Grossniklaus, 2003	Promoter analysis
pEG101 (C-term)	Earley et al., 2006	Protein localization
pEG104 (N-term)	Earley et al., 2006	Protein localization
pGWB411 (C-term)	Nakagawa et al., 2007	Gene overexpression
pGWB412 (N-term)	Nakagawa et al., 2007	Gene overexpression

Plant transformation

Wild-type Col-0 plants were transformed using *Agrobacterium tumefaciens* strain GV3101 carrying different binary vectors using the floral dip method (Clough and Bent, 1998). T_1 seeds obtained from the dipped plants were plated on media containing carbenicillin (10 mg/L) to prevent *Agrobacterium* growth, and hygromycin (30 mg/L, pMDC164), glufosinate ammonium (BASTA®, 10 mg/L, pEG101 and pEG104) or kanamycin (50 mg/L, pGWB411 and pGWB412), to select transfromants (Figure 8). Table 5 summarizes the transgenic lines of *Arabidopsis* generated in this study.

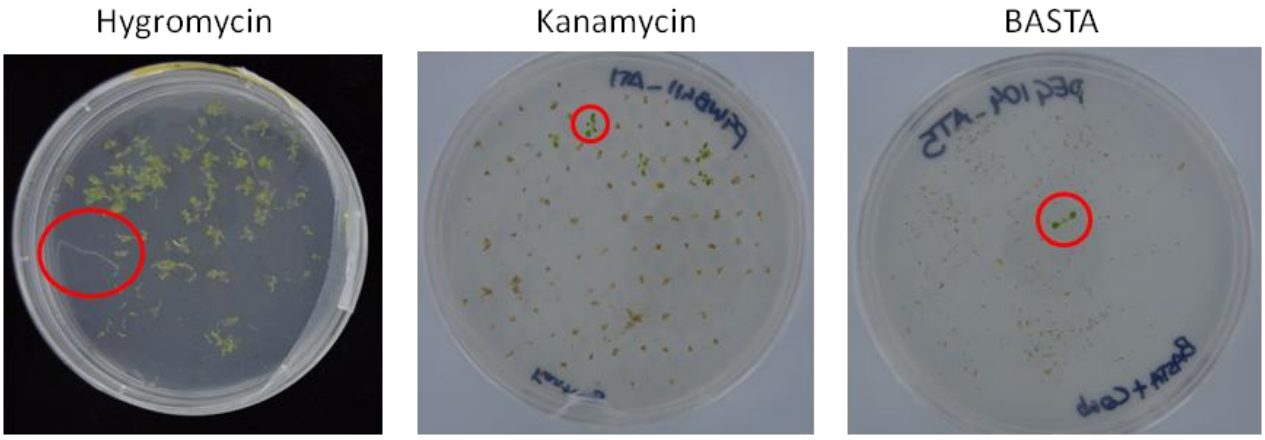

Figure 8. Transformed lines selection. Seeds of dipped Arabidopsis Col-0 plants were sown on 30 mg/L hygromycin, where resistant seedlings show longer epicotyls and roots, or on 50 mg/L kanamycin and 10 mg/L glufosinate ammonium (BASTA), where resistant plants present green expanded cotyledons.

Table 5. List of transgenic *Arabidopsis* lines obtained in the present study

Transgenic line	Vector	Construct	Use
DRT111 prom	pMDC164	DRT111prom-GUS	Promoter analysis
DRT111 OX	pGWB411	35S::DRT111-FLAG	Overexpression
	pGWB412	35S::FLAG-DRT111	Overexpression
DRT111 YFP	pEG101	35S::DRT111-YFP	Protein localization
	pEG104	35S::YFP-DRT111	Protein localization
TIP41-like prom	pMDC164	TIP41-likeprom-GUS	Promoter analysis
TIP41-like OX	pGWB411	35S::TIP41-like-FLAG	Overexpression
	pGWB412	35S::FLAG-TIP41-like	Overexpression
TIP41-like YFP	pEG101	35S::TIP41-like-YFP	Protein localization
	pEG104	35S::YFP-TIP41-like	Protein localization
SIN-like prom	pMDC164	SIN-likeprom-GUS	Promoter analysis
SIN-like OX	pGWB411	35S::SIN-like-FLAG	Overexpression
	pGWB412	35S::FLAG-SIN-like	Overexpression
SIN-like YFP	pEG101	35S::SIN-like-YFP	Protein localization
	pEG104	35S::YFP-SIN-like	Protein localization

2.2.6 GUS assay

Seedlings and tissues of *Arabidopsis* transgenic plants transformed with the construct (DRT111/TIP41-like/SIN-like) prom::GUS were used for GUS activity measurements. The histochemical detection of GUS activity was performed as described by Sunkar *et al*. (2006). GUS staining patterns were confirmed by observing five different T_2 transgenic lines.

2.2.7 Confocal Imaging

Confocal microscopy analyses were performed on an Inverted Z.1 microscope (Zeiss, Germany) equipped with a Zeiss LSM 700 spectral confocal laser scanning unit (Zeiss, Germany). Samples were excited with a 488 nm, 10 mW solid laser with emission split at 505 nm for yellow fluorescent protein (YFP) and at 531 nm for propidium iodide detection and with a 405 nm, 10 mW solid laser with emission split at 420 nm for DAPI detection. Propidium iodide staining was carried out as described previously (Sassi et al., 2012). DAPI (1µg/mL) was dissolved in VECTASHIELD Mounting Media.

2.2.8 Production of recombinant His-AtRGGA protein

To produce His-tagged AtRGGA in *Escherichia coli*, the coding sequence was amplified and cloned between SalI and NotI restriction sites of pET28a vector. Primers used are listed in Table 6. All constructs were sequenced to rule out the presence of mutations introduced by PCR. The His-tagged AtRGGA from pET28a constructs was overexpressed following transformation in *E. coli* BL21 (DE3). Cells were recovered and lysed by 1 mg mL^{-1} lysozyme after growth at 37°C on Luria-Bertani medium supplemented with 50 mg L^{-1} kanamycin. Induction was carried out by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside to a culture at an optical density measured at 600 nm of 1 and then incubating the culture at 37°C for 4 h. Protein constructs were purified by nickel affinity chromatography as described by the manufacturer (Qiagen). The purified protein was analyzed by SDS-PAGE as described by Laemmli (1970).

2.2.9 RNA EMSA

Total RNA (700 ng) extracted from control and salt stress-treated plants was labeled with biotin using the RNA 3' End Biotinylation Kit (Pierce Biotechnology) following the manufacturer's instructions. One microliter of a 1:20 dilution (approximately 30 ng) of the labeled RNA was used for each EMSA reaction. Similar amounts of labeled RNA were also used for poly(A^+) and poly(A^-) RNA, which were prepared using the mRNA Isolation Kit (Roche Applied Science). 5S, 5.8S, 18S and 25S rRNAs were *in vitro* transcribed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). Primers used are listed in Table 6. RNA was incubated with 7.5 µg of His-tagged AtRGGA or His-tagged PYR1. When present, unlabeled RNA was used as a competitor at 560 ng (approximately 160-fold). The RNA EMSA was carried out with the LightShift Chemiluminescent RNA EMSA Kit (Pierce Biotechnology). The binding reaction was analyzed by gel electrophoresis on a native 6% polyacrylamide gel in 0.53 Tris-borate/EDTA buffer and transferred to a nylon membrane.

Table 6. List of primers used for EMSA

Name	Sequence 5'-3'	Application
Rgga Fw Sal I	ACGCGTCGACAGATGGCAACTTTGAACCCTTTTG	Cloning in pET28a
Rgga Rev NotI	ATAAGAATGCGGCCGCCTTGCCCCCAAGAGATGG	Cloning in pET28a
18S FOR	TAATACGACTCACTATAGGGTACCTGGTTGATCCTGCCAG	*In vitro* transcription
18S REV	CAATGATCCTTCCGCAGGTTCACCTACGGAAACC	*In vitro* transcription
25S FOR	TAATACGACTCACTATAGGGGCGACCCCAGGTCAGGCGGG	*In vitro* transcription
25S REV	TCGAATCTTAGCGACAAAGGGCTGAATCTCAGTGG	*In vitro* transcription
5.8S FOR	TAATACGACTCACTATAGGGAAAACGACTCTCGGCAACGG	*In vitro* transcription
5.8S REV	TTGTGACACCCAGGCAGACGTGCCCTCGGCCGG	*In vitro* transcription
5S FOR	TAATACGACTCACTATAGGGGGATGCGATCATACCAGCACT AATGC	*In vitro* transcription
5S REV	GAGGGATGCAACACGAGGACTTCCCGGGAGGTC	*In vitro* transcription

2.2.10 Immunoblotting

To detect the FLAG-RGGA fusion protein in overexpressing lines and after RNA Immunopurification, immunoblotting was performed using α-FLAG antibody (Sigma-Aldrich). Total proteins were extracted from 100 mg of tissue using 2x sample buffer (200 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 5% ß- mercaptoethanol). Samples were heated at 95°C for 5 min and subjected to SDS-PAGE followed by transfer on nitrocellulose membranes (Amersham, GE Healthcare). A 1:5.000 dilution of α-FLAG antibody in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) was used for immunoblotting following the manufacturer's instructions. Immunoreactive bands were detected by the ECL Western blotting detection system (GE Healthcare).

2.2.11 RNA immunoprecipitation

Pulverized leaf tissues (100mg) from Col-0 and *AtRGGA* overexpressing plants were used for RIP assay as described in Sorenson and Bailey-Serres (2015). The protocol is summarized in Figure 9. 50 µL of Protein G Dynabeads (Life Technologies) were coated with 5 µg of the α-FLAG or α-HA (Sigma) antibodies for specific and non-specific immunoprecipitations respectively. For competitive elution, 3× FLAG peptide (Sigma-Aldrich) were used for α-FLAG antibody. Immunoprecipitated RNA was extracted from 90 µL of the eluate using TRIzol Reagent (Life Technologies), while 10 µL of the eluate were used for immunoblot. IP RNA quality and size distribution were evaluated using the Agilent 2100 Bioanalyzer, RNA 6000 Pico Chip (Agilent Technologies).

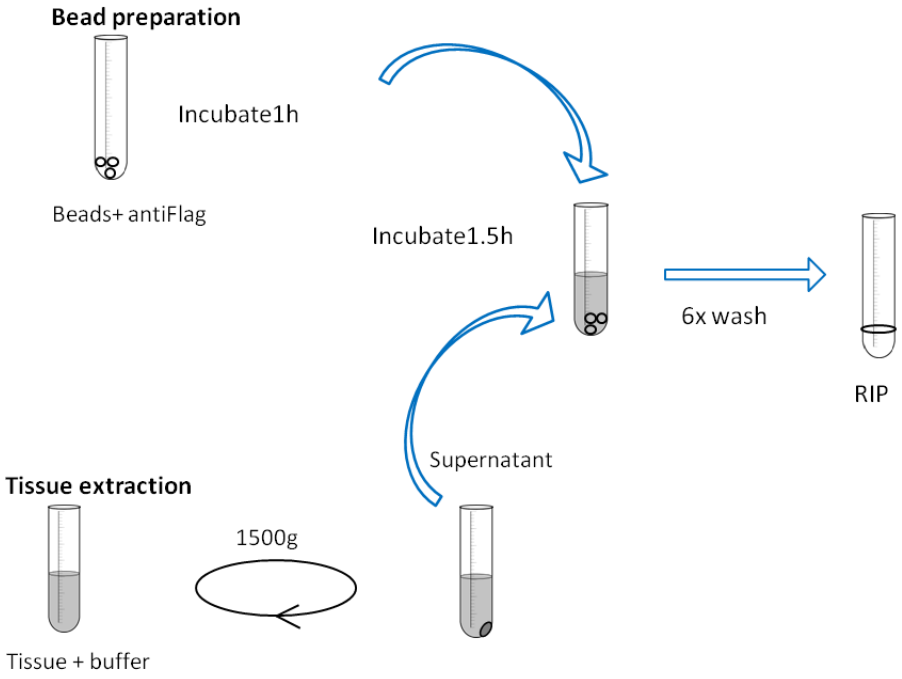

Figure 9. RNA immunoprecipitation. Schematic representation of the RIP method, including bead preparation using the anti-flag antibody, tissue extraction, immunoprecipitation and elution.

2.2.12 Yeast two hybrid

For yeast two hybrid assay (Figure 10), AtRGGA was cloned in frame with the *GAL4* DNA binding-domain of the bait plasmid pGBKT7 (Clontech) digested with EcoRI and SalI (Table 7). The AtRGGA::pGBKT7 obtained plasmid was used to transform Y2HGold Yeast Strain (Clontech), using the Lithium acetate/Polyethylene glycol method (Bai and Elledge, 1996). The Arabidopsis cDNA library constructed in the prey vector pGADT7RecAB (Clontech), such that the proteins encoded by the inserts were fused with the *GAL4* activation-domain, was already available in the CNR-IBBR lab. The overnight culture of Y2HGold (AtRGGA::pGBKT7) was transformed with 380 µg of the cDNA library::pGADT7 using the previously described method (Bai and Elledge, 1996), and plated on SD/Leu−,Trp−,Ade−,His− medium (7.5g/L Yeast Nitrogen Base, 0.75g/L Amino acid mix lacking Leu ,Trp ,Ade and His, 20g/L glucose, pH 5.8). The transformation efficiency was calculated as CFU/volume of yeast transformed cells plated on –Leu/-Trp medium. Colonies growing on SD/Leu−,Trp−,Ade−,His− medium were transferred on SD/Leu−, Trp− Ade− His− /Aureobasidin A medium for a more stringent selection. The colonies showing fastest growth rate were picked and suspended in 50µL of DNAse free distilled water. 1µL was used for colony PCR using vector specific primers (Table 7) .

The selected prey plasmids were recovered from Liticase-treated cells (Sigma-Aldrich) using QIAprep Spin Miniprep Kit (Qiagen) and transformed into *E. coli* DH5α.

To confirm the interaction, the prey plasmids isolated from *E.coli* were then co-transformed into Y2HGold together with AtRGGA::pGBKT7 or with the empty vector pGBKT7, as negative control. An equal amount of overnight-grown yeast culture was dropped onto selective media SD/Leu−, Trp− to guarantee the presence of both vectors, and on SD/Leu−, Trp− Ade− His− /Aureobasidin A, to verify the interaction. Prey plasmids showing interaction with AtRGGA::pGBKT7 and not with empty pGBKT7 were considered as true interactors and identified by DNA sequencing..

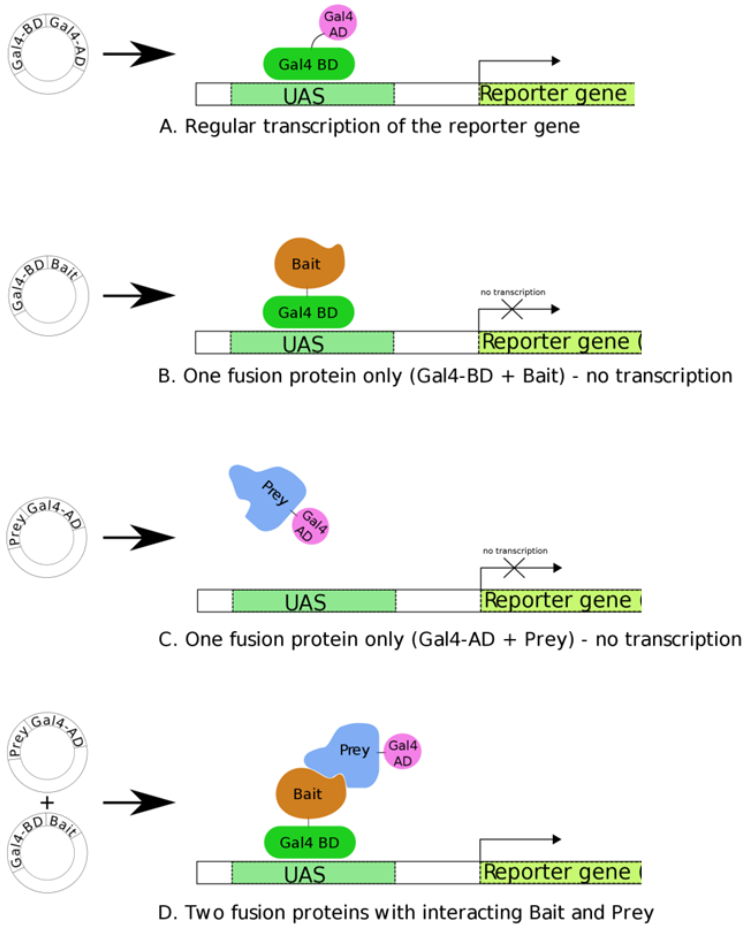

Figure 10. Yeast two hybrid principle. Two proteins are expressed in yeast, with the bait fused to the Gal4 DNA-binding (GAL4 BD) domain, and the prey fused to the Gal4 transciption activation domain (GAL4 AD). In Yeast, activation of the reporters under the control of the GAL4 upstream activating sequence (UAS) only occurs in a cell that contains proteins which interact and reconstitute a functional GAL4. (https://en.wikipedia.org/wiki/Two-hybrid_screening)

Table 7. List of primers used for YeastTwoHybrid

Name	Sequence 5'-3'
ECORI_RGGA_ATG	AGTACGAATTCATGGCAACTTTGAACCCTTT
SalI_RGGA_STOP	ATCCGTCGACTTACTTGCCCCCAAGAGA
pGAD for	AAAGAGATCTTTAATACGACTCACTATAGG
pGAD rev	AGATGGTGCACGATGCACAGTT

2.3 Results

2.3.1 From microarray to functional genetics

In a previous study, several genes whose expression was differentially regulated in *Solanum tuberosum* culture cells adapted to high concentrations of polyethylene glycol (PEG) were identified in the laboratories of CNR-IBBR (Ambrosone et al., 2012; Ambrosone et al., in preparation). Fifty promising candidates for further functional analyses in *Arabidopsis thaliana* were selected among the 544 genes with altered expression during PEG adaptation, based on: i) predicted functional category; ii) previously uncharacteryzed for involvement in abiotic stress responses; iii) high degree of sequence similarity between the gene identified in *Solanum tuberosum* and the putative orthologue in Arabidopsis. The BLAST algorithm (Altschul et al., 1990) was used to determine homologies with Arabidopsis genes with known or unknown function. When the nucleotide identity percentages with the highest scoring gene were lower than 50 %, the protein sequences were used. Only proteins sharing a sequence identity higher than 40 % were selected (Table 8). The genes were assigned to functional categories according to the Functional Catalog (Fun-Cat) classification system (Ruepp et al., 2004). Functional categories representing regulatory processes such as “signal transduction mechanism”; “protein with binding function” were enriched in the selected genes (Figure 11).

Table 8. List of Arabidopsis selected genes and their distribution in functional categories (Fun-Cat annotation). Fold change values (ratio) in adapted cells *vs.* control untreated cells obtained by the potato microarray analysis and the identity percentage (% identity) between potato and Arabidopsis proteins are indicated. Locus number and name, where present, are indicated. Blue line point out the three genes selected after phenotyping.

Ratio	% identity	Locus	Name	Description
		Unclassified protein		
5,83	67	AT4G36660	/	Unknown
4,25	63	AT1G36380	/	Unknown
4,09	69	AT3G07900	/	molecular_function unknown
3,79	94	AT1G77710	ATCCP2	phosphatidylinositol biosynthetic process
		Cellular communication, signal trasduction mechanism		
3,24	47	AT3G58310	/	molecular_function unknown
		Protein with binding function		
5,65	81	AT4G16520	ATG8F	Autophagy
4,27	45	AT2G18190	/	Oxidative stress
4,09	76	AT5G56140	/	RNA-binding
3,86	64	AT4G14300	/	RNA binding
3,32	62	AT3G05420	ACBP4	Acyl-CoA binding protein, response to ethylene stimulus
3,21	59	AT1G43560	ATY2	protein disulfide oxidoreductase activity
3,02	62	AT5G25060	RRC1	RNA binding regulation of alternative splicing
2,98	67	AT1G30480	DRT111	RNA binding
2,93	70	AT4G27000	ATRBP45C	RNA binding
3,25	71	AT4G17510	UCH3	ubiquitin thiolesterase activity
2,8	56	AT2G25490	EBF1	negative regulation of ethylene mediated Pathway
		Cell cycle and DNA processing		
5,26	39	AT2G47980	SCC3	sister-chromatide cohesion protein
		Catalytic process		
3,74	50	AT1G61820	BGLU46	beta glucosidase, hydrolase activity
3,74	70	AT4G17040	CLPR4	inducing stress acclimatization
3,63	75	AT3G61790	/	Seven-in-absentia protein,
3,51	48	AT4G20860	/	oxidoreductase activity,FAD binding
3,28	69	AT2G24200	LAP1	response to salt stress
2,84	46	AT4G29270	/	acid phosphatase activity
		Secondary metabolism		
6,16	67	AT3G03710	RIF10	Involved in response to phosphorus (P) starvation
		Nucleic acid metabolism		
2,91	42	AT5G49530	/	SIN-like family protein
2,79	78	AT1G66740	AtSP7	ANTI- SILENCING- FUNCTION
		Transcription		
2,39	84	AT5G56030	HSP90	EARLY-RESPONSIVE TO DEHYDRATION
		Unclassified protein		
0,04	62	AT5G51200	EMB3142	Unknown
0,05	60	AT1G49975	/	photosynthesis
0,06	48	AT5G48880	PKT1/2	acetyl-CoA C-acyltransferase activity
0,14	68	AT1G65270	/	Unknown
0,19	85	AT5G64130	/	cAMP-regulated phosphoprotein 19-related protein
		Transcription		
0,04	40	AT1G53910	RAP2.12	Encodes a member of the ERF (ethylene response factor)
0,07	40	AT2G40750	WRKY54	member of WRKY Transcription Factor
0,11	61	AT3G60800	/	DHHC-type zinc finger family protein
0,12	60	AT5G65670	IAA9	auxin induced gene
0,16	89	AT5G13080	WRKY75	cellular response to water deprivation
0,3	42	AT1G01260	JAM2	DNA binding transcription factor - ABA response
0,25	51	AT1G75390	ATBZIP44	regulation of transcription
		Interaction with the environment		
0,12	40	AT1G14210	/	response to salt stress
0,22	48	AT2G03440	ATNRP1	response to cold, response to heat
0,24	42	AT4G32280	IAA29	response to auxin stimulus
		Protein with binding function or cofactor requirement		
0,08	85	AT5G19090	/	metal ion transport
0,28	70	AT3G06190	ATBPM2	Encodes a member of the MATH-BTB domain proteins
0,23	62	AT1G32790	CID11	RNA-binding protein
		Cellular comminication, signal trasduction mechanism		
0,18	53	AT2G35050	/	protein serine/threonine/tyrosine kinase activity
0,06	66	AT1G42540	ATGLR3.3	calcium ion transport
0,11	54	AT1G05460	SDE3	Encodes a protein with similarity to RNA helicases
0,2	67	AT4G34270	/	TIP41-like family protein
0,21	44	AT5G63930	/	

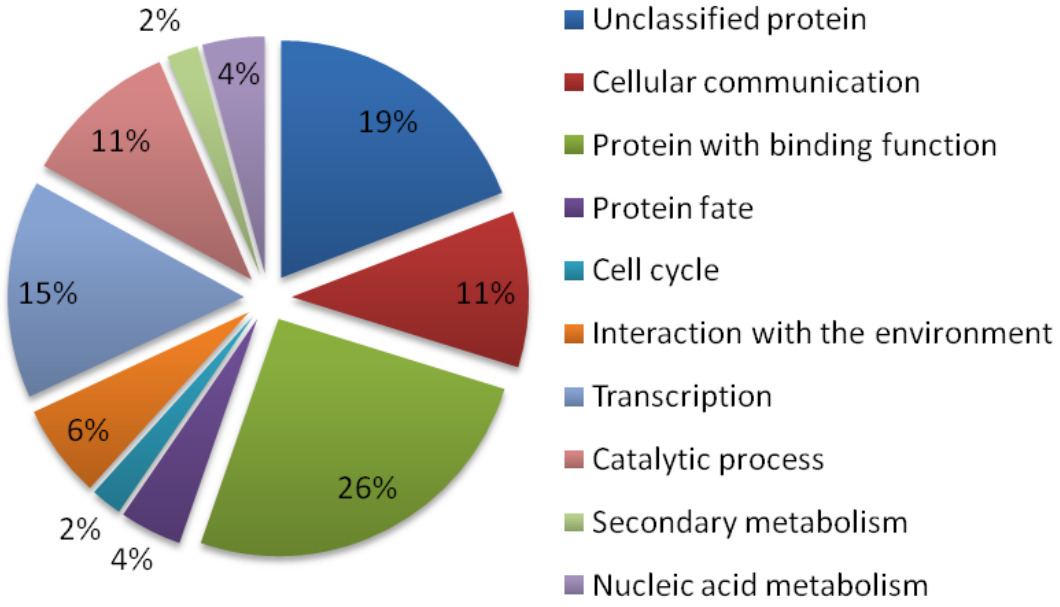

Figure 11. Distribution of 50 selected genes into functional categories, using the Functional Catalogue (Fun- Cat) annotations.

2.3.2 Knockout mutants: selection of homozygous lines

To evaluate the involvement of the selected genes in responses to abiotic stress, a loss-of function approach was pursued. Fifty *knockout* mutants, predicted to carry a 4 Kb T-DNA insertion in the transcribed regions of the target genes, were identified within the TAIR collection (https://www.arabidopsis.org/) and ordered from Nottingham Arabidopsis Stock Centre (Table 9). Mutants carrying the T-DNA insertion within the predicted transcripts were not available for 4 genes, therefore promoter or intron insertions were selected.

The predicted insertion site and the zigosity status of the T-DNA were verified by PCR using the T-DNA express tool for design of primers (Materials and Methods, paragraph 2.2.3). Using this method, 1000 plants were screened (20 individuals per gene) to identify individuals homozygous for the presence of the T-DNA (Figure 12). The PCR analysis confirmed presence of the T-DNA insertion in the predicted regions for 38 mutants. In particular, more than two homozygous individuals were identified for 87% of the 36 mutants carrying the T-DNA insertion within the transcribed region; while five homozygous individuals with a T-DNA insertion in the putative promoter area were selected for N665483 and eight individuals for the N659880 with intron insertion (Table 9). Remaining 10 putative insertion mutants did not contain a T-DNA insertion in the region of interest.

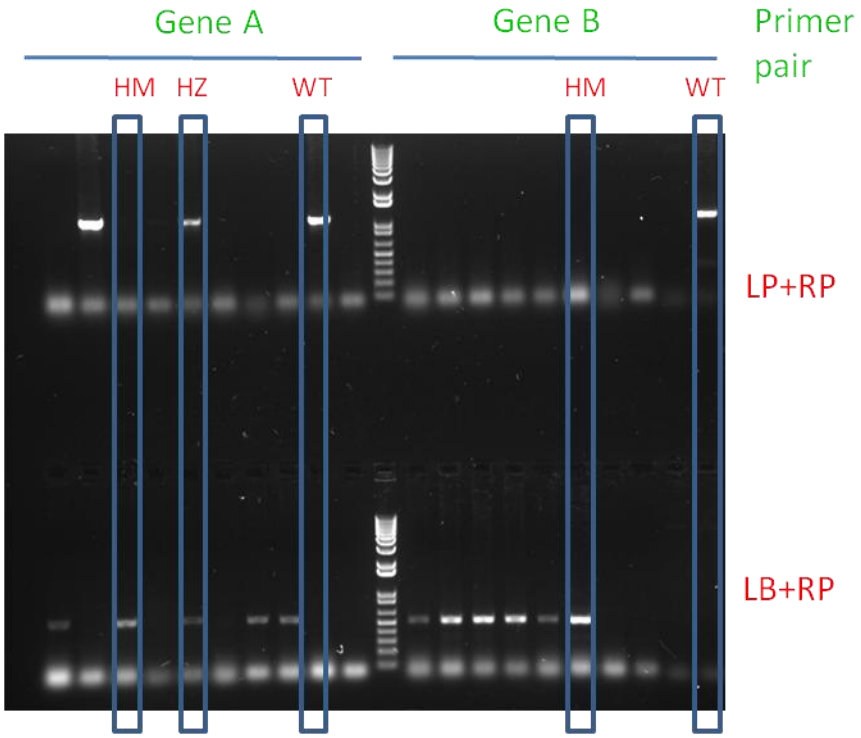

Figure 12. Genotyping of 2 of 50 knockout mutants. Isolation of homozygous (HM) T-DNA insertion lines by PCR amplification using T-DNA left border (LB) and gene (LP-RP) specific forward and reverse primers. HZ: Heterozygous lines for the T-DNA insertion. WT: Wild Type (Columbia) used as negative control. 1Kb Plus DNA ladder was used as a molecular size marker.

Table 9. List of Arabidopsis *knockout* mutants identified within the TAIR collection. HM indicates the number of homozygous individuals isolated by PCR and used in phenotype screening. Blue line point out the three genes selected after phenotyping.

Locus	Name	insertion site	SALK	NASC	HM
AT4G36660	/	exon	SALK_052218	N552218	0
AT1G36380	/	exon	SALK_150032	N650032	5
AT3G07900	/	exon	SALK_065949	N686155	8
AT1G77710	ATCCP2	exon	SALK_151519	N651519	5
AT3G58310	/	exon	SALK_013540C	N671231	4
AT4G16520	ATG8F	exon	SALK_057021	N653221	7
AT2G18190	/	exon	SALK_064794C	N659242	10
AT5G56140	/	exon	SALK_123494	N623494	5
AT4G14300	/	exon	Wisc413-416I9	N854650	5
AT3G05420	ACBP4	exon	SALK_040164C	N674601	9
AT1G43560	ATY2	exon	SALK_028065	N528065	1
AT5G25060	RRC1	exon	SALK_102739	N602739	3
AT1G30480	DRT111	exon	SALK_066706	N664667	9
AT4G27000	ATRBP45C	exon	SALK_063484	N656570	7
AT4G17510	UCH3	exon	SALK_140822	N640822	0
AT2G25490	EBF1	exon	SALK_020997	N520997	1
AT2G47980	SCC3	exon	SALK_095771C	N658342	1
AT4G17040	CLPR4	exon	SALK_090614	N590614	2
AT3G61790	/	exon	SALK_018558C	N658116	5
AT4G20860	/	exon	SALK_131268	N631268	4
AT2G24200	LAP1	exon	SALK_110626	N610626	0
AT4G29270	/	intron	SALK_053381C	N659880	8
AT3G03710	RIF10	exon	SALK_104980	N604980	1
AT5G49530	SIN-like	exon	SALK_133036C	N660190	5
AT1G66740	AtSP7	promoter	SALK_123420	N668973	0
AT1G61820	BGLU46	exon	SAIL_360_B01	N877264	2
AT5G56030	HSP90	exon	SALK_064707C	N672556	0
AT5G51200	EMB3142	exon	SALK_055559	N555559	4
AT1G49975	/	promoter	SALK_021405C	N665483	5
AT5G48880	PKT1/2	exon	SALK_144455	N644455	0
AT1G65270	/	exon	SALK_097162C	N663425	5
AT5G64130	/	exon	SALK_031836	N531836	2
AT1G53910	RAP2.12	exon	SALK_152421	N652421	1
AT2G40750	WRKY54	exon	GK-642C12	N461572	3
AT3G60800	/	exon	SALK_026159	N526159	2
AT5G65670	IAA9	exon	SALK_069445C	N681249	4
AT5G13080	WRKY75	promoter	SALK_048763	N548763	0
AT1G01260	JAM2	exon	SALK_051573	N551573	0
AT1G14210	/	exon	SALK_055304	N555304	2
AT2G03440	ATNRP1	exon	SALK_069833C	N672654	0
AT4G32280	IAA29	exon	SALK_091933C	N663323	3
AT5G19090	/	exon	SAIL_899_D10	N840594	0
AT3G06190	ATBPM2	exon	SALK_121270C	N665071	3
AT2G35050	/	exon	SALK_107170C	N670017	5
AT1G42540	ATGLR3.3	exon	SALK_099757C	N663463	7
AT1G05460	SDE3	exon	SALK_099137	N599137	4
AT4G34270	TIP41-like	exon	SALK_006384C	N654482	6
AT1G75390	ATBZIP44	exon	SAIL_15_H08	N870155	0
AT5G63930	/	exon	SAIL_147_G05	N871551	0
AT1G32790	CID11	exon	SALK_139651	N639651	2

2.3.3 Phenotype screening: candidate genes selection

In order to identify genes with a functional role in adaptation to osmotic stress, progenies of the 38 confirmed knockout homozygous lines were subjected to a large-scale phenotype screening. Different parameters such as seed germination, root growth and plant survival in the presence of NaCl or ABA were evaluated to assess involvement in stress responses and sensitivity to the universal stress hormone, respectively.

To screen for both sensitive and tolerant mutants, gradual concentrations of NaCl or ABA, such that were un-inhibitory or partially inhibitory to the wild type Col-0, were used.

At the germination stage, Col-0 displayed sensitivity after 2 days of exposure to high concentration of NaCl (100mM) and ABA (2μM) with 88% and 12% of germinated seeds respectively, compared with 100% of seeds sown on GM or 50mM of NaCl and 97% on 0.5μM of ABA.

At the whole plant level, stress caused shoot and root growth reduction. In particular, after 7 days of exposure to high salt (180mM NaCl) or ABA (100μM), 42% and 30% of Col-0 seedlings showed signs of necrosis or bleaching, respectively. In the presence of 120 mM NaCl, the elongation of wild-type roots was 33% of roots on control media, while roots on 80mM NaCl containing media retained 74% of their ability to elongate. Similarly, after 12 days of exposure to 50μM or 20μM ABA, the primary root length was reduced of 28% and 54% compared to control condition.

Knockout lines showing clear, discernible phenotypes compared to Col-0 in at least one of the conditions used were considered promising and subjected to a second independent replicate to confirm the observed phenotypes and decrease noise due to inter-experimental variations. Using this strategy, we selected three mutants showing the most reproducible and dramatic differences (Figure 13).

N664667 contained a T-DNA insertion in *DRT111* (*At1g30480*) a predicted RNA-binding protein, and is hereafter referred to as *drt111*. *drt111* mutants were insensitive to ABA (2 μM) at the germination stage. After 4 days of treatment, 41 % of seeds developed plants with cotyledons fully expanded compared to 12 % of Col-0 seeds. The second selected mutant was N654482, containing a T-DNA insertion in *TIP41-like* (*At4g34270*) a predicted component of the TOR pathway, hereafter referred to as *tip41-like*. *tip41-like* plants were hyper-sensitive to multiple stresses, especially to ABA treatment, with only 31% of plants surviving long-term (7 days) exposure to ABA (100 μM), compared to 54% of Col-0 plants. Similarly, in presence of 0.5 μM of abscisic acid, after 2 days of incubation, only 76% of mutant seeds germinated compared to 98% of wild type seeds. Finally, N660190, mutated in *SIN-like* (*At5g49530*) a putative subunit of RNA polymerase III, hereafter indicated as *sin-like*. s*in-like* showed a significant reduction (≥75%) in root growth compared to wild type in all tested conditions, including the controls.

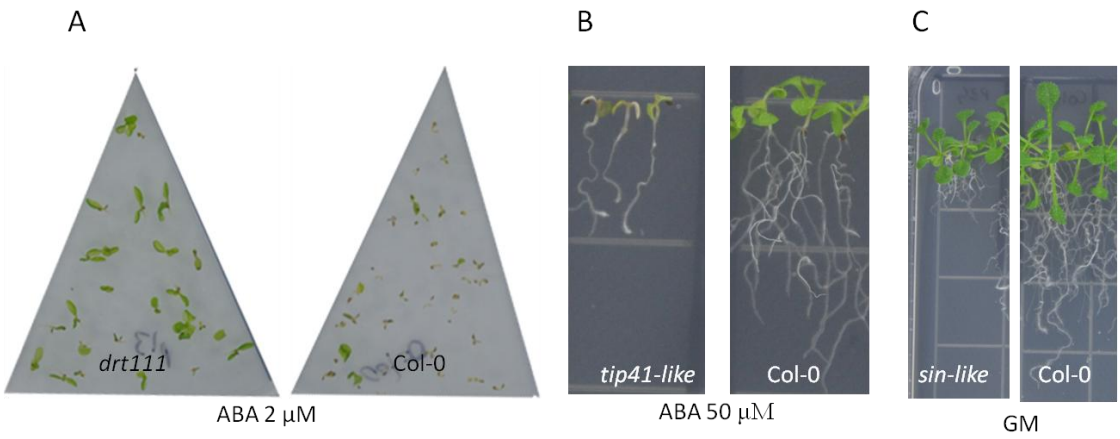

Figure 13. Phenotypes of three selected *knockout* mutants. A) Germination analysis of *drt111* mutants compared to Col-0 after 4 days of exposure to ABA (2µM). B) 14-day old *tip41-like* and Col-0 seedlings germinated for 4 days on GM and transferred to medium containing ABA (50µM). C) 14-day old *sin-like* and Col-0 seedlings germinated on GM medium.

The 3 genes in which a T-DNA insertion caused a dramatic phenotype were thus considered worth pursuing and subjected to functional analysis. This included:

- a bioinformatic analysis, to compare the gene initially identified in potato with the Arabidopsis orthologue and the presence of functional domains in the predicted protein;

- an expression analyses, to identify tissues/developmental stages/ treatments inducing expression of the target genes;

- a protein localization studies, to identify the subcellular compartment in which the target proteins are active;

- a phenotype analysis on overexpressing and knockout lines, to identify the processes affected by the target genes.

2.3.4 The RNA-binding protein DRT111: Bioinformatic analysis

The *Solanum tuberosum* protein sequence of LOC102603413 shares 67% sequence identity with the protein encoded by the locus *At1g30480* of *Arabidopsis thaliana*, which consequently was hypothesized to be the Arabidopsis orthologue. The alignment was obtained using the multiple alignment tool MUSCLE (Figure 14). A bioinformatic analysis using the web tool InterPro (www.ebi.ac.uk/interpro/) indicated that the DRT111 protein contains a G-patch domain (aa 212-260; IPR000467) and RNA recognition motif domain at the C-terminus (aa 282-372; IPR000504)

(Figure 15). The Arabidopsis protein was previously annotated as DRT111 (DNA-damage-repair/toleration 111) based on its ability to rescue the mutagen-sensitive phenotype of the *Escherichia coli* recG mutant (Pang et al., 1993), and also known as RSN2 (REQUIRED FOR SNC4-1D 2; Zhang et al., 2014). DRT111 shares significant similarity with other plant proteins. In particular, the BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/) was able to identify 80% of sequence identity with the *Brassica rapa* protein, 73% identity with a sequence from *Vitis vinifera* and 63% with *Zea mays* and *Solanum lycopersicum* proteins, indicating that DRT111 is highly conserved in plants.

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)

S.tuberosum      MLGGLYGDLPPPSSSGDDDKPTNATTANVWSCSAKMAPSALRKP--FAPPQTLLRPQPKP
A.thaliana       MLGGLYGDLPPPT---DDEKPS-GNSSSVWSSSTKMAPPTLRKPPAFAPPQTILRPLNKP
                 ************:   **:**: ..::.***.*:****.:****  ******:***  **

S.tuberosum      KPQPSAAPKPHPSIPLDENTNPNPTFATTSFQPALVAVTSSVLEEYDPARPNDYEDYRRE
A.thaliana       KPIVSAPYKPPPPSNSSQSVLIPANESAPSHQPALVGVTSSVIEEYDPARPNDYEEYKRE
                 **  **. ** *.   .:..   .. ::.*.*****.*****:***********:*.**

S.tuberosum      KKRKQMEAEVRRELEERERKERERERREEKEKREKERE--------LNISGEEAWRRRAAM
A.thaliana       KKRKATEAEMKREMDKRRQEDEERDKRERERREEKERERDNSDPSRLNISGEEAWKRRAAM
                 ****  ***:.**:::* .:: **:. *.*:******        *********.*****

S.tuberosum      SGGGGGGSAPPRSPSPPSG-NEFSIGRSESGGLGLGAEGKMTAAQRMMAKMGWKEGQGLG
A.thaliana       SGGGSGGKG--RSSSPPGNVDGFSIGKSETSGLGVGAGGQMTAAQRMMAKMGWKQGQGLG
                 ****.**..  **.***.. : ****.**:.***:** *:***************:*****

S.tuberosum      KLEQGITTPLMAKKTDKRGGVIVASEAKQQQQQQQQQAPEKKVKSVNFNMPPTRVVLLRN
A.thaliana       KSEQGITTPLMAKKTDRRAGVIVNA-----SENKSSSAEKKVVKSVNINGEPTRVLLLRN
                 * **************.*.**** :     .:::...* :* *****:*  ****:****

S.tuberosum      MVGPGEVDDDLEGEVAEECSKFGTVTRVLIFEITETNFPHEEAVRIFIQFERAEHATKAL
A.thaliana       MVGPGQVDDELEDEVGGECGKYGTVTRVLIFEITEPNFPVHEAVRIFVQFSRPEETTKAL
                 *****:***:**.**. **.*:*************.***  ******:**.*.* :****

S.tuberosum      IELEGRFFGGRIVHACFYDEERFGNNELAPMPGEIPGF
A.thaliana       VDLDGRYFGGRTVRATFYDEEKFSKNELAPVPGEIPGY
                 ::*:**:**** *.* *****.*.:*****:******:

Percent Identity  Matrix - created by Clustal2.1


  1: S.tuberosum  100.00   67.82
  2: A.thaliana    67.82  100.00
```

Figure 14. Sequence alignment of the Arabidopsis and potato DRT111 proteins. Alignment was generated using MUSCLE (3.8). An "*" (asterisk) indicates positions which have a conserved residue; A ":" (colon) indicates residues with strongly similar properties. A "." (period) indicates residues with weakly similar properties. Bottom: Alignment score.

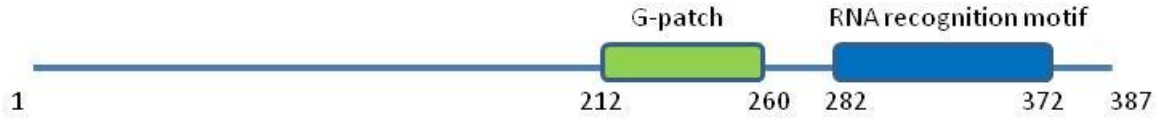

Figure 15. Schematic representation of *Arabidopsis thaliana* DRT111 protein domain organization. The boxes indicate the location of the G-patch domain (InterPro: IPR000467) and RNA recognition motif (InterPro: IPR000504).

2.3.5 DRT111 is expressed in stomata and trichomes and is stress-inducible

In silico analysis of *Arabidopsis* microarray data through the Electronic Fluorescent Pictograph (eFP) browser, revealed that *DRT111* is highly expressed in dry seeds with lower levels in developing siliques, suggesting that *DRT111* is up-regulated at later stages of seed maturation. The gene is also expressed at a high level in the shoot apex and flower stage 9 with a gradual decrease of transcript levels from flower developmental stage 10 to 15 (Figure 16).

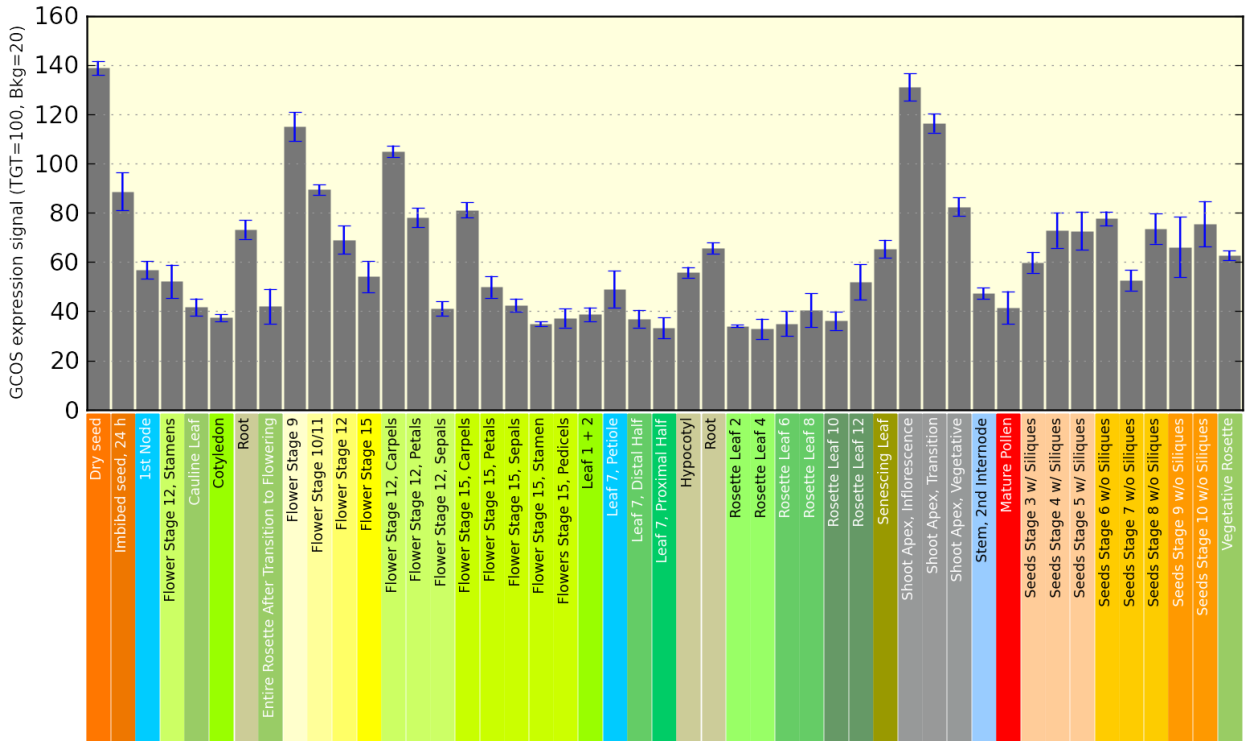

Figure 16. Expression levels of *DRT111* in different tissues based on Arabidopsis microarray data displayed in the eFP browser (according to Schmid et al. 2005). Data are normalized by the GCOS method, TGT value of 100.

In order to study the expression of *DRT111* in different plant tissues and developmental stages, transgenic *Arabidopsis* plants expressing the β-glucoronidase (*GUS*) gene driven by the *DRT111* promoter were generated. The genomic 2 kb fragment upstream of the protein-coding sequence was

arbitrarily defined as the promoter and cloned into pDONR207. After recombination, a DRT111Prom- pMDC164 plasmid was obtained carrying the putative DRT111 promoter upstream of the GUS reporter gene. The obtained plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. The DRT111Prom-GUS was transformed into *A. thaliana* using *Agrobacterium*-mediated transformation by the floral dip method. T_1 seeds harvested from the dipped plants were sown on hygromycin-containing media and transformed lines were selected. T_2 hygromycin-resistant plants were stained using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The blue colour observed either in plant tissues or in cells indicated the presence of the GUS protein. GUS distribution was observed both in seedlings and in adult plants in the vasculatures and in cells surrounding the trichomes. A strong staining of stomata was observed in leaves of adult plants, not in seedlings (Figure 17), indicating the expression of *DRT111* in guard cells and a possible involvement in stomatal movements.

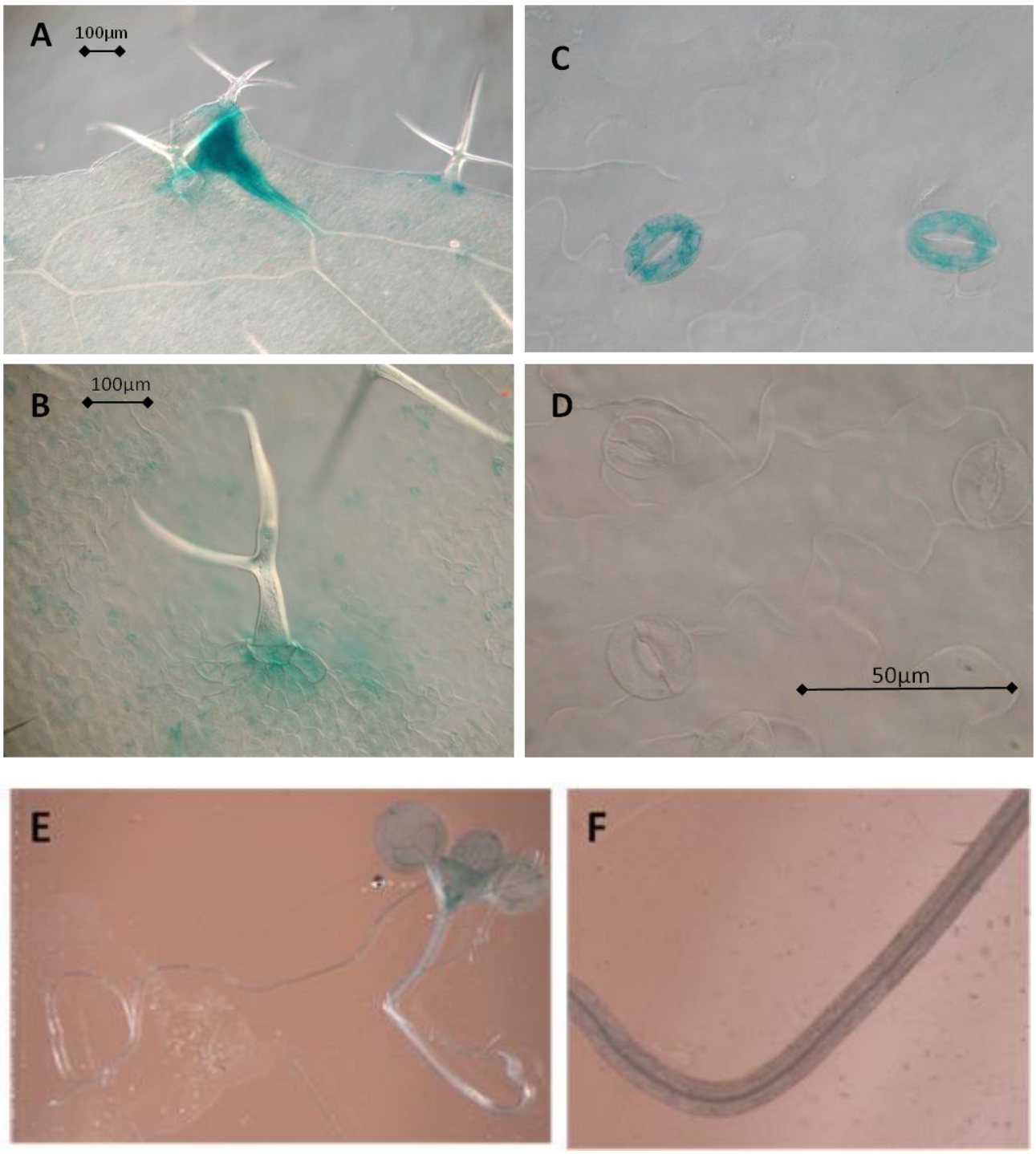

Figure 17. Histochemical localization of GUS activity in transgenic Arabidopsis plants expressing the GUS reporter gene under the control of the *DRT111* promoter. Leaves of adult plants and seedlings were stained. Close up views of trichomes (A,B) and stomata in adult plant (C) and 10-day old seedlings (D). Whole seedling (E) and root (F) are also shown.

To verify the possible regulation of *DRT111* by osmotic stress in Arabidopsis, the regulation of gene expression in seedlings during long-term exposure to different stress treatments was investigated. A time course was performed on 12-day-old seedlings exposed to NaCl (120mM),

ABA (10μM) and PEG (35% W/V) for 2, 5 and 7 days. The *DRT111* expression levels were analyzed by qRT-PCR (Figure 18). After 2 days of exposure to NaCl and ABA, an increase in expression was observed. After 5 days of exposure, *DRT111* was up-regulated in ABA- and PEG-treated samples. After 7 days, values of expression reverted to those of untreated samples.

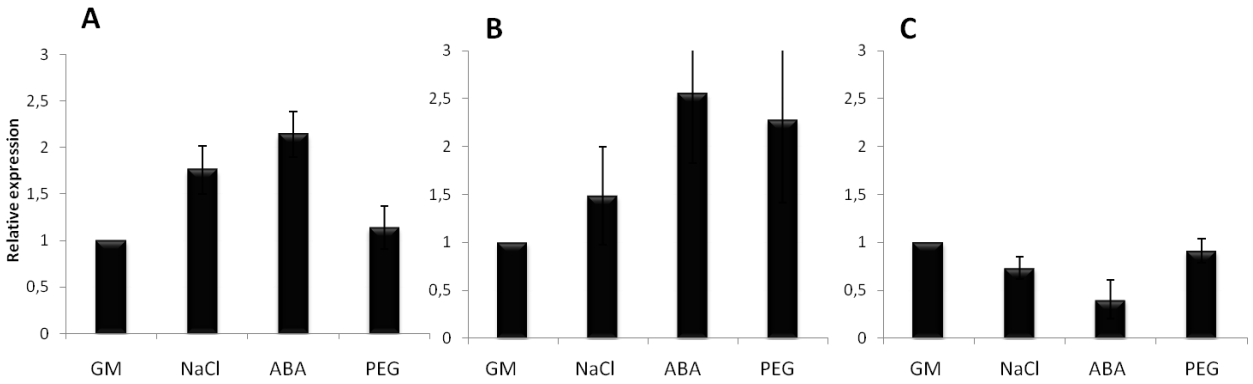

Figure 18. Relative expression of *DRT111* measured by qRT-PCR in 7-day-old seedlings of *A. thaliana* after 2 days (A), 5 days (B) and 7 days (C) of exposure to PEG (35% w/v), NaCl (120mM) or ABA (10 μM). Elongation Factor *EF1α* was used as reference gene and data were normalized using RNA from untreated seedlings. Error bars indicate standard deviation of three biological replicates.

2.3.6 DRT111 protein localizes to the nucleus

To analyze DRT111 protein subcellular localization, transgenic plants overexpressing fusion proteins between YFP and DRT111 were generated. The coding sequence of *DRT111* was cloned in the entry vector pDONR207. Recombination was performed with LR reaction and two binary plasmids were obtained: DRT111-pEG101, to produce a C-terminal fusion DRT111-YFP; and DRT111-pEG104 to produce a N-terminal fusion YFP-DRT111. The obtained plasmids were transformed into *A. tumefaciens* strain GV3101. A PCR positive colony was liquid cultured and used for the genetic transformation of *Arabidopsis thaliana* using the floral dip method. T_1 seeds obtained from the dipped plants were plated on glufosinate ammonium (BASTA®) and putative transformed lines were selected. Thanks to a collaboration with Prof. Giorgio Morelli (CREA-NUT, Rome), 5-day old seedlings of three different T_2 transgenic YFP-DRT111 lines were observed by confocal laser scanning microscopy following a short incubation in propidium iodide to counterstain cell walls. As shown in Figure 19, in cells of the root apex, where vacuoles are less developed, a clear YFP signal was observed in the nucleus of plants expressing N-terminal fusions of YFP with DRT111. Similar results with lower signal intensity were obtained using transgenic plants expressing a C-terminal fusion (data not shown). A complete overlap of the YFP signal with

the 4′,6-Diamidino-2-phenylindole (DAPI), a nuclear tracker, confirmed the subcellular localization in the nucleus (Figure 20).

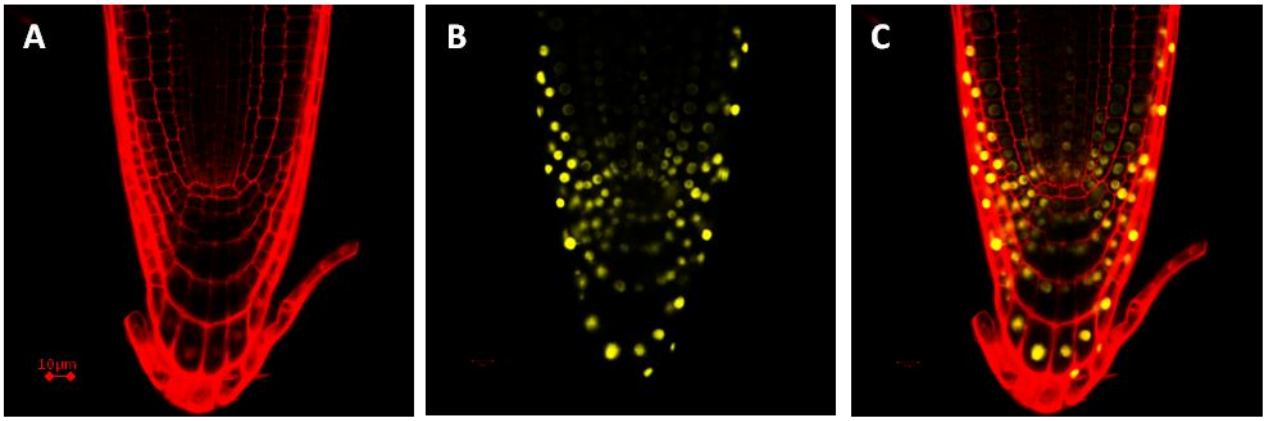

Figure 19. Subcellular localization of DRT111 protein in Arabidopsis root apex. Confocal microscopy visualization of 5-day-old transgenic Arabidopsis plants expressing a YFP-DRT111 fusion protein. Propidium iodide staining (A), YFP fluorescence (B), and merged images (C) are shown. Scale bar: 10µm

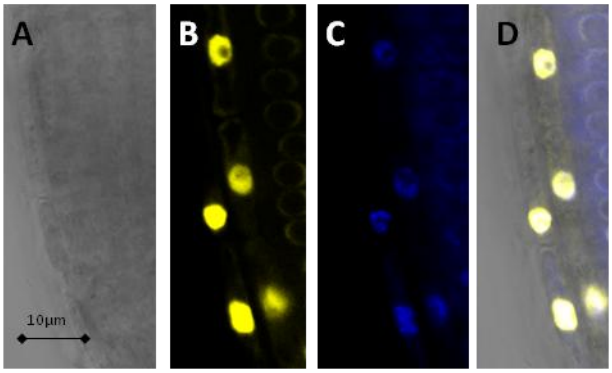

Figure 20. DRT111 protein localization. Confocal microscopy visualization of 5-day-old Arabidopsis root (A). YFP fluorescence from YFP-DRT111 fusion protein (B; yellow) and nuclear stain DAPI (C; blue) and merge images (D). Bar: 10µm

2.3.7 Generation and phenotyping of *DRT111* overexpressing plants

To gain insights into the function of *DRT111*, a gain-of-function strategy was adopted, in addition to the analysis of loss-of-function mutants. Transgenic plants overexpressing *DRT111* were generated, placing the coding sequence of *DRT111* downstream of the strong constitutive promoter CaMV 35S and in fusion (N- or C-term) with the FLAG tag. The DRT111:pDONR207 entry vector (obtained as described in Materials and Methods 2.2.5) was used for LR reaction with the pGWB411 and pGWB412 destination vectors. Two binary plasmids were obtained: DRT111-

pGWB411, to produce a C-terminal fusion DRT111-FLAG; and DRT111-pGWB412 to produce a N-terminal fusion FLAG-DRT111. Both vectors were used for *Agrobacterium*-mediated transformation of Arabidopsis plants. T_1 seeds obtained from transformed plants were screened on selective medium containing kanamycin. Twelve 35S::DRT111-FLAG and eight 35S::FLAG-DRT111 transformed lines were selected and T_2 seeds were used for segregation analysis. Five lines per construct showed a segregation ratio close to 3:1, indicating the presence of a single T-DNA insertion locus. T_3 seeds from five individuals of the three above mentioned lines were plated on kanamycin and homozygous lines were selected to be used for further analyses.

Increased expression of DRT111 causes ABA-sensitivity

The large-scale phenotype screening highlighted the insensitivity to elevated concentration of ABA of *drt111* knockout mutants (SALK_066706) at the germination stage (Paragraph 2.3.3). Therefore, to investigate on the DRT111 involvement in seed germination, phenotype analyses in the presence of elevated concentration of abscisic acid were conducted comparing knockout mutants to wild-type Col-0 and DRT111-overexpressing plants. Seeds of two different overexpressing lines and *drt111* were sown on ABA-containing media and the germination percentage was scored. After 2 days of exposure to 5µM of ABA, an average of 30% of *drt111* presented radicle emergence, compared to only 14% of Col-0 (Figure 21). In contrast, seeds of overexpressing plants showed high sensitivity to the presence of exogenous abscisic acid with only 9% (FLAG-DRT111) and 2% (DRT111-FLAG) of seeds germinated after 6 days compared to 45% and 40% of *drt111* and Col-0 respectively.

Based on these results, we demonstrate that an increased expression of DRT111 results in a hypersensitivity to ABA-treatments during seed development, showing an opposite phenotype compared to *drt111*.

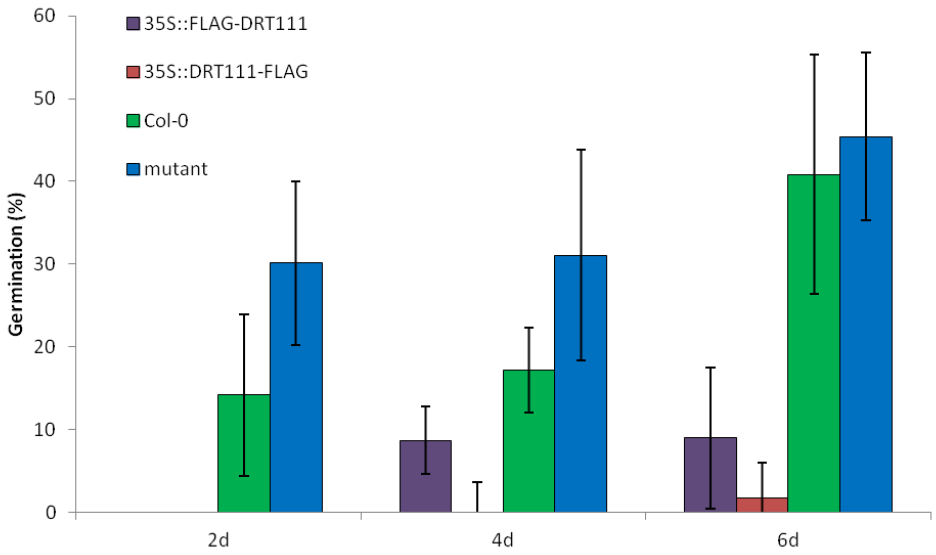

Figure 21. Germination analysis of *DRT111 knockout* mutants and overexpressing plants compared with wild-type (Col-0) in presence of ABA (5 µM). Germination was scored in terms of radicle emergence 2, 4 and 6 days after stratification. Error bars indicate standard deviation of three biological replicates.

drt111 mutants have reduced leaf water loss

The possible role of DRT111 in regulation of stomatal movements was evaluated measuring the decline in fresh weight of detached leaves over time. Both *drt111* and two DRT111-overexpressing lines were compared to Col-0. Leaves from 3-week old plants were detached and the loss in fresh weight was monitored during a time course of 6 hours. As shown in Figure 22, Col-0 and 35S::FLAG-DRT111 plants had lost a similar amount of water, retaining 27% and 29% of fresh weight, respectively after 6 hours. Interestingly, leaves detached from knockout plants retained a higher amount of water, having lost 42% of their initial fresh weight, while 35S:: DRT111-FLAG plants showed a dramatic water loss (14%), thus suggesting a DRT111 role in stomatal movements in drought stress conditions.

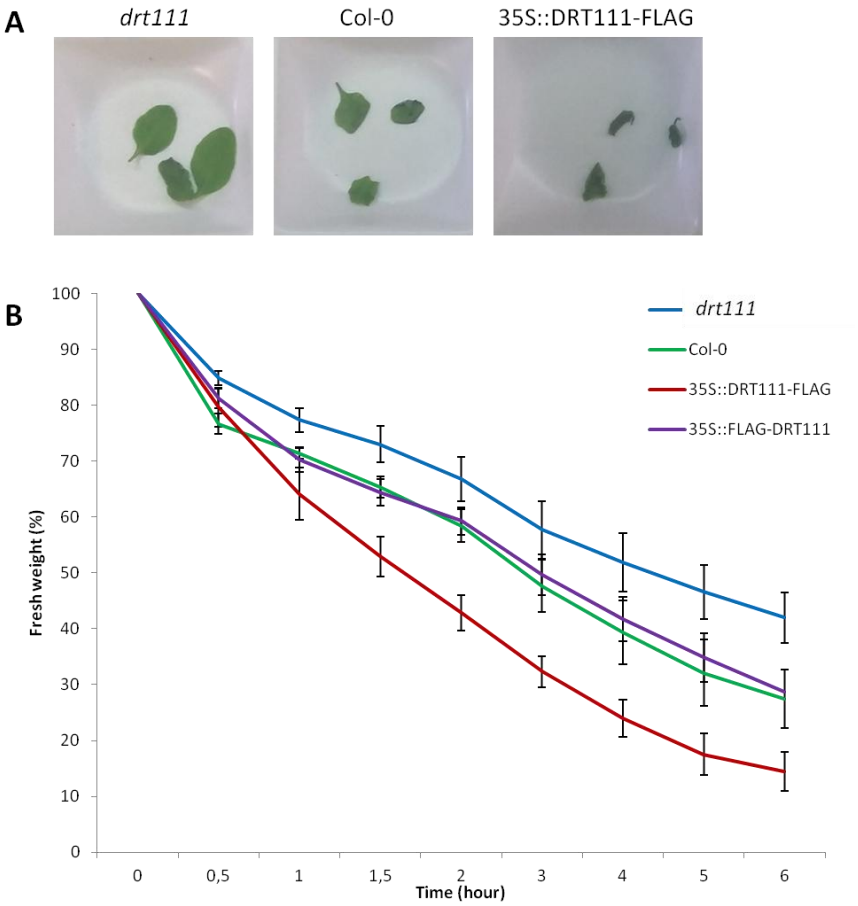

Figure 22. Water loss of leaves detached from *drt111* mutants, *DRT111* overexpressing plants and Col-0. A) Leaves of the three genotypes 6h after detachment. B) Data represent percentages of initial weight lost at different time points. Error bars indicate standard deviation of three biological replicates.

2.3.8 The putative component of TOR pathway, TIP41-like: bioinformatic analysis

The Arabidopsis TIP41-like protein sequence (locus *At4g34270*) was aligned with *Solanum tuberosum* protein (locus LOC102590132) using the MUSCLE tool (Figure 23). Sequence identity between the two encoded proteins was higher than 67%. Similarity search using BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/) in the NCBI database also showed a high match with proteins from several plant species, such as *Brassica rapa* (89%), *Vitis vinifera* (71%), *Solanum lycopersicum* (67%) (data not shown), indicating that TIP41-like is highly conserved in plants. On the basis of sequence alignment, a conserved region was found (aa 60-253; IPR007303; PF04176), whose annotation in the Pfam database (http://pfam.xfam.org) identified this protein as a member of TIP41-like family, a negative regulator in yeast systems of the TOR signaling pathway, involved in a cell-growth program in response to nutrients and stimulus.

Figure 23. Sequence alignment of the Arabidopsis and potato TIP41-like proteins. Alignment was generated using MUSCLE (3.8). An "*" (asterisk) indicates positions which have a conserved residue; A ":" (colon) indicates residues with strongly similar properties. A "." (period) indicates residues with weakly similar properties. Bottom: Alignment score.

2.3.9 *TIP41-like* is expressed in vascular tissues and is stress-inducible

Analysis of global gene expression data displayed in the Arabidopsis eFP browser, showed that TIP41-like is expressed in all plant organs, with slightly higher values in dry seeds and roots (Figure 24). A virtually complete absence of transcript is observed in the mature pollen, suggesting that *TIP41-like* could be down-regulated during pollen development and/or post-pollination events.

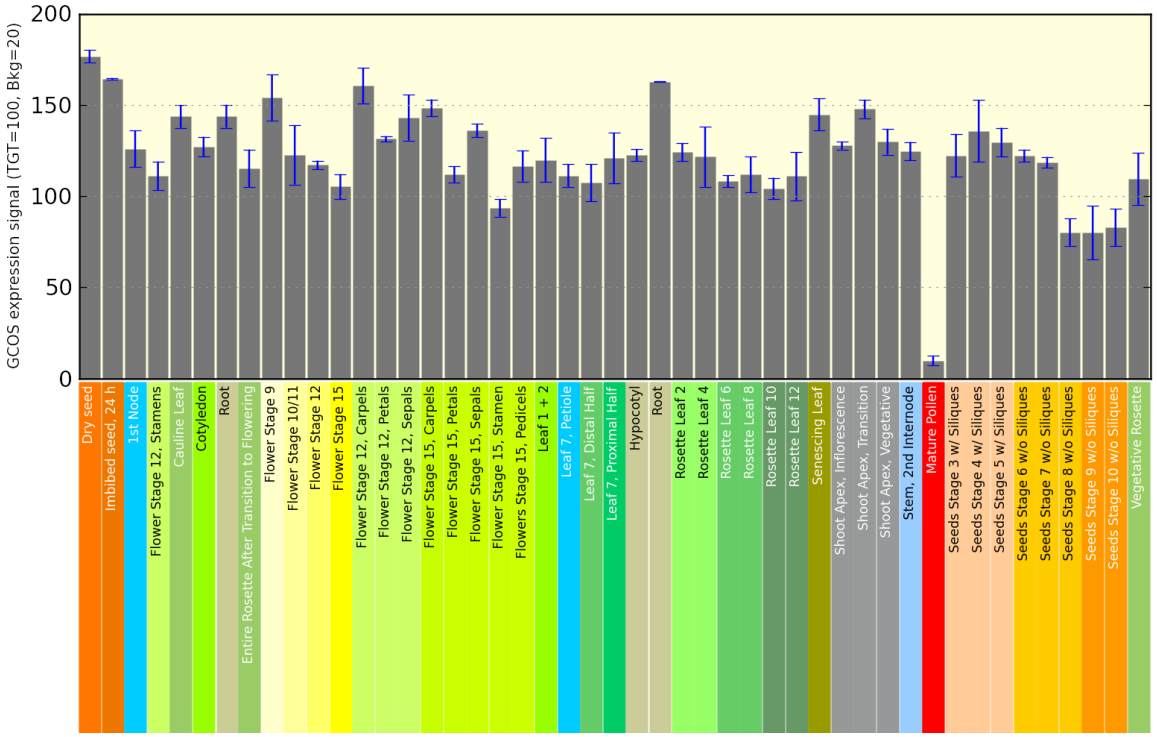

Figure 24. Expression levels of *TIP41-like* based on Arabidopsis microarray data displayed in the eFP browser (according to Schmid et al. 2005). Data are normalized by the GCOS method, TGT value of 100.

To gain insights into the function of *TIP41-like* in plants, its expression pattern was analysed, using transgenic *Arabidopsis* plants expressing the β-glucoronidase (*GUS*) gene driven by the *TIP41-like* promoter. The 2 Kb fragment upstream from the *TIP41-like* start codon was PCR amplified and cloned in the entry vector pDONR207. After sequencing, the plasmid was used for the LR reaction with the destination vector pMDC164 and introduced into *Arabidopsis thaliana* through floral-dip method. T_1 seeds from the dipped plant were sown on hygromycin-containing media and transformed lines were selected. The T_2 transgenic plants expressing the GUS reporter gene driven by the putative promoter of *TIP41-like* were stained using X-Gluc to visualize the spatial and temporal patterns of activity of the TIP41-like promoter. As shown in Figure 25, GUS activity was visualized both in seedlings and in adult plants in vascular tissues.

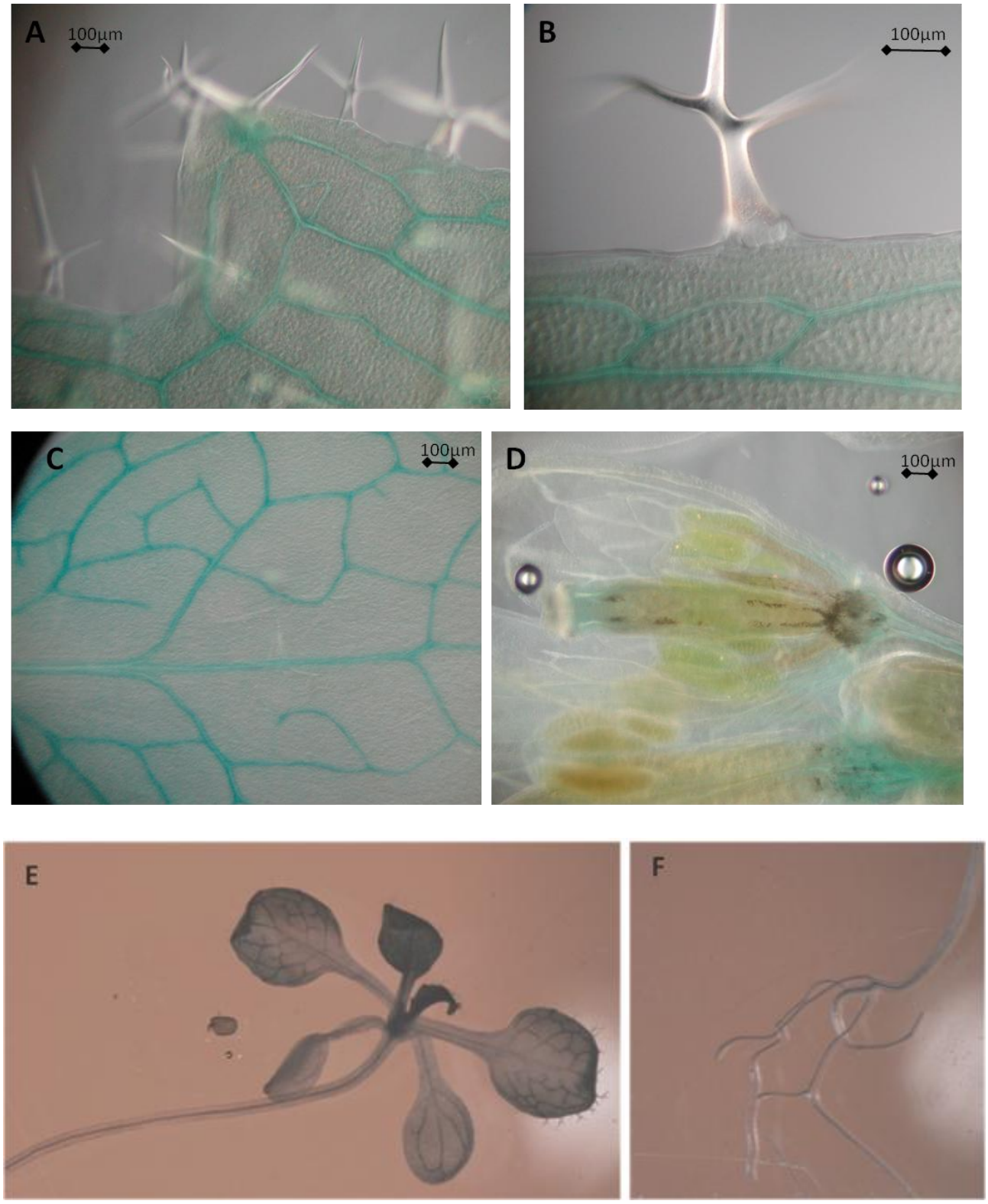

Figure25. Histochemical localization of GUS activity in transgenic Arabidopsis plants expressing the GUS reporter gene under the control of the *TIP41-like* promoter. Leaves of adult plants (A,B) and 10-day old seedlings (C) inflorescence (D), whole seedling (E) and root (F) were stained.

To verify whether *TIP41-like* is also inducible by stress in Arabidopsis, the gene expression in seedlings after long-term exposure to different treatments was examined as described for *DRT111*

(paragraph 2.3.5). As shown in Figure 26, NaCl and ABA treatments induced an increase of gene expression compared to the control at 2 days. The induction was observed at lower levels also after 5 days of exposure to PEG, while expression values were similar to those of control plants after 7 days, indicating that Tip41-like transcript abundance is transiently increased by stress-treatments (Figure 26).

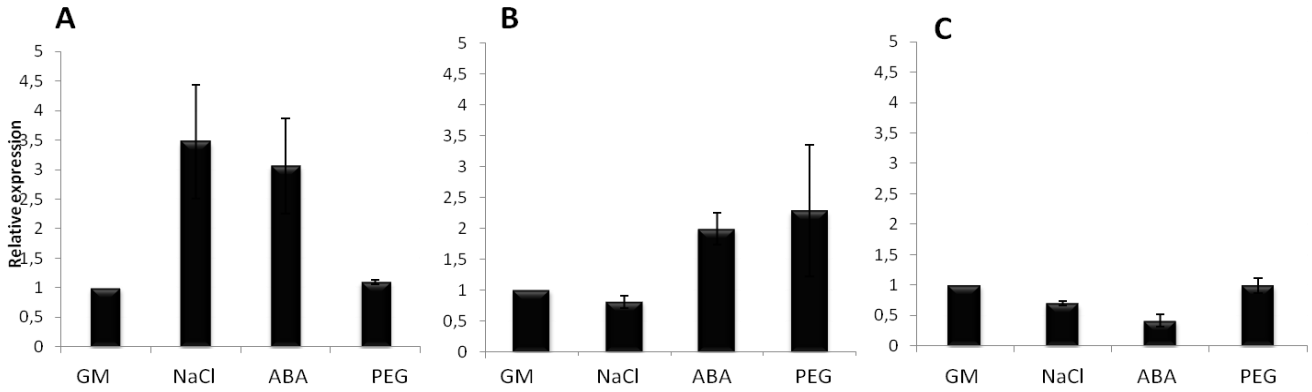

Figure 26. Gene expression of *TIP41-like* measured by qRT-PCR in 7-day-old seedlings of *A. thaliana* after 2 days (A), 5 days (B) and 7 days (C) of exposure to PEG (35% w/v), NaCl (120mM) or ABA (10 µM). Elongation Factor *EF1α* was used as reference gene and data were normalized using RNA from untreated seedlings. Error bars indicate standard deviation of three biological replicates.

2.3.10 Cytoplasmic localization of TIP41-like protein

In order to investigate the subcellular localization of the protein encoded by *TIP41-like*, transgenic plants overexpressing a fusion protein between TIP41-like and YFP were generated as described above (paragraph 2.3.6).

Three transgenic T_2 lines expressing both C-terminal and N-terminal YFP fusion were visualized by confocal microscopy. The YFP signal was detected only using the fusion protein TIP41-like-YFP (C-terminal fusion). As shown in Figure 27, in cells of the root apex, a clear YFP signal was observed in the cytoplasmic area. The localization of the reporter was also compared with DAPI (Figure 28). Interestingly, in 5-day old plants the fusion protein is highly expressed in the quiescent center (QC) compared to 14-day old seedlings where an uniform signal was detected.

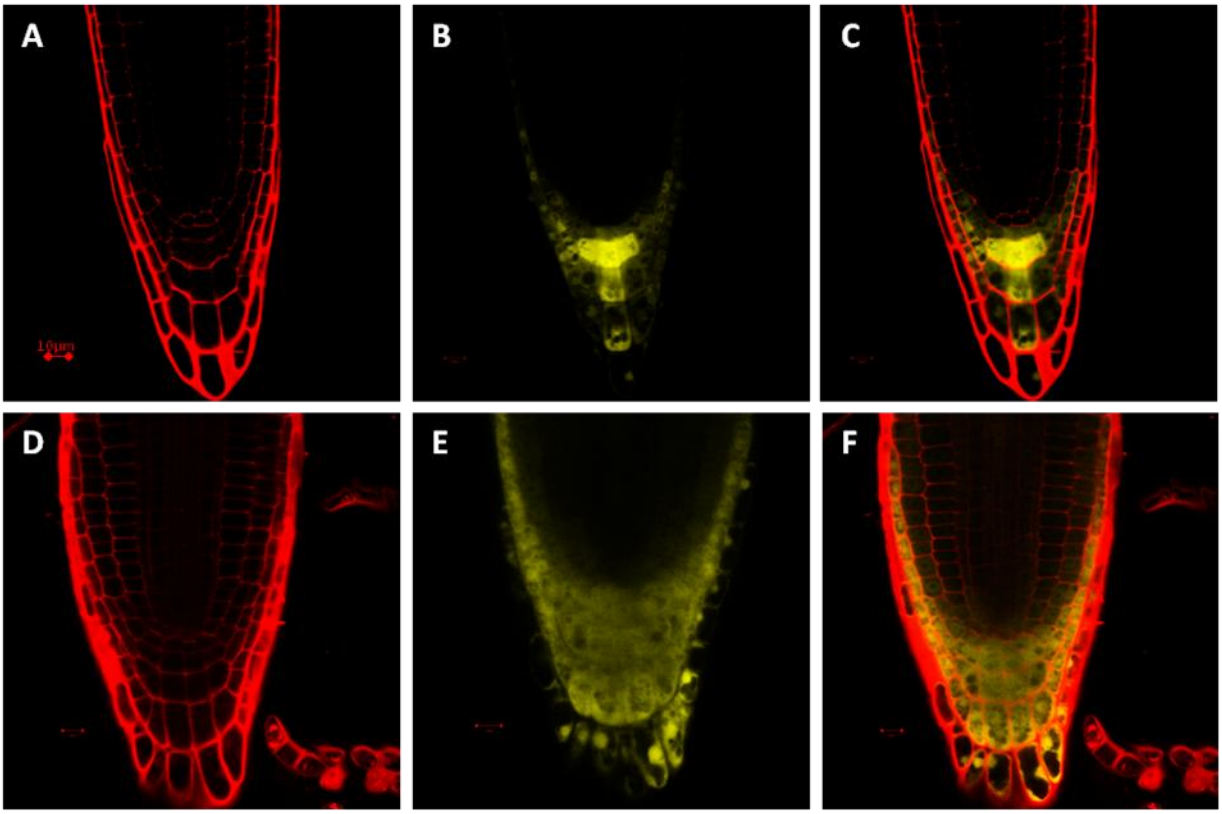

Figure 27. Subcellular localization of TIP41-like protein in root apex of 5-day old (A;B;C) and 14-day old (D;E;F) Arabidopsis seedlings. Confocal microscopy visualization for transgenic Arabidopsis plants expressing a TIP41-like-YFP fusion protein. Propidium iodide staining (A;D), YFP fluorescence (B;E), and merged images (C;F) are shown. Bar: 10μm

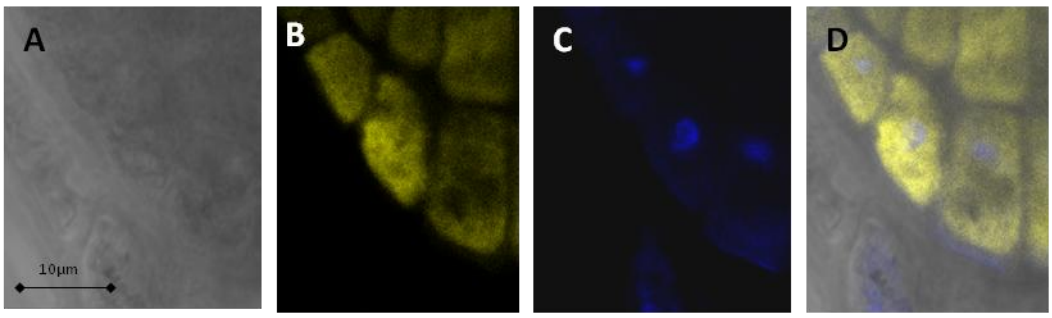

Figure 28. TIP41-like protein localization. Confocal microscopy visualization of 5-day-old Arabidopsis root (A). YFP fluorescence from YFP-DRT111 fusion protein (B; yellow), nuclear stain DAPI (C; blue) and merge images (D). Bar: 10μm

2.3.11 Generation and phenotyping of *TIP41-like* overexpressing plants

Transgenic plants overexpressing *TIP41-like* were generated, in order to investigate on the gene function during stress response. The coding sequence of *TIP41-like* was cloned into the pDONR207 entry vector and placed downstream of the strong constitutive promoter CaMV 35S by LR reaction with the pGWB411 and pGWB412 destination vectors. Two binary plasmids were obtained: TIP41-like-pGWB411, to produce a C-terminal fusion 35S::TIP41-like-FLAG; and TIP41-like-pGWB412 to produce a N-terminal fusion 35S::FLAG-TIP41-like. Both vectors were used to transform

Arabidopsis plants. T_1 seeds obtained from dipped plants were plated on kanamycin. Fourteen 35S::TIP41-like-FLAG and seventeen 35S::FLAG-TIP41-like transformed lines were selected. T_2 seeds were used for segregation analysis. Six 35S::FLAG-TIP41-like lines showed 3:1 segregation ratio, indicating the presence of one single T-DNA insertion locus, while only one 35S::TIP41-like-FLAG line was selected. T_3 seeds from five individuals of the three above mentioned lines were plated on kanamycin and homozygous lines were selected to be used for further analyses.

The abolished expression of TIP41-like causes hypersensitivity to ABA

To characterize the role of TIP41-like in the ABA-dependent mechanisms of response to environmental stresses, the *tip41-like* insertion mutant (SALK_006384) and transgenic TIP41-like overexpressing plants were used for phenotype analyses in the presence of abscisic acid at different developmental stages.

At the germination stage, *tip41-like* displayed a higher sensitivity to ABA compared with wild-type Col-0. In particular, while 100% of wild type and 73% of 35S::TIP41-like-FLAG seeds presented fully expanded cotyledons after 7 d of exposure to ABA (0.5 µM), only 46% of mutant seeds were germinated. In contrast, seeds of over expressing plants (35S::FLAG-TIP41-like) did not show any significant differences in their ability to germinate compared with the wild type (Figure 29).

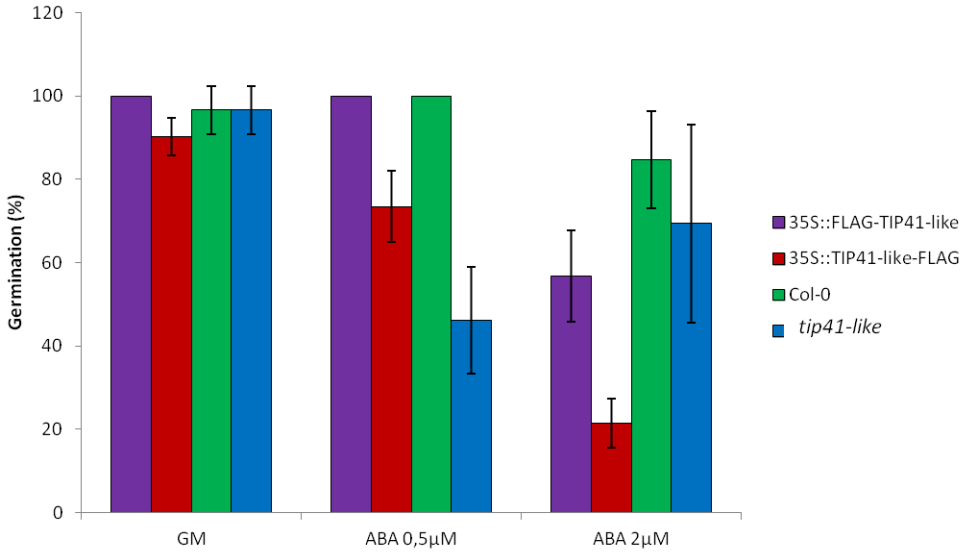

Figure 29. Germination analysis of TIP41-like knockout mutants and transgenic plants compared with wild-type (Col-0) in the presence of ABA (0.5 and 2µM). Germination was scored in terms of fully expanded cotyledons 7 days after stratification. Error bars indicate standard deviation of three biological replicates.

Survival tests showed differences in the ability to withstand stress conditions of mutant and overexpressing plants compared with controls. After 6 days of exposure to ABA, the knockout showed different survival percentages from the wild type (Table 10). More than 27% of knockout seedlings displayed signs of necrosis or bleaching, compared with 14% of Col-0 seedlings and 10% of 35S::FLAG-TIP41-like line seedlings. While 35S::TIP41-like-FLAG line did not show any significant difference compared to mutant, with only 75% of plants surviving long-term exposure.

Table 10. Survival test of *tip41-like*; Col-0 and TIP41-like overexpressing plants. Seedlings were germinated for 5 days on GM and transferred to medium containing 50 μM ABA. Survival was scored daily in terms of absence of necrotic or bleached leaves. Data are means of three biological replicates.

	Plant survival (%)			
Genotype	3d	4d	5d	6d
mutant	83,3	75	75	72,2
Col-0	88,6	88,1	85,1	85,1
35S::FLAG-TIP41-like	91,7	89,9	89,9	89,9
35S::TIP41-like-FLAG	81,8	75,8	75,8	75,8

Root growth experiments on plates showed a hypersensitivity of *tip41-like* mutants to the presence of ABA in the medium (20 μM), with an 60% of reduction in root length compared to wild type while overexpressing plants did not display significant differences (Figure 30).

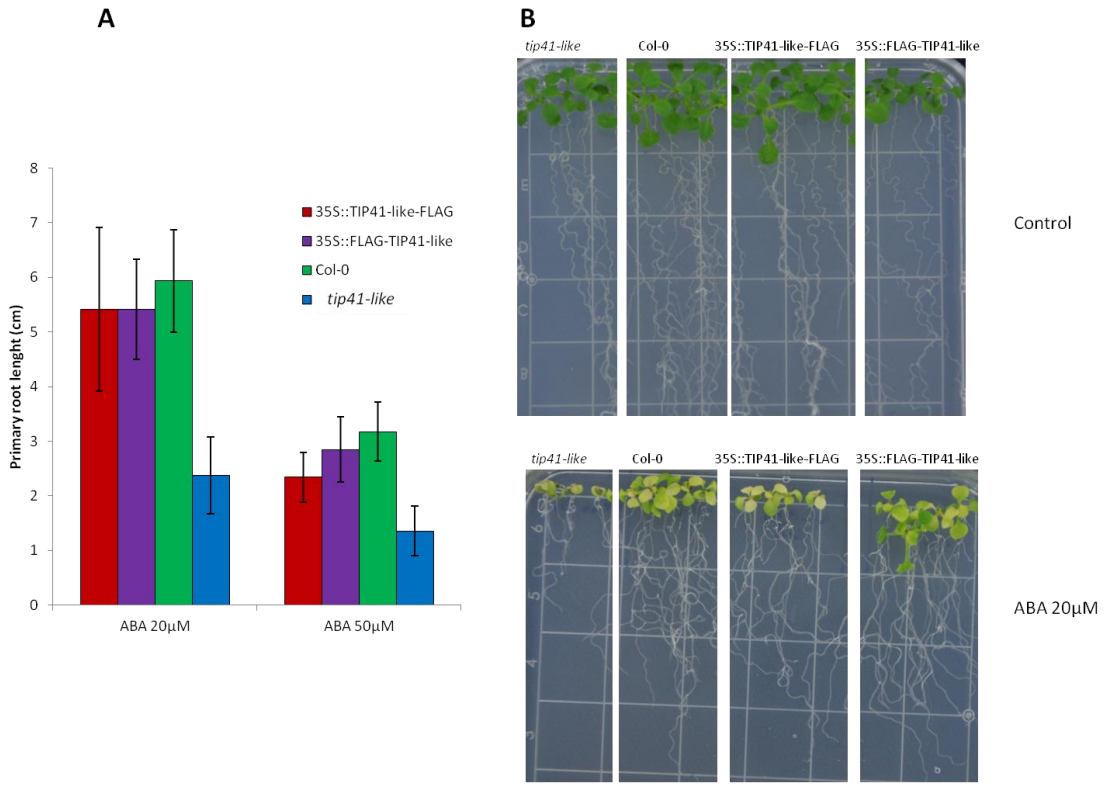

Figure 30. A) Quantification of primary root length of TIP41-like knockout mutants and transgenic plants compared with wild-type (Col-0). 14-day old seedlings germinated for 4 days on GM and transferred to medium containing 20 or 50 µM ABA. Error bars indicate standard deviation of three biological replicates. B) Photograph of seedlings grown as described in A.

Stomatal movements are an ABA-regulated process, which regulates the water lost through transpiration. In order to measure the decline of fresh weight caused by transpiration over time, leaves of 3-week old TIP41-like overexpressing plants and knockout mutants were detached and the weight loss was monitored during a time course of 6 hours (Figure 31). After 1 hour no significant difference was observed in the water loss rate compared to the wild type. Leaves of 35S::FLAG-TIP41-like line retained a higher amount of water, with a reduction of 66% of their initial fresh weight compared to 58% of Col-0 after two hour. The knockout mutant as well as the other overexpressing line (35S::TIP41-like-FLAG), had lost a similar amount of water with 17% and 19% of fresh weight, compared to 27% of Col-0 after six hours, indicating that the abolished gene expression confers an increase stomatal sensitivity in response to dehydration stress.

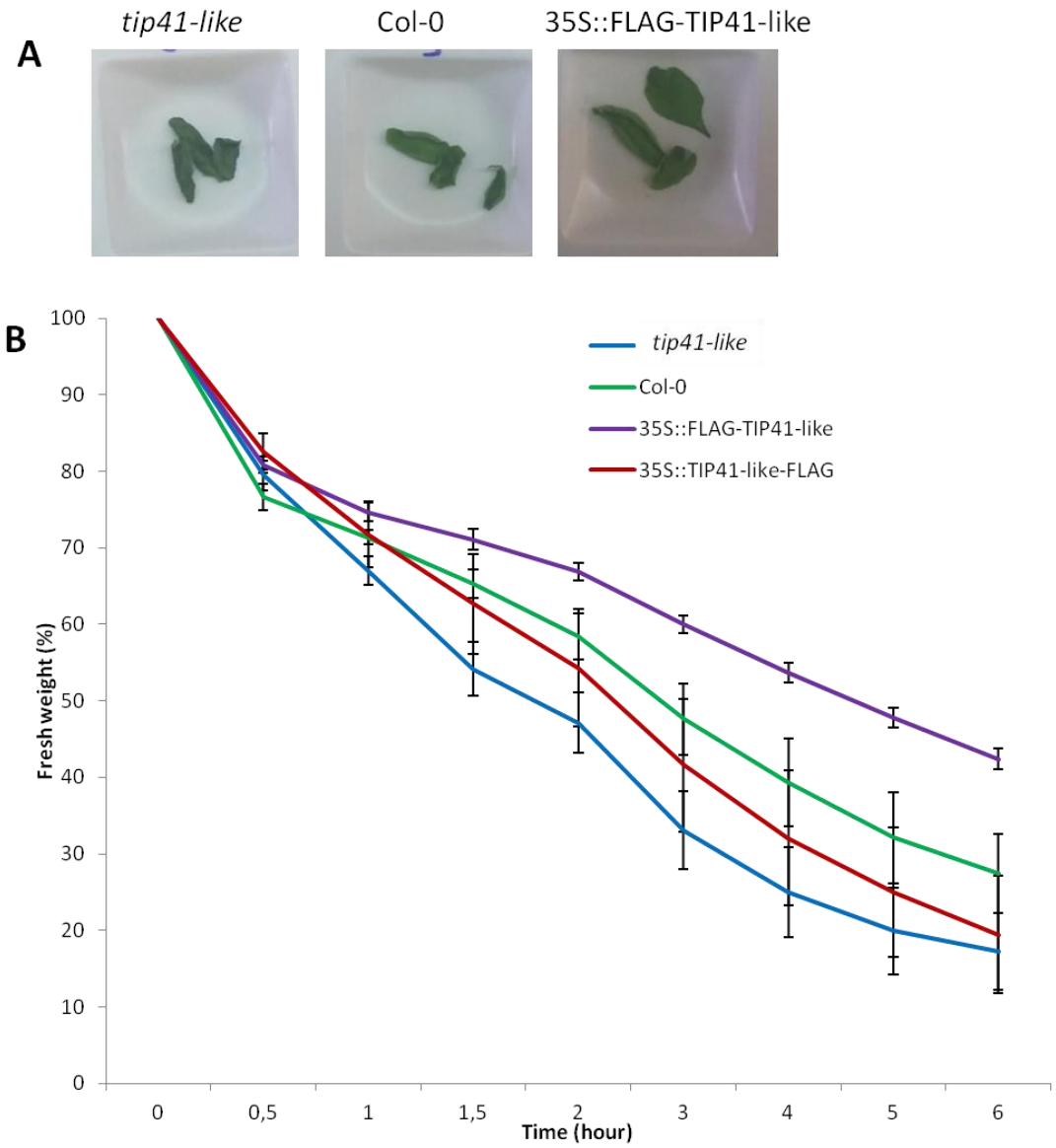

Figure 31. Water loss of leaves detached from *tip41-like* mutants, TIP41-like overexpressing plants and Col-0. A) Leaves of the three genotypes at the end of experiment. B) Data represent percentages of initial weight lost at different time points. Error bars indicate standard deviation of three biological replicates.

2.3.12 The putative subunit of RNA POL III, SIN-like: bioinformatic analysis

The potato protein encoded by the locus LOC102594031 was aligned with the Arabidopsis SIN-like protein (locus *At5g49530*). As shown in Figure 32, the two proteins share 42% sequence identity.

The Arabidopsis SIN-like protein was analyzed using the bioinformatic tool InterPro in order to predict the presence of domains and important sites. SIN-like was identified as a specific peripheric component of RNA polymerase III complex (IPR006886) due to the presence of the Sin_N conserved region (aa 114-483; PF04801). All the proteins identified as putative SIN-like orthologs in other species also contain the conserved region Sin_N (PF04801). The BLASTP algorithm was

used to assign sequence identity percentage. In particular, the Arabidopsis protein shares 73% of sequence identity with a *Brassica rapa* protein, 44% with *Vitis vinifera* and 41% with a *Solanum lycopersicum* protein (data not shown).

Figure 32. Sequence alignment of the Arabidopsis and potato SIN-like proteins. Alignment was generated using MUSCLE (3.8). An "*" (asterisk) indicates positions which have a conserved residue; A ":" (colon) indicates residues with strongly similar properties. A "." (period) indicates residues with weakly similar properties. Bottom: Alignment score.

2.3.13 SIN-like is expressed in stomata and slightly induced by osmotic stress

In silico analysis of expression based on publically available microarray data indicates that *SIN-like* is highly expressed in the shoot apex at flowering stage. In particular, in open flowers, transcript levels are high in carpels, but decrease in the outer floral organs, such as sepals, petals, and stamens. *SIN-like* is also expressed at a high level in dry seeds, with a slight decrease when imbibed, and in senescing leaves (Figure 33).

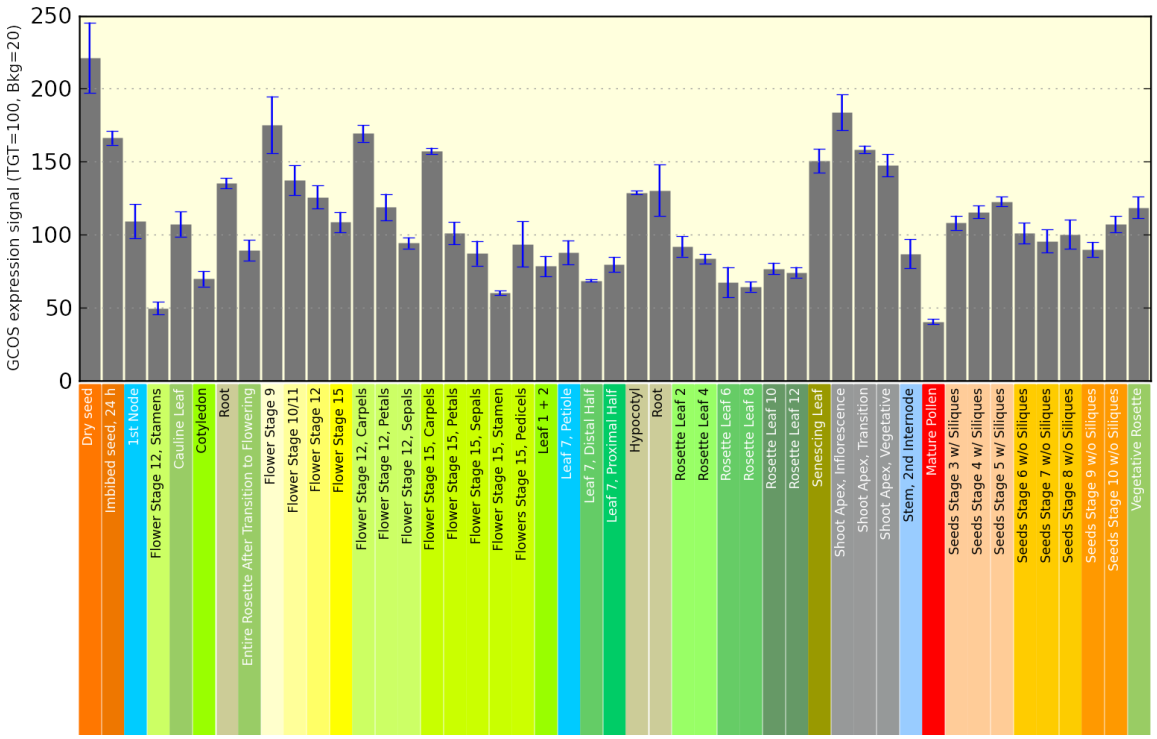

Figure 33. Expression levels of *SIN-like* based on Arabidopsis microarray data displayed in the eFP browser (according to Schmid et al. 2005). Data are normalized by the GCOS method, TGT value of 100.

To investigate the promoter activity of *SIN-like*, transgenic Arabidopsis plants expressing the gene encoding a β-glucuronidase (GUS) reporter enzyme driven by the *SIN-like* promoter were generated. Transgenic T_2 plants were stained using X-Gluc. The expression of the GUS gene driven by the promoter of *SIN-like* was observed in all the analysed tissue of seedlings, especially in the thick inner wall of guard cells. Same patterns of activity of the *SIN-like* promoter were observed in adult plants (Figure 34).

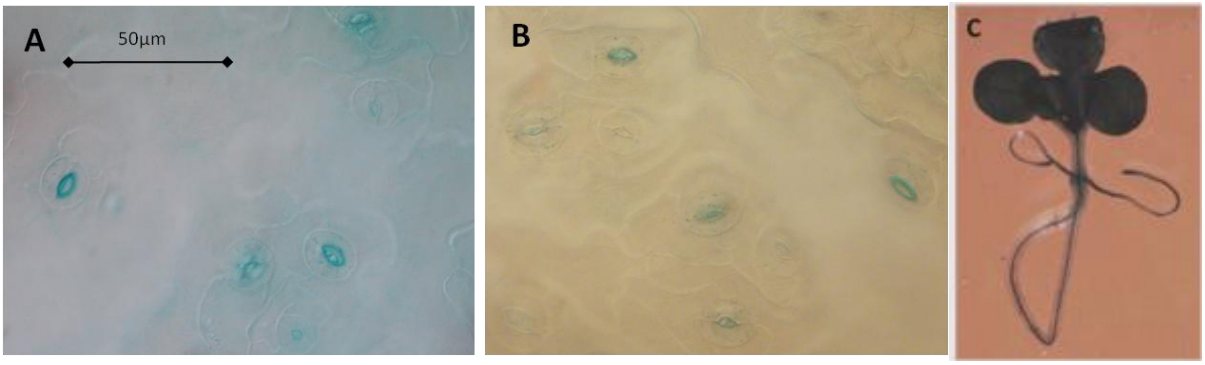

Figure 34. Histochemical localization of GUS activity in transgenic Arabidopsis plants expressing the GUS reporter gene under the control of the *SIN-like* promoter. Leaves of adult plants (A) and 10-day old seedlings (B) and whole seedling (C) were stained.

To investigate whether *SIN-like* was also induced by osmotic stress in Arabidopsis, the regulation of gene expression in seedlings during long-term exposure to different stress treatments was evaluated. *SIN-like* expression levels under salt stress were similar to the control, while ABA and PEG treatments induced a slight regulation of gene expression after 5 days (Figure 35). Overall no significant regulation of gene expression caused by stress was observed.

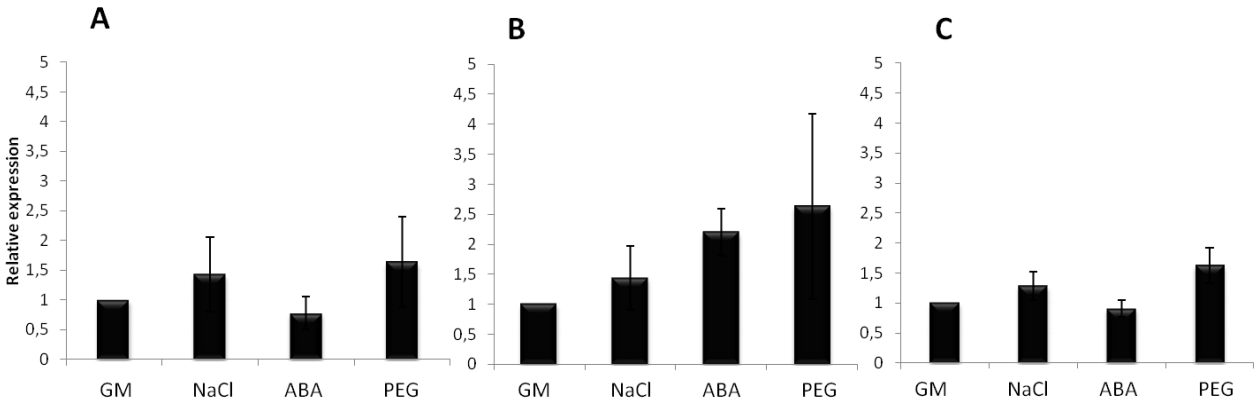

Figure 35. Gene expression of *SIN-like* measured by qRT-PCR in 7-day-old seedlings of *A. thaliana* after 2 days (A), 5 days (B) and 7 days (C) of exposure to PEG (35% w/v), NaCl (120mM) or ABA (10 µM). Elongation Factor *EF1α* was used as reference gene and data were normalized using RNA from untreated seedlings. Error bars indicate standard deviation of three biological replicates.

2.3.14 Nuclear localization of SIN-like protein

Transgenic plants overexpressing a YFP SIN-like fusion protein were generated. The coding sequence of *SIN-like* was cloned into pDONR207 vector and recombined by LR reaction into the pEG101 destination vector, in order to produce a C-terminal fusion protein (SIN-like-YFP); and pEG104 vector to obtain a N-terminal fusion protein (YFP-SIN-like). Stable transgenic lines were selected on BASTA containing media and 5-day old seedlings of three transgenic T_2 lines were analyzed, using confocal microscopy. The intensity of the YFP signal was higher using N-terminal

fusion YFP-SIN-like. Interestingly, the fusion protein was localized mainly in the nucleus, with lower cytoplasmic diffusion in the quiescent center (QC) (Figure 36). The position of the nucleus was confirmed by nucleus-specific staining with 4′,6-Diamidino-2-phenylindole (DAPI) and merging of the YFP and DAPI fluorescence images (Figure 37).

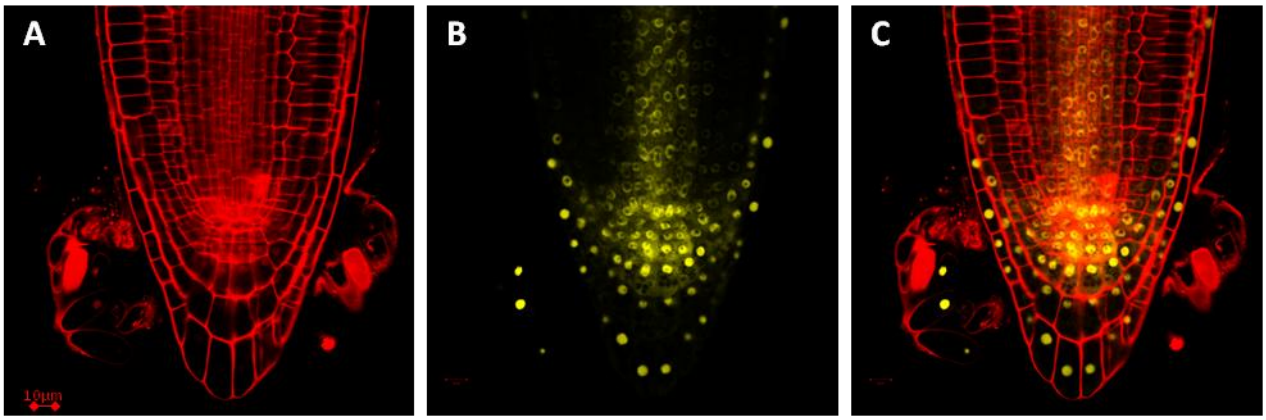

Figure 36. Subcellular localization of SIN-like protein in root apex of 5-day old Arabidopsis seedlings. Confocal microscopy visualization for transgenic Arabidopsis plants expressing a YFP-SIN-like fusion protein. Propidium iodide staining (A), YFP fluorescence (B), and merged images (C) are shown. Scale bar: 10µm

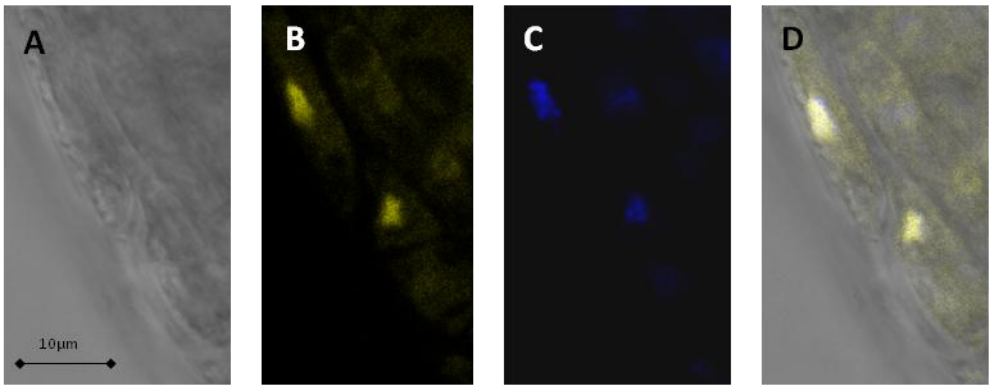

Figure 37. SIN-like protein localization. Confocal microscopy visualization of 5-day-old Arabidopsis root (A). YFP fluorescence from YFP-SIN-like fusion protein (B; yellow) and nuclear stain DAPI (C; blue) and merge images (D). Scale bar: 10µm

2.3.15 Generation and phenotyping of SIN-like overexpressing plants

Large-scale phenotype screening showed that Arabidopsis *sin-like* mutants display severe reduction of root growth (Paragraph 2.3.3). To gain insights into the role of *SIN-like* in root growth, a gain-of function strategy was adopted. Transgenic Arabidopsis plants carrying the coding sequence of *SIN-like* under the control of the strong constitutive promoter CaMV 35S were generated in fusion (both N- and C-terminal) with FLAG tag as previously described (Paragraph 2.3.7). T_2 seeds of five

35S::SIN-like-FLAG lines and twelve 35S::FLAG-SIN-like lines were used for segregation analysis. Three lines per construct had a segregation ratio equal to 3:1, indicating that one single T-DNA insertion had occurred. T_3 seeds from five individuals per construct were plated on kanamycin and four homozygous lines were selected. A higher expression of the transgene was observed in the line #1 of 35S::SIN-like-FLAG by western blot analysis (Figure 38), which, therefore, was used for further analysis.

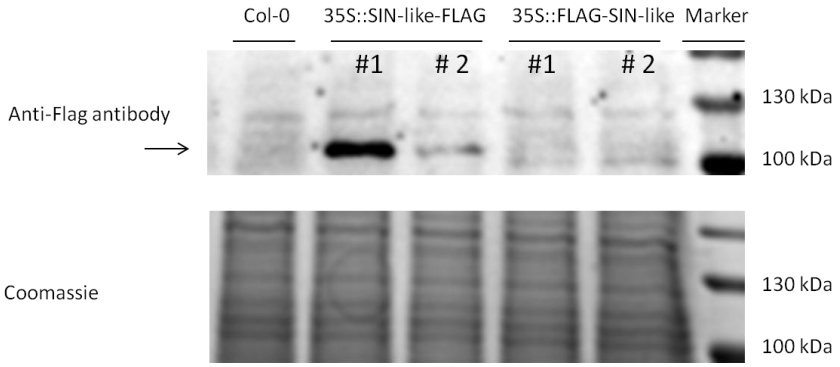

Figure 38. Screening by western blot analysis of SIN-like overexpressing lines. The SDS-polyacrylamide gel was stained with Coomassie and western blot was performed with anti-FLAG antibodies. Col-0 was used as a negative control. Arrow indicates SIN-like-FLAG protein. The 35S::SIN-like-FLAG line #1 was selected for further analyses. Marker: Page Ruler Protein Ladder

SIN-like overexpressing plants and knockout mutants were compared to wild-type Col-0, measuring the root length of 14-day old seedlings. As shown in Figure 39, *sin-like* showed a significant reduction (72%) in root growth compared to wild type, while the overexpressing line did not show any significant difference, suggesting that an increased expression of *SIN-like* does not alter root development.

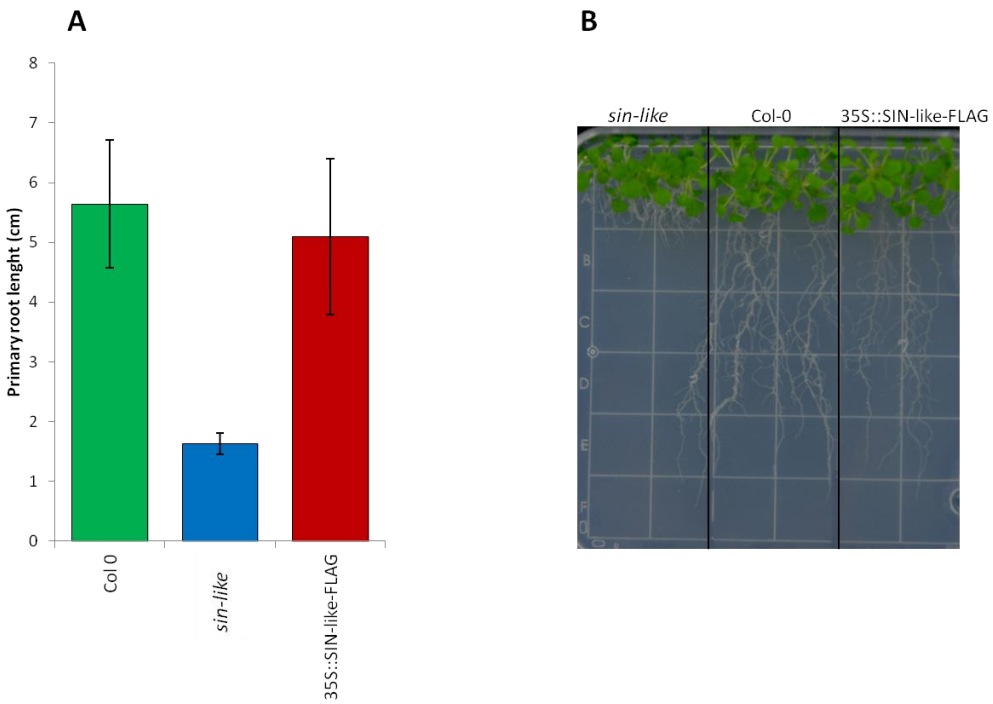

Figure 39. Quantification of primary root length of SIN-like knockout mutants and transgenic plants compared with wild-type (Col-0). A) Root length measurement of 14-day old seedlings grown on GM. Error bars indicate standard deviation of three biological replicates. B) Photograph of 14-day old seedlings on GM.

Among the differentially expressed genes in *S.tuberosum* adapted cells, the Arabidopsis ortholog *AtRGGA*, encoding a glycine-rich RNA-binding protein, was previously partially characterized in responses to drought and salt stress at CNR-IBBR. Here we analyze the protein role in the RNA regulatory mechanisms.

2.3.16 AtRGGA is up-regulated by long-term exposure to ABA and PEG

To investigate whether *AtRGGA* is induced by stress treatments in Arabidopsis, gene expression in seedlings exposed to NaCl and PEG stress was analyzed. ABA treatments were also included to assess a possible involvement of the hormone in the regulation of *AtRGGA* transcript abundance. In seedlings, 24-h treatments with different concentrations of NaCl caused a slight down-regulation of *AtRGGA* expression, while a 2d exposure to ABA and PEG induced an up-regulation (Figure 40), indicating that *AtRGGA* transcript abundance is reduced by salt stress in the short term but increased over longer periods of exposure to ABA and osmotic stress.

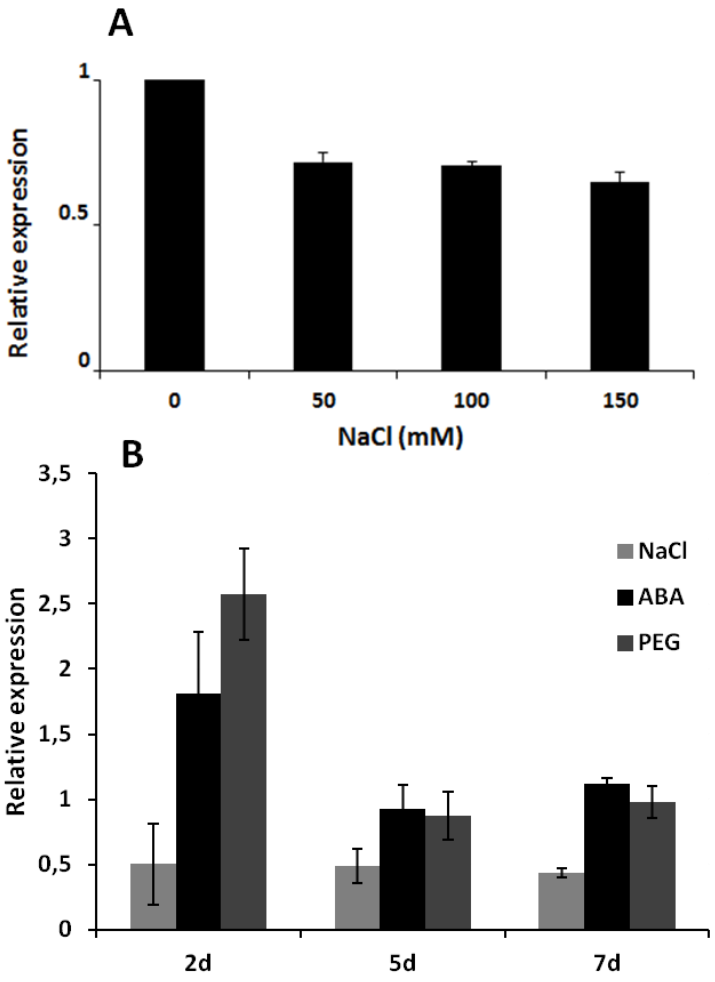

Figure 40. Gene expression of *AtRGGA* measured by qRT-PCR in 12-d-old seedlings of *A. thaliana* treated for 24h with NaCl (0, 50, 100, 150mM) (A) and after 2, 5 and 7 days of exposure to PEG (35% w/v), NaCl (120mM) or ABA (10 µM) (B). Elongation Factor *EF1α* was used as reference gene and data were normalized using RNA from untreated seedlings. Error bars indicate standard deviation of three biological replicates.

2.3.17 The modified expression of AtRGGA has an impact on stress tolerance

To characterize the role of *AtRGGA* in plant responses to salt and drought stress, a T-DNA insertion mutant in which *AtRGGA* gene expression is abolished (SALK_143514, *rgga*) and transgenic plants over-expressing a FLAG-RGGA fusion protein, already available in the CNR-IBBR laboratory, were used. To assess a role of RGGA in ABA-dependent mechanisms of response to environmental stresses, root growth and survival tests in presence of ABA were performed. Root growth experiments on plates showed a hypersensitivity of *rgga* to the presence of ABA in the media, while over-expressing plants did not display significant differences as compared to Col-0 (Figure 41). In terms of survival to ABA exposure, 10-day of treatment highlighted significant differences between genotypes. 35S::FLAG-RGGA plants showed a higher ability to tolerate the presence of 50 µM ABA in the media, with 60% of plants still surviving after 10-day of exposure to the hormone, while the mutant only had about 20% of the individuals still surviving and wild-type had about 47% (Figure 42). These experiments, together with additional ones described in Ambrosone et al (2015) show that *AtRGGA* contributes to molecular mechanisms resulting in stress tolerance in *Arabidopsis.*

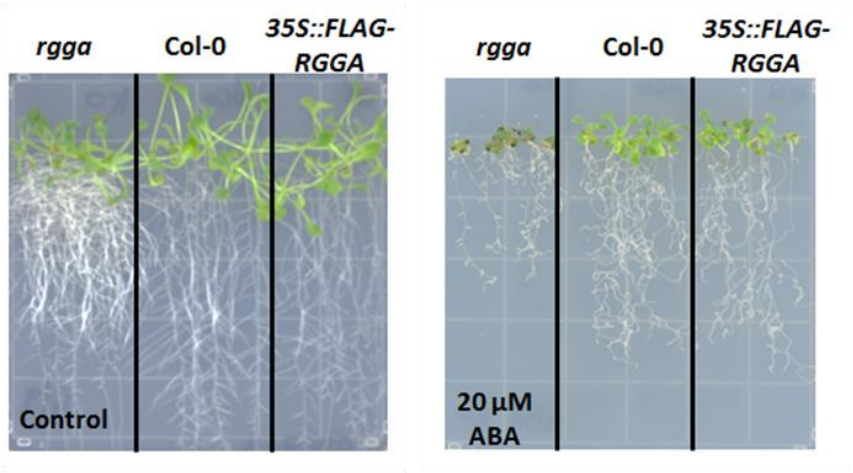

Figure 41. Phenotypes of *AtRGGA* knockout and overexpressing plants compared to wild-type Col-0. 14-day old seedlings germinated for 4 days on GM and transferred to control GM medium or medium containing 20µM ABA.

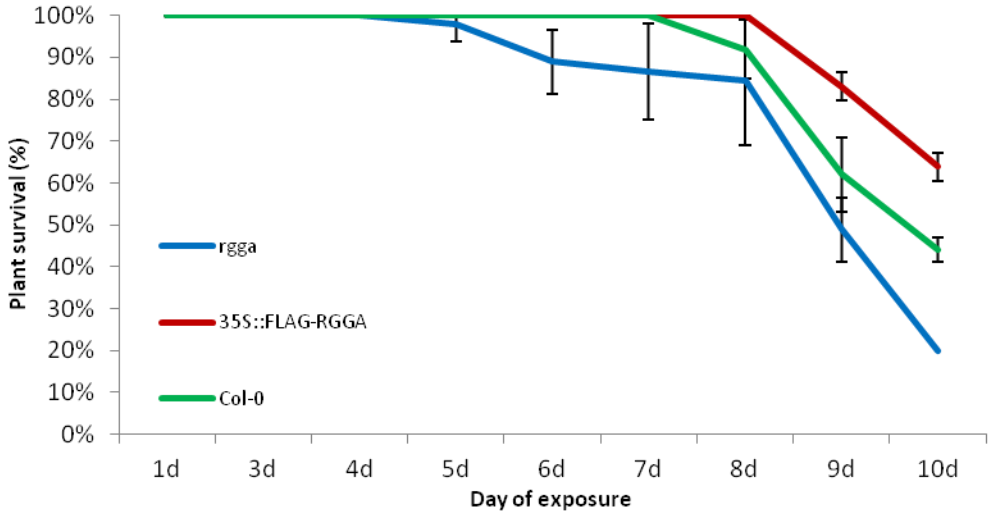

Figure 42. Survival test of *AtRGGA* knockout and overexpressing plants compared to wild-type Col-0. 18-d-old seedlings germinated on GM and transferred to ABA (50 µM) medium. Survival was scored daily in terms of absence of necrotic or bleached leaves. Error bars indicate standard deviation of three biological replicates

2.3.18 AtRGGA binds RNA *in vitro*

AtRGGA is annotated as a putative RNA-binding protein. To verify the assigned function, an RNA ElectroMobility Shift Assay (EMSA) was performed using recombinant, His-tagged AtRGGA (His-RGGA) and total RNA. RNA was labelled with biotin and incubated with or without His-RGGA prior to electrophoresis in native conditions. A recombinant version of the PYR1 ABA receptor, His-PYR1, was used as a negative control. As shown in Figure 43, an RNA mobility shift was specifically observed when RNA was incubated with AtRGGA, indicating that AtRGGA was capable of binding RNA, and the binding was competed by adding an excess of unlabeled RNA, thus showing that AtRGGA binds to RNA *in vitro*. The delayed fragment was observed in the case of RNA extracted from both control and stress treated plants, indicating that the RNA(s) recognized by AtRGGA is/are expressed in both conditions (Figure 43A; lanes 3,6).

To assess the specificity of the binding, poly(A^+) and poly(A^-) RNA fractions were used for RNA EMSA. An Oligo dT-bound resin was used to isolate the mature mRNA [poly(A^+)] from total RNA extracted from Arabidopsis seedlings. The unbound non-adenylated RNA species were used as poly(A^-) fraction.

A band shift after incubation with His-RGGA was observed when poly(A^-) RNA was used, indicating that AtRGGA binds to one or more RNAs contained in the poly(A^-) RNA fraction (Figure 43B).

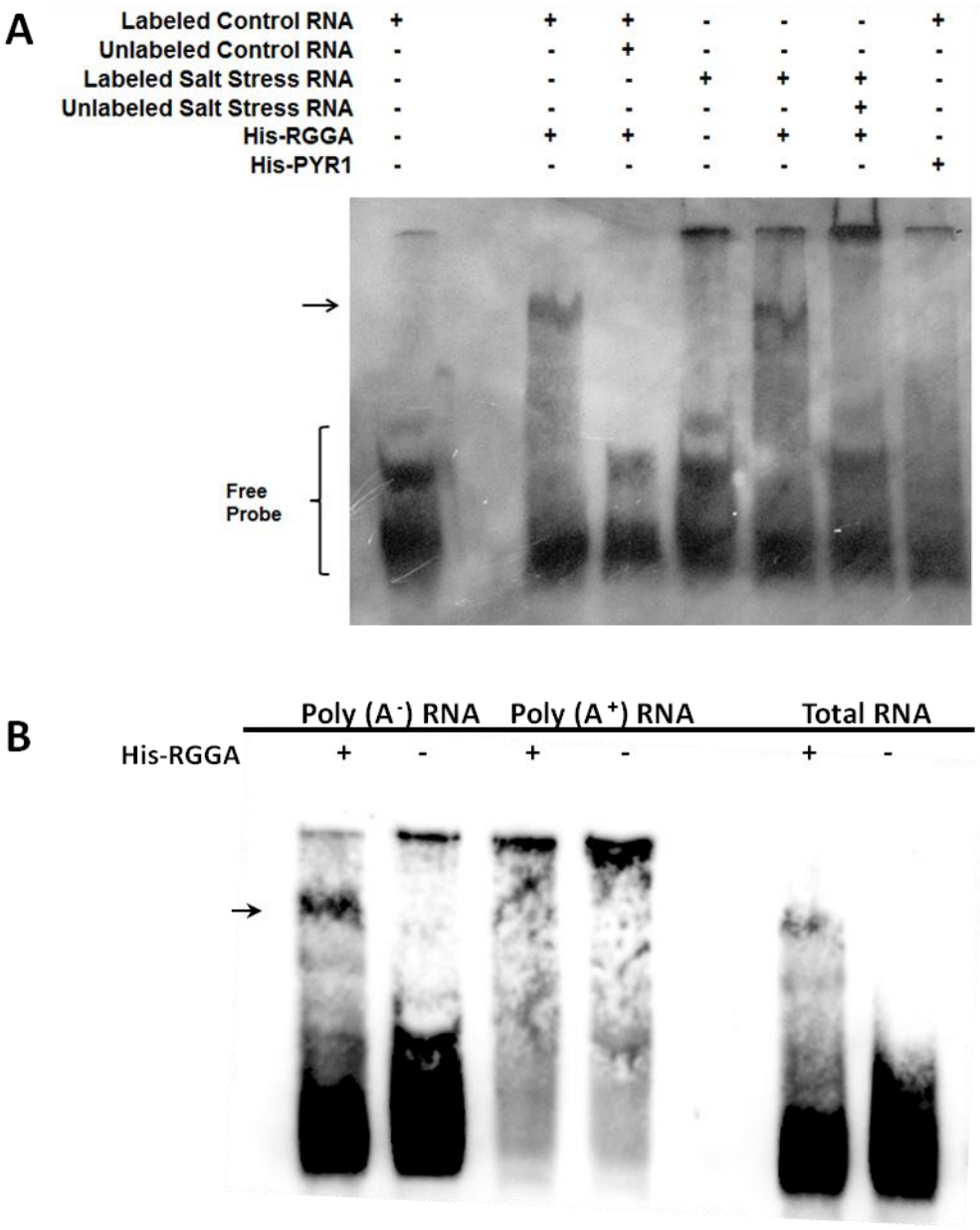

Figure 43. A) EMSA of Arabidopsis RNA incubated with recombinant AtRGGA (His-RGGA). RNA was extracted from NaCl-treated (Salt Stress RNA) or untreated (Control RNA) plants and labeled with biotin. Unlabeled RNA (160-fold) was used as a competitor. Recombinant PYR1 (His-PYR1) served as a negative control. B) EMSA of Arabidopsis total, poly(A+), and poly (A-) RNA incubated with or without recombinant AtRGGA (His-RGGA). The brackets indicate labeled RNA, and the arrows indicate RGGA-bound RNA

Since ribosomal RNAs (rRNAs) are the most abundant non-adenylated RNAs, and because they have a major role in the post-transcriptional regulation of expression, we considered them obvious candidates for the binding to RGGA. To test each cytoplasmic rRNA, sequences encoding 5S, 5.8S, 18S and 25S were cloned in pGEM T-easy and *in vitro* transcribed (Figure 44). Each rRNA was then used for EMSA with recombinant AtRGGA. As shown in Figure 45, a band shift in the presence of AtRGGA was observed for 5S and 5.8S rRNAs and not in the case of 18S and 25S rRNAs.

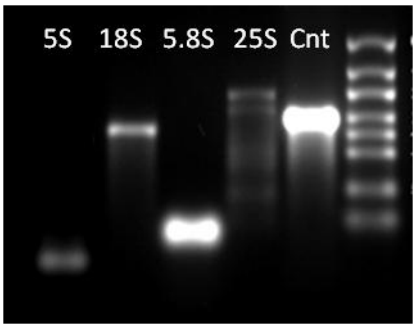

Figure 44. Electrophoresis profile of *in-vitro* transcripted Arabidopsis ribosomal RNAs (5S; 5.8S; 18S and 25S) on formaldehyde-agarose (1%) gel. Control template (Cnt) coding for of 2.2kb RNA transcript was used as positive control. Right lane shows RNA ladder

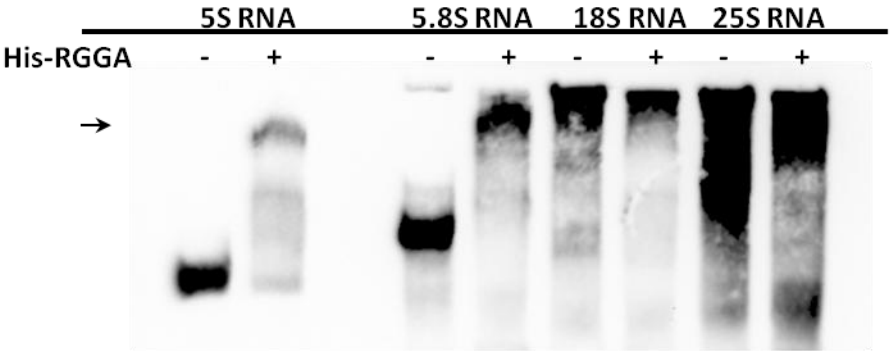

Figure 45. Electromobility shift assay (EMSA) of Arabidopsis *in-vitro* transcribed 5S, 5.8S, 18S and 25S RNA incubated without or with recombinant AtRGGA (His-RGGA). The arrow indicates RGGA-bound RNA.

2.3.19 AtRGGA binds RNA *in vivo*

In order to verify that AtRGGA binds RNA *in vivo*, RNA immunoprecipitation (RIP) was performed during the visit to the Laboratories of Prof. Julia Bailey-Serres (University of California, Riverside, USA). The RIP requires the use of a specific antibody bound to beads to pull-down protein-RNA complexes (detailed description in Materials and Methods section 2.2.11). Therefore, we used transgenic plants transformed with a 35S::FLAG-RGGA vector and confirmed the expression of the FLAG-RGGA fusion protein in different generations of Arabidopsis. As shown in Figure 46, all tested plants expressed the fusion protein, however, a higher expression of the transgene was observed in plants of the T_3 generation, which were therefore used for subsequent experiments.

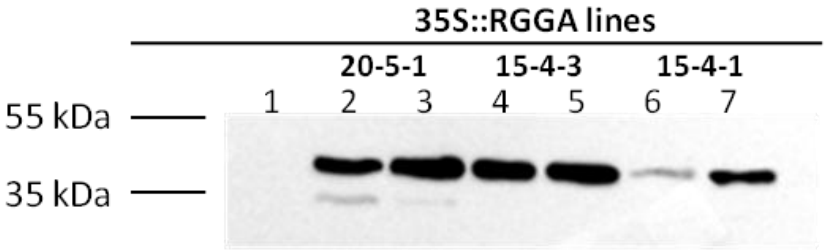

Figure 46. Western blot analysis of FLAG-RGGA expression in three lines of transgenic Arabidopsis plants (35S::RGGA) using anti-FLAG antibody. T_3 generation plants (lane 3, 5 and 7) were compared to T_4 generation plants (lane 2, 4 and 6). Col-0 (lane 1) was used as a negative control.

To investigate if the ability of AtRGGA to bind RNAs is dependent on stress condition, RIP was performed using extracts from 10-day old seedlings grown in control condition or exposed to salt stress (120mM NaCl) (RGGA FLAG RIP). A RIP of the same tissue samples with a nonspecific antibody (anti-HA) was performed to evaluate background RNA association with the IP matrix (RGGA HA RIP). Col-0 plants, lacking the antigen, served as additional negative control (Col-0 FLAG RIP). After RIP, western blot analysis using anti-FLAG antibody confirmed the presence of FLAG-RGGA in the RGGA FLAG RIP immunopurified fraction (Figure 47).

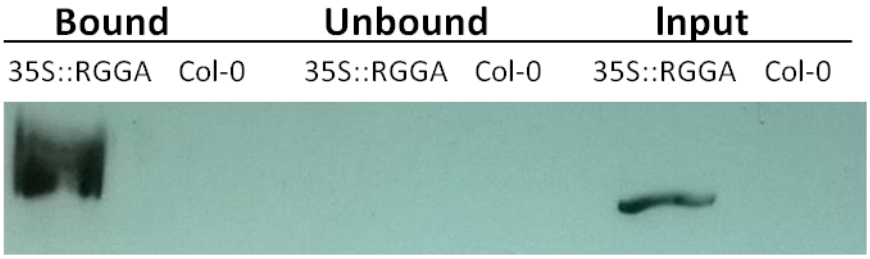

Figure 47. Western blot analysis after immunoprecipitation (IP) of RGGA protein from transgenic Arabidopsis plants (35S::RGGA). The Input extract was compared to the post-IP unbound extract, and the IP sample to check the efficiency of the IP. Col-0 was used as negative control.

The different RIP fractions were run on Bioanalyzer pico-chips together with total RNA for comparison. Presence of RNA in the RGGA FLAG RIP fraction but not in the Col-0 FLAG RIP or in the RGGA HA RIP indicated that RGGA binds to RNA *in vivo* (Figure 48). The 5S, 5,8S rRNAs were detected in the RGGA immunoprecipitate. 18S and 25S RNAs were also identified, indicating that RGGA is capable to bind ribosomal RNAs *in vivo*. No obvious difference was observed between control and stress RNA profiles. Taken together, the results demonstrate that AtRGGA is capable of efficiently binding RNA *in vitro* and *in vivo*, both in control and during salt stress conditions.

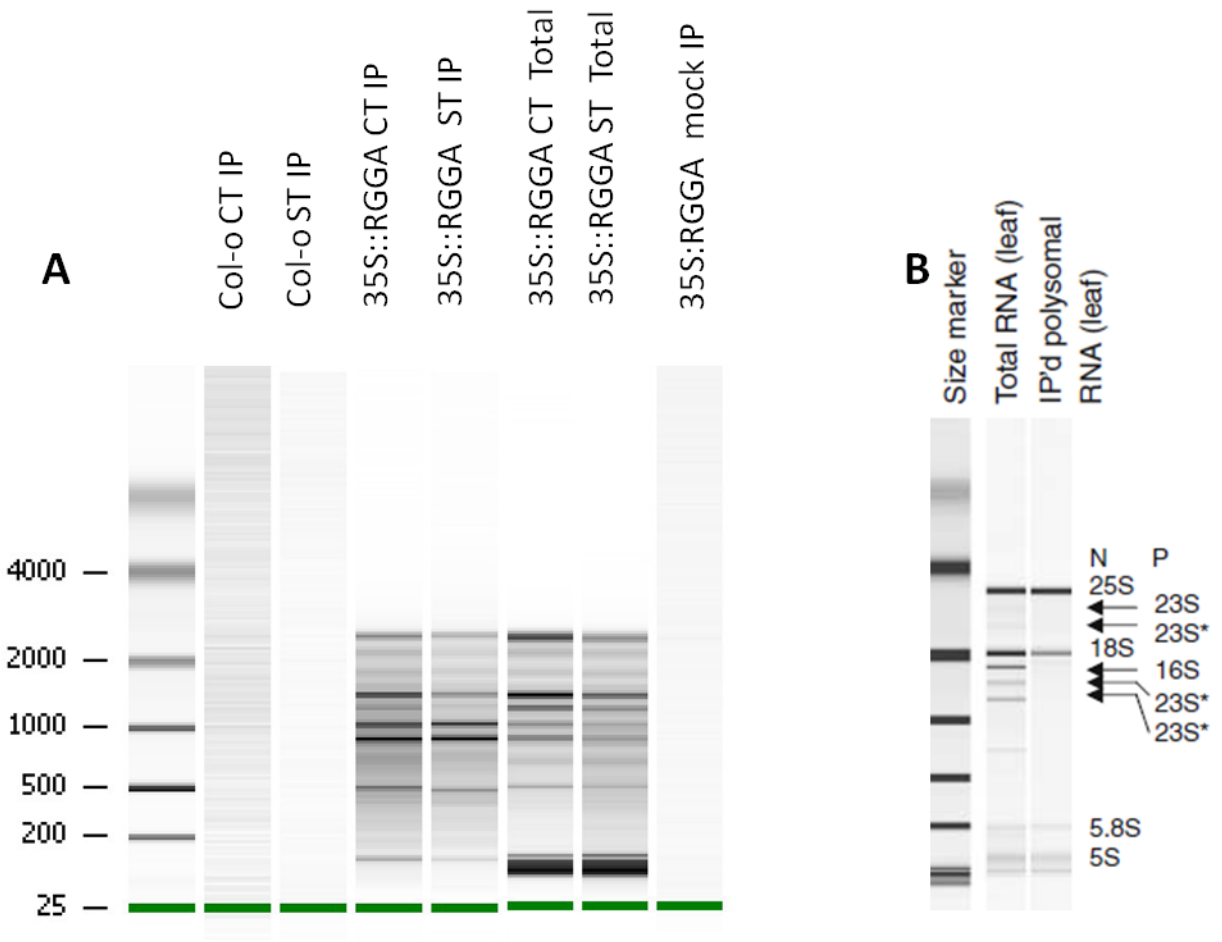

Figure 48. Bioanalyzer RNA profile from Total RNA (Total), RNA from mock IP using a nonspecific antibody (mock IP), and RNA extracted from RNP complexes eluted following RIP for RGGA protein (IP) using anti-flag antibody. Tissue of Columbia (Col-0), lacking the antigen, was also immunopurified as negative control. RNAs were extracted from leaves of transgenic Arabidopsis plants (35S::RGGA) after 48h of exposition to 120mM NaCl (ST) compared to control condition (CT). B) Example Bioanalyzer RNA profile. RNA N, nuclear rRNAs; P, plastid rRNAs (23S, 16S) and their degradation products (23S*).

2.3.20 Identification of interacting partners of AtRGGA

To identify putative AtRGGA interacting proteins, a yeast two-hybrid (Y2H) screening was performed. To produce the bait, the coding sequence of *RGGA* was cloned in vector pGBKT7 downstream of the GAL4 DNA-binding domain. An Arabidopsis cDNA library constructed in pGADT7 vector, such that the proteins encoded by the inserts were fused to the 3' end of the GAL4 activation-domain, was used as a prey. *Saccharomyces cerevisiae* strain GOLD harbouring four reporter genes (HIS3, ADE2, MEL1, and AUR1-C) that are activated in response to bait-prey interaction was used for sequential transformation, introducing first the bait plasmid containing AtRGGA. Large-scale transformation was performed to introduce the prey library in cells containing the bait plasmid. The transformed cells were plated on selective medium lacking the aminoacids Tryptophan and Leucine for selection of clones containing the two plasmids, and

Histidine, Adenine for the selection of colonies in which an interaction had putatively taken place. In order to obtain high transformation efficiency (≥1 x 10^6 independent clones, putatively saturating the cDNA library) two independent transformation reactions were carried out, with ~ 2×10^5 and ~9×10^6 transformants respectively. 6892 positive clones able to grow on selection media were obtained. These were streaked on medium containing Aureobasidin A and aminoacids supplements lacking Tryptophan, Leucine, Histidine and Adenine, where the growth could be considered as a confirmation of the interaction between the two recombinant proteins. 356 positive clones showing the fastest growth were selected. Colony PCRs on 356 positive clones were performed using vector specific primers to check the insert size in the prey vector, in order to identify groups of clones which may contain the same insert (Figure 49).

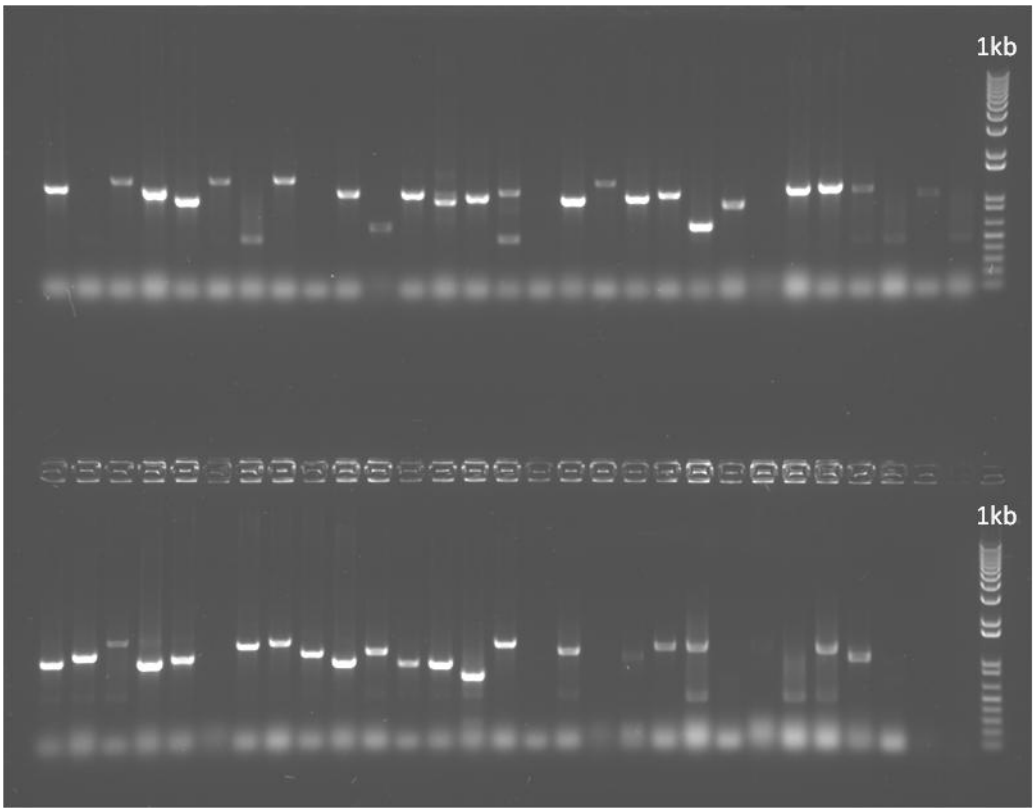

Figure 49. Yeast colony PCR on 58 of 356 colonies. Determination of positive genes size using electrophoresis of PCR products. Lane 30 and 60: 1kb plus DNA ladder

The prey plasmids from these colonies were recovered and transformed into *E. coli* for amplification. They were then reintroduced into GOLD, together with AtRGGA or with the empty vector pGBKT7 to remove false positives able to activate the transcription of reporter genes in the absence of an interacting partner. Using this strategy, we could identify six putative AtRGGA interactors able to confer in yeast the ability to grow on selective media when co-transformed with

RGGA pGBKT7 and not with empty pGBKT7 (Figure 50). Two of six encode RNA-binding proteins, RANBP1 and a member of the Arabidopsis Pumilio family of proteins, APUM24. The last four clones identified encode a translation elongation factor with SPOC domain, a small ubiquitin-like modifier (SUMO) protein, SUMO5, ACP1 and ZCF12, an acyl-carrier and a kinesin-like protein respectively (Table 11).

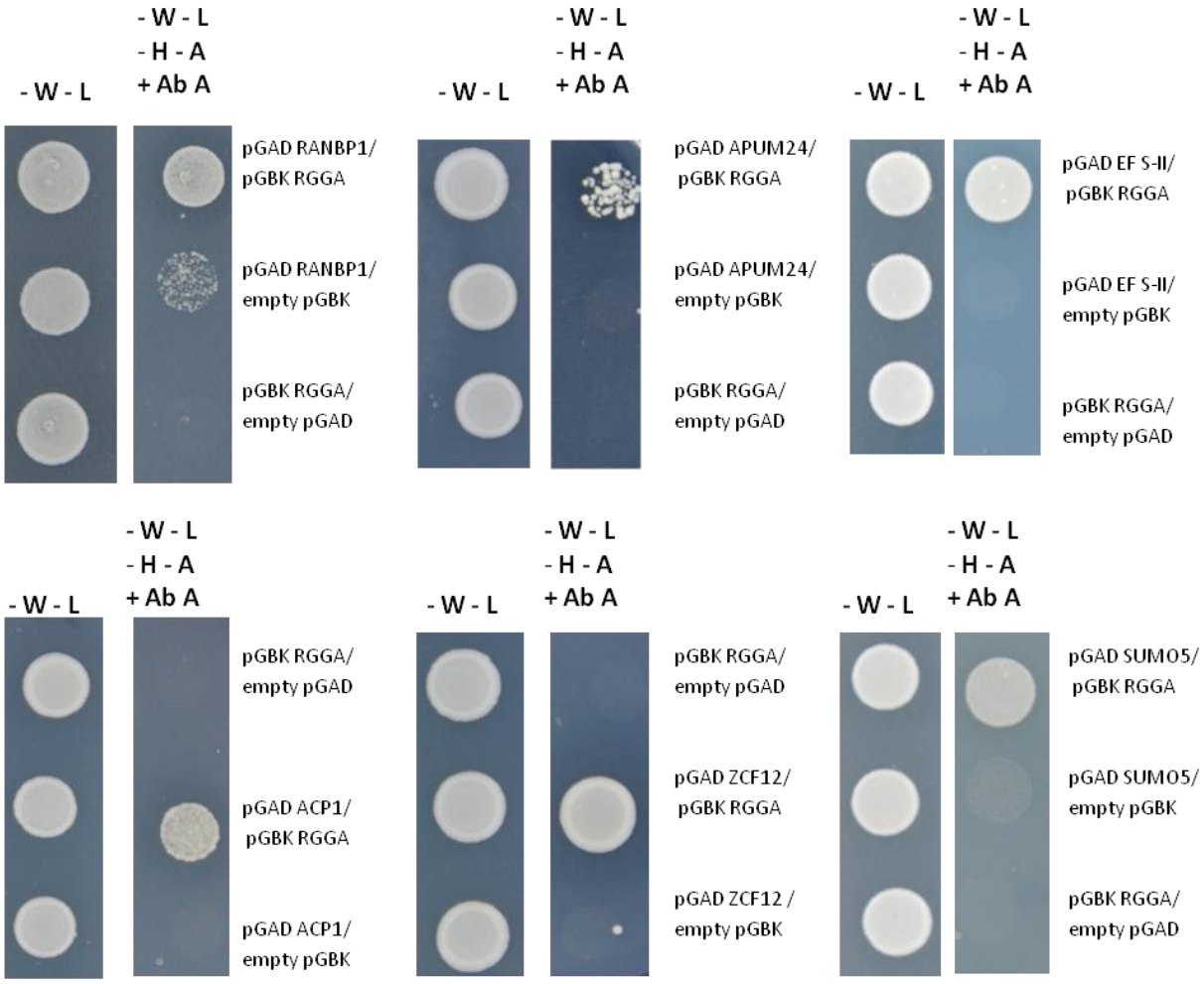

Figure 50. Yeast two-hybrid assay between AtRGGA and candidate interactors. An equal amount of cells was dropped onto selective media to demonstrate the stringency of binding between proteins. Colonies growing on SD/-W,-L were successfully transformed with both vectors. Growth on SD/-W,-L,-H,-A,+Ab A is a sign of positive interactions. The empty vector pGADT7 and pGBKT7 were used as negative controls.

Table 11. Identified protein partners of RGGA by Yeast Two Hybrid assay

Name	Locus	Description	Gene Onthology
RANBP1	AT5G58590	Ran-binding protein 1 homolog	mRNA transport, protein import into nucleus, translocation
APUM24	AT3G16810	Arabidopsis Pumilio (APUM) proteins containing PUF domain	RNA binding. PUF proteins regulate both mRNA stability and translation through sequence-specific binding to the 3' UTR of target mRNA transcripts
ACP1	AT3G05020	acyl carrier protein expressed in leaves, roots, and dry seeds	ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process
ZCF125	AT1G59540	kinesin-like protein	ATP binding, microtubule motor activity
EF S-II	AT5G25520	SPOC domain / Transcription elongation factor S-II protein	Transcription, DNA-templated, translational elongagtion
SUMO5	AT2G32765	Small ubiquitin-like modifier (SUMO) protein	Protein sumoylation

2.4 Discussion

2.4.1 DRT111 participates in stress-induced inhibition of seed germination

Although the physiological role of the phytohormone abscisic acid (ABA) in the maintenance of seed dormancy is well characterized, some of the molecular players involved in the signalling and implementation of these processes remain to be identified. In this study, we have provided evidence that DRT111 is involved in the ABA signaling pathway during seed germination.

The Arabidopsis *DRT111* is the ortholog of the potato LOC102603413 gene (Figure 14) whose expression was observed to be differentially regulated in *Solanum tuberosum* culture cells during gradual exposure to increasing concentrations of polyethylene glycol (PEG) (Leone et al, 1994; Ambrosone et al., 2011). In Arabidopsis, DRT111 (DNA-DAMAGE-REPAIR/TOLERATION PROTEIN 111), also known as RSN2 (REQUIRED FOR SNC4-1D 2) owes its name to an early paper showing that the expression of DRT111 in *E. coli* was able to partially rescue the DNA-damage resistance to mutants lacking recombination-intermediate-resolution activities (Pang et al., 1993). Here, we have shown that the Arabidopsis *DRT111* is up-regulated upon long-term exposure to ABA and PEG (Figure 18) and is expressed in specific tissues that respond to osmotic stress conditions, such as thricomes and stomata (Figure 17), suggesting the DRT111 role in stress adaptation. Trichomes positively influence the water retention in leaves through the increased reflection of solar radiation, reducing the leaf temperature and, consequently, the transpiration rate (Gianoli and Gonzalez-Teuber, 2005). Therefore, trichome density together with stomata closure have proved to be an important mechanism to minimize water loss (Gianoli and Gonza´lez-Teuber, 2005; Skirycz et al., 2010).

Consistent with *DRT111* expression in guard cells, knockout mutants show slower water loss rate in detached leaf assays as compared with wild-type plants (Figure 22). It is well known that water loss is mainly dependent on stomatal movements, a process mediated by ABA (Verslues et al., 2006). The lower transpiration rate shown by *drt111* is indicative of a defect in ABA biosynthesis or, more likely, ABA sensitivity.

In accordance with the water loss results, a significant insensitivity to ABA of *drt111* is also observed at the seed germination stage (Figure 21). The loss-of-function mutants are better able to withstand ABA treatments, showing a higher germination rate compared to wild type in the presence of the hormone. Conversely, overexpression of *DRT111* in Arabidopsis resulted in plants hypersensitive to ABA during germination. In Arabidopsis, at this stage ABA controls seed maturation and dormancy by inhibiting germination and reserve mobilization. ABA signaling in germinating seeds is associated with the expression of many regulatory genes, such as LEC1, LEC2

(LEAFY COTYLEDON1 and 2), FUS3 (FUSCA3), and ABIs (ABSCISIC ACID INSENSITIVE proteins) (Kroj et al., 2003; To et al., 2006). The two protein phosphatases 2C (PP2Cs) ABI1 and ABI2 are negative regulators in the ABA signalling pathway during seed germination, seedling growth and stomatal closure (Downton et al., 1988;Ma et al., 2009; Park et al., 2009). ABI3, ABI4 and ABI5 encode three different transcription factors of the B3, AP2 and bZIP domain families, respectively and regulate ABA-inducible gene expression in which results in seed germination inhibition (Finkelstein and Lynch, 2000).

Among these important transcription factors, ABI3 has been considered the major regulator of seed maturation. The abolished expression of ABI3 reduces both seed dormancy and thermoinhibition of germination (Tamura et al., 2006). In Arabidopsis, ABI3 is repressed by the chromatin-remodeling factor PICKLE (Perruc et al., 2007). In addition, alternative splicing also has an important role in controlling gene expression. Sugliani et al. (2010) detected two ABI3 transcripts, ABI3-α and ABI3-β, which encode full-length and truncated proteins, respectively. ABI3-α carries a cryptic intron, which is spliced out in ABI3-β transcript. Alternative splicing of ABI3 is regulated during development with an ABI3-β accumulation at the end of seed maturation.

An homologous of the human protein RBM5, SUA (SUPPRESSOR OF ABI3-5), reduces splicing of the cryptic ABI3 intron, leading to a decrease in ABI3-β and to a fast up-regulation of active ABI3-α in ripe seeds, which is necessary to inhibit the seed maturation during germination. In addition to its function in the alternative splicing of ABI3, SUA was also shown to be required for the control of alternative splicing of SNC4 and CERK1 (CHITIN ELICITOR RECEPTOR KINASE1), (Zhang et al., 2014), involved in the bacterial infections resistance (Bi et al., 2010; Gimenez-Ibanez et al., 2009). Interestingly, analysis of suppressor mutants of *snc4-1D* identified *DRT111* as another conserved splicing factor, which is required for the constitutive defence responses in snc4-1D together with *SUA*. In *sua* and *drt111* mutants, *SNC4* splicing is altered leading to reduction of *SNC4* transcripts. Further analysis provided evidence that SUA and DRT111 are also required for the correct splicing of *CERK1*, which encodes a receptor-like kinase that functions as a receptor for chitin (Zhang et al., 2014). The requirement of DRT111 or SUA for correct alternative splicing of *SNC4* and *CERK1,* together with the report that SUA is involved in splicing of ABI3 suggest that DRT111 may have overlapping/complementary functions and that DRT111 may also function in pre-mRNA splicing of *ABI3* in a manner similar to that of SUA.

The SUA protein has a conserved domain architecture, with two RNA recognition motifs and a G-patch domain, which are also found in DRT111 (Figure 15). The RNA recognition motif domains are contained in many eukaryotic proteins involved in RNA regulation. Also the G-patch domain has a specific function in RNA processing and, in particular, it might mediate a distinct type of

RNA-protein interaction. Based on the presence of these functional motifs, DRT111 could bind directly to specific RNA targets. Moreover, the DRT111 protein is localized into the nucleus (Figure 19), similar to the GFP signal observed in nucleus expressing the SUA:GFP fusion protein (Sugliani et al., 2010), characteristic of RNA maturation components.

The evidence presented in this section suggests that *DRT111* could contribute to pre-mRNA splicing of ABI3, promoting the dormancy and playing an important roles in the regulation of seed germination (Figure 51). Future experiments which will include a detailed analysis of ABI3 splicing variants in wild-type and *drt111* will prove this hypothesis.

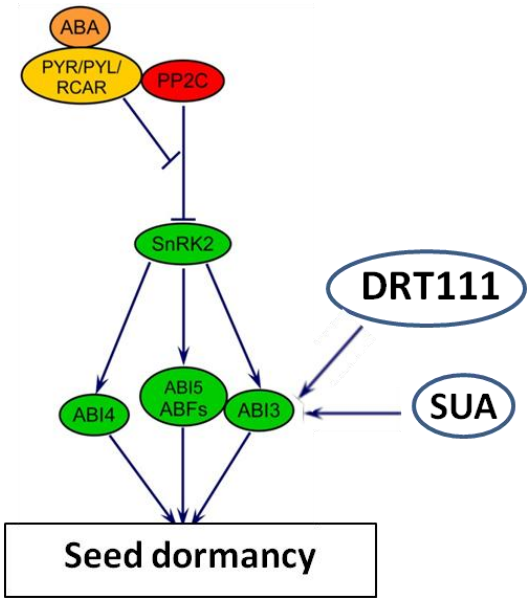

Figure 51. Model for ABA signaling in plants, showing how DRT111 regulates ABI3. PYR: pyrabactin resistance; PYL: PYR-like; RCAR: regulatory components of ABA receptors; PP2C: protein phosphatase 2C; SnRK2: SNF1-related protein kinase2; ABF: ABA responsive element binding factor; ABI3/4/5: ABA insensitive 3/4/5; SUA: SUPPRESSOR OF ABI3-5.

2.4.2 TIP41-like is a component of ABA-mediated mechanisms of stress responses

The regulation of growth in response to environmental cues is the main process that helps plants to survive stress conditions. The TOR (TARGET OF RAPAMYCIN) kinase plays a central role in modulation of cell growth in response to unfavourable conditions as main coordinator of nutrient, energy and stress signalling networks. (Wullschleger et al., 2006; Robaglia et al., 2012; Xiong and Sheen, 2014). TOR is a Ser/Thr protein kinase evolutionarily conserved among all eukaryotes. It was identified in *Saccharomyces cerevisiae* through genetic screening of mutants resistant to rapamycin, an immunosuppressant that inhibits human T cell proliferation (Heitman et al., 1991). In yeast, the main channels for TOR signalling are the PP2A (Protein phosphatase 2A) interacting proteins TAP42 and TIP41. Homologs of these proteins, TAP46 and TIP41-like, are present in

plants and are also substrates of TOR kinase. Although the molecular functions and the dynamic regulatory mechanisms of TOR kinase are well characterized in yeast and mammals, little was known about its role in plants until recent breakthroughs. This study offers some important insights into the function of the Arabidopsis TIP41-like in the ABA signaling pathway.

The embryo lethality of Arabidopsis *tor* knockout mutants and the natural resistance of plants to rapamycin (Menand et al., 2002; Ren et al., 2011) leave largely uncharacterized the regulatory mechanisms of the TOR kinase in plants. In yeast and mammals, TOR forms at least two large protein complexes with different structures and functions, TORC1 and TORC2 (TOR complex 1 and 2) (Wullschleger et al, 2006). In plants, the components of the TOR kinase complexes have not been precisely established (Figure 52). Several TORC1 conserved elements have been identified. By contrast, specific components of TORC2 complex seem to be absent in plants. Unique TOR complexes may form in plants to serve specialized functions.

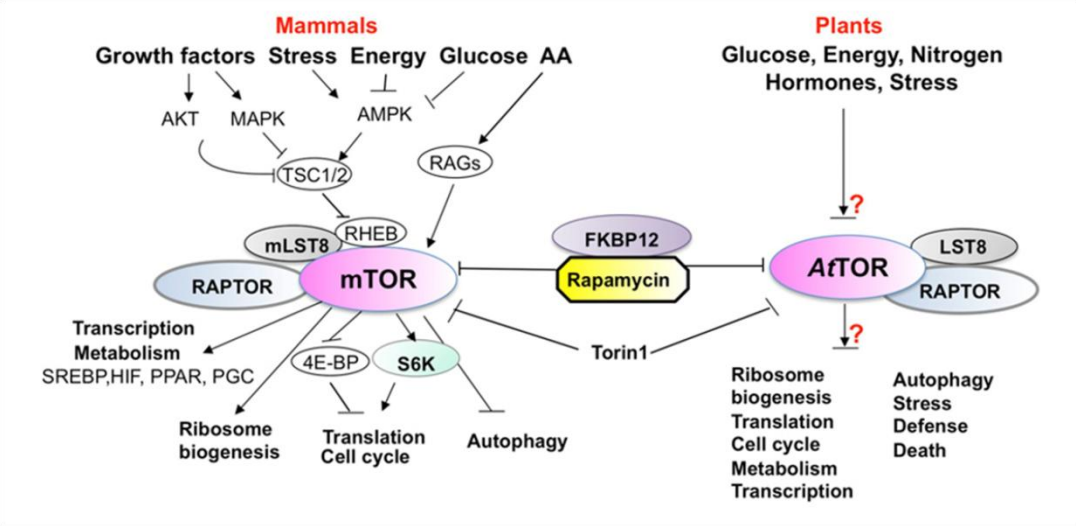

Figure 52. TOR signaling networks in mammals and plants (Xiong and Sheen, 2014). AA: Amino acid; SREBP: sterol regulatory element-binding protein; HIF: hypoxia-inducible factor; PGC: peroxisome proliferator-activated receptor-g coactivator; PPAR: peroxisome proliferator-activated receptor; TSC1/TSC2, tuberous sclerosis1/tuberous sclerosis2

TOR signaling regulates cell growth and proliferation by promoting anabolic processes, such as translation, transcription and ribosome biogenesis, and negatively regulating autophagy. Interestingly, recent studies suggest the TOR involvement in the regulation of the metabolic adjustment of cells to osmotic stress in Arabidopsis. Deprost et al. (2007) have identified an inverse correlation between the expression level of TOR and the sensitivity of the primary root length to salt stress, indicating that a constitutive TOR expression may reduce the effect of osmotic stress on root growth.

TIP41-like encodes a small protein of 290 aa. Recent studies hypothesized a peroxisomal localization of TIP41-like based on the presence at the C-terminus of a Peroxisome targeting signal

(Kataya et al., 2015). However, experimental evidence showed that the full-length TIP41-like protein targets to the nucleus and cytosol, with a possible masking of the Peroxisome targeting domain (Kataya et al., 2015). Accordingly, our results show that TIP41-like localizes in the cytoplasm (Figure 27).

Promoter activity as well as *in silico* expression analyses show that *TIP41-like* is widely expressed in Arabidopsis adult plants and seedlings, with high activity in vascular tissues (Figure 25). On the basis of its ubiquitous expression, *TIP41-like* was frequently used as a reference gene in expression analysis (Czechowski et al. 2005). Interestingly, the *Solanum tuberosum* ortholog (Figure 23) is regulated in culture cells adapted to polyethylene glycol (PEG) (Ambrosone et al., 2011). Similarly, long-term exposure to NaCl and ABA induces significant gene up-regulation in Arabidopsis (Figure 26), indicating that *TIP41-like* is stress-inducible.

tip41-like knockout mutants are hypersensitive to ABA compared with wild type both at the germination and seedling stage (Figure 29; Table 10). In particular, the abolished expression of *TIP41-like* seems to influence root growth in response to ABA with a severe reduction of primary root length compared to wild type (Figure 30). Recently, Kataya and colleagues (2015) observed that the abolished expression of *TIP41-like* leads to a slower seedling growth, delayed flowering and accelerated senescence. Since it is well known that ABA has a major role in the promotion of leaf senescence (Nooden, 1988; Liang et al., 2014; Song et al. 2016), the results of Kataya and colleagues (2015) might be interpreted as a further confirmation that *tip41-like* mutants present an alteration in ABA biosynthesis/perception. Consistently, water loss analysis on detached leaves, indicative of the efficiency of stomatal closure, another ABA-mediated process (Verslues et al., 2006), showed that *tip41-like* leaves lose water more rapidly than wild-type (Figure 31).

In yeast and human, TIP41 was reported to bind PP2A (Sents et al. 2013; Lillo et al. 2014), an important Ser/Thr phosphatase involved in the osmotic stress response in mammalian culture cells (Parrott and Templeton, 1999). In yeast the inactivation of PP2A by TOR has been well characterized (Rohde et al., 2001; Düvel et al., 2003), while a similar regulation in response to various environmental cues is not yet determined in plants. The PP2A is holoenzyme composed of three subunits (A, B and C) with different cellular functions. In Arabidopsis, more than 20 genes encode PP2A subunits, leading a large number of possible PP2A dimer or trimer combinations with different substrate specificities (Farkas et al., 2007).

The PP2A-associated protein, TAP46, is phosphorylated by the TOR kinase and regulates the PP2A activity via binding the catalytic subunit PP2Ac. Silencing of TAP46 causes a dramatic reduction in plant growth and development with decrease of protein synthesis rate, indicating that TAP46 acts as a positive effector of the TOR pathway (Ahn et al., 2011).

Recently, the TAP46 and PP2A involvement in the ABA-regulated seed germination were also studied. Both TAP46 and PP2A interact with ABA INSENSITIVE5 (ABI5) (Hu et al., 2014). ABI5 encodes an ABA-regulated transcription factor that acts in the last step of ABA signaling pathway in seed maturation and seedling development (Finkelstein et al., 2002). The *abi5* knockout mutants display insensitivity to ABA. Based on this evidence, it was shown that ABI5 is stabilized by ABA-induced phosphorylation, and quickly degraded upon removal of ABA after PP2A dephosphorylation (Lopez-Molina et al., 2001). Interaction with TAP46 protects ABI5 from PP2A activity, thus maintaining ABI5 in the active form.

Interestingly, the phenotypes of *TIP41-like* depletion reported here extensively overlap those described for *TAP46* over-expressing lines, such as a low germination in presence of ABA. Since in yeast TIP41 binds and inhibits TAP42 (the Arabidopsis TAP46), negatively regulating the TOR pathway, we propose that a similar role is carried out in plants by TIP41-like. This protein may act as a negative regulator of TAP46 in the stabilization of ABI5 during seed germination (Figure 53). Because *tip41-like* mutants present additional phenotypes other than at the germination stage, it is likely that several pathways are affected by TIP41 in addition to the ABI5 regulation. Future experiments will address this hypothesis by identifying downstream components of the TOR signaling pathway in order to have a global view of the mechanisms regulated by TIP41 during osmotic stress in plants.

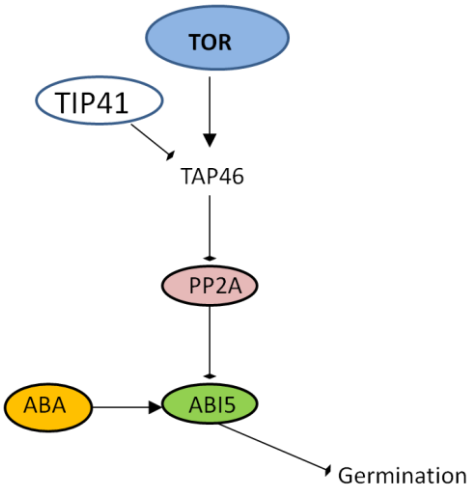

Figura 53. Model for TOR signaling during seed germination in Arabidopsis. TIP41 negatively regulates TAP46 that stabilizes ABI5 and protects ABI5 from the action of PP2A.

2.4.3 A proposed role of SIN-like in root development

Transcription requires the action of three multisubunit enzymes, the DNA-dependent RNA polymerases Pol I-III. Pol I transcribes 45S rRNA, precursor of 5.8S, 18S and 25S ribosomal RNAs. Pol II is required for the transcription of mRNAs and noncoding RNAs. Pol III transcribes mainly 5S rRNA, tRNAs and several small RNAs. In plants, there are two additional RNA polymerases, Pol IV and Pol V, involved in the biogenesis of small interfering RNAs and regulation of gene silencing processes (Haag and Pikaard, 2011). Although researchers were largely focused on the roles played by Pol II-transcribed RNAs, recent studies reveal that the well known transcription mechanisms of Pol III are more complex than formerly assumed (Dieci et al., 2007; Acker et al., 2013), suggesting the possible roles of Pol III-transcribed RNAs in regulating responses to stimuli (Hu et al., 2012).

Knockout mutations in the subunits of Pols are generally lethal. Therefore, few studies address the functions of plant Polymerases. Here, we have characterized an Arabidopsis Pol III subunit mutant, *sin-like*, in response to stress.

The "SIN-like" name is due to the presence of the Sin_N conserved region. In Drosophila, this region was identified in the interactor (SXL Interactor) of the RNA binding protein SXL (sex lethal) that acts as a regulator of both alternative pre-mRNA splicing and translation (Dong and Bell , 1999).

The Arabidopsis *SIN-like* encodes the orthologue of human RPC5, specific periphery component of Pol III. Despite limited sequence similarity, RPC5 is identified as orthologue of *Saccharomyces cerevisiae* RPC37 (Ream et al.2015). Both RPC37 and RPC5 dimerize in yeast and human with RPC53. The dimer is anchored to the Pol III core and acts integrating a protein network for initiation and termination (Hu et al., 2002; Wu et al., 2011).

Upadhya et al. (2002) identified in *S. cerevisiae* an important repressor of Pol III, MAF1. MAF1 is a downstream effector of several stress and nutrient signalling pathways, including the target of rampamycin (TOR) pathway and the DNA damage and secretory signaling pathways (Cieśla and Boguta., 2008; Michels et al., 2010), suggesting that Pol III activity may be inhibited under several stresses as a result of the activation of these pathways.

Mutation in *SIN-like* leads to phenotypes that could be associated with altered cell development. Arabidopsis *sin-like* mutants show stunted roots, with a relatively normal shoot growth (Figure 39). The induction of the *S. tuberosum* ortholog (LOC102594031) in culture cells adapted to polyethylene glycol (PEG) (Leone et al, 1994; Ambrosone et al., 2011), suggested the involvement of *SIN-like* and consequently Pol III, in abiotic stress responses. The Arabidopsis gene is widely expressed in all tissues including guard cells (Figure 34), raising the hypothesis of a role of the gene

in the regulation of gas exchange. However, in detached leaf assays, where the water loss is mainly dependent on stomatal movements (Verslues et al., 2006), *sin-like* leaves lose a similar amount of water to wild type (data not shown). Moreover, the regulation of gene expression in response to stress treatments was not observed in Arabidopsis (Figure 35). Even though we could not confirm that *SIN-like* is involved in cellular stress responses, the impact of an abolished expression of *SIN-like* on root architecture may have important consequences on the whole-plant responses and ability to tolerate stress conditions. In response to environmental cues, plants modify the root architecture to increase water uptake efficiency (Lynch 1995; Den Herder et al., 2010). This process is controlled by several genetic components as well as hormone balance. In particular, ABA and the auxin indole-3-acetic acid (IAA) represent the major regulators of growth, development and tropism of roots. In Arabidopsis, ABA and auxin pathway are mediated by both common and independent mechanisms, with recent suggestions of a crosstalk between the two pathways (Casimiro et al., 2003; Rock and Sun, 2005). Therefore, the downstream effects of ABA and auxin signals are particularly studied.

Recently, a long non-coding RNA (lncRNA) was suggested to regulate PIN2 (PIN-FORMED2), an auxin efflux carriers essential in the distribution of IAA. The long non-coding RNAs are mainly transcribed by Pol II. Recently, also the Pol IV action in the biogenesis of several lncRNAs was demonstrated (Li et al., 2015). Interestingly, the Pol IV/V-transcribed APOLO lncRNA is involved in root development (Bazin and Bailey-Serres. 2015). Plants with reduction in APOLO transcription, indeed, show altered primary root growth and gravitropism, possible due to altered expression of PID (PINOID), a kinase required for the polar localization of PIN2 (Huang et al., 2010; Bazin and Bailey-Serres, 2015).

The severe reduction in *sin-like* roots could be explained by an alteration of RNAs transcribed by Pol III. Concordantly, Johnson et al. (2016) noted a similar phenotype in another Pol III subunit mutant, *nrpc7-1*. The gene encodes the Arabidopsis orthologue of yeast subunit Rpc25. *nrpc7-1* plants present significantly shorter roots than wild type plants. The mutant also displays delayed emergence of the first true leaves and small siliques. The authors attributed these phenotypes mainly to altered small RNA stability, alternative splicing defects, and abundances of a number of RNA molecules.

In Arabidopsis, *nrpc7-1* is the first and only Pol III subunit mutant reported thus far. Therefore, our work makes a notable contribution to functional analyses of Pol III. Future studies will focus on the link between the *sin-like* mutant phenotype and the change of the stability of RNAs transcribed by Pol III.

2.4.4 The role of AtRGGA in post-trascriptional regulation of gene expression during osmotic stress

In Ambrosone et al., (2015), we provided evidence that the Arabidopsis AtRGGA is involved in tolerance to drought and salt stress. AtRGGA is expressed in several Arabidopsis tissues, including tissues that perceive or respond to osmotic stress conditions, such as roots and stomata, and tissues that undergo extensive dehydration processes, such as pollen (Ambrosone et al., 2015). AtRGGA is up-regulated in Arabidopsis seedlings upon long-term exposure to PEG and ABA (Figure 40). Moreover, the *rgga* knockout plants are hypersensitive to ABA treatments while the overexpressing plants appear to be better able to withstand ABA treatments (Figure 41,42), indicating that AtRGGA participates in ABA-dependent mechanisms of response to salt and drought stress.

AtRGGA encodes a cytoplasm-localized protein (Ambrosone et al., 2015) with several Gly/Arg (RGG) motifs, the Stm1 N-terminal and HABP4_PAI-RBP1 domains characteristic of RNA and nucleic acid-binding proteins. Stm1 is a yeast G4 quadruplex and purine motif triplex nucleic acid-binding protein of *Saccharomyces cerevisiae* that has been shown to associate with telomeric Y' DNA and ribosomes (Van Dyke et al., 2004). HABP4 binds hyaluronan as well as RNA, while the human PAI-1 mRNA-binding protein binds the type 1 plasminogen activator inhibitor and has been suggested to be involved in the regulation of mRNA stability (Huang et al., 2000; Heaton et al., 2001). As expected from the gene annotation, we show that recombinant AtRGGA is capable of efficiently binding RNA *in vitro* (Figure 43). The similarity with the Stm1, HABP4, and PAI1 proteins, together with the cytoplasmic localization of the protein, suggest that AtRGGA might affect post-transcriptional regulation mechanisms such as the control of RNA stability, storage, or translation efficiency rather than the synthesis or nuclear processing of RNAs. In humans and yeast, RGG motif-containing proteins have emerged as key players involved in the post-transcriptional regulation of gene expression, affecting RNA stability as well as RNA translation to protein through interaction with the translation initiation factor eIF4G, which recruits ribosomes to mRNAs (Rajyaguru et al., 2012; Walsh and Mohr, 2014). While the possibility that poly(A^+) RNAs are also bound by AtRGGA could not be ruled out, we observed binding *in vitro* to poly(A^-) RNA and, more in detail, to the small ribosomal RNAs 5S and 5.8S (Figure 45), suggesting that AtRGGA may bind to the ribosomes to modify translation efficiency and/or the stability of ribosome-bound mRNAs. However, *in vitro* assays help identifying the RNA-protein complexes, but may fail to reproduce the real physiological interaction, which can be evaluated only using *in vivo* methods. We therefore performed the immupurification of RNA-protein complexes *in planta*. The analysis confirmed the specificity of AtRGGA-rRNA interaction and revealed that AtRGGA is able of binding all RNA

components of the ribosomes *in vivo* (Figure 48). Interestingly, the profile of the RNAs extracted from AtRGGA-containing complexes suggests that additional RNAs are bound, possibly including mRNAs. RNA sequencing is now in progress to establish the pool of RNAs bound by AtRGGA *in vivo*.

Using yeast two-hybrid assay we identified several putative AtRGGA interactors. In accordance with our hypothesis, the identified protein partners are mainly involved in RNA processing, transport and ribosome biogenesis (Table 11).

One of the AtRGGA interactors, RANBP1, is an RNA-binding protein with a charatheristic RAN-binding domain. RANs are small GTP-binding protein mainly involved in nuclear import and export of protein and RNA (Haizel et al., 1997). These proteins are localized to the cytoplasmic side of the nuclear pore complexes, in accordance with the AtRGGA perinuclear localization (Ambrosone et al., 2015). The directionality of nucleocytoplasmic transport is determined by the high presence of Ran GTP-binding in the nucleus that establishes a gradient with the high Ran-GDP in the cytoplasm (Stewart, 2007). In Arabidopsis, over-expression of RanBP1 causes hypersensitivity to auxin with consequently altered root growth probably due to an impaired import of proteins that suppress auxin action (Kim et al., 2001). However, little is known about the biological functions of RanBPs in plant growth and development. An interesting hypothesis could be that AtRGGA regulates transport of stress-related mRNAs when associated to RanBP1.

In addition, a kinesin-like protein, ZCF125, was identified as an interactor of AtRGGA. Kinesin proteins act as molecular motors that directionally transport diverse cargos along microtubules, including organelles, protein complexes and mRNAs (Hirokawa, 1998; Hirokawa et al., 2009). ZCF125 could, thus, be involved in the sorting of AtRGGA/RNA complexes within the cells.

The screening identified also a member of SUMO family, *SUMO5*. SUMO genes encode small ubiquitin-like modifier proteins that alter function and/or cellular distribution of different target proteins by covalent attachment. In particular, in human and yeast, these proteins regulate several cellular processes including nucleocytoplasmic transport, transcriptional regulation and RNA transport (Geiss-Friedlander and Melchior, 2007). Interestingly, SUMO modification of proteins is important in responses to environmental stimuli (Saitoh and Hinchey, 2000; Goodson et al., 2001). SUMO1 and SUMO2 have been shown to have a role in abiotic stress response such as heat shock, H_2O_2 and ethanol (Kurepa et al., 2003). However, SUMO5 is poorly characterized. This protein may regulate the turnover, activity or subcellular localization of AtRGGA in response to stress.

Indeed, we also identified as putative interactors of AtRGGA two proteins that are localized to the nucleus, which may be an indication that in certain conditions AtRGGA undergoes a redistribution from the cytoplasm to the nucleus. Here, AtRGGA probably acts regulating the transcription,

through interaction with the elongation factor S-II. The transcription elongation factor S-II was originally identified in *S. cerevisiae* as a specific protein stimulating the RNA polymerase II (Nakanishi et al.,1992). In particular, S-II confers yeast resistance to 6-azauracil, an inhibitor of enzymes involved in purine and pyrimidine biosynthesis, by stimulating transcription elongation of azuracil-suppressor (Shimoaraiso et al., 2000). Furthermore, Koyama et al., (2003) suggested that S-II is essential for the survival of yeast under oxidative stress by transcriptional mRNA proof-reading during elongation. Therefore, it might be possible that S-II, when associated with AtRGGA, increases the transcription elongation of genes involved in stress response.

A member of PUF proteins, APUM24, was also identified as AtRGGA interactor. PUF proteins are a large family of RNA-binding proteins. Binding is mediated by the conserved PUF domain, which recognizes specific RNA sequences. These proteins regulate both stability and translation binding to the 3'UTR of target mRNA transcripts (Wang et al., 2002; Francischini and Quaggio, 2009). Interestingly, microarray analysis show changes in APUMs expression in response to several environmental stimuli, such as drought and salinity. Therefore, they might regulate their target mRNAs in response to abiotic or biotic stimuli (Francischini and Quaggio, 2009). APUM24, together with APUM23, differs from most other Arabidopsis Pumilio proteins based on its subcellular localization. Indeed, while other PUMs are cytoplasmic, APUM23 and APUM24 are localized in the nuclear area, and concentrated in the nucleolus, the specialized structure responsible for ribosome biogenesis. Abbasi et al., (2011) suggest the interaction of APUM24 with APUM23, whose expression in microarray studies changed in response to ABA. APUM24 may interact with APUM23 to mediate its role in the control of plant development via rRNA processing and consequently ribosome biogenesis (Abbasi et al., 2010). Thus, AtRGGA, through interaction with APUM24, may regulate ribosome biogenesis in certain conditions.

Figure 54 summarizes, based on the RNA binding assays and protein interaction studies, our proposed roles of AtRGGA in transcriptional and post-transcriptional control of gene expression during osmotic stress. Future functional analysis of the reported AtRGGA interactors, will prove this hypothesis.

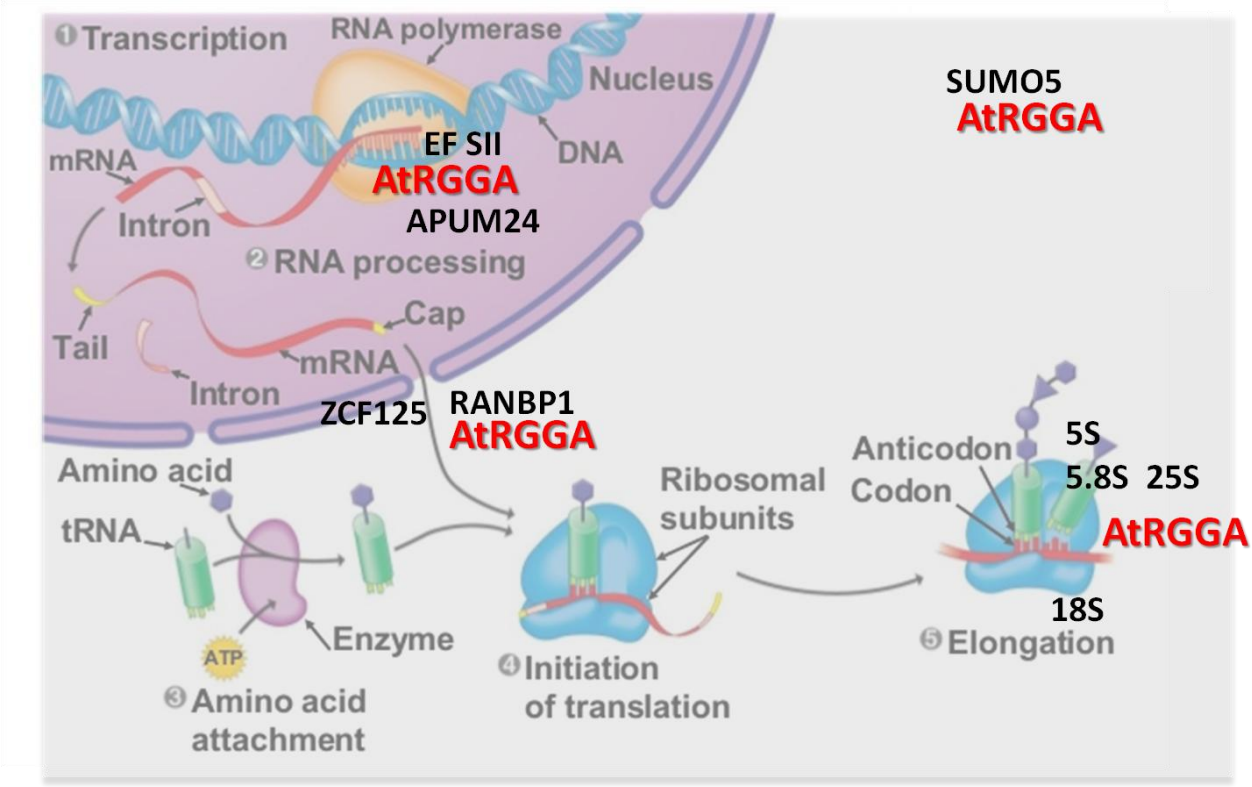

Figure 54. Proposed roles of AtRGGA in transcriptional and post-transcriptional control of gene expression during osmotic stress. In nucleus, AtRGGA interacts with the transcriptional elongation factor S-II regulating the transcription and modifies RNA stability and/or ribosome biogenesis when associated to the PUF protein APUM24. In the cytoplasmic side of the nuclear pore complexes, AtRGGA interacts with RANBP1 and ZCF125 to regulate the transport of stress-related mRNAs and, then, their translation when associated to ribosomal rRNAs. Finally, activity, localization or turn-over of AtRGGA may be regulated by the interaction with SUMO5. This scheme was prepared by modifying a figure from http://www.slideshare.net/mooshoo1/10-lecture-presentation.

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3. Transcriptomic changes induced by progressive drought stress and rehydration in tomato

3.1 Introduction

Drought is a major environmental stress causing severe reduction in crop yields. In a scenario of severe climate changes and increased global food demands (FAO, 2009), improving crop tolerance to drought is essential to ensure environmental sustainability and food security (Passioura, 2007).

The development of tolerant crops requires the understanding of physiological and molecular responses involved in adaptation to harsh environments and the identification of associated gene networks (Collins et al., 2008). Physiological and biochemical responses include stomata closure, modulation of photosynthetic performances, accumulation of osmolytes and growth retardation (Bosco de Oliveira, 2012). Stomata represent the first barrier plants employ to avoid dehydration, with the trade-off of a reduced CO_2 supply to the mesophyll which results in a reduced photosynthesis rate, further inhibited by metabolic impairments as water deficit intensifies (Chaves et al., 2009). Along with plant responses to water deficit, understanding the recovery of photosynthesis upon rehydration is of paramount importance to understand water stress effects on photosynthesis (Flexas et al., 2004; Lutfor Rahman *et al.*, 1999).

Impact of drought on gene expression has been intensely analysed in numerous species such as Arabidopsis (Ding et al., 2012. Sakuraba et al., 2015), rice (Huang et al., 2014. Oono et al., 2014), maize (Kakumanu et al., 2012.), sorghum (Dugas et al., 2011; Sekhwal et al., 2015), poplar (Barghini et al., 2015), and tomato (Gong et al., 2010.) by high-throughput transcriptomics.

A predominant role in driving drought-induced changes in gene expression is played by the hormone abscisic acid (ABA). The mechanisms of ABA perception and signal transduction are the subject of intense research and major breakthroughs have included the identification of a family of cellular receptors (Ma et al., 2009; Park et al., 2009). An increase in ABA changes the hydraulic regulation of stomata (Chaves et al., 2009), resulting in stomata closure under adverse hydraulic conditions by controlling the biochemistry of guard cells and decreasing water permeability within the leaf vascular tissue (Monnet et al., 2012). To avoid cellular dehydratation, plants also increase the amino acid proline under stress condition. Proline is essential both as component of protein and as free amino acid, facilitating water uptake and reduces the accumulation of Na^+ and Cl^- (Ashraf and Foolad 2007).

Tomato (*Solanum lycopersicum* L.) is one of the major horticultural crops and an important dietary source of vitamins A and C as well as carotenoids such as lycopene (Canene-Adams et al., 2005). Tomato is also considered a fleshy fruit plant model system with several investigation tools available, including the sequenced genome (Sato et al., 2012) and its large open source genomic repository (Suresh et al., 2014). Although tomato is cultivated worldwide, it is considered sensitive to stresses of biotic and abiotic nature (Rai et al., 2013, Kissoudis et al., 2015). Most of modern tomato cultivars are

very sensitive to water deficit, which results in reduced seed development and germination, reduced vegetative growth and impaired reproduction (Nuruddin, 2003; Bartels and Sunkar, 2005; Rai et al., 2013). The average water footprint per Kg of tomato has been calculated in 215 liters, 30% of which are supplied by irrigation (Mekonnen and Hoekstra, 2011). Therefore, it is an essential strategy to develop drought tolerant, higher yielding varieties to cope with the increasing demand for tomato (Solankey et al., 2014). Valuable donors of positive traits for response to environmental stresses include wild-relatives of tomato, such as *Solanum pennellii*, a drought tolerant species. Comparative transcriptomics revealed distinct patterns of expression between *S. pennellii* and the *S. lycopersicum*. The wild relative showed a number of gene expression patterns more suitable for stress tolerance, such as a higher expression of several genes involved in waxes deposition, possibly accounting for the thicker cuticle of the wild species compared to cultivated tomato. By contrast, domesticated tomato was selected for a number of fruit traits and post-harvest quality (Koenig et al., 2013).

Specific gene expression studies in response to water stress have been carried out in leaves using microarray approaches (Gong et al., 2010) identifying differentially expressed transcripts of genes involved in energy, plant hormones, and cation transporters (Sadder et al., 2014) and a number of transcription factors and signalling proteins (Gong et al., 2010). However, studies that integrate the different levels of response to drought stress in tomato are under-represented.

Recently, a growing number of statistical algorithms allows several approaches for gene differential expression detection. Coexpression cluster analysis allows the identification of distinct expression profiles, and is a powerful tool to investigate mechanisms of transcriptional regulation, identifying behaviours and trends in gene expression as well as novel putative regulatory motifs (Rapaport et al., 2013).

The goal of our research was the characterization of physiological, biochemical and molecular processes occurring in tomato in response to limiting water input, in order to dissect the complexity of the molecular events occurring in response to drought. Genome-wide comparisons of stress responses across different cycles of drought and rehydration were carried out using Illumina deep sequencing of RNA populations followed by cluster analysis to identify stress-induced patterns of expression. The work here described is part of the manuscript by Iovieno and colleagues (2016).

3.2 Materials and Methods

3.2.1 Plant materials, growth conditions and stress treatments

Seeds of cultivar M82 (accession LA3475) supplied by the Tomato Genetics Resource Center (TGRC, http://tgrc.ucdavis.edu/) were germinated in soil in a semi-controlled greenhouse. When seedlings had developed 2 true leaves (25 days after sowing), they were transplanted in pots, filled with soil (one plant per pot) and fertilized after seven days with Nitrophoska gold (Compo Agricoltura, Cesano Maderno, Italy). Plants were well irrigated for thirty days prior to start the stress treatments. Then plants were equally divided into control and stress treatment, 9 replicates per treatment, and arranged in a randomized block design.

As described in Iovieno et al (2016), two cycles of water deficit were performed by water withholding until soil water content of stress pots was less than 1/3 of control pots, inducing a nearly complete closure of the stomata. This corresponded to 16 and 6 days of water withholding in the first and second cycle of drought, respectively. Between these two stress cycles, plants were well irrigated allowing a full recovery of soil water content and stomatal conductance. Control plants were well watered throughout the entire experimental period.

During the experiment, the soil water content (θ, m^3/m^3) was determined from dielectric measurements performed by a Time Domain Reflectometer (TDR100 Campbell Scientific Inc. Logan, UT) and applying the Topp's equation. The 14,2 cm trifiliar probes were placed in 3 pots per treatment.

Leaf samples for molecular and biochemical analyses were collected at three different time point of the experiment.

3.2.2 Gas exchange analysis

Gas exchange analyses as well as the soil water content measurements described above were performed in collaboration with the physiology group of CNR-ISAFoM (Dr. P. Giorio and Dr. R. Albrizio). Net photosynthetic CO_2 assimilation rate (A, µmol m^{-2} s^{-1}) and stomatal conductance to water vapour (g_s, mol m^{-2} s^{-1}) were measured on a fully expanded, well-exposed top leaf on 5-6 plants per treatment between 10:00 am and 1:00 pm. Measurements were carried out using a portable open-system gas-exchange and modulated fluorometer analyser Li-6400XT (Li-Cor Biosciences), with CO_2 inside leaf chamber set to 400 µmol CO_2 mol^{-1} air. An artificial light source LED with emission peaks centred at 635 nm in the red and at 465 nm in the blue provided a PPFD equal to 2000 µmol (photons) m^{-2} s^{-1} (90% red, 10% blue).

3.2.3 Proline and ABA content measurements

Leaf samples were collected by excising the leaf at the petiole from three biological replicates. Two technical replicates were performed for each sample. Proline content was determined according to the method of Claussen (2005). 250 mg of finely ground leaf tissue were suspended in 1.5 mL of 3% sulphosalicylic acid and filtered through a layer of glass-fiber filter (Macherey-Nagel, Ø 55mm, Germany). 1mL of Glacial acetic acid and 1 mL ninhydrin reagent (2,5 g ninhydrin/100 mL of a 6:3:1 solution of glacial acetic acid, distilled water and 85% ortho-phosphoric acid, respectively) were added to 1 mL of the clear filtrate. The mixture was incubated for 1 hr in a boiling water bath. The reaction was terminated at room temperature for 5 min. Readings were taken immediately at a wavelength of 546 nm. The proline concentration was determined by comparison with a standard curve.

For ABA measurements, 150 mg of fine powder were extracted in distilled, autoclaved water with constant shaking at 4°C overnight in the dark. The supernatant was collected after centrifugation (10000 x g for 10 min) and diluted 50-fold with TBS buffer (50 mM TRIS, 1 mM MgCl2, 150 mM NaCl, pH 7.8). Subsequently, ABA was analysed by indirect enzyme-linked assay (ELISA) using the Phytodetek ABA test kit (Agdia) following the manufacturer's instructions. Colour absorbance following reaction with substrate was read at 405 nm using a plate autoreader (1420 Multilabel Counter Victor^{3}TM, PerkinElmer).

3.2.4 Statistical Analyses

The statistical significance of soil water content, gas-exchange and fluorescence parameters, ABA and proline contents between water treatments was evaluated through Student's t-test.

3.2.5 Isolation of RNA, cDNA synthesis and qRT-PCR

Total RNA was extracted from leaf tissues using TRIzol Reagent (Life Technologies). RNA quantity was measured spectrophotometrically by NanoDrop ND-1000 Spectrophotometer (NanoDropTechnologies), and integrity was verified on a denaturing formaldehyde gel.

For qRT-PCR validations, reverse transcription was performed using 1 µg of DNase-treated total RNA and using SuperScript II Reverse Transcriptase™ (Life Technologies). qRT-PCR reactions were performed using an ABI 7900 HT (Applied Biosystems) and Platinum SYBR Green qPCR SuperMix (Life Technologies). Primers sequences are listed in Table 12. Preparation of reactions was automated using the Liquid Handler Robot Tecan Freedom Evo.

For each target, reactions were performed in triplicate on two or more independent biological replicates. All figures show one series, with the error bars based on technical repeats. Quantification of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). As calibrators were used control leaf samples of plant grown in control conditions and the housekeeping EF1-α gene was used as an endogenous reference gene (Nicot et al., 2005) for the normalization of the expression levels of the target genes. For each sample, the mRNA quantity was calculated relative to the calibrator sample for the same gene.

Table 12. List of primers used for gene expression analysis

Name	Sequence 5'-3'
qRT-NCED For	CATAATCGAAAACCCGGATG
qRT-NCED Rev	AACTTTTGGCCATGGTTCAG
qRT-P5CS For	AACTGAGCTTGATGGCAAGG
qRT-P5CS Rev	ACCAGAGGCTGAGCTGATGT
qRt-EF1α For	CTCCATTGGGTCGTTTTGCT
qRt-EF1α Rev	GGTCACCTTGGCACCAGTTG
Solyc03g116390.2 For	TTTGGCATGGCTGATACTGA
Solyc03g116390.2 Rev	GCAGCTGCTAAGCACACAAG
Solyc02g093180.2 For	CCGTTGGTTTCCTGAATTTG
Solyc02g093180.2 Rev	GAATTGGCTTTGGCTTTGAG
Solyc06g072130.2 For	GGATCTGTTCTTGCCCTTGA
Solyc06g072130.2 Rev	AACAGCCGGGTTGATATGAC
Solyc05g015490.2 For	AAGAGCTTGGGCGATAAGTG
Solyc05g015490.2 Rev	ACACAAACAAACCGGGTCAT
Solyc02g084850.2 For	CTCAAGGCATGGGTACTGGT
Solyc02g084850.2 Rev	GTTGTCCAGGCATCTTCTCC
Solyc03g113930.1 For	TTTCCTCCCGAAGATCCTTT
Solyc03g113930.1 Rev	TTCCTTCTTCATCCCTGGAA
Solyc09g092690.2 For	AGGGCGAAATTGCTCTGATA
Solyc09g092690.2 Rev	TCCCAGGATTCCTTCTCCTT
Solyc12g010980.1 For	GTCGCTGCATCAGATCGTAA
Solyc12g010980.1 Rev	TCGTCTCCTCCCCATTCTAA
Solyc05g014000.2 For	TTGTTGGCGATGTGATCCTA
Solyc05g014000.2 Rev	CCTGGCTTTGGATTTACTGG
Solyc05g054820.1 For	AAATGGCGGTGAAAACACTC
Solyc05g054820.1 Rev	TCTTTGCAACGATCTCAGGA
Solyc12g094440.1 For	CCGATGCGGTAAAAAGAGAG
Solyc12g094440.1 Rev	GATCCTTCCCTCCAATCACA
Solyc01g008510.2 For	CAGGAGAACCAGGAACGAAG
Solyc01g008510.2 Rev	AAGCCCATTCCCTTCTTGAT
Solyc03g082420.2 For	CCGGCGATAACAAAGATACG
Solyc03g082420.2 Rev	TTCTCATCGGTGACATTGGA
Solyc11g020330.1 For	TCATCACTAAGCGCAGATGG
Solyc11g020330.1 Rev	CGCGAGCAGAGTGTTTTCTA

3.2.6 Library Preparation and Sequencing

RNA pools of three biological replicates were used for all RNA-Seq experiments. The total RNA was DNase treated and purified using the RNeasy Plant Mini kit (Qiagen) following manufacturer's

protocol (Qiagen). RNA samples were analyzed quantitatively and qualitatively by NanoDrop ND-1000 Spectrophotometer (NanoDropTechnologies) and by Bioanalyzer (Agilent Technologies).
cDNA libraries were prepared with 1 ug of starting total RNA and using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA), according to TruSeq protocol. Library size and integrity were determined using the Agilent Bioanalyzer 2100. Each library was diluted to 2 nM and denatured. 8 pM of each library was loaded onto cBot (Illumina) for cluster generation with cBot Paired End Cluster Generation Kit (Illumina) and sequenced using the Illumina HiSeq 1500 with 100bp paired-end reads in triplicate obtaining ~14 million reads for replicate. The sequencing service was provided by Genomix4life Ltd (http://www.genomix4life.com) at laboratory of Molecular Medicine and Genomics (University of Salerno, Italy).

3.2.7 RNAseq analysis

The RNA-seq data analysis was prerformed by Prof. Chiusano group (Univeristy of Naples "Federico II"). The cleaning of the raw sequences from the RNAseq data was made using the Trim Galore package (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). In the first step, low-quality bases were trimmed from the 3' end of the reads. In the second step, Cutadapt (Martin, 2011) removed adapter sequences; default settings for paired-end was used. The quality check of the remaining sequences was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The 1) cleaned pairs, and 2) the high quality single reads obtained after the cleaning step, were used as input for the mapping to the tomato genome (version 2.40), independently. Bowtie version 2.1.0 (Langmead and Salzberg, 2012) and Tophat version 2.0.8 (Kim et al., 2013) were used for mapping. Paired and single reads, uniquely mapped, were counted, independently, per gene available from the iTAG annotation, version 2.3, using the HTSeq-count (http://www.huber.embl.de/users/anders/HTSeq/) version 0.5.4p1, in "union" default mode setting.
In order to define the set of expressed genes, raw read counts were normalized to RPKM (Reads per Kilobase per Million) and genes above the 1 RPKM cut-off were kept for the subsequent analyses. Differential expressed genes (DEGs) were found performing the negative binomial test implemented in the DESeq package (Anders and Huber, 2010) version 1.10.1, at a false discovery rate threshold (FDR) 0.01.
The k-means (MacQueen, 1967) cluster analyses were then performed on the expression level (log2 RPKM) for DEGs detected in all the stages, using 20 cluster, a number defined by the Elbow method (Thorndike, 1953), i.e. minimizing the within group variance at different cut-offs. GO enrichments were estimated via the goseq Bioconductor package (Young et al., 2010) (FDR <= 0.05) on each

detected cluster possessing similar expression profiles in all the stages. As goseq requires gene length data, median transcript length per gene was obtained by parsing with a custom R script cDNA fasta files, as obtained from Ensembl Plants repository. GO annotations for tomato genes were obtained via BLAST2GO (Conesa et al., 2005) against NR databases and default settings. Unless otherwise stated, further graphical outputs were obtained with R custom scripts.

3.3 Results

3.3.1 Experimental outline

To gain a comprehensive understanding of mechanisms activated by dehydration and rehydration events in crops, an experimental plan based on tomato plants of the M82 genotype subjected to cycles of drought stress and rewatering was developed at CNR-IBBR, Portici (NA). Soil water status was monitored throughout the experiment as a measure of the progression of drought stress, and recovery under rewatering (Figure 55A). When water was withheld, pots subjected to drought stress underwent a continuous decline in soil water content (θ), which was significantly lower compared to control pots starting 2 days from the beginning of water withholding. Drought continued until θ was approximately 22% of the control pots. This was the maximum stress point (Dr1) for the 1st cycle of drought. Reinstatement of irrigation allowed an immediate full recovery of θ to control values, which were maintained for seven days, until the end of rewatering (RW). A rapid decline in θ was observed when a second cycle of drought stress was imposed and values similar to those of Dr1 were measured 6 days after the beginning of drought treatment (Dr2). A graphic representation of the progression of the experiment is shown in Figure 55B.

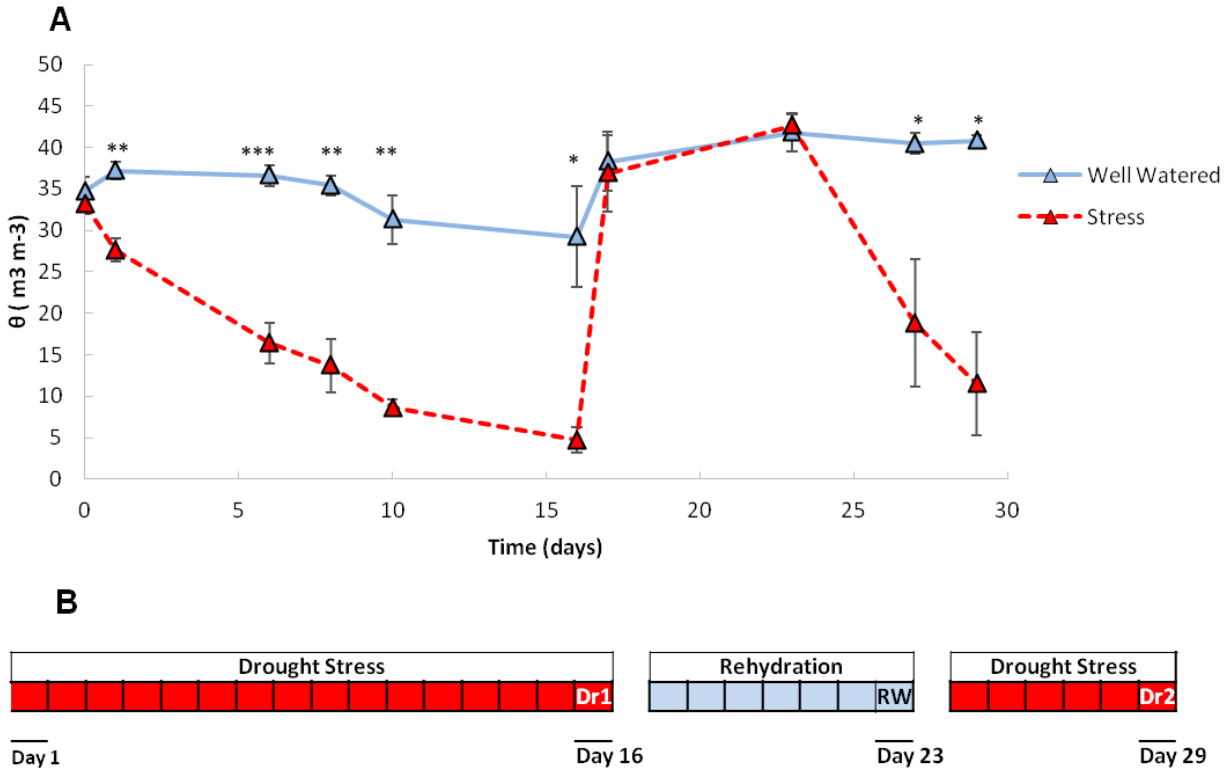

Figure 55. Experimental outline. A) Volumetric soil water content (θ) throughout the progression of the experiment. Values represent average measurements ± SD of 3 replicates. Asterisks denote significant differences according to Student's t-test between well-watered and stressed pots. *, ** and *** indicate significantly different values in drought stress compared to well-watered pots at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. B) Schematic representation of the experimental design highlighting the points Dr1 (16 days of irrigation withhold), RW (7 days of irrigation) and Dr2 (6 days of irrigation withhold).

3.3.2 Drought stress led to a decrease of leaf gas exchange

In collaboration with CNR-ISAFoM, Ercolano (NA), we measured leaf gas exchange parameters to assess the impact of drought stress and rehydration on the physiology of tomato. Net CO_2 assimilation rate (*A*) and stomatal conductance to water vapour (g_s) decreased significantly in the stressed plants compared to well-watered plants. At Dr1, CO_2 assimilation (*A*) decreased to a minimum of 2.2 µmol m^{-2} s^{-1} corresponding to 10% of the CO_2 assimilation rate measured in controls. Similar patterns were observed for g_s, which in the stressed plants at Dr1 was as low as 0.030, compared to 0.710 mol m^{-2} s^{-1} found in the fully watered plants. A moderate recovery of both *A* and g_s was observed one day after rewatering when soil water content was fully restored (Table 13). Both parameters rose to values comparable to those of the controls at RW. Under the second treatment CO_2 assimilation and stomatal conductance decreased more rapidly as compared to the previous stress cycle, reaching minimum average values of 4.0 µmol m^{-2} s^{-1} for *A* and 0.070 mol m^{-2} s^{-1} for g_s at Dr2 (Table 1).

Table 13. Influence of drought stress on leaf gas exchanges. CO_2 assimilation (*A*, µmol m-2 s-1) and stomatal conductance (g_s, mol m-2 s-1) were measured at the points Dr1 (16 days of irrigation withhold), RW (7 days of irrigation, 23 days from the beginning of the experiment) and Dr2 (6 days of irrigation withhold). Average values ± SD (n≥5) are shown. An "*" (asterisk) indicates significantly different values in drought stress compared to well-watered plants at p=0.001, according to Student's t-test.

Days	Treatment	A (µmol m-2 s-1)	gs (mol m-2 s-1)
16=Dr1	Well-watered	20.8±0.4	0.71±0.089
	Water stress	2.2±1.1	0.03±0.014
23= RW	Well-watered	18.1±3.5	0.635±0.092
	Water stress	22±2.1	0.558±0.058
29=Dr2	Well-watered	15.5±2	0.603±0.098
	Water stress	2.81±2.9*	0.04±0.023

3.3.3 Proline and ABA accumulation in drought stressed tomato

Well known metabolic alterations induced by drought stress include leaf accumulation of the osmolyte proline (Claussen, 2005) and the hormone ABA (Sharp and LeNoble 2002). We therefore measured proline and ABA content at several time points in our experiment by a spectrophotometer and ELISA assay, respectively. At Dr1, the amount of proline in the stressed plants was about 10 fold higher than the control. Proline amount in the stressed plants decreased in response to rewatering. However, values were still higher than controls for both genotypes at RW. The accumulation of proline reached the highest observed values of 4.5 at Dr2 (Figure 56A).

Ten days of drought stress was not sufficient to elicit accumulation of leaf ABA as compared to the controls (Figure 2B). As the stress became more severe leaf ABA content was as high as 15367 picomols/g fresh weight at Dr1. At the end of second drought cycle (Dr2), ABA content was 10 fold higher than that measured at RW (Figure 56B).

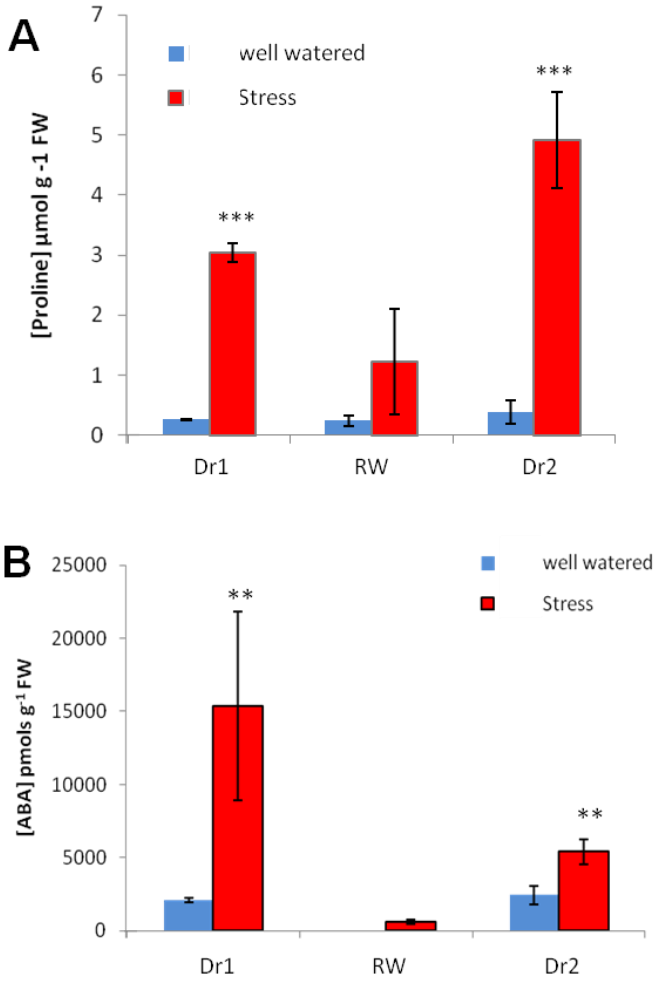

Figure 56. Quantification of Proline (A) and ABA content (B) in leaves during drought cycles (Dr1 and Dr2) and rewatering (RW); Asterisks denote significant differences according to Student's t-test between well-watered and stressed pots. *, ** and *** indicate significantly different values in drought stress compared to well-watered pots at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

To investigate the correlation between proline and ABA accumulation and transcription of related biosynthetic genes we measured the gene expression of two rate-limiting steps. Expression of *Solyc08g043170.2.1* and *Solyc07g056570.1.1* encoding a Pyrroline-5-carboxylate synthetase (*P5CS*) and a 9-cis-epoxycaratenoid dioxygenase (*NCED*) respectively was evaluated by qPCR. In Dr1, *P5CS* was induced, while in RW and Dr2 no significant up-regulation was observed (Figure 57A). *NCED* was induced at comparable levels at Dr1 and Dr2, while at RW expression levels were similar to the controls (Figure 57B).

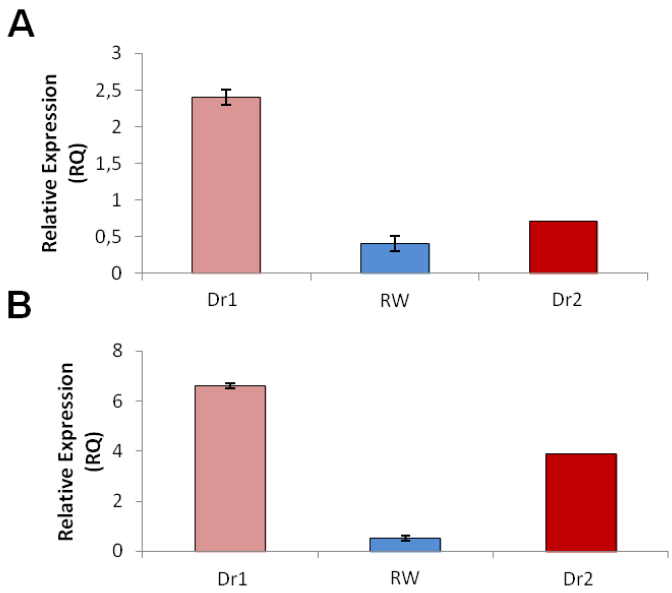

Figure 57. Expression analysis of *P5CS* (A) and *NCED* (B) by qRT-PCR in tomato plants after 14 days of drought stress (Dr1), 7 days of rewatering (RW) and 6 days of second cycle of drought (Dr2). RNA from well-watered control plants was used as calibrator sample.

3.3.4 Transcriptomic perturbations in response to drought stress and rehydration

To identify genes whose expression was altered by drought stress and rewatering in leaves, which could result in the observed physiological alterations, we carried out transcriptome sequencing. We used the Illumina platform on RNA samples extracted from leaves of well-watered plants (WW) as well as in Dr1, RW and Dr2. In collaboration with Prof. Chiusano (University of Naples "Federico II"), RNA sequencing derived data were subjected to gene expression analyses followed by clustering of genes showing similar trends of expression by comparison of the four conditions, and by gene ontology (GO) enrichment analysis. By comparing the different treatments, 966 genes that showed differential expression in at least one of the comparisons were identified, which were therefore considered as Differentially Expressed Genes (DEGs).

The analysis highlighted that a large number of DEGs were down-regulated during drought stress. Comparative analysis of drought stressed (Dr1 and Dr2) *vs.* watered plants (WW and RW) revealed 119 DEGs common to all 4 comparisons. These included several histone encoding genes (e.g. *Solyc10g008910*), cell wall modifying enzymes (e.g. *Solyc04g082140*) as well as heat shock proteins (e.g. *Solyc11g020330*).

In order to classify transcripts based on their behavior in WW, Dr1, RW and Dr2, a cluster analysis was performed using normalized expression values of the DEGs in each of the experimental conditions. Twenty clusters were identified which grouped transcripts with similar expression trends. RNA sequencing results and the cluster analysis were validated using qRT-PCR on genes selected

from different clusters (Table 14). Figure 58A and B show normalized expression values from RNA sequencing and qRT-PCR experiments, respectively. As shown in Figure 58C, a good correlation was observed between the two sets of results.

Table 14. The 14 DEGs selected to validate RNA-seq analysis.

	Locus	Cluster	Description
1	Solyc03g116390.2	7	Late embryogenesis abundant protein
2	Solyc02g093180.2	7	N-hydroxycinnamoyl/benzoyltransferase 3
3	Solyc06g072130.2	7	Aquaporin
4	Solyc05g015490.2	7	Non-specific lipid-transfer protein
5	Solyc02g084850.2	7	Unknown Protein
6	Solyc03g113930.1	20	Class IV heat shock protein
7	Solyc09g092690.2	20	Peptidyl-prolyl cis-trans isomerase
8	Solyc12g010980.1	1	Acyltransferase-like protein
9	Solyc05g014000.2	1	Pectate lyase
10	Solyc05g054820.1	17	Exocyst complex protein EXO70
11	Solyc12g094440.1	18	High mobility group family (Fragment)
12	Solyc01g008510.2	20	Photosystem II 5 kDa protein%2C chloroplastic
13	Solyc03g082420.2	3	Heat shock protein
14	Solyc11g020330.1	3	Class IV heat shock protein

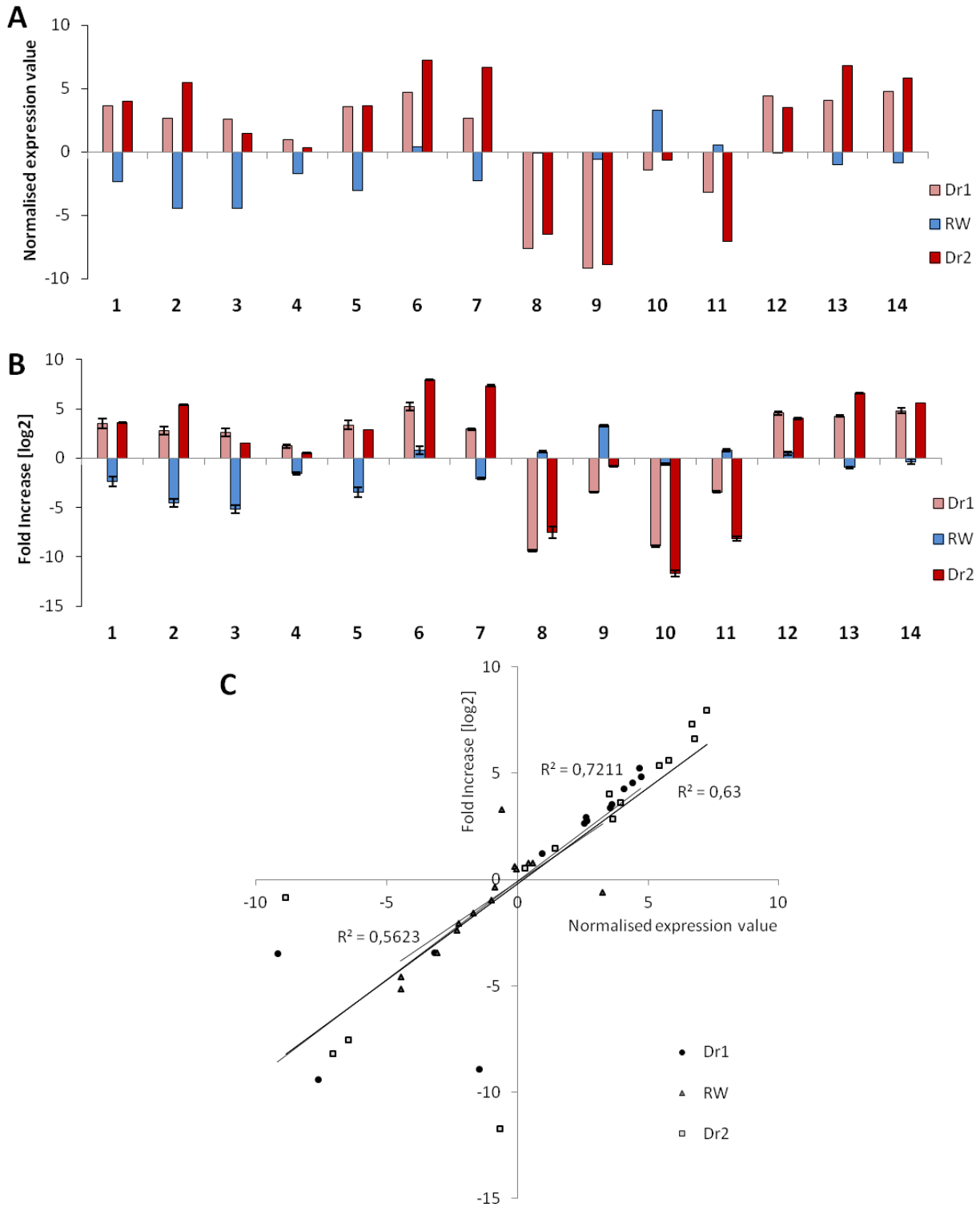

Figure 58. qRT-PCR validation of RNA-sequencing data on 14 selected genes. (A) Expression value detected by RNA sequencing method. (B) Expression analysis conducted by qRT-PCR. Data have been plotted on a log2 scale. (C) Correlation between RNA sequencing and qRT-PCR data. The normalised expression value obtained with RNA sequencing (x axis) were compared to the log2 of fold increase by qRT-PCR (y axis). Dr1: 16 days of drought stress; RW: 7 days of re-irrigation and Dr2: 6 days of second drought stress. RNA from well-watered control plants was used as calibrator sample.

Seven clusters of DEGs were selected for further investigation based on their similar expression patterns. Among them, clusters 1, 2, 14, 17, and 18 included genes with a higher expression level in

WW and RW, while the remaining two clusters 7 and 20 were composed of transcripts with higher expression in Dr1 and Dr2 stressed plants (Figure 59A). Interestingly, clusters containing genes repressed during drought contained several histone variants and chlorophyll binding proteins. Several heat shock proteins and a heat shock factor also appeared to be induced by drought. GO enrichment analyses were performed on clusters 1, 2, 14, 17, and 18 and clusters 7 and 20 independently (Figure 59B). These analyses showed that genes related to photosynthetic light harvesting (such as Chlorophyll a/b binding protein, *Solyc08g067320*) and to modification of cell wall (i.e. Pectinesterase, *Solyc09g075350*) were down-regulated in Dr1 and Dr2. Several genes encoding sucrose and starch metabolic processes were also down-regulated.

GO categories enriched in clusters 7 and 20, instead, were more specifically related to stress, including classes such as response to water stimulus (members included dehydrin, *Solyc01g109920.2*) and water deprivation (including genes such as 2 NAC domain encoding IPR003441-*Solyc12g013620.1/Solyc07g063410.2*). Transcripts coding for proteins involved in protein folding (Peptidyl-prolyl cis-trans isomerase *Solyc09g092690.2* and heat shock protein *Solyc03g117630.1*) were also induced by water stress.

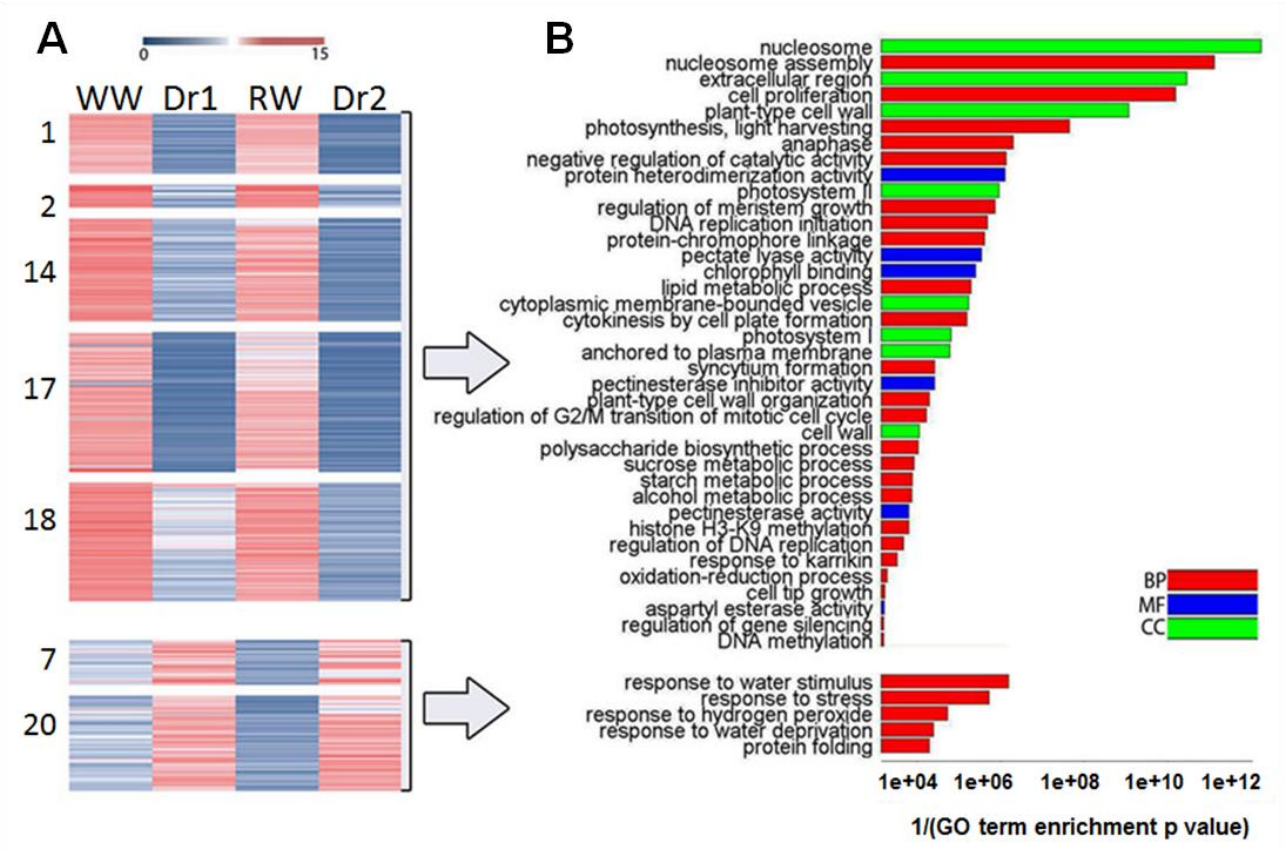

Figure 59. A) Heatmap of selected clusters of Differentially Expressed Genes showing their expression behavior. Red and blue indicate higher and lower expression values, respectively. B) Barplot showing GO Enrichment Analyses (goseq R package, FDR ≤ 0.05) of clusters 1, 2, 14, 17, 18 and 7, 20 independently, plotting GO terms (y axis) and the reciprocal of enrichment p value (x axis). Colors indicate GO ontology: red for Biological Process (BP), blue for Molecular Function (MF) and green for Cellular Component (CC). WW: well-watered plants; Dr1: 16 days of drought stress; RW: 7 days of re-irrigation and Dr2: 6 days of second drought stress.

3.4 Discussion

3.4.1 Comprehensive picture of tomato responses to drought and recovery

In the present study, we have provided a detailed picture of physiological, metabolic and molecular adjustments employed by adult plants of tomato when exposed to events of prolonged water stress, whose imposition was assessed by detailed monitoring of soil water content (θ, Figure 55) and stomatal conductance (g_s ,Table 13). Leaf water status was strongly impaired by drought stress, as indirectly confirmed by the very low values in both these two parameters observed at Dr1 and Dr2. Easlon and Richards (2009) reported at water content of about 30% of fully irrigated soil low values of both gs and CO_2 assimilation (*A*), quite comparable to our data at Dr1 when θ ranged around 30% of well irrigated treatments (Table 13). Lutfor Rahman et al. (1999) found in four tomato genotypes that after ca. 10 days of interrupted irrigation, soil water content and g_s decreased to values quite similar to our data. On this basis, we can admit a condition of severe water stress when plants approached Dr1 or Dr2. These values are consistent with the effects observed in response to rewatering. Upon rehydration, a prompt recovery of *A* in response to stomata reopening would exclude the impairment of the photosynthetic machinery (Cornic, 2000). We found a moderate recovery in both *A* and g_s after one day of soil rewatering. Chaves et al. (2009) reviewed that a recovery in *A* of about 50% within a day from rewatering indicates severe water stress and that a few more days are required to reestablish the photosynthetic machinery as we observed in our experiments.

A significant accumulation of ABA and proline was observed in Dr1 and Dr2 compared to control and rewatering conditions (Figure 56), further indicating a condition of severe water stress (Claussen, 2005).

Interestingly, ABA values were not higher in Dr2 compared to Dr1, in disagreement with a previous report in which successive cycles of drought induced higher levels of ABA accumulation (Muñoz-Mayor et al., 2012). This discrepancy could be dependent on the different genotypes used (Amjad et al., 2014) or it could be an indication that drought stress intensity was different in the two conditions.

At the molecular level, several transcription factors were up-regulated during drought stress. Tomato heat stress transcription factor *HsfA3* was up-regulated in drought stress conditions, which probably accounts at least partially for the observed up-regulation of several members of the Heat shock protein family.

Two isoforms of subunit A of Nuclear factor Y (NF-Y), encoding orthologues of Arabidopsis *NF-YA7* and *NF-YA10* were also induced by drought stress. NF-Y is a heterotrimetric transcription factor whose subunit A, encoded in *Arabidopsis* by a 10 member gene family, is responsible for binding to

DNA promoter sequences containing the CCAAT-box. Over-expression of NF-YAs including NF-YA7 and NF-YA10 causes a dwarf phenotype and an increase in stress tolerance. Expression of NF-YA transcripts is stress-inducible and is inhibited in *Arabidopsis* in control conditions by miR169, a microRNA present in several isoforms (Li et al., 2008; Leyva-González et al., 2012).

Interestingly, a different mechanism was described in tomato, where a positive correlation was described between miR169c expression and drought stress tolerance (Zhang et al., 2011). Expression of miR169c was induced by drought stress and down-regulated by *SlNF-YA1, SlNF-YA2, SlNF-YA3* and an additional target not described in Arabidopsis, *SlMRP1*. Over-expression of miR169 caused increased drought tolerance, resulting at least partially from a decreased stomatal opening and water loss (Zhang et al., 2011). Drought stress-induced up-regulation of miR169 was also observed in rice and *Glycine max*, possibly indicating that the duration and intensity of the drought stress as well as the plant physiological stage in which the stress is experienced can result in different outcomes of miR169 and its relative targets expression (Ferdous et al., 2015).

The presence of high levels of ABA (Figure 56) and of several targets of the ABA transduction pathway among the drought up-regulated genes, including *RD29B* (*Solyc03g025810.2*) and several LEA proteins indicates that such pathway is active in tomato after prolonged drought stress.

However, the concomitant up-regulation of inhibitors of the ABA signaling cascade such as putative orthologues of Arabidopsis *PP2CA* (Solyc03g096670.2), *MFT* (*SELFPRUINING 2G, Solyc02g079290.2*), and *AFP3* (*Solyc05g012210.2*; Garcia et al., 2008) indicates that a negative feedback loop is also in place. A similar situation was observed in *Arabidopsis* plants exposed to moderate drought stress, where the up-regulation of effectors of the ABA response as well as of negative regulatory components was observed (Clauw et al., 2015).

Drought stress caused induction of 3 NAC domain-containing transcription factors, two of which encode *JA2* (*Solyc12g013620*) and *JA2like* (*JA2L, Solyc07g063140*), recently shown to have antagonistic roles in stomatal movements in tomato during pathogen attack (Du et al., 2014). *JA2* is itself induced by ABA and promotes stomatal closure through induction of expression of the ABA biosynthetic gene *NCED1*. In contrast, *JA2L* is induced by the bacterial virulence factor Coronatine and is proposed to have a role in stomatal reopening by regulating the expression of genes involved in Salicylic acid metabolism (Du et al., 2014). The presence of both *JA2* and *JA2L* in our list of drought induced genes indicates that these two TFs might also be involved in abiotic stress-triggered stomatal movements and represents a further indication that antagonistic pathways balance responses and concur to the final balance of physiological adjustments.

The GO Enrichment analysis of the categories over-represented in clusters showing interesting patterns allowed for the identification of specific functions regulated by drought stress (Figure 59).

Several GO categories related to photosynthesis, such as "photosystem I", "photosystem II", "chlorophyll binding" and "photosynthesis, light harvesting" were enriched in clusters comprising genes with higher expression in well-watered rather than drought stressed samples, possibly indicating a reduced synthesis of components of the photosynthetic machinery. This correlated with the low photosynthetic assimilation rate observed in drought stressed plants compared to the controls or rehydrated plants. A similar down-regulation of photosynthetic genes was observed in a progressive drought stress treatment on Arabidopsis plants, while in a situation of moderate drought stress the photosynthesis rate and expression of photosynthetic genes were not affected (Harb et al., 2010), suggesting that the mode of drought stress application and the intensity of the stress influence the impact on the photosynthetic machinery. Concomitant with the downregulation of components of the photosystem under drought, we observed an upregulation of a FtsH homologue (*Solyc03g112590.2*) with a predicted chloroplast target peptide. FtsHs are ATP-dependent zinc metalloproteases, which, in chloroplasts, have been suggested to be involved in the turnover of the oxidized D1 protein of the photosystem II (PSII) reaction center during recovery from photoinhibition (Lindahl et al., 2000; Bailey et al., 2002). Recently, Zhang and colleagues (2014) observed a progressive and constant upregulation of FtsH in *Medicago truncatula* subjected to drought stress and suggested a role in the repair of PSII damages resulting from drought-induced oxidative stress (Zhang et al., 2014).

Reduction of leaf growth occurs as a result of reduced cell division and/or cell expansion. Analysis of the GO enrichment categories suggests that both cell division and expansion are affected in tomato during drought stress. Categories such as “Cell Proliferation”, “Anaphase”, “Regulation of DNA replication” were enriched in well-watered rather than drought stressed samples, indicating that cell division could be repressed during drought.

Histones were among the gene families with more down-regulated members. Histone expression is regulated during the progression of the cell cycle and tightly connected to DNA replication (Meshi et al., 2000; Rattray and Muller, 2012). The observed repression of the expression of several H3 and H4 isoforms, concomitant with repression of genes such as a DNA topoisomerase, 2 DNA polymerase, Single-stranded DNA-binding replication protein A large subunit, Single-stranded DNA binding protein p30 could be an indication of a reduction in cell division, which could be one of the factors leading to growth reduction in stress conditions. The observed repression of histone expression under drought is consistent with a recent report in rice showing that expression of several histone isoforms was reduced upon salt and drought treatment (Hu and Lai, 2015).

Among the GO categories enriched in gene clusters down-regulated by drought stress we identified the cellular compartment "plant-type cell wall", "cell wall" and the molecular function "pectate lyase

activity" and "pectinesterase activity", suggesting that cell wall modifying activities are repressed after prolonged drought stress.

Together, the observed results give a comprehensive picture of whole plant responses to drought stress and recovery. Based on the cluster analysis of the obtained differentially expressed genes, future work will be directed toward a functional characterization of genes with a previously uncharacterized involvement in drought response. Their probable roles in adaptation mechanisms make them interesting targets for future applications.

3.5 References

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4. Conclusions

The present work was aimed to contribute to the knowledge of molecular mechanisms involved in the plant response to osmotic stress, using genomic approaches and specific advanced molecular technologies. In particular, we used *Arabidopsis thaliana* as tool for i) the detailed analysis of AtRGGA, a previously identified gene in the lab of CNR-IBBR and ii) the characterization of three genes whose function in stress response was so far unknown. These were: - the splicing factor *DRT111* (At1g30480), - the negative regulator of TOR pathway *TIP41-like* (At4g34270), and - the subunit of Pol III *SIN-like* (At5g49530). Furthermore, this study also gave a complete picture of the mechanisms employed to maintain a cellular and whole plant homeostasis in one of the major crop species, *Solanum lycopersicum* during drought stress.

In *Arabidopsis,* the results can be summarized as follows:

- *DRT111* is induced upon long-term exposure to ABA and PEG and is mainly expressed in trichomes and stomata, organs controlling transpiration. Germination analysis on knockout mutants and *DRT111* overexpressing plants as well as the reported association with another important splicing factor SUA and the protein localization into the nucleus, suggest that DRT111 may be involved in pre-mRNA splicing of ABI3, regulating ABA-related seed germination.
- *TIP41-like* is constitutively expressed in vascular tissues and is up-regulated by long-term exposure to NaCl and ABA. The abolished expression of *TIP41-like* leads to ABA hypersensitive phenotypes at germination and seedling stage as well as reduction in root development. On the basis of our results and literature evidences, we suggest that this cytoplasmic protein may act as a negative regulator of another component of TOR pathway, TAP46 in the stabilization of ABI5 during seed germination, and possibly affects several pathways in the ABA-mediated response to osmotic stress.
- *SIN-like*, constitutively expressed in all tissues, encodes a nuclear protein. Knockout mutants show severe reduction in root growth. Therefore, we suggested that the mutation in *SIN-like* leads to phenotypes mainly associated with altered cell development that could be explained by an alteration of RNAs transcribed by Pol III.
- The *AtRGGA* gene is induced by long-term exposure to PEG and ABA. Phenotype analyses performed on plants with modified *AtRGGA* expression indicate that the gene is involved in tolerance to drought and salt stress. AtRGGA protein binds ribosomal RNAs *in vitro* and *in vivo.* Finally, using the yeast two-hybrid method we identified several putative AtRGGA partners that are mainly involved in RNA processing, transport and ribosome biogenesis.

Therefore, we conclude that AtRGGA plays an important role in transcriptional and post-transcriptional control of gene expression during osmotic stress.

The analysis of physiological, biochemical and molecular responses in tomato plants induced by two cycles of prolonged drought stress and one of rewatering, gave a comprehensive picture of whole plant responses to drought. The results obtained can be summarized:

- Drought leads to a decrease of leaf gas exchanges, such as stomatal conductance and CO_2 assimilation, and a consistent accumulation of proline and ABA to maintain a cellular and whole plant homeostasis;
- Global transcriptome profiling showed 966 Differentially Expressed Genes (DEGs). Cluster analysis of the DEGs, indicated that water stress mainly results in down-regulation of gene expression with only a small subset of up-regulated genes. Gene Onthology (GO) categories such as cell proliferation and cell cycle were significantly enriched in the down-regulated fraction of genes upon drought stress, indicating that cell division could be repressed leading to growth reduction during drought. While, the categories of up-regulated fraction were more specifically related to stress, including classes such as response to water stimulus and water deprivation.

  We conclude that the physiological responses and gene expression are closely interconnected. Therefore, analysis of these results has given interesting candidate genes that could play novel roles in drought tolerance and adaptation.

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