Novel mechanisms and transcription factors involved in the control of stomatal behavior in *Arabidopsis thaliana*

Nuevos mecanismos y factores de transcripción involucrados en el control del comportamiento estomático en *Arabidopsis thaliana*

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Memoria presentada por **Tommaso Legnaioli**, Licenciado en Biología Molecular, para optar al grado de **Doctor por la Universidad de Barcelona**

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I. INTRODUCTION

The importance of stomatal function

The increase in water use efficiency in order to maintain or improve crop yields in times of global warming and progressive reduction of water resources is one of the major challenges faced by agricultural sciences in the last decades (Battisti & Naylor, 2009; Boyer, 1982; Cominelli et al, 2009). Addressing this issue at a plant physiology level lead us to consider which are the mechanisms plants use to survive and to optimize growth under restrictively dry environmental conditions. Most of plants water losses are due to transpiration because of their crucial need of carbon dioxide from the external atmosphere in order to perform photosynthesis. The stomatal pores are the main gates between the apoplast and the external atmosphere, driving over 95% of plants water losses (Schröder et al, 2001). The regulation of stomatal pore aperture is, therefore, the main tool plants use to control gas exchanges with the external atmosphere. A stomate is constituted by two guard cells surrounding the stomatal pore: an increase of the turgor of these bean shaped cells implies the widening of the stomatal aperture, their deflation, in turn, reduces the size of the pore. Stomatal movements are therefore meant to regulate the balance between water retain and CO_2 assimilation. Accordingly to its pivotal function, stomatal behaviour relies on the integration of a huge number of physiological and external signals into a complex network capable of responding to a wide spectrum of environmental conditions. The comprehension of these mechanisms is therefore considered a critical step towards the achievement of an improvement in plants water use efficiency (Cominelli et al, 2009; Ingram & Bartels, 1996; Schröder et al, 2001).

Mechanisms of Abscisic acid-mediated stomatal movements

Biosynthesis, catabolism and transport of ABA

Abscisic acid (ABA) plays a key role in the induction of stomatal closure by a mechanism of action that has been partially discovered and characterised (Joshi-Saha et al, 2011; Raghavendra et al, 2010; Umezawa et al, 2010; Weiner et al, 2010).

The biosynthetic pathway that converts the carotenoid precursor Zeaxanthin into Abscisic acid is still partially unclear but it has been suggested that the limiting reactions are the ones catalyzed by 9-cis-epoxycarotenoid dioxygenases (NCEDs). Abscisic acid levels in the plant are also regulated by catabolism through ABA 8'-hydroxylation, catalyzed by the enzymes of the CYP707A family (Nambara & Marion-Poll, 2005). In response to drought stress, the transcription of *NCED*s is induced and ABA is synthesized in roots and vascular tissues of the leaves and transported through the plant xylematic vessels and the apoplast towards the stomatas; conversely, rehydration induces *CYP707A*s transcription and ABA degradation (Kanno et al, 2010; Seo & Koshiba, 2011).

In *Arabidopsis* it has been demonstrated that ABA is released in the apoplast through the action of specific carriers like the ABA transporter *At*ABCG25 (Kuromori et al, 2010). In the same model plant, recent studies have revealed that ABC transporters also account for abscisic acid uptake by the guard cells (Kang et al, 2010; Kanno et al, 2012).

ABA perception and signal transduction

After decades of research in the field, in 2009, two studies provided the lacking keystone to understand the mechanism of intracellular perception of

ABA in *Arabidopsis thaliana*. Both groups described multimeric complexes of variable composition as ABA receptors. The novel players placed by these studies on the field of ABA binding and sensing are the elements of a protein family counting 14 members in *Arabidopsis thaliana*, which are characterised by their homology with STeroidogenic Acute Regulatory lipid Transfer (START) proteins (Ma et al, 2009; Park et al, 2009). The PYR/PYL/RCAR proteins (PYRABACTIN RESISTANCE/ PYRABACTIN RESISTANCE 1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR) were identified as ABA receptors on one side through the use of pyrabactin, a molecule mimicking ABA (Park et al, 2009) and, at the same time, by screening for interactors using a protein phosphatase from clade A of family 2C (clade A PP2C) (Ma et al, 2009). The interaction of these receptors with ABA seems in fact to disrupt a dimeric form, favouring the contact with a coreceptor: a member of the clade A PP2C (Dupeux et al, 2011). These phosphatases had already been described as negative regulators of ABA signalling since the nineties through the discovery of ABA INSENSITIVE (ABI) *gain of function* mutants corresponding to the genes *ABI1* and *ABI2* (Leung et al, 1994; Leung et al, 1997; Meyer et al, 1994) and later with the discovery of the *loss of function* mutant HYPERSENSITIVE TO ABA 1 (HAB1) (Sáez et al, 2004). Structural studies delineated the physical interactions between ABA, PYR/PYL/RCAR and PP2C explaining that the binding of abscisic acid resulted in an inactivation of the protein phosphatase. This inactivation is the unleashing event of ABA signal transduction cascade (Miyazono et al, 2009; Nishimura et al, 2009; Yin et al, 2009). *Arabidopsis thaliana* counts with a huge amount of possible combinations between PYR/PYL/RCARs and PP2Cs and the different combinations of receptor complexes present different characteristics in ABA selectivity and sensitivity, possibly deriving in different ways of activation of the pathway (Szostkiewicz et al, 2010).

The following steps in abscisic acid signalling are mediated by various kinases: some of which respond to ABA through the second messenger Ca^{2+} (CALCIUM-DEPENDENT PROTEIN KINASES), while others represent the master switch upstream of any secondary signal. The latter were identified as

key players in the early phase of ABA response in *Vicia faba* (Li & Assmann, 1996; Li et al, 2000) and later on in *Arabidopsis* (Mustilli et al, 2002) and classified as type 2 Snf1-related kinases (SnRK2). Further works explained that PP2Cs negative action on ABA signalling is due to their repressing action on SnRK2 activity (Umezawa et al, 2009; Vlad et al, 2009; Yoshida et al, 2006)as well as on CALCIUM-DEPENDENT PROTEIN KINASES (CPK) (Lee et al, 2009a). The SnRK2 kinases family counts 10 members in *Arabidopsis*, being 3 of theme (SnRK2.2, SnRK2.3 and SnRK2.6/SRKE/OST1) the triggers of most of ABA responses in the plant (Fujii et al, 2011; Fujii & Zhu, 2009). *In vivo* and *in vitro* data suggest that ABI1, PYR/PYL/RCARs and SnRK2s interact in what seems to be a signalosome in charge of triggering ABA signalling transduction pathway (Fujii et al, 2009; Nishimura et al, 2010).

Molecular and biophysical basis of stomatal opening and closure

The stomatal pore open state requires an elevated turgor of the guard cells through the action of the H^+-ATPases that polarize the plasma membrane allowing an inward flux of K^+, which reduces water potential thus sustaining osmotic pressure (Shimazaki et al, 2007). The inward rectifying channel responsible for the observed K^+ flux was identified in KAT1, whose activity is blocked through phosphorylation by OST1 kinase in response to ABA signalling (Sato et al, 2009). Another important target of OST1 is AtrbohF, a catalytic subunit of a membrane NADPH oxidase (Kwak et al, 2003; Sirichandra et al, 2009). This phosphorylation event triggers the activity of the enzyme, which leads to the rapid increase in citoplasmic Reactive Oxigen Species (ROS) often called oxidative burst. Following the ROS production and diffusion, Ca^{2+} is released in the cytoplasm from intracellular compartments and from the apoplast (Cho et al, 2009); consequently, CALCIUM-DEPENDENT PROTEIN KINASES (CPKs) are activated.

A major contribution to the biophysics of stomatal closure is also given by the outwards anionic currents observable in the guard cells in response to ABA. The closing stimulus generates, in fact, a first rapid (R-type) anion current that is

followed by a second slow one (S-type). The R-type ion current is mediated in part by the ALLUMINIUM-ACTIVATED MALATE TRANSPORTER 12 (AtALAMT12), although the mechanism underlying the activation of these channels by ABA is yet unknown (Meyer et al, 2010). On the other hand, the S-type anion current in guard cells has been related with SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), which has at least 2 functional homologues in *Arabidopsis*: SLAC1-HOMOLOG (SLAH) 1 and 3 (Negi et al, 2008; Vahisalu et al, 2008). The activity of SLAC1 is gated by phoshporylation events, representing an important control switch for the transduction of the closing signal. The channel is, in fact, activated by OST1 (Geiger et al, 2009) and its activity is further induced in response to Ca^{2+}, probably by a mechanism involving CPK21 and CPK23 (Geiger et al, 2010). Conversely, SLAC1 is blocked by the activity of ABI1 and ABI2 phosphatases (Geiger et al, 2010; Geiger et al, 2009). It has been reported that one of the homologues of SLAC1, SLAH3, accounts for part of the S-type anion current and that its activity is induced by CPK21 (Geiger et al, 2011; Ma & Wu, 2007). Moreover, the S-type current is also favoured by the activity of two more CPKs: CPK3 and 6 (Mori et al, 2006). The anion currents mentioned above contribute to the depolarization of the membrane observed in response to ABA although a major contribute is also given by the blockage of the active transport of H+ out of the membrane. A screening for impaired stomatal response to drought led to the discovery of *ost2* (OPEN STOMATA 2) mutant, presenting a constitutively active H^+-ATPase that resulted in constant membrane polarization thus impeding stomatal closure (Merlot et al, 2007). The activity of this ATPase is normally blocked in response to ABA through the action of another Ca^{2+} dependent kinase, PROTEIN KINASE SOS2-LIKE 5 (PSK5) (Fuglsang et al, 2007). The depolarization of the membrane results in an alkalinization of the citosol, which leads to the activation of a GATED OUTWARDLY-RECTIFYING K^+ CHANNEL (GORK). This channel produces an outward K+ current that compensates the charge unbalance due to the exit of anions and contributes to reduce the osmotic pressure sustaining the guard cells turgor thus ultimately favouring their shrinkage and the closure of the stomatal pore (Hosy et al, 2003; Li et al, 2006).

Other proteins have been implied in ABA perception and signalling in guard cells that don not fit in the pathway described above. It is the case of the GPCR-type G proteins GTG1 and GTG2, previously proposed as abscisic acid receptors; which supported the hypothesis of extracellular reception and explained the involvement of G-protein mediated signalling in ABA response (Pandey et al, 2009). Another controversial putative receptor is CHLH/GUN5/ABAR, the H subunit of a magnesium-chelatase, whose ability of binding ABAR is yet discussed (Shen et al, 2006; Tsuzuki et al, 2011; Wu et al, 2009a), though it is now clear that it takes part in the process of ABA signal transduction and ultimately leads to transcriptional activation of ABA responsive genes through the inhibition of three WRKY transcription factors (Shang et al, 2010; Tsuzuki et al, 2011).

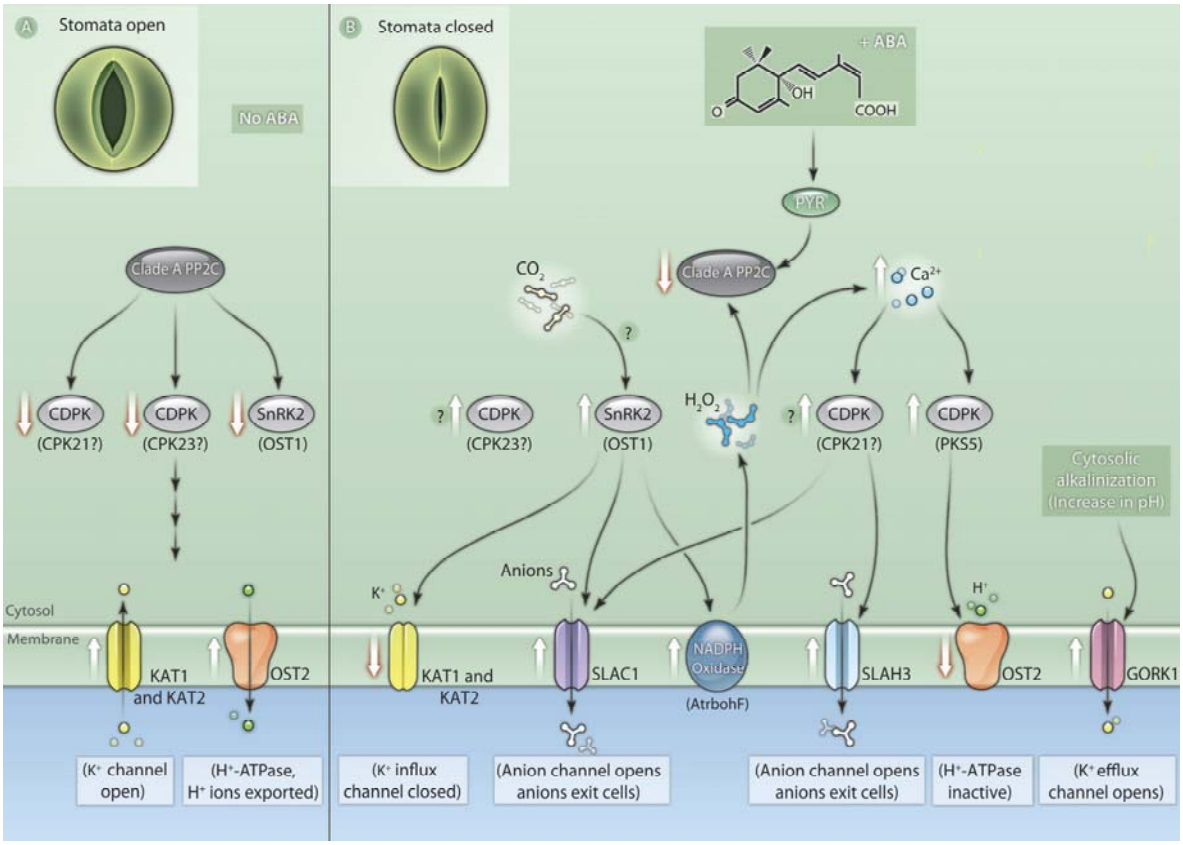

Figure 1: Scheme of the molecular mechanisms underlying stomatal behaviour. From Joshi-Saha et al, 2011; Science Signalling.

The transcriptional response to ABA and drought

Several studies have put in evidence the huge impact that abscisic acid signalling has on *Arabidopsis* transcriptome; in addition the transcriptional regulation has a huge impact on the ability of the plant to resist to drought stress (Fujita et al, 2011; Shinozaki et al, 2003).

Most of the transcriptional responses unleashed by ABA in guard cells involve genes displaying ABA responsive elements (ABRE) in their promoters (Busk & Pagès, 1998; Giraudat et al, 1994; Hattori et al, 2002; Zhang et al, 2005). Abscisic acid transcriptional responsiveness was connected to the presence of an ABRE and one or more *coupling elements* (CE) such as DEHYDRATION RESPONSIVE ELEMENTS/C-REPEAT (DRE/CRT) (Hobo et al, 1999; Narusaka et al, 2003; Shen et al, 2006; Skriver et al, 1991). Furthermore the effectiveness of couples of ABRE *cis*-elements was demonstrated in *Arabidopsis* and rice (Gómez-Porras et al, 2007).

The characteristic sequence of the ABRE (PyACGTGG/TC) presents an ACGT core that is the typical binding sequence for basic leucine zipper domain (bZIP) transcription factors in plants (Foster et al, 1994). The transcription factors responsible for ABRE related regulation of transcription belong to group A of the bZIP family (Choi et al, 2000; Uno et al, 2000). These transcription factors can be divided in two groups: those responsible for ABA signalling in the seed: the members of the ABI5/AtDPBF family; and those expressed in the vegetative tissues in response to stress: the four *ABF/AREB*s (*ABSCISIC ACID RESPONSIVE ELEMENTS BINDING FACTORS*) (Kim, 2006). In particular, *ABF1* seems to respond to cold, while *AREB1/ABF2*, *AREB2/ABF4* and *ABF3* are induced in response to osmotic stresses such as drought or salt, as well as in response to ABA (Fujita et al, 2005; Yoshida et al, 2010). Interestingly, it has been shown that these three transcription factors require ABA in order to achieve their full activation (Fujita et al, 2005; Yoshida et al, 2010). Moreover, it has been demonstrated that SnRK2s are implied in ABA dependent phosphorylation of AREB1/ABF2 in *Arabidopsis* connecting its function directly to the abscisic acid signalling core (Furihata et al, 2006). This mechanism has

also been described in wheat (Johnson et al, 2002) and in rice (Kobayashi et al, 2005). Also, the ABA-dependent phosphorylation of AREB/ABFs by SnRK2s has also been demonstrated in vivo (Kline et al, 2010). It is clear now that *AREB1/ABF2*, *AREB2/ABF4* and *ABF3* play a pivotal role in *Arabidopsis* response to dehydration, since their over-expression increase drought resistance and ABA sensitivity (Kang et al, 2002) and their triple mutation produces the opposite effects (Yoshida et al, 2010). At the transcriptomical level, the triple mutant obtained in *Arabidopsis* presents a response to ABA similar to the *SnRK2*s triple mutant, thus underlying the tight functional relation between these two groups of ABA signalling effectors (Fujita et al, 2011; Fujita et al, 2009b; Yoshida et al, 2010). Besides the AREB/ABF, a great number of transcription factors from different families and plant species has been characterised for their impact on plants response to ABA and osmotic stresses like dehydration or salt toxicity (Fujita et al, 2011), like, for example, DRE/CRT BINGING PROTEINs (DREB1/CBFs), a group of transcription factors that were characterised for their specific binding to DRE/CRT *cis*-elements (A/GCCGAC) (Agarwal et al, 2006; Kikis et al, 2005) and were shown to interact with members of AREB/ABF group (Lee et al, 2010).

A global overview of ABA and osmotic stress responsive transcriptome led to the estimation that around 10% of the known coding transcripts of Arabidopsis thaliana are regulated by abscisic acid (Nemhauser et al, 2006; Seki et al, 2002). Microarray data suggest that ABA induced genes in vegetative tissues include *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) proteins; protein kinases and phosphatases involved in signalling; transporters and enzymes involved in secondary metabolism such as the biosynthesis of osmoprotectants and a wide variety of TRANSCRIPTION FACTORs. On the other hand, the repressed genes correspond to proteins involved in growth and development such as ribosomal components, cell wall expansion associated enzymes, chloroplast and plasma membrane proteins (Fujita et al, 2011; Fujita et al, 2009b; Matsui et al, 2008; Nemhauser et al, 2006; Yoshida et al, 2010). Transcriptomics studies directed specifically to guard cells allowed to understand that, although ABA response maintains its core in this cell type, the

transcriptional profile presents significant differences with other analysed tissues. The subset of guard cell specific ABA responsive transcripts is enriched in elements that may mediate the crosstalk with other hormones, in Ca^{2+}-related genes, in components of light signalling that regulate stomatal movements and in sugar transporters that influence osmotic potential. Moreover, apart from the canonical ABA related *cis* elements, a new guard cell-specific motif (GTCGG) was detected (Wang et al, 2011a).

A wider transcriptomic approach, the whole genome tiling array, revealed that abiotic stress conditions and ABA signalling affect a considerable amount (5-10%) of the observed non-protein-codifying transcriptional units (non-AGI TUs). In most cases the ABA/stress-responsive observed non-AGI TUs do not present any ABRE in their promoting regions. Interestingly, the 80% of these TUs produce one of the strands of a double stranded sense-antisense transcript (SAT) (Matsui et al, 2008; Zeller et al, 2009). While the overall function of these SATs remains unclear, an increasing number of small RNAs is being related to the response to abiotic stresses. In particular, several micro RNAs (miRNA), conserved among different species of plants, have been characterized for their action in abiotic stress signalling through the identification of their targets (Covarrubias & Reyes, 2010; Khraiwesh et al, 2012).

The transcriptional regulation mediated by ABA and drought stress relies also on chromatin modification and remodelling mechanisms, which are believed to be implied in the priming of the response (Kim et al, 2010; Luo et al, 2012). This is the case of histone deacetylation, which affects ABA response both at the level of HISTONE ACETYL TRANSFERASES (HATs) (Zhou et al, 2009) and of HYSTONE DEACETYLASES (HDAs) (Sridha & Wu, 2006). Another example is the ATP-dependent chromatin remodelling complex SWITCH SUBUNIT 3 B (SWI3B), whose activity favours the expression of ABA responsive genes and that is in turn repressed by HAB1 (Sáez et al, 2008).

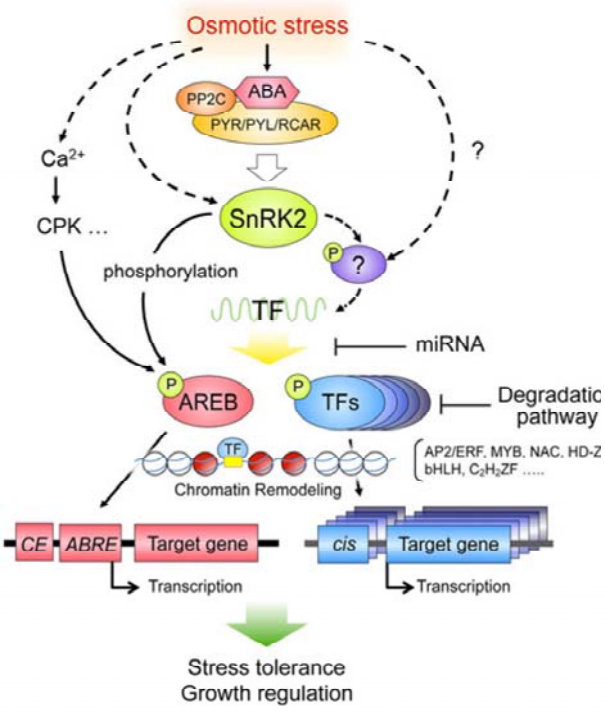

Figure 2: Schematic representation of the mechanisms responsible for ABA-dependent transcriptional changes.

From Fujita et al, 2011; Journal of plant research.

Overview of transcription factors, drought responses and stomata

We therefore know that the core of ABA signalling is brought to the transcriptional level by the AREB/ABFs, nevertheless, the action of these TFs unleashes several other transcriptional responses that are still poorly characterised. Aside of AREB/ABFs, another important category of TFs involved in ABA response is the DRE/CRT BINGING PROTEINs (DREB1/CBFs). These APETALA2/ETHILENE RESPONSIVE FACTORs (AP2/ERFs) were characterised for their specific binding to DRE/CRT *cis*-elements (A/GCCGAC) (Agarwal et al, 2006; Kizis et al, 2001). The 4 member of *DREB1* gene family show transcriptional activation in response to ABA: *DREB1D*/*CBF4* transcription is enhanced by osmotic stress, while *DREB1A-C*/*CBF1-3* respond to cold (Haake et al, 2002; Knight et al, 2004). Other member of the family, *DREB2A* and *DREB2B*, are induced in response to osmotic stress and high temperature (Liu et al, 1998; Nakashima et al, 2000; Sakuma et al, 2006). Finally DREB1A/CBF3, DREB2A and DREB2C have been reported to interact physically with AREB/ARF transcription factors (Lee et al, 2010). The AP2/ERF transcription factor family also presents negative regulators of ABA signalling like RAP2.1, down-regulator of drought and cold responses (Dong & Liu, 2010),

ABA REPRESSOR 1 (ABR1) (Pandey et al, 2005), and ERF7 (Song et al, 2005). The latter has been shown to produce stomatal aperture. Moreover ERF7 appears to be regulated through SnRK3.1-mediated phosphorylation and to interact with ABI2: strengthening the idea of its implication in ABA signalling down-regulation (Guo et al, 2002). Conversely, another AP2 TF, ARIA INTERACTING DOUBLE AP2 DOMAIN PROTEIN (ADAP) may be part of a transcriptional activation complex including ARM REPEAT PROTEIN INTERACTING WITH ABF2 (ARIA) and AREB1/ABF2 (Lee et al, 2009b). Moreover, RAP2.6 binds to CE elements regulating osmotic stress and ABA responses (Zhu et al, 2010) integrating theme with jasmonate response (Wang et al, 2008) and bacterial pathogen defense (He et al, 2004). Important roles in drought response are also played by other types of transcription factors: the MYB transcription factor MYB102 functions in response to wounding induced dehydration by binding to ABRE-CE1 sequences (Denekamp & Smeekens, 2003). MYB41, a transcription factor sharing high homology with MYB102, is expressed in response to drought and ABA and mediates cuticle and cell wall modifications induced by abiotic stress (Cominelli et al, 2008). Another family member, the ABA-induced MYB44, participates in stomatal response to ABA by down-regulating PP2Cs consequently increasing drought tolerance (Jung et al, 2008) as well as down-regulating Jasmonic acid signalling (Jung et al, 2010). In the same protein family, the two close homologues MYB60 and MYB61 are specifically expressed in guard cell: the first was shown to be expressed in response to blue light and to induce stomatal opening (Cominelli et al, 2005), while the second has the opposite effect in absence of light (Liang et al, 2005). MYB96, in turn, participates in ABA signalling favouring the expression of *RESPONSIVE TO DESSICATION 22* (*RD22*) (Seo et al, 2009). Another MYB transcription factor capable of influencing stomatal behaviour is MYB15. The over-expression of this transcription factor, in fact, increases stomatal ABA sensitivity thus improving drought tolerance (Ding et al, 2009). The couple of TFs MYB2 and MYC2 also participate in ABA-mediated the activation of *RD22* (Abe et al, 2003). The bHLH family TF MYC2 is also believed to be a key piece in the integration of the signalling pathways of several hormones in response to light, biotic and abiotic stress (Fujita et al, 2009a). Further more, the ABA

INDUCIBLE BHLH transcription factor (AIB) takes part in ABA mediated drought response (Li et al, 2007). Members of the ARABIDOPSIS NAC TRANSCRIPTION FACTORS (ANAC) family also contribute to drought response. In particular *ANAC019*, *ANAC055*, *RD26*/ *ANAC072* and *ATAF1* are up-regulated by ABA and dehydration (Fujita et al, 2004; Tran et al, 2004). While ANAC019, ANAC055 and RD26/ ANAC072 are positive regulators of ABA signalling (Fujita et al, 2004; Jensen et al, 2010; Tran et al, 2004); ATAF1, displays a similar function although it seems to down-regulate biotic stress responses (Jensen et al, 2008; Wu et al, 2009b). Moreover *ANAC019* and *ANAC055* modulate ABA and jasmonate signalling regulating the E3 ligase activity of the RING finger protein RINGH2 FINGERA2A (RHA2a) (Bu et al, 2008; Bu et al, 2009). Moreover, three members of class I HD-Zip transcription factors, are transcribed in response to ABA and dehydration: HOMEOBOX PROTEIN 6 (HB6) (Söderman et al, 1999), HB7 (Söderman et al, 1996) and HB12 (Henriksson et al, 2005). The first one was also shown interact with ABI1 and act as negative regulator of ABA signalling (Himmelbach et al, 2002). The C2H2 group of TFs, in turn, counts with two ABA and drought responsive genes: *ZINC FINGER PROTEIN 2* (*AZF2*) and *SA AND ABA DOWNREGULATED ZINC FINGER GENE* (*SAZ*). The first one is induced by ABA, salinity and dehydration by a mechanism involving ABI1 (Sakamoto et al, 2004), and is a transcriptional repressor that down-regulates ABA signalling (Drechsel et al, 2010). Conversely, *SAZ* expression is inhibited, among other stimuli, by ABA, drought and SA, while its function is antagonistic to AREB/ABF-mediated ABA signalling (Jiang et al, 2008). Another mediator of AREB/ABF signalling is ABA OVERLY SENSITIVE MUTANT 3/WRKY TF 63 (ABO3/WRKY63). This protein binds to *AREB1*/*ABF2* promoter in vivo impairing its expression in response to ABA, further more *abo3* mutant displays enhanced ABA sensitivity (Ren et al, 2010). Other WRKY transcription factors: WRKY40, WRKY 18 and WRKY 60, are ABA signalling inhibitors antagonized by the action of ABAR/CHLH/GUN5 (Shang et al, 2010). Finally, a NF-Y family TF, *NUCLEAR FACTOR Y SUBUNIT A5* (*NFYA5*), is induced by ABA and dehydration and controls stomatal aperture. Its expression is, in turn regulated by miRNA169 (Li et al, 2008).

These and other transcription factors have been related with drought response and guard cell turgor control. All together these data reveal the importance of transcription factors from many different families in the control of stomatal aperture.

External stimuli influencing stomatal behaviour

A broad range of environmental information converge on the guard cells where it is integrated in the signalling systems described above in order to optimize the resources usage and minimize the risks.

CO_2

One of the signals influencing guard cells state is the concentration of CO_2. Stomata close in response to high level of carbon dioxide and open when the concentration of this basic reagent for photosynthesis is low (Hu et al, 2010; Negi et al, 2008; Xue et al, 2011). Carbon dioxide is thought to move through plasma membranes by diffusion as well as by mean of specific aquaporins (Uehlein et al, 2003). Decades ago, it was observed that carbonate concentration affects stomatal state (Mrinalini et al, 1982): the condensation of water and CO_2 into HCO_3^- is catalyzed by carbonic anhydrases, which have recently been implied in the stomatal response to carbon dioxide (Hu et al, 2010). The anion channel SLAC1 has been identified as a target of this regulation (Negi et al, 2008) and HCO_3^- is considered essential in this CO_2 mediated activation (Xue et al, 2011). Another important player in this signalling pathway is OST1, which may have a role in it that is functionally independent from ABA receptors (Xue et al, 2011).

Foliar pathogens

Another factor capable of inducing stomatal closure is the presence of foliar pathogens that use stomatal pores as a way to overcome the physical barrier of plant cuticle (Grimmer et al, 2012; Zeng et al, 2010). It has been discovered that several pathogen-microbe associated molecular patterns (MAMP-PAMPs) induce stomatal closure (Melotto et al, 2006). These molecules are generally conserved among pathogenic as well as non-pathogenic species (Mackey & McFall, 2006). Examples of PAMPs are flg22 (from flagellin), elf18 (from ELONGATION FACTOR EF-Tu) and Peptidoglycan, which are typically bacterian; chitin and xylanase from fungi, and glucan and elicitin from oomycetes (Boller & Felix, 2009). The stomatal closure is due to independent pathways that are activated by pathogen related receptors (PRR), each pathogen may activate several PRRs through several PAMPs. The case of flagellin and the related PAMP flg22 is one of the most studied. FLAGELLIN SENSITIVE2 (FLS2) is the PRR responsible for flg22 responsive stomatal closure (Melotto et al, 2006; Zeng & He, 2010). It is known that these signalling pathways share components with abiotic stress response such as the synthesis of ABA, the oxidative burst, the synthesis of NO, the G-protein signalling and the regulation K^+ channels activity (Melotto et al, 2006; Neill et al, 2008; Zhang et al, 2008). On the other hand MAMP-PAMPs response involve also specific components such as the hormone Salycilic Acid (SA), which is epistatic to ABA (Melotto et al, 2006; Zeng & He, 2010). The innate immunity signalling also relies on a MAP kinase phosphorylation cascade triggered by MEKK1 that involves MKK4 and 5 as well as MPK3 and 6. This cascade also leads to transcriptional regulation through the action of WRKY29 and WRKY22 transcription factors (Asai et al, 2002). Moreover, the stomatal closure seems to require the modulation of the activity of H^+-ATPases OST2 and AHA2 (Liu et al, 2009). Moreover the regulator of plant immune response RIN4 (RPM1 INTERACTING FACTOR 4) has been described as OST2 activator, thus favouring guard cell swelling and stomatal opening (Liu et al, 2009). Several plant pathogen use virulence factor in order to open stomata and favour the infection. A well-known example is CORONATINE (COR), produced by several

strains of *Pseudomonas syringae* that are capable of bypassing the flg22-induced stomatal closure (Freeman & Beattie, 2009; Liu et al, 2009; Melotto et al, 2006). The action of CORONATINE probably involves RIN4 (Liu et al, 2009) and is also directed to K^+ channels (Melotto et al, 2006; Zhang et al, 2008).

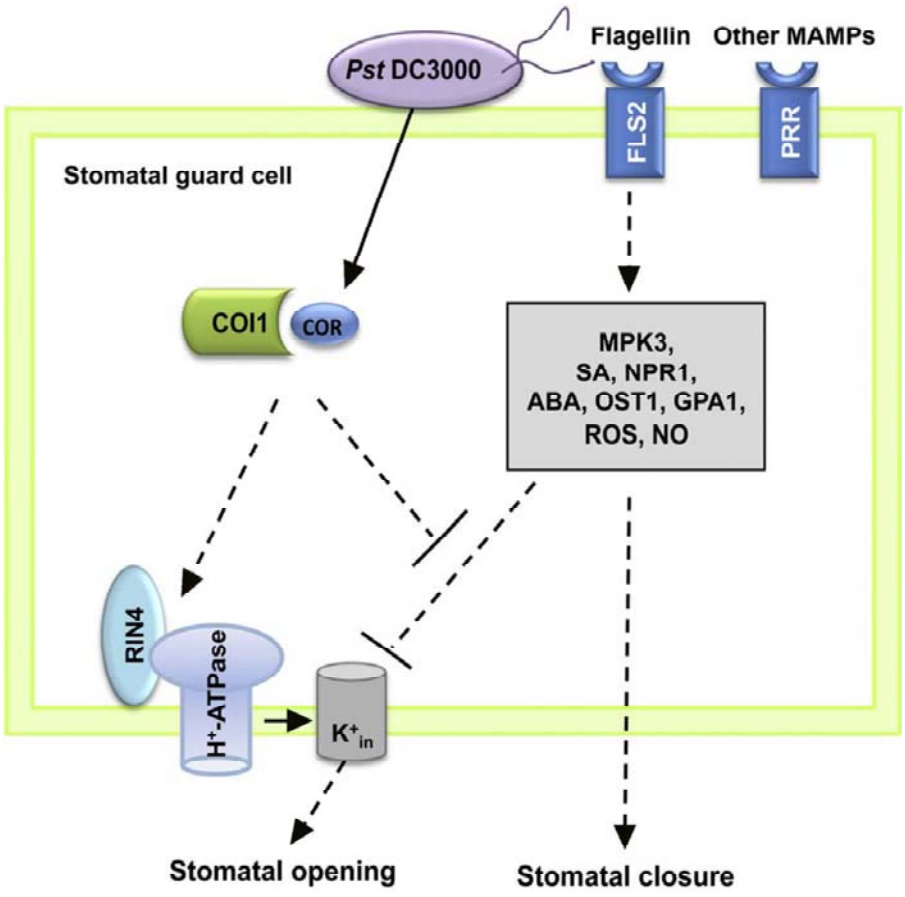

Figure 3: Scheme depicting the molecular response to *Pseudomonas syringae* in guard cells.

From Zeng et al, 2010; Current Opinion in Biotechnology.

Light

One of the key environmental signals regulating stomatal state is light. Light is in fact the main source of energy for plants because of its essential function in photosynthesis. That is why guard cells are prompt to detect and elaborate luminic signals and why such signals constitute a strong stimulus to stomatal opening and therefore CO_2 absorption (Chen et al, 2012; Shimazaki et al, 2007). The stomata open in response to blue light through the activation of membrane H+-ATPases due to its C-terminus phosphorylation and binding by a 14-3-3 protein (Emi et al, 2001; Kinoshita & Shimazaki, 1999; Kinoshita &

Shimazaki, 2002). The phototropins, as well as PHOT1 and PHOT2, participate in stomatal opening in response to blue light in *Arabidopsis* (Kinoshita et al, 2001). This signalling pathway is activated by light dependent autophosphorylation of PHOT1 and PHOT2 (Inoue et al, 2008). PHOT1 activity requires the presence of ROOT PHOTOTROPISM2 (RPT2) (Inada et al, 2004); PHOT2 is, in turn, negatively regulated by PROTEIN PHOSPHATASE 2A (PP2A) (Tseng & Briggs, 2010). Both photoreceptors induce the release of Ca^{2+} from the plasma membrane into the citosol independently. PHOT1 functions under lower light fluence rate, while PHOT2 induces Ca^{2+} release also from the endoplasmic reticulum and the vacuole in response to higher fluence rates. This mechanism requires the action of PHOSPHOLIPASE C (PLC) and the production of tri-phosphate inositol ($InsP_3$) (Harada et al, 2003). In *Arabidopsis* thaliana the perception of blue light, especially at fluence rates higher than 1µmol m^{-2} s^{-1}, relies also on the CRYPTOCHROMES: CRY1 and CRY2 (Mao et al, 2005; Talbott et al, 2003). These proteins also participate in stomatal aperture in response to red light (Boccalandro et al, 2012). The red component of light is capable of induce stomatal opening though with much lower efficiency then blue light (Kinoshita et al, 2001). Nevertheless red light is responsible for a great increase efficiency in blue light-dependent stomatal opening (Kinoshita et al, 2001). It has been suggested that this contribution is due to the photosynthetic activation permitted by red light, which would provide higher levels of ATP and lower levels of CO_2 (Shimazaki et al, 2007). However it has been shown that reduction of Rubisco activity and consequently photosynthetic rate did not affect red light effects on stomatal conductance (Baroli et al, 2008). The stomatal response to red light is mediated by PHYTOCHROME B (PHYB) and, in its absence, by PHYA (Wang et al, 2010a). There is evidence that the PHYTOCHROME mediated pathway also participates in blue light signalling (Wang et al, 2010a), possibly through a crosstalk with CRY signalling, given the known physical and functional interactions (Ahmad et al, 1998; Más et al, 2000). The nuclear protein CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) antagonizes light signalling in guard cells through direct contact with the cryptochromes and indirectly down-regulating phototropin and phytochrome-mediated pathways (Mao et al, 2005; Wang et al, 2010a; Wang et al, 2001;

Yang et al, 2001). The effects of light on stomata are also due to transcriptional regulators: it is the case of *MYB60*, a guard cell-specific TRANSCRIPTION FACTOR whose transcription is enhanced in response to blue light through the action of CRY, PHYA and PHYB (Cominelli et al, 2005; Wang et al, 2010a). While MYB60 has been described for its positive action on stomatal opening (Cominelli et al, 2005), its homolog *MYB61*, expressed in guard cells mainly in absence of light, seems to have the opposite function (Liang et al, 2005).

The circadian clock

Living in an environment presenting periodical changes due the hearth's rotation on its axis has led most of the living species to develop and conserve a molecular mechanism prompt to predict and anticipate such changes. The mechanism underlying the adaptation to daily fluctuating environmental parameters is commonly known as circadian clock. This mechanism is self-sustainable, therefore it subsists in constant condition, as well as capable of buffering environmental changes while keeping its pace. The common structure of a molecular clock involves three main parts: input pathways, that are meant to regulate the pace of the molecular oscillation in response to external stimuli such as light or temperature; a core oscillator, that generates the rhythmicity generally through the action of interconnected negative feedback loops, and output pathways, that integrate temporal information into physiology and metabolism generating observable oscillatory phenomena. One of the characteristics of a circadian clock-driven oscillatory phenomenon is its persistence in constant conditions, also called *free running*. Moreover the time taken for its cyclical repetition in *free running*, called *period*, is about 24 hours and this pace is kept at different temperatures. Nevertheless, the oscillation should be able to adjust its moments of maximum and minimum, its *phase*, in response to environmental changes in parameters such as light or temperature (Bell-Pedersen et al, 2005). The processes regulated by the endogenous clock

in plants involve, among others: development (Dowson-Day & Millar, 1999; Nozue et al, 2007; Ruts et al, 2012; Yazdanbakhsh et al, 2011), leaf movement (Millar et al, 1995), photoperiodic regulation of flowering time (de Montaigu et al, 2010; Imaizumi, 2010; Song et al, 2010; Zhao et al, 2012), CO_2 fixation (Warren & Wilkins, 1961), central and secondary metabolism (Blasing et al, 2005; Fukushima et al, 2009; Kolosova et al, 2001), hormone biosynthesis and responses (Covington & Harmer, 2007; Mizuno & Yamashino, 2008; Novakova et al, 2005; Thain et al, 2004), abiotic stress responses (Bieniawska et al, 2008; Fowler et al, 2005; Kidokoro et al, 2009; Nakamichi et al, 2009), intracellular Ca2+ concentrations (Johnson et al, 1995; Xu et al, 2007), ROS homeostasis and response (Lai et al, 2012), water uptake (Takase et al, 2011) and defence against pathogens (Bhardwaj et al, 2011; Burgess & Searle, 2011; Wang et al, 2011b). At a transcriptional level, it was observed that about 25% of *Arabidopsis thaliana* transcripts oscillate diurnally in constant conditions (Covington et al, 2008; Hazen et al, 2009), taking in account several possible conditions of diurnally changing temperature and light, 90% of the mRNAs display an oscillatory trend (Michael et al, 2008b). Such pervasive influence, constitute an adaptive advantage in terms of fitness, photosynthetic capacity, biomass accumulation and survival (Dodd et al, 2005; Green et al, 2002) as well as in the optimization of nightly starch utilization (Graf et al, 2010). Moreover, a population genetics approach showed that the optimal functionality of the circadian clock is a criterion of selective pressure in *Arabidopsis* (Yerushalmi et al, 2011).

The molecular mechanism

Decades of study of the circadian clock of *Arabidopsis thaliana* have led to the identification of several genes implied in this mechanism (McClung, 2011; Nagel & Kay, 2012; Nakamichi, 2011; Troncoso-Ponce & Mas, 2012). The key components of the clock can be classified on the base of their *phase* of expression and action during the subjective day of the plant (Nakamichi, 2011). The transition from night to day is marked by the action of two highly homologous MYB transcription factors: CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), which are

partially redundant but both essential for proper clock functionality (Alabadí et al, 2002; Mizoguchi et al, 2002; Schaffer et al, 1998; Wang & Tobin, 1998). The CCA1-LHY binding sites are the CCA1-binding sites (AAC/AAATCT) and the EVENING ELEMENTS (AAAATATCT) (Alabadí et al, 2001; Wang & Tobin, 1998). The CCA1 and LHY interact in vivo constituting a "morning complex" (Lu et al, 2009) that is a transcriptional repressor, inhibiting the expression of most of the evening-night circadian genes, in some cases in association with DE-ETIOLATED1 (DET1) (Lau et al, 2011). On the other hand, these MYB TRANSCRIPTION FACTORs promote the transcription of the *PSEUDO RESPONSE REGULATOR*s (*PRR*) *7* and *9* (Farré et al, 2005; Portolés & Más, 2010). CCA1 activity is also regulated by phosphorylation by CK2 in a temperature dependent manner (Portolés & Más, 2010), partially explaining the accelerating effects of CK2 regulatory subunits (CKB3 and 4) on the clock (Perales et al, 2006; Sugano et al, 1998; Sugano et al, 1999). The action of CK2 on CCA1 is somehow necessary for clock functioning in Arabidopsis (Daniel et al, 2004; Lu et al, 2011) but apparently not in rice (Ogiso et al, 2010). The homologous of CCA1/LHY REVEILLE (RVE) 1 and 7 have been implied in clock output (Kuno et al, 2003; Rawat et al, 2009), while another homologous, RVE5, is responsible for *PRR5* activation (Farinas & Más, 2011; Rawat et al, 2011). The circadian transcription factors expressed later in the subjective day are *PRR9*, peaking in the early morning, followed by *PRR7*, expressed until midnight, and *PRR5*, expressed from midday to midnight (Eriksson et al, 2003; Matsushika et al, 2000). These PRRs act as transcriptional repressors of *CCA1* and *LHY* (Farré et al, 2005; Nakamichi et al, 2010) as well as drivers of clock output pathways (Nakamichi et al, 2012). PRR5 is also a repressor of *REV8*, its activator (Farinas & Más, 2011; Rawat et al, 2011); while PRR9 constitutes a positive feedback loop with LIGHT-REGULATED WD1 (LWD1), a circadian protein involved in the transcriptional activation of *CCA1*, *LHY*, *PRR5* and *TIMING OF CAB EXPRESSION 1* (*TOC1*) (Wang et al, 2011c; Wu et al, 2008). The latter is the most known among the evening/night transcriptional regulators. *TOC1* was first described as through the short period phenotype of its *loss of function* mutant *toc1-1* (Millar et al, 1995); once the corresponding gene was cloned (Strayer et al, 2000), it was observed that the transcription of *TOC1*

under the control of its own promoter caused period lengthening, while the over-expression produced arrhythmicity (Más et al, 2003a). *TOC1* transcription is repressed by CCA1-LHY-DET1 complex (Alabadí et al, 2001; Lau et al, 2011). TOC1 itself functions as a transcriptional repressor of several clock genes (Gendron et al, 2012; Huang et al, 2012; Pokhilko et al, 2012). The repression of *CCA1* probably occurs in cooperation with CCA1 HIKING EXPEDITION (CHE), a TCP (TEOSINTE BRANCHED 1, CYCLOIDEA and PCF) circadian transcription factor (Pruneda-Paz et al, 2009). Other targets of TOC1 are the *PRR*s *5*, *7* and *9* as well as the evening genes *EARLY FLOWERING 4* (*ELF4*), *LUX ARRHYTHMO* (*LUX*) and *GIGANTEA* (*GI*) (Huang et al, 2012). *LUX ARRHYTHMO*/*PHYTOCLOCK* (Onai & Ishiura, 2005) is an evening gene whose transcription is repressed by both TOC1 and the "morning complex" (Gendron et al, 2012; Hazen et al, 2005; Helfer et al, 2011; Huang et al, 2012; Portolés & Más, 2010). This transcription factor is believed to be the DNA binding subunit of an "evening complex", which also includes ELF3 and ELF4 (Chow et al, 2012; Nusinow et al, 2011). This complex directly inhibits the transcription of *PRR9* (Chow et al, 2012; Helfer et al, 2011) as well as the expression of *PHYTOCHROME INTERACTING FACTORS* (*PIF*) *4* and *5*, being the latter the unleashing event of the circadian pattern of hypocotyl growth (Nusinow et al, 2011). Moreover LUX represses its own transcription (Helfer et al, 2011) and is also a positive regulator of the transcription of *CCA1* and *LHY*, probably through an indirect mechanism (Hazen et al, 2005). The other components of the "evening complex", ELF3 and ELF4 were shown to be repressors of *PRR7* (Dixon et al, 2011). Further more ELF3 seems to have regulatory functions on the complex: it is, in fact, implied in nuclear recruitment of ELF4 (Herrero et al, 2012) and in PHYTOCHROME signalling interaction with the clock (Kolmos et al, 2011). Recent computational modelling of Arabidopsis circadian clock has stressed the functional importance of the transcriptional repressions operated by TOC1 and by the "evening complex": summed to the critical role of the "morning complex" they constitute a three components *repressilator* circuit. This structure optimizes the clock robustness as well as the possibilities of fine tuning mechanisms (Pokhilko et al, 2012).

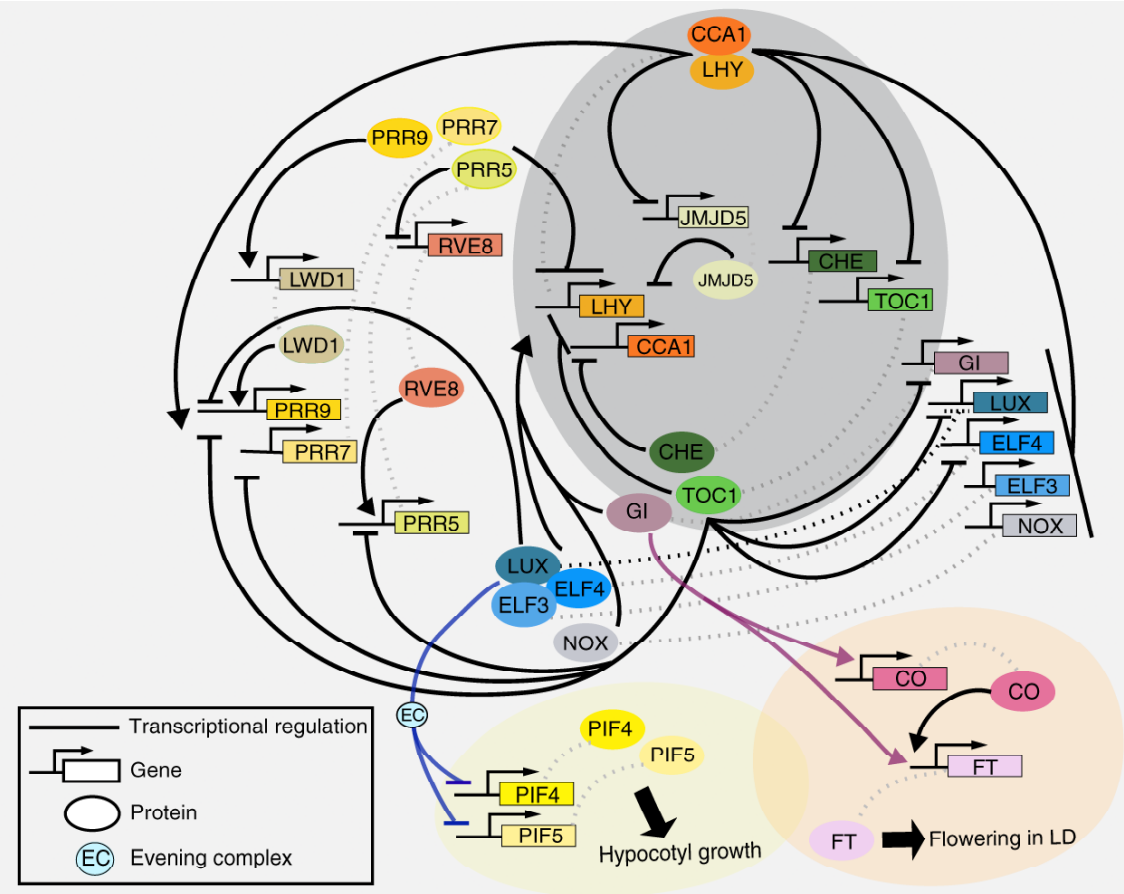

Figure 4: Scheme representing the network of transcriptional regulation constituting the core oscillator of *Arabidopsis thaliana*.

From Nagel and Kay, 2012; Current Biology.

The transcriptional control of circadian genes also implies several chromatin-remodelling events. Histone H3 acetylation and trimethylation regulate the circadian expression of *TOC1*, *CCA1* and *LHY* (Perales & Más, 2007; Song & Noh, 2012). Moreover, the monoubiquitylation of histone H2B affects the expression of circadian genes (Himanen et al, 2012). Intriguingly, the putative histone demethylase JUMONJI DOMAIN CONTAINING PROTEIN 5 (JMJD5) seems to have a clock related function synergical to TOC1 (Jones & Harmer, 2011). A global evidence of the importance and diffusion of histone modification for the regulation of clock genes expression in *Arabidopsis* has been recently produced by Malapeira and collaborators (Malapeira et al, 2012).

An important step of regulation of circadian mRNA levels is the alternative splicing: the accumulation of non-productive splicing isoforms of *LHY*, *PRR7*, *PRR3* and *TOC1* transcripts has been observed in response to temperature descent; on the other hand, in the same conditions mRNA levels of *CCA1*, *PRR9* and *PRR5* increase as their non-productive isoforms disappear (Filichkin et al, 2010; James et al, 2012). The alternative splicing of CCA1, LHY and other circadian homologues has been related to temperature compensation (Filichkin & Mockler, 2012; Park et al, 2012). A possible agent of this regulatory mechanism is the PROTEIN ARGININE METHYL TRANSFERASE 5 (PMRT5), whose functions have been related to alternative splicing as well as *Arabidopsis* circadian clock (Hong et al, 2010; Sánchez et al, 2010). Interestingly it has been shown that mutation of a spliceosomal gene affected clock function (Jones et al, 2012). The SNW/SKI-INTERACTING PROTEIN (SKIP) has also been related with splicing regulation of circadian genes (Jones et al, 2012). Another circadian clock regulated protein that may play a role in this is GLYCIN-RICH RNA BINDING PROTEIN 7/COLD CIRCADIAN CLOCK RNA BINDING 2 (GRP7/CCR2) (Heintzen et al, 1997). This protein has been shown to regulate the stability of its own transcript promoting alternative splicing leading to non-sense decay. This mechanism constitutes a *slave* oscillator, whose pace is regulated by the *master* clock core (Schöning et al, 2007). This secondary oscillator seems to influence several clock outputs (Fu et al, 2007; Kim et al, 2008; Schöning et al, 2007).

The circadian oscillator also relies on a post-traductional layer of regulation. TOC1 and PRR5 are targeted for degradation by E3 ubiquitin ligases through physical interaction with ZEITLUPE (ZTL) (Fujiwara et al, 2008; Kiba et al, 2007; Más et al, 2003b), FLAVIN BINDING KELCH F-BOX 1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2) (Baudry et al, 2010). On the other hand, the stability of TOC1 is favoured by the interaction with PRR3 (Para et al, 2007) and the nuclear import favoured by the interaction with PRR5 (Wang et al, 2010b). Another element of control of circadian proteins stability is *GI*: a late day-expression gene repressed by the “morning complex” and DET1 (Lau et al, 2011). GI protein has various functions: in the clock core, it promotes daytime

TOC1 degradation by stabilizing ZTL in response to blue light (Kim et al, 2007). At the output level, it has a key role in photoperiodic regulation of flowering, in fact, GI enhances the transcription of the flowering promoting gene *CONSTANS* (*CO*) and stabilizes CO protein by impeding its FKF1-mediated degradation (Fowler et al, 1999; Suárez-López et al, 2001; Valverde et al, 2004). It has been recently reported that GI also participates in CO-mediated activation of *FLOWERING LOCUS T* (*FT*) that triggers flowering (Sawa & Kay, 2011).

The circadian regulation of stomata

As many other important functions in the plant, the stomatal state is influenced by the circadian clock. It has been shown in several plants that stomatal conductivity and aperture fluctuates throughout the day and these fluctuations persist in constant conditions both in light and darkness (Correia et al, 1995; Holmes & Klein, 1986; Kaiser & Kappen, 1997; Kumar et al, 2012; Lebaudy et al, 2008; Martin & Meidner, 1971; Somers et al, 1998). This oscillation is reflected in the circadian pattern of parameters like CO_2 fixation rate (Warren & Wilkins, 1961), water uptake (Takase et al, 2011) and foliar pathogen sensitivity (Bhardwaj et al, 2011). The circadian regulation of stomatal conductivity might partially be supported by the oscillation of the endogenous amount of ABA (Novakova et al, 2005) as well as on the fluctuation of its second messengers: intracellular Ca^{2+} concentrations (Johnson et al, 1995; Xu et al, 2007) and ROS levels and sensitivity (Lai et al, 2012). On the other hand, ABA has been reported as a disruptor of periodicity in circadian genes oscillations (Hanano et al, 2006), suggesting that the clock and abscisic acid signalling regulate each other. Evidences of this functional interaction have been observed widely at a transcriptomic level. It has been calculated that 68% of circadian oscillating transcripts are implied in stress responses (Kreps et al, 2002); on the other hand, a significant proportion of ABA-responsive genes display daily fluctuations (Mizuno & Yamashino, 2008). Finally, it has been shown that the

transcriptional response to drought changes significantly throughout the day in both *Populus* and *Arabidopsis* (Wilkins et al, 2010; Wilkins et al, 2009). Moreover, the ABA responsive transcription factor *DRE*/*CRT BINGING PROTEIN 1C* (*DREB1C*) is implied in cold and drought response and is directly inhibited by PHYTOCHROME INTERCATING FACTOR 7 (PIF7), which accounts of both TOC1-mediated circadian regulation and PHYA-mediated red light response (Kidokoro et al, 2009). Further more, *DREB1* transcription factors transcription is also enhanced by the "morning complex" (Dong et al, 2011) and their cold-mediated induction is gated by the clock through a PRR5, 7 and 9 mediated inhibition (Nakamichi et al, 2009). The importance of a proper circadian regulation of stomatal conductance is reflected in the peculiarities observed in the oscillator of guard cells. In this cell type, the fluctuations of circadian transcripts and proteins show a slight difference compared to other leaf-cell types. These differences might be the cause of the alteration in *phase* and *period* length observed in *free running* conditions (Yakir et al, 2011). A crucial mechanism for circadian modulation of stomatal closure involves the clock-regulated gene *FT*. In fact it has been recently demonstrated that the closed-stomata phenotype of *phot1phot2* double mutant is reverted by a null mutation in *ELF3* gene. The stomatal opening was due to an increase in H^+-ATPase activity. The target of ELF3 transcriptional inhibition is *FT*. Interestingly, the knock out mutation *ft-1* recovers *phot1phot2* phenotype, demonstrating that FT is a key piece in modulation of photoperiodic flowering as well as on the blue light mediated stomatal aperture. The genetic interacrion described above proves that these processes are modulated by the circadian clock during the last light hours of the day through the ELF3-mediated inhibition of *FT* transcription (Kinoshita et al, 2011). The biological meaning of such regulatory circuit could be the extension of the opening hours favouring a higher photosynthesis in a time window in which humidity generally decreases with a reduced the risk of dehydration (Hubbard & Webb, 2011). Another interesting circadian regulation of stomatal behaviour is constituted by the action of GRP7/CCR2, the circadian regulated component of *slave* oscillator, which acts as an inductor of stomatal closure through a mechanism of mRNA export from the nucleus (Kim et al, 2008). All together this data suggest that stomatal movements are modulated by

the circadian clock through several mechanisms and that in such modulation a specially relevant role is played by transcriptional regulation.

II. OBJECTIVES

Our work is focused on the mechanisms plants use to optimize the use of their water resources.

The aim of our work is to generate advances in the comprehension of the transcriptional networks modulating stomatal behaviour in *Arabidopsis thaliana*.

In particular we wish to identify unknown regulatory elements and mechanisms affecting stomatal behaviour:

In the first chapter of this thesis we focus on the transcriptional mechanisms underlying circadian modulation of ABA sensitivity and stomatal behaviour, given the relevance of this functional interaction for plants water use efficiency. In particular we seek to understand the role of the core oscillator component TOC1 in the circadian influence over ABA signalling. At the same time we are interested in the characterization of ABA effects on this circadian gene.

The second chapter is dedicated to the search of transcription factors capable of influencing stomatal behaviour. Our strategy is based on the use of a battery of *Arabidopsis thaliana* lines that over-express transcription factors through ß-estradiol inducible promoter. In our work we aim to find a reliable protocol for coupling ß-estradiol induction with a screening procedure capable of detecting alterations in stomatal closure or opening. Our final goal would then be to identify and characterize new transcription factors implicated in the control or modulation of stomatal behaviour. This screening could therefore lead to a better comprehension of the stomatal regulation mechanisms as well as to the development of potentially useful biotechnological tools for crop improvement.

III. RESULTS

Chapter 1

Perspectives on TOC1, the circadian clock and stomatal ABA responses

Plants, as sessile organisms, have evolved a number of ways to rapidly sense and adjust to the changing environmental conditions. One of the most studied mechanisms involved in these adjustments is the biological or circadian clock, which is able to maintain biological rhythms with a period of approximately 24 h (Harmer, 2009; Más, 2005; McClung, 2008). Circadian clocks were suggested to confer adaptive advantages to organisms (Dodd et al, 2005; Green et al, 2002; Michael et al, 2003; Ouyang et al, 1998) by means of not merely responding to the external signals but also anticipating the predictable environmental variations that occur during the day–night transitions. The presence of an endogenous timing system thereby allows the synchronization of biological activities to occur at specific phase relationships with the environment, permitting the temporal separation of incompatible metabolic events (Harmer, 2009; Hotta et al, 2007; Más, 2005; McClung, 2008).

Intensive research efforts have been devoted in past years to decipher the molecular and biochemical mechanisms underlying circadian clock function. In plants, as in many other organisms, the circadian rhythmicity seems to rely on multiple negative feedback loops at the core the oscillator (Bell-Pedersen et al, 2005; Wijnen & Young, 2006). Molecular-genetic approaches in *Arabidopsis thaliana* have aided in the identification of clock components and mechanisms of regulation within the circadian oscillator (Más, 2008). The reciprocal regulation between a pair of Myb transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) (Wang & Tobin, 1998) and LATE ELONGATED HYPOCOTYL (Schaffer et al, 1998) with a pseudo-response regulator TIMING OF CAB EXPRESSION 1 (TOC1 or PRR1) (Makino et al, 2000; Strayer et al, 2000) was initially proposed as a core feedback mechanism important for clock function (Alabadí et al, 2001). Computer modelling and experimental validation have subsequently confirmed the existence of additional, interconnected multiple

loops that confer robustness and flexibility to the oscillatory activities (Locke et al, 2006; Rand et al, 2004; Zeilinger et al, 2006).

Despite the increasing knowledge of the molecular networks comprising the *Arabidopsis* circadian oscillator, little is known about the precise biochemical and molecular function of TOC1 within the clock. *TOC1* mutant and RNAi plants display a short-period phenotype for clock-controlled gene expression as well as day length-insensitive flowering time (Strayer et al, 2000). The constitutive high expression of *TOC1* leads to arrhythmia under constant light conditions in a number of clock outputs (Makino et al, 2002; Más et al, 2003a). TOC1 was also proposed to function between the environmental signals and the circadian and photo-morphogenic outputs (Más et al, 2003a). Further studies have evidenced that a very precise regulation of TOC1 gene and protein rhythmic expression is essential for proper clock function. Different mechanisms contribute to this regulation including changes in chromatin structure (Perales & Más, 2007), transcriptional regulation (Alabadí et al, 2001) and protein degradation by the proteasome pathway (Más et al, 2003b), which together, accurately control the 24h rhythmic oscillation of *TOC1* expression and function.

The use of genome-wide approaches has provided some clues into the global transcriptional networks controlled by the clock. Circadian clock regulation of the *Arabidopsis* transcriptome is highly extensive although the precise fraction of the genome that is circadian regulated highly varies among the different studies (Covington & Harmer, 2007; Covington et al, 2008; Dodd et al, 2007; Edwards et al, 2006; Harmer et al, 2000; Hazen et al, 2009; Michael et al, 2008a; Michael & McClung, 2003; Michael et al, 2008b; Mizuno & Yamashino, 2008; Schaffer et al, 2001). A recent study has integrated the information from multiple circadian datasets to get a better estimation of the expressed gene fraction that is circadianly regulated (Covington et al, 2008). The use of tiling arrays has also identified the circadian regulation of intergenic regions, introns and natural antisense transcripts, which has extended the pervasiveness of clock function far beyond protein coding genes (Hazen et al, 2009). The functional clustering of clock-regulated genes has also provided insights into metabolic and physiologic pathways that are under circadian control. Indeed,

clock-regulated genes are over-represented among several plant hormone and stress-responsive pathways. This is consistent with the diurnal variations of phytohormone abundance (Robertson et al, 2009) and with the gated regulation of plant hormone sensitivity by the clock (Covington & Harmer, 2007; Dodd et al, 2007).

One of the hormones regulated by the circadian clock is abscisic acid (ABA). This phytohormone is essential in the regulation of many plant growth and development processes as well as in the control of plant responses to stressful environments (Finkelstein et al, 2002; Leung & Giraudat, 1998; Zhu, 2002). The significant number of ABA-related genes that are controlled by the clock has been reported in various studies (Covington & Harmer, 2007; Dodd et al, 2007; Mizuno & Yamashino, 2008). Furthermore, the regulation between ABA and clock signalling pathways is bidirectional as treatment with ABA lengthens the circadian period of gene promoter activity (Hanano et al, 2006). Evidence of a feedback loop between cADPR and the circadian oscillator also suggests a mechanism by which the circadian coordination of ABA-transducing components might be involved in clock-mediated regulation of plant responses to ABA (Dodd et al, 2007). The influence of the clock is not limited to gene expression but is also evidenced in the circadian regulation of physiological processes controlled by ABA. For instance, under water deficit conditions, ABA induces the closure of the stomatal pore, and this closure was found to be gated by the clock. Indeed, ABA is less effective at closing the stomata in the morning than in the afternoon (Correia et al, 1995), which may ensure that stomata are closed in the heat of the afternoon if water supply is limited (Robertson et al, 2009).

The cellular changes in ABA concentration (Verslues & Zhu, 2007) trigger a downstream network of signalling cascades that ultimately regulate vegetative and reproductive processes and result in enhanced plant tolerance to the environmental stress (Hirayama & Shinozaki, 2007). Essential for ABA signalling is the perception of hormone changes by ABA receptors (Hirayama & Shinozaki, 2007). Among the possible hormone sensors, recent reports have proposed that regulators of ABA-related protein phosphatases 2C may function as ABA

receptors within the ABA signaling pathway (Ma et al, 2009; Park et al, 2009). The H subunit of the magnesium-protoporphyrin IX chelatase (ABAR/CHLH/GUN5, At5g13630) was arguably proposed to function as an ABA receptor because of its ABA-binding activity and the ABA-related phenotypes in germination, stomatal closure and responses to water-deficit conditions of ABAR mis-expressing plants (Shen et al, 2006). In broad bean (*Vicia faba*), ABAR is also involved in ABA-induced stomatal signalling and, equally to its homologous in *Arabidopsis*, was proposed to specifically bind ABA (Zhang et al, 2002). However, the possible function of ABAR as a receptor was questioned in a study reporting that in barley (*Hordeum vulgare L.*), the magnesium chelatase large subunit does not bind ABA (Muller & Hansson, 2009). This issue was addressed in a recent study by using a newly developed ABA-affinity chromatography technique, which convincingly showed the specificity of the ABA binding to ABAR (Wu et al, 2009a). Furthermore, the authors also assigned ABA-related functions to particular ABAR protein domain (Wu et al, 2009a). Altogether, the results in *Arabidopsis* consign an important role for ABAR within the ABA signalling pathway.

Here, we uncover ABA-related phenotypes for *TOC1* mis-expressing plants and confirm a role for ABAR in the ABA signalling pathway. *TOC1* is induced by ABA, and this induction is gated by the clock and determines the timing of TOC1 binding to the *ABAR* promoter. Molecular-genetic studies show the existence of a negative feedback loop in which TOC1 negatively regulates the expression of *ABAR*, whose activity is in turn necessary for *TOC1* activation by ABA. Our studies suggest that proper timing of this feedback loop is important for ABA-mediated changes in gene expression and plant responses to drought conditions.

Genome-wide analysis of TOC1 transcriptional networks

To gain insights into the mechanisms of TOC1 function in the clock, we performed genome-wide transcriptomic analysis of wild-type (WT) and TOC1 over-expressing plants (TOC1 cDNA fused to YFP, herein denominated TOC1-

ox) (Más et al, 2003a) synchronized under 12 h light:12 h dark (LD) cycles for 10 days. Statistical analysis showed a total of 245 significantly increased and 160 significantly decreased genes in TOC1-ox compared with WT (Annexed table A). We also analysed transcriptomic data of WT and TOC1 mutant plants (*toc1-2*) (Strayer et al, 2000) synchronized under LD cycles followed by 2 days under constant light (LL) conditions and found that about 75 genes were downregulated and 105 upregulated in *toc1-2* samples (Annexed table B).

Using a recently published circadian dataset (Hazen et al, 2009) we found that about 50% (in TOC1-ox, Fisher's exact test P=2,2 x 10^{-16}) and over 40% (in *toc1-2*, Fisher's exact test P=1,8 x 10^{-7}) of the mis-regulated genes showed circadian fluctuations in mRNA abundance (Figure 1A and Table 1). Similar or even higher percentages were obtained when other circadian datasets (Covington et al, 2008) were examined (Table 1).

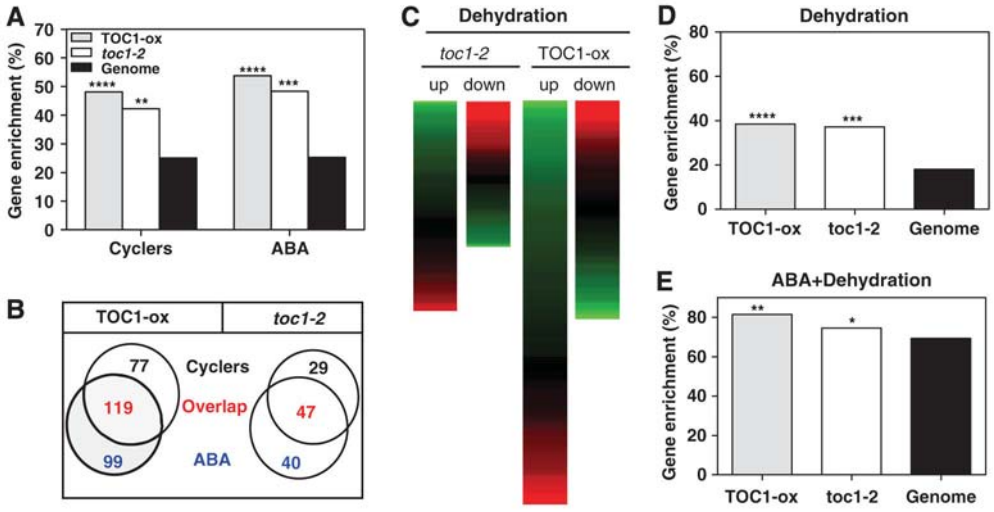

Figure 1: Genome-wide analysis of TOC1 transcriptional networks. (A) Percentages of cyclers and ABA-related genes at the whole-genome level, and in TOC1-ox and *toc1-2* transcriptomic datasets (**P-value<10^{-8}; ***P-value<10^{-11}; ****P-value<10^{-15}). (B) Venn diagrams showing the overlap between cyclers and ABA-related genes in TOC1-ox and *toc1-2* transcriptomic datasets. (C) Hierarchical clustering of *toc1-2* and TOC1-ox mis-regulated genes with transcripts involved in dehydration responses. Percentages of dehydration (D) at the whole-genome level, and in TOC1-ox and *toc1-2* transcriptomic datasets, and among these, the ABA-related genes (E) (*P-value<10^{-3}; **P-value<10^{-6}; ***P-value<10^{-11}; ****P-value<10^{-15}).

Hormone/ treatment/condition	TOC1-ox		*toc1-2*	
	Enriched genes (%)	P-value	Enriched genes (%)	P-value
Cyclers (Hazen et al, 2009)	48,14	$2,2 \times 10^{-16}$	42,22	$1,7 \times 10^{-7}$
Cyclers (Covington et al, 2008)	55,17	$2,2 \times 10^{-16}$	59,22	$5,6 \times 10^{-9}$
ABA (Matsui et al, 2008)	53,82	$2,2 \times 10^{-16}$	48,33	$6,0 \times 10^{-12}$
ABA (Matsui et al, 2008) Cyclers (Hazen et al, 2009)	54,58	$1,36 \times 10^{-7}$	54,02	$6,2 \times 10^{-4}$
Drought (Matsui et al, 2008)	38,51	$2,2 \times 10^{-16}$	37,22	$3,4 \times 10^{-10}$

Table 1: Meta-analysis of *TOC1* mis-regulated genes with publicly available datasets of cycling, ABA- and drought-related genes.

Among all of the mis-regulated genes, we were expecting to find a significant proportion of clock-related genes not directly regulated by TOC1. Noticeably, meta-analysis of mis-regulated genes using databases that contain a broad spectrum of transcriptomic profiles (https://www.genevestigator.com/gv/index.jsp) also showed a significant overlap with transcripts involved in ABA signalling pathways (Figure 2).

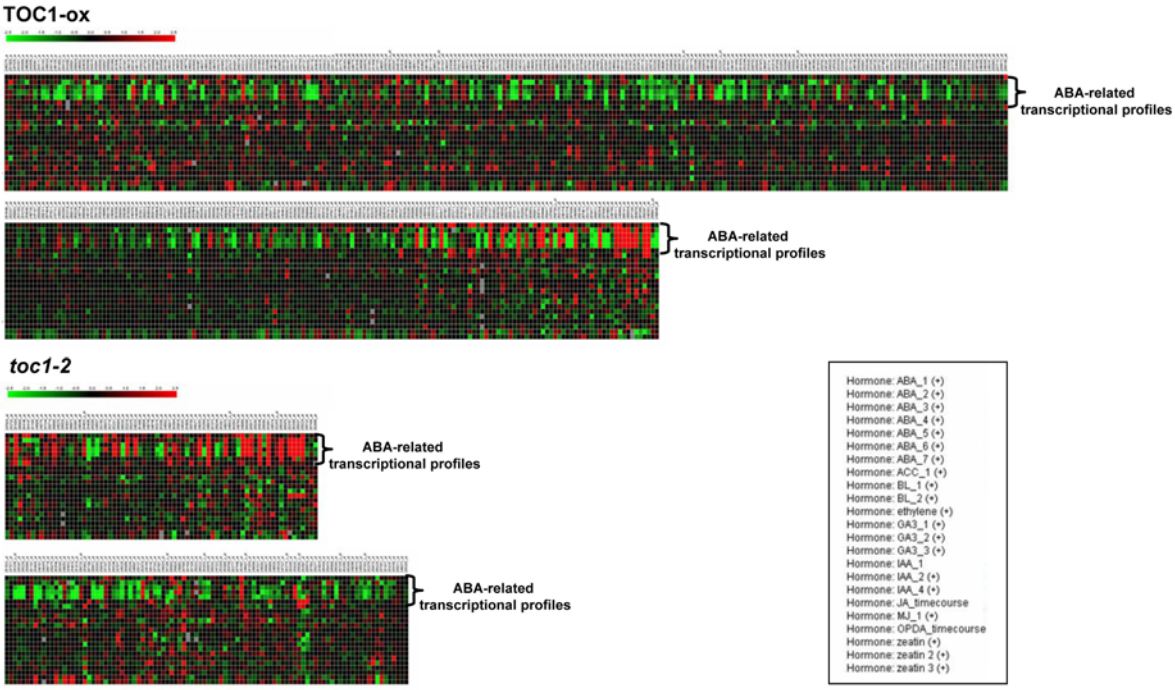

Figure 2: Meta-analysis of up- and down-regulated genes in TOC1-ox and *toc1-2* using transcriptomic profiles from experiments with different hormone treatments. Details about the experimental conditions and treatments in each dataset are described in https://www.genevestigator.com/gv/index.jsp.

Functional clustering using specific ABA-related datasets (Matsui et al, 2008) confirmed the highly significant number of ABA-responsive genes in TOC1-ox (53,82%, Fisher's exact test $P=2,2 \times 10^{-16}$) and in *toc1-2* (48,33%, Fisher's exact test $P=6,0 \times 10^{-12}$) (Figure 1A; Table 1). Both, upregulated and downregulated set of genes were found to be associated with ABA transcriptional networks (Figure 3A).

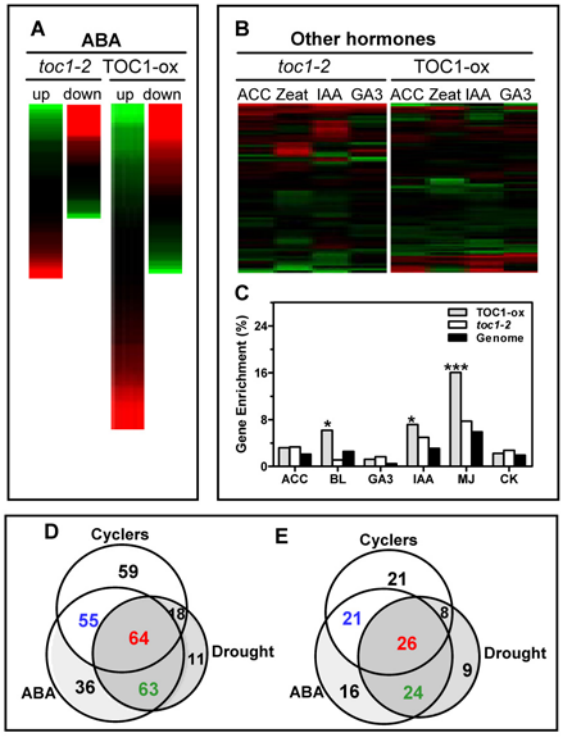

Figure 3: Genome-wide analysis of TOC1 transcriptional networks. (A) Hierarchical clustering with transcripts regulated by ABA and (B) other hormones (gibberellic acid [GA3], 1-Aminocyclopropane-1-carboxylic Acid [ACC], indole acetic acid [IAA] and Zeatin [Zeat]). High and low expression is depicted as a $\log_2$ ratio with maximum and minimum scale of ±3. (C) Percentages of hormone-related genes at the whole-genome level and in TOC1-ox and *toc1-2* transcriptomic datasets. Venn diagrams showing the overlap between cyclers, ABA- and drought-related genes in TOC1-ox (D) and *toc1-2* (E) transcriptomic datasets. (*P-value<10^{-3}; **P-value< 10^{-8}; ***P-value<10^{-11}).

The list of mis-regulated genes included relevant components of the ABA signalling pathway (Finkelstein et al, 2002) such as ABA1, Histone H1-3, RD29B, several late embryogenesis abundant proteins and ABAR/CHLH/GUN5 (Annexed tables C and D). We also checked whether clock dysfunction in TOC1 mis-expressing plants was affecting other hormone transcriptional networks. However, functional clustering analysis showed that, with the exception of methyl jasmonate, no other hormone pathways were as significantly affected as the ABA (Figure 3B and C). Furthermore, a significant proportion of these ABA-related genes show a circadian oscillation (54,58% in TOC1-ox, Fisher's exact test P=1,36 x 10^{-7} and 54,02% in *toc1-2*, Fisher's exact test P=0,6 x 10^{-4}) (Figure 1B and Table 3).

Our data analysis also showed that about 38% of the mis-regulated genes in TOC1-ox (Fisher's exact test P=2,2 x 10^{-16}) and in *toc1-2* (Fisher's exact test P=3,4 x 10^{-10}) were related to plant responses to dehydration (Figure 1D and Table 1). These percentages are higher than the 18% of the genes that are associated with drought responses in the genome (Matsui et al, 2008). This

is noteworthy because the plant response to changes in water status is one of the many processes controlled by ABA. Consistently, the analysis of common elements in the promoters of the mis-expressed genes also showed a significant over-representation of motifs implicated in molecular responses to dehydration (Annexed table E). Furthermore, two- and three-fold comparisons of circadian, ABA and drought-related genes showed that 81,41% (in TOC1-ox) and a 74,62% (in *toc1-2*) of the drought-related genes are regulated by ABA (Figure 1E). We also found an important overlapping among ABA and drought genes that were regulated by the circadian clock in both TOC1-ox (Figure 3D) and *toc1-2* (Figure 3E) datasets. Analysis by quantitative PCR (Q-PCR) confirmed the mis-expression of some selected genes in TOC1-ox and *toc1-2* mutant plants (Figures 4 and 5). When other relevant dehydration-related genes were analysed by Q-PCR, we also found a significant mis-expression, suggesting that the drought-related transcriptional networks altered in TOC1 mis-expressing plants might be even more important than estimated by our microarray data. Altogether, these data led us to further examine the possible link between TOC1 and plant responses to drought mediated by ABA.

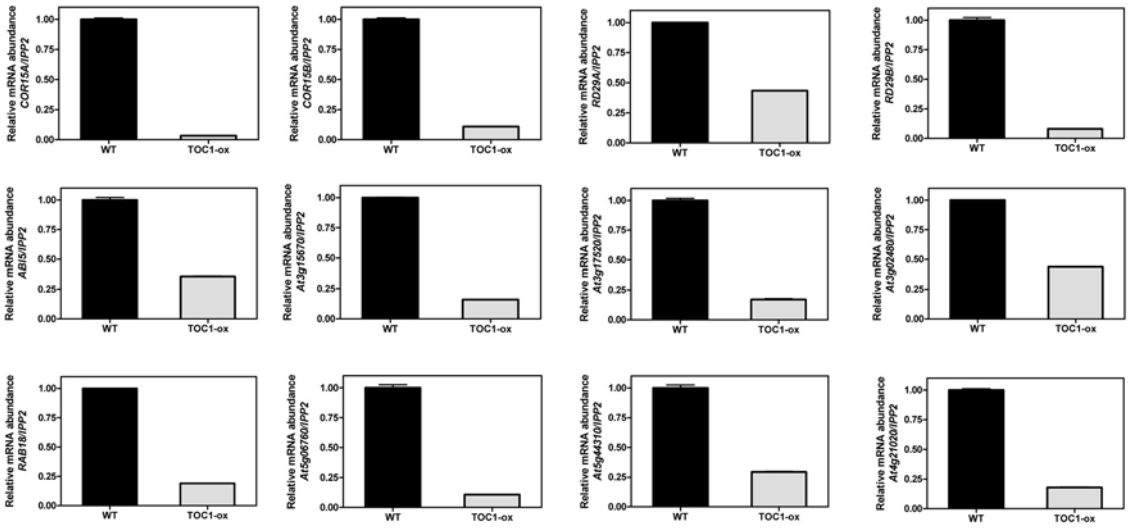

Figure 4: Comparison of relative expression of ABA-related genes and genes involved in dehydration responses in WT and TOC1-ox plants. Analysis of mRNA abundance in WT and TOC1-ox plants was performed by Quantitative-PCR (QPCR). mRNA was quantified relative to *IPP2*. Data are plotted relative to the value of WT plants.

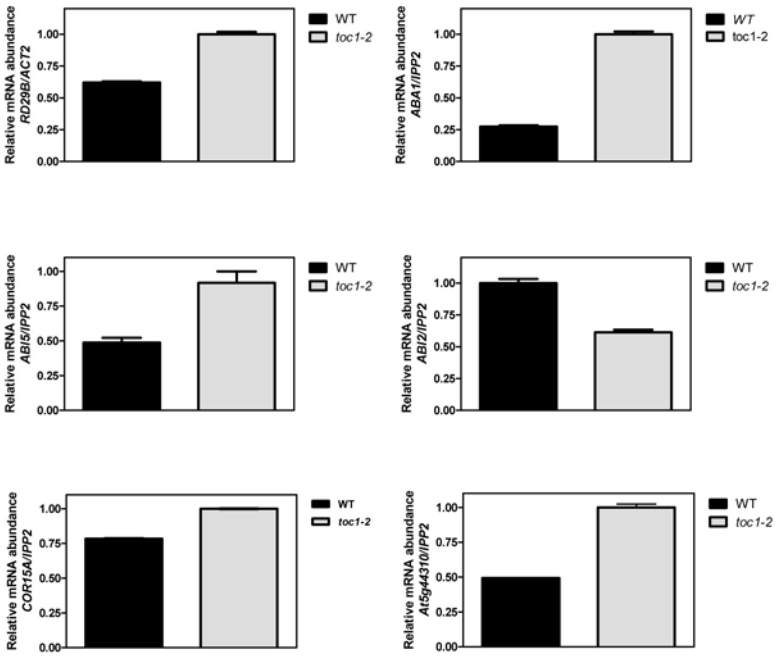

Figure 5: Comparison of relative expression of ABA-related genes in WT and *toc1-2* mutant plants. Analysis of mRNA abundance was performed by Quantitative-PCR (Q-PCR). mRNA was quantified relative to *IPP2*. Data are plotted relative to the maximum value.

ABA-mediated responses to drought are impaired in *TOC1* mis-expressing plants

If the changes in gene expression correlate with functional alterations in physiological responses, then plants mis-expressing TOC1 should display ABA-related phenotypes. Data analysis showed that many stress-responsive genes known to be involved in dehydration tolerance (Ingram & Bartels, 1996; Thomashow, 1999) were downregulated in TOC1-ox plants, suggesting that TOC1-ox plants might have reduced tolerance to drought. We therefore analysed responses to drought stress in WT, TOC1-ox, *toc1-2* and *TOC1 RNAi* plants (Más et al, 2003a) by examining percentages of recovery after dehydration using seedlings grown on agar plates (Fujita et al, 2005). Our results showed that most TOC1-ox plants were unable to recover from the drought stress, exhibiting a reduced survival rate (2%) after re-watering (Figure 6A and B). In contrast, approximately 50% of the *toc1-2* (not shown) and 52% of *TOC1 RNAi* plants completely recovered the drought (Figure 6A and B) whereas WT plants showed intermediate phenotypes and about 15% recovered after re-watering (Figure 6A and B). Thus, over-expression of TOC1 significantly

reduced the plant tolerance to drought (P-value<0,0001) whereas *toc1-2* and *TOC1 RNAi* plants responded better than WT to water-deficit conditions (P-value<0,0001). The survival rates of *TOC1 RNAi* plants on dehydration are a long-term response that is unlikely because of altered clock phase in these plants.

The altered tolerance of *TOC1* mis-expressing plants to water stress might be due to deficient stomatal closure under dehydration conditions. Thus, we next examined stomatal guard cell movement that is controlled by both ABA and the circadian clock (Robertson et al, 2009). The experiments were performed at midday, a time when stomata are more responsive to ABA (Robertson et al, 2009). As expected, ABA treatment in WT leaves caused significant stomatal closure (Figure 6C and D). However, this effect was not as pronounced in TOC1-ox whereas stomata in *TOC1 RNAi* (not shown) and *toc1-2* mutant leaves were more effectively closed (Figure 6C and D). Compared with WT, the stomatal response to ABA was significantly altered in TOC1-ox (P-value<0,001), *TOC1 RNAi* (P-value<0,01) and *toc1-2* (P-value<0,01). Consistent with these results, the stomatal conductance of TOC1-ox plants was not as responsive to the treatment with ABA as in WT plants (Figure 6E) whereas *toc1-2* mutant plants showed an increased response (Figure 6E). Statistical analysis verified the significance of the differential ABA effects, with P-values below 0,001. If the stomatal closure and conductance are impaired in TOC1 over-expressing plants, then the leaf water-loss rates might also be affected. Thus, we next measured the water-loss rates of detached rosettes in TOC1 mis-expressing plants. Our studies showed that 3 h after detachment, the leaves of *TOC1 RNAi* plants showed a 40% of water loss in contrast to the 54 and 75% of WT and TOC1-ox plants, respectively (Figure 6F). These differences were observed throughout the time course analysis (Figure 6F).

Collectively, our results indicate that proper *TOC1* expression is important for stomatal function and regulation of leaf transpiration rates, a notion that is reinforced by the opposite phenotypes of *TOC1* over-expressing and mutant plants. In addition to the previously reported and well-characterized clock phenotypes, our study shows an unpredicted role for TOC1 in the control of

ABA-mediated plant responses to drought.

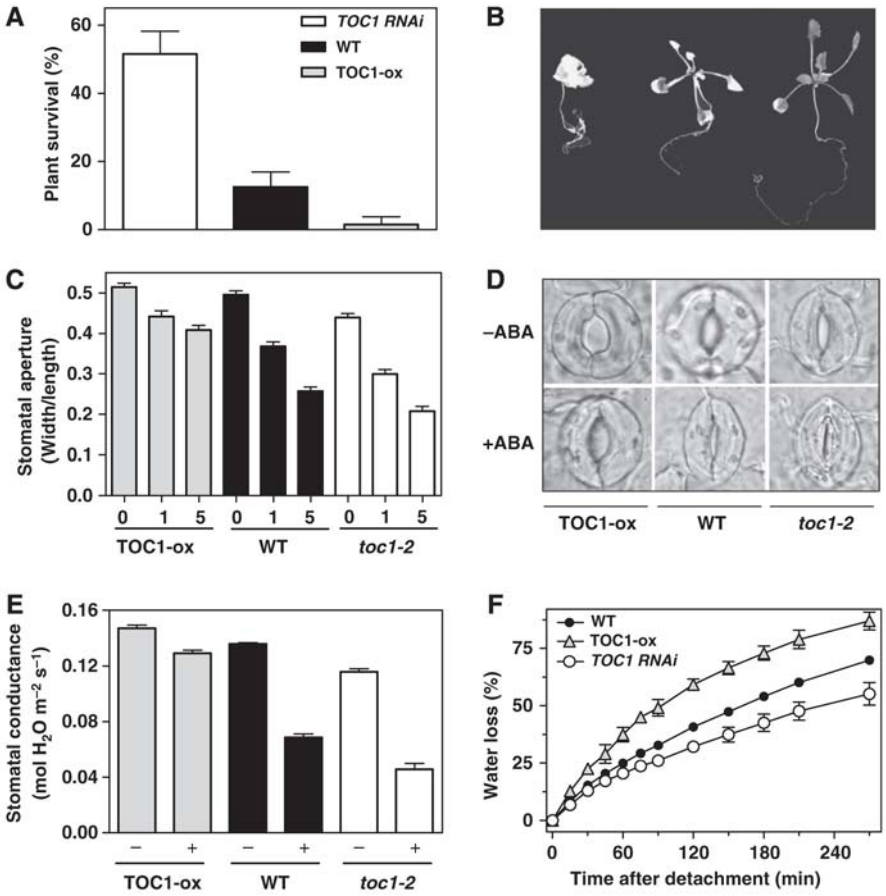

Figure 6: Altered responses to drought conditions of plants mis-expressing *TOC1*. (A) Plant survival to dehydration stress on agar plates. Data are means ± s.e.m. of duplicate experiments with at least 25 plants per genotype. (B) Representative photographs of TOC1-ox (left), WT (middle) and *TOC1 RNAi* (right) plants of the dehydration experiments. (C) Stomatal aperture in rosette epidermis of TOC1-ox, WT and *toc1-2* plants. Stomatal dimensions were measured after incubation for 2 h in a buffer containing 0, 1 or 5 mM ABA. Data are means ± s.e.m. of duplicate experiments with at least 100 stomata per genotype and per treatment. (D) Representative images by light microscopy of the stomata guard cells. (E) Stomatal conductance of TOC1-ox, WT and *toc1-2* mutant plants. Gas exchange measurements were performed with at least 10 rosettes for genotype. (F) Water-loss rates of detached rosettes from WT, TOC1-ox and *toc1-2* plants. Data are means ± s.e.m. of triplicate measurements with at least five rosettes for genotype at each time point. Compared with WT, the phenotypic differences were statistically significant with P-values<0,01 in all cases.

TOC1 regulates the diurnal and circadian expression of *ABAR*

One of the mis-regulated genes in TOC1 microarray datasets was *ABAR/CHLH/GUN5* (Shen et al, 2006; Wu et al, 2009). This was of interest because ABAR was initially reported as an ABA receptor and because preliminary studies in the laboratory had linked TOC1 with ABAR (Riechmann JL and Mas P, unpublished results). Although a recent report in barley has questioned the function of ABAR in ABA signalling (Muller & Hansson, 2009), our phenotypic studies using different *Arabidopsis ABAR RNAi* lines with decreased patterns of *ABAR* expression (Figure 7A) were consistent with previous reports (Shen et al, 2006; Wu et al, 2009a). Indeed, compared with the effect of ABA on WT plants (Figure 7B and C), the stomata in different *ABAR RNAi* lines were almost insensitive to the treatment with the hormone (Figure 7B and C). Differences were statistically significant in the absence (P-value<0,0001) and in the presence of ABA (P-value<0,0001). Consistent with these and the previously described phenotypes (Shen et al, 2006; Wu et al, 2009a), our studies also showed that the water-loss rates of *ABAR RNAi* plants were significantly higher than in WT (P-value<0,005 at all time points) (Figure 7D). Furthermore, comparisons of *ABAR* mRNA expression with the severity of water-loss phenotypes in different RNAi lines showed a significant inverse correlation between *ABAR* mRNA abundance and water-loss rates (Figure 7E). Finally, our studies also showed that the impaired stomatal closure and waterloss rates also correlated with altered responses to dehydration conditions (Figure 7F) with a reduced survival percentage of the different *ABAR RNAi* plants. Altogether, our results are in concordance with the previous study in *Arabidopsis* and verify a role of *ABAR* within the ABA signalling pathway in *Arabidopsis*.

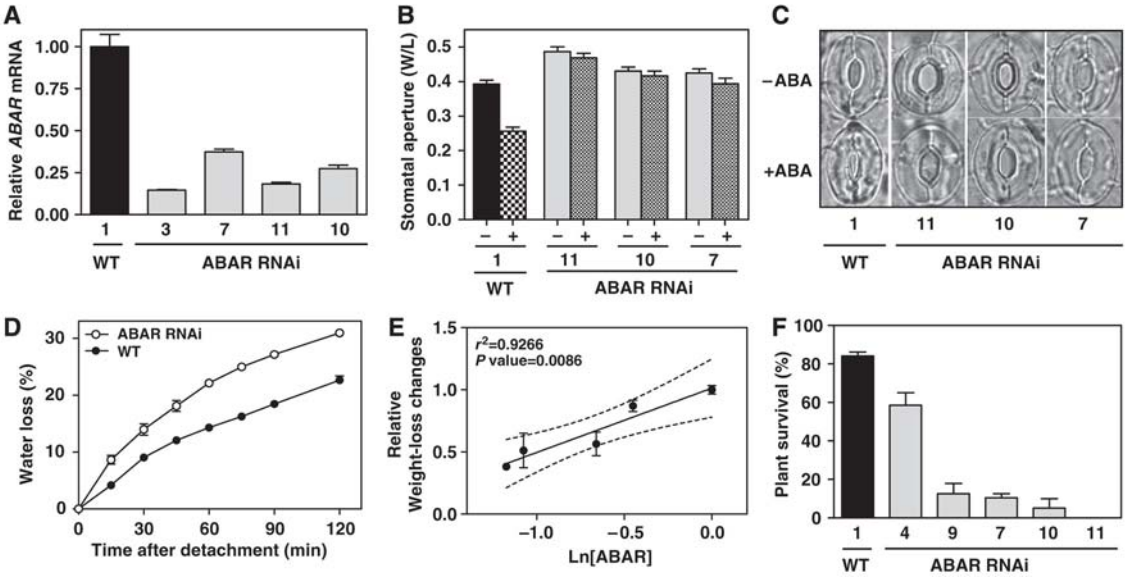

Figure 7: Altered responses to drought conditions of plants mis-expressing *ABAR*. (A) Analysis by Q-PCR of *ABAR* mRNA expression in WTand several *ABAR RNAi* lines. (B) Stomatal aperture in rosette epidermis after incubation for 2 h in a buffer containing 0 or 5 mM ABA. Data are means ± s.e.m. of duplicate experiments with at least 100 stomata per genotype and per treatment. (C) Representative images by light microscopy of the stomata guard cells. (D) Water-loss rates of detached rosettes from WTand *ABAR RNAi* plants. Data are means ± s.e.m. of triplicate measurements with at least five rosettes for genotype at each time point. (E) Correlation between *ABAR* mRNA abundance and weight-loss changes of detached rosettes. Weight-loss changes were plotted relative to the WT value. (F) Plant survival to dehydration stress on agar plates. Data are means ± s.e.m. of duplicate experiments with at least 25 plants per genotype. Compared with WT, the phenotypic differences were statistically significant with P-values<0,005 in all cases.

On the basis of the ABA-related phenotypes and the pattern of *ABAR* expression in TOC1 microarrays, we next explored the possible link between the circadian clock and *ABAR* expression. To do so, we first performed time course analyses over a diurnal cycle of WT plants grown under short-day (ShD) and long-day (LgD) conditions. Our results showed that *ABAR* transcript abundance rhythmically oscillated with a peak of expression close to dawn (Figure 8A). Similar oscillatory patterns were observed when *ABAR* expression was examined under LL conditions (Figure 8B) indicating that the circadian clock controls the expression of *ABAR*.

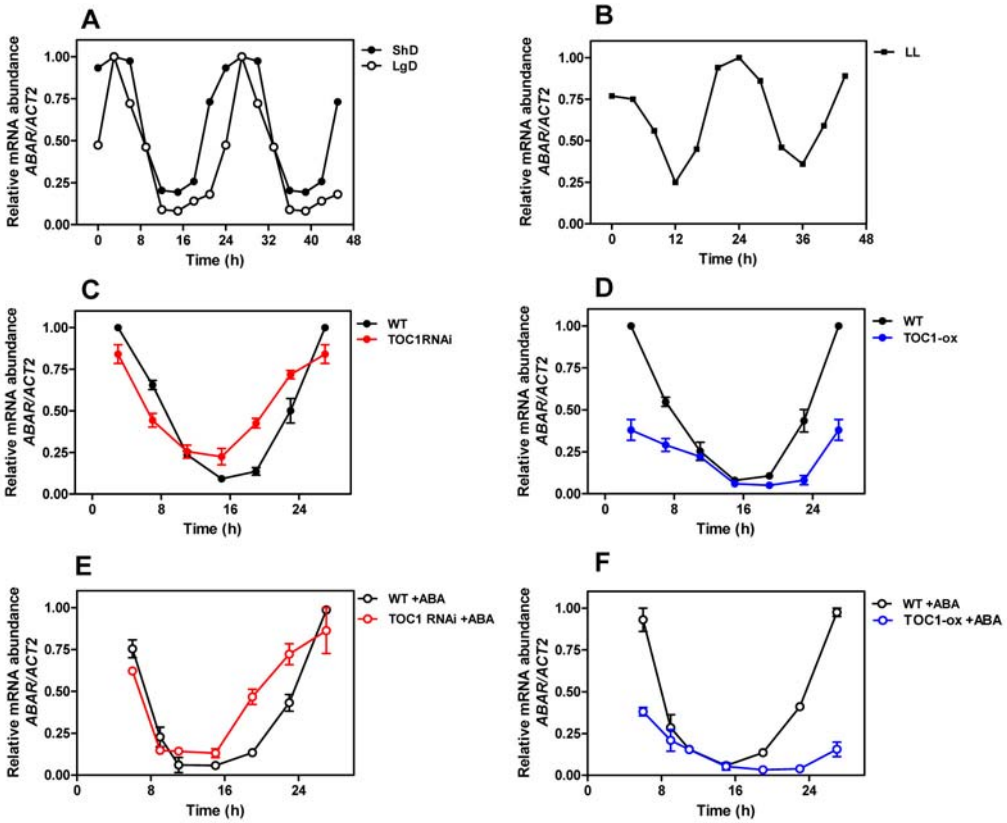

Figure 8: The diurnal expression of *ABAR* is regulated by TOC1. (A) Diurnal waveform of *ABAR* expression examined by Northern blot in WT plants grown under Short-Day (ShD, 8h light:16h dark) and Long-Day (LgD, 16h light:8h dark) conditions. (B) Circadian waveform of *ABAR* expression examined by Northern blot in WT plants synchronized under 12h light:12h dark (LD) cycles followed by 2 days under constant light conditions (LL). (C, D) Diurnal waveform of *ABAR* expression in *TOC1 RNAi* and TOC1-ox plants grown under 12h light:12h dark cycles. (E, F) Effects of ABA treatment on *ABAR* expression in *TOC1 RNAi* and TOC1-ox plants grown under 12h light:12h dark cycles.

As TOC1 is an essential component of the *Arabidopsis* circadian system, we reasoned that the diurnal and circadian expression of *ABAR* might be controlled by TOC1. Indeed, our results showed that the waveform of *ABAR* mRNA was altered in *TOC1 RNAi* and TOC1-ox plants under LL. The phase of *ABAR* expression in *TOC1 RNAi* was advanced, leading to higher mRNA abundance at times when *ABAR* expression is at its minimum in WT plants (Figure 9A). The waveform of *ABAR* expression in *TOC1 RNAi* plants also suggests that *ABAR* might be complexly regulated in the absence of a functional

TOC1. In TOC1-ox plants, *ABAR* expression was clearly affected, with very reduced abundance throughout the circadian cycle (Figure 9B). Changes in *ABAR* expression were also observed in TOC1-ox and *TOC1 RNAi* plants growing under LD cycles (Figure 8C and D). The alterations in *ABAR* expression were also observed when seedlings were treated with ABA (Figure 8E and F).

Together, these results indicate that the circadian clock controls the timing of *ABAR* expression. Our studies also assign a key function for TOC1 in modulating the phase and amplitude of *ABAR* diurnal and circadian waveform. The circadian period and/or diurnal phase changes in *toc1-2* and TOC1-ox plants might affect the proper expression of *ABAR* and other ABA/drought-related genes, and this misregulation may alter the proper timing of plant responses to drought.

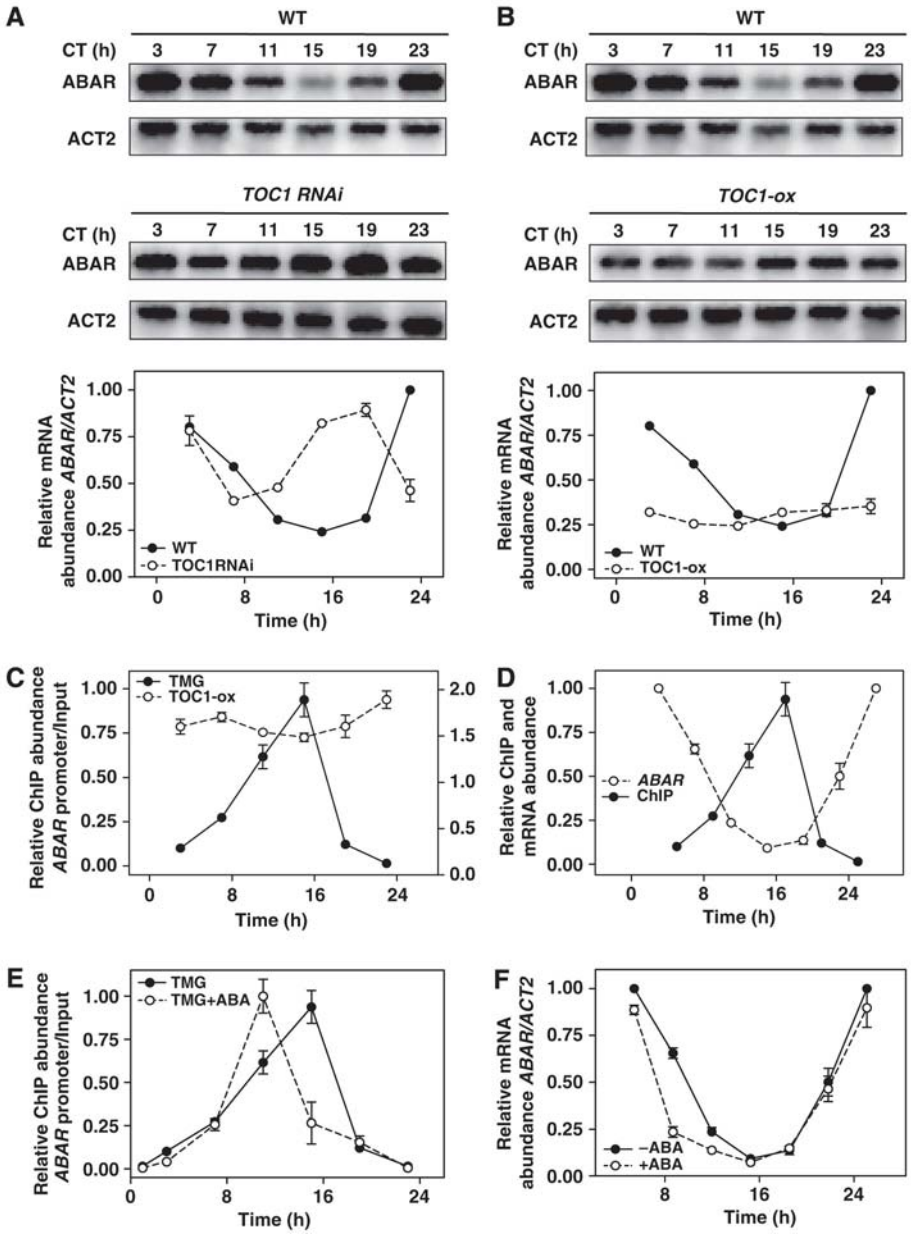

Figure 9: TOC1 binds to the *ABAR* promoter and regulates *ABAR* circadian expression. (A, B) Northern blot analysis *of TOC1 RNAi* and TOC1-ox plants synchronized under LD cycles followed by 2 days under LL. The waveforms of *ABAR* expression after mRNA quantification are shown below the blots. CT, circadian time. (C) ChIP assays with TOC1-ox (right axis) and TMG plants (left axis) after TOC1 immunoprecipitation with an antibody to YFP followed by Q-PCR amplification of the ABAR promoter. (D) Comparison of the antiphasic waveforms of *ABAR* expression by northern blot and TOC1 binding to the *ABAR* promoter by ChIP assays. (E) Effects of ABA on the waveform of TOC1 binding to the *ABAR* promoter. (F) Effects of ABA on the *ABAR* mRNA expression. ChIP abundance was plotted relative to the maximum value.

TOC1 is physically associated to the ABAR promoter

As DNA-binding events are crucial in ABA signalling pathways (Busk & Pagès, 1998), we next explored whether regulation of *ABAR* expression occurred through binding of TOC1 to the *ABAR* locus. Chromatin immunoprecipitation (ChIP) assays were performed with TOC1-ox and TOC1 minigen plants (TMG, expressing the *TOC1* gene under its own promoter) (Más et al, 2003a). The TMG plants more closely resemble the WT conditions (as compared with the arrhythmic TOC1-ox) although the TMG clock runs slower than in WT, with a delayed phase of rhythmic gene expression (Más et al, 2003a). Time course experiments throughout the 24 h cycle showed significant amplification, consistent with the *in vivo* binding of TOC1 to the *ABAR* promoter (Figure 9C). Further analysis showed a rhythmic oscillation in the binding, with a peak at a time when *ABAR* expression is minimal (Figure 9C and D). Our results showed that TOC1 binding is antiphasic to *ABAR* mRNA expression (Figure 9D) and this is consistent with the inverse correlation between abundance and phenotypes of *TOC1* and *ABAR*. The binding seems to be restricted to the *ABAR* promoter, and more specifically located around the transcription start site (Figure 10). No evident amplification was observed with primers flanking a region in the fourth exon of the *ABAR* gene (Figure 10).

Binding ChIP assays with TOC1-ox plants showed significant amplification of the *ABAR* promoter at all times examined, indicating that TOC1 protein remains bound throughout the cycle (Figure 9C). This constant binding (as opposed to the oscillatory waveform in TMG plants) correlates with a constant repression of *ABAR* (compare Figure 9B and C) and might account for the ABA-related phenotypes observed in TOC1-ox plants. On the basis of this notion, we expected that treatment with ABA should affect TOC1 binding and/or *ABAR* expression. To check this possibility, we next performed a time course of ChIP assays in TMG samples treated with the hormone. We also compared by northern blot the pattern of *ABAR* expression in ABA-treated and untreated plants. Our results showed a transient change in the phase of TOC1 binding to the *ABAR* promoter in ABA-treated TMG plants (Figure 9E). Concomitantly, we also observed a change in *ABAR* mRNA waveform, with a significant acute

repression (P-value<0,005) around midday, the time window when TOC1 is preferentially bound to the *ABAR* promoter (Figure 9F). These results indicate a temporal coincidence between the ABA-mediated regulation of *ABAR* expression and TOC1 binding to the *ABAR* promoter. The specificity of this association was confirmed by analysing TOC1 binding to other relevant ABA-related loci. Our results showed that with the exception of *ABAR*, none of the selected loci was amplified (Figure 11). Binding was not observed even after treating the seedlings with ABA (Figure 12).

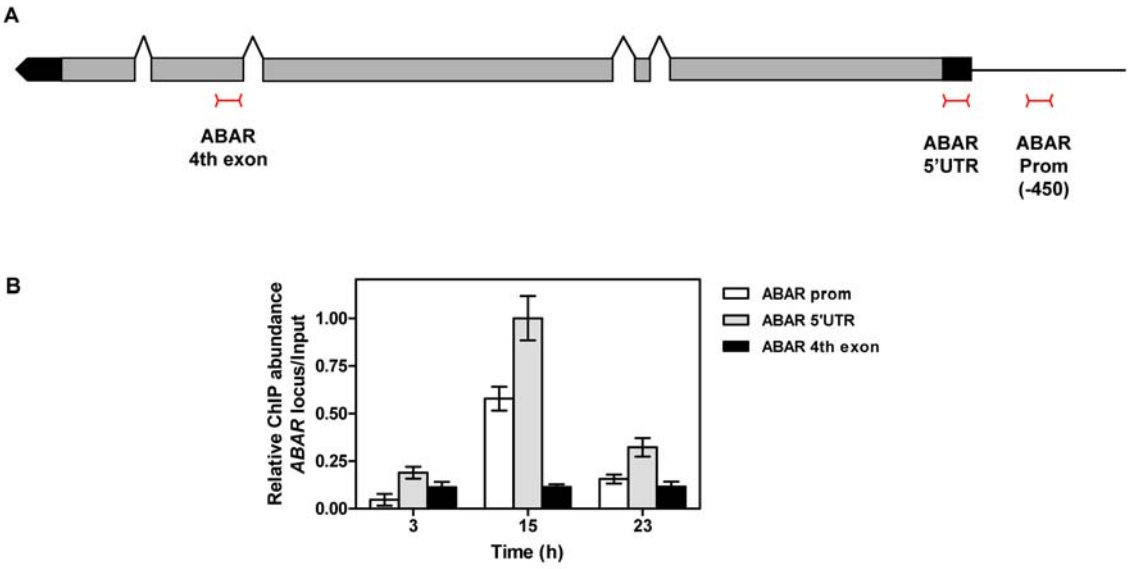

Figure 10: Analysis of TOC1 binding to the *ABAR* locus. (A) Schematic representation of the *ABAR* genomic structure. Red lines indicate the position of the regions chosen for amplification in the ChIP assays. *ABAR* Promoter (around 450 bases from the ATG); *ABAR* 5'UTR (around the *ABAR* transcription start site); ABAR 4th exon (around the 4th exon of the gene). (B) QPCR reflecting the binding of TOC1 to the *ABAR* locus in TMG plants synchronized under LgD cycles (16h light:8h dark) and then transferred for two days under constant light conditions. ChIP assays were performed after TOC1 immunoprecipitation with an antibody to YFP followed by amplification of the different regions of *ABAR* locus. Samples were analyzed at the indicated circadian times (CT3, CT15 and CT23).

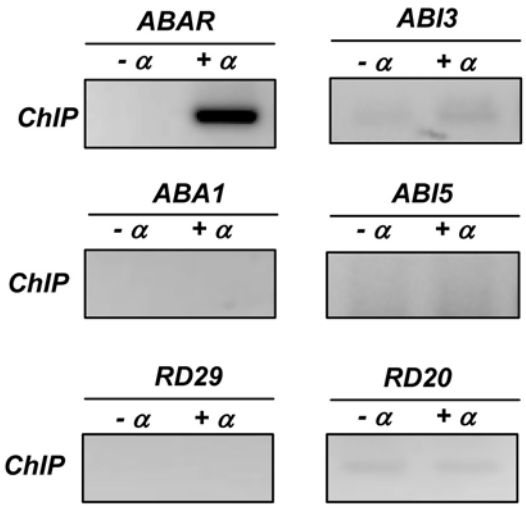

Figure 11: ChIP assays of TOC1 binding to the *ABAR* promoter with TMG plants after TOC1 immunoprecipitation with an antibody to YFP followed by amplification of several promoters of genes involved in ABA signalling pathways. As control, samples were similarly processed in the absence of antibody (-α).

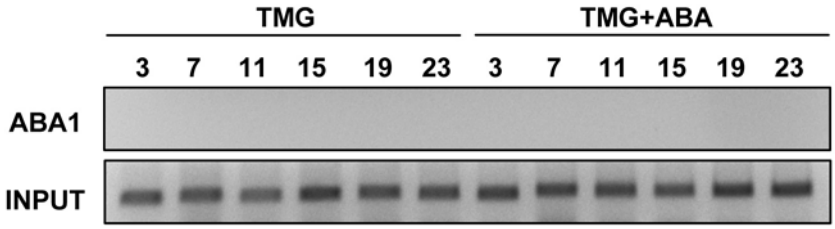

Figure 12: ChIP assays with TMG plants after TOC1 immunoprecipitation with an antibody to YFP followed by amplification of the *ABA1* promoter. TOC1 binding to the *ABA1* promoter was analyzed in the presence or in the absence of ABA.

Altogether, our results show that TOC1 binds to the *ABAR* promoter. This binding is regulated not only by the circadian clock but also by the ABA, which advances the phase of TOC1 binding and acutely represses *ABAR* expression around midday. On the basis of the phenotypes of *ABAR* and *TOC1* mis-expressing plants, this regulation is most likely important for ABA-mediated plant

responses to drought.

TOC1 expression is acutely induced by ABA and this regulation is gated by the clock and requires a functional ABAR

Relevant to our studies was the observation that *TOC1* was included in the microarray dataset of genes regulated by ABA (Matsui et al, 2008). To examine this new regulatory mechanism, we monitored bioluminescence rhythms of plants expressing the luciferase gene (*LUC*) driven by the *TOC1* promoter (*TOC1:LUC*) in the presence of ABA. Our results showed that ABA treatments administered during the subjective day acutely induced *TOC1:LUC* expression (Figure 13A). The magnitude of induction was not constant but progressively increased throughout the day, reaching maximum values at 5–10 h after the subjective dawn (Figure 13A). Administration of ABA during the subjective night had no clear immediate consequences on *TOC1:LUC* amplitude or phase (Figure 13B). On subsequent days, the extended time with the hormone led to a decreased amplitude and slight delay in the phase of *TOC1:LUC* expression. The time-of-day-dependent regulation of *TOC1* expression by ABA indicates that the acute response is gated by the clock and correlates with the timing of TOC1 binding and changes in *ABAR* expression by ABA.

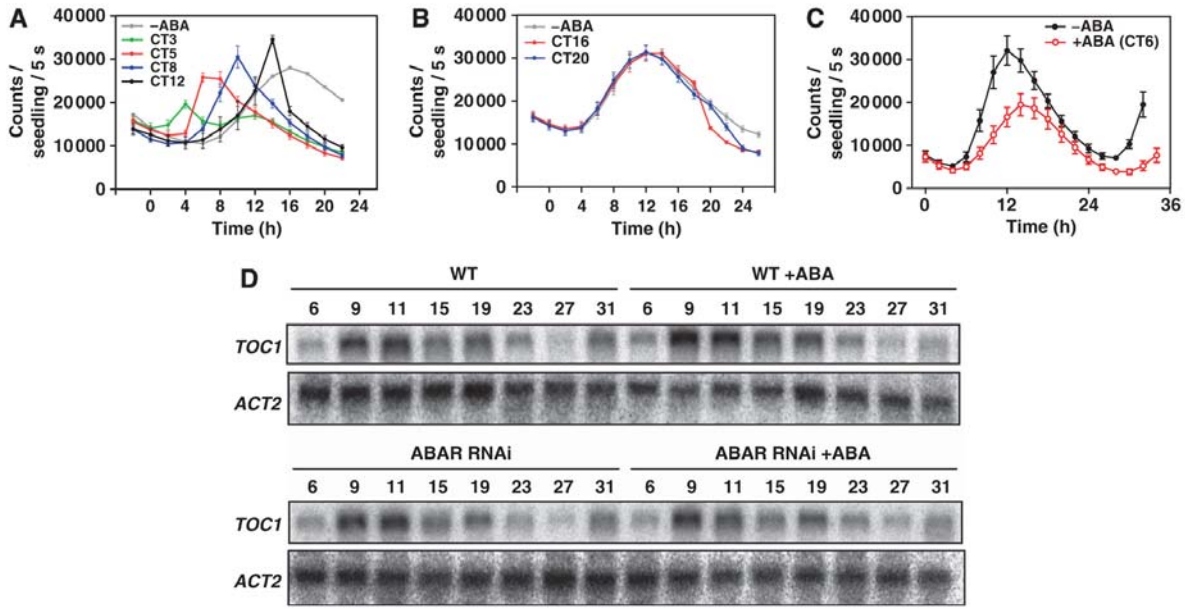

Figure 13: *TOC1* is acutely induced by ABA and this regulation is gated by the clock and requires a functional ABAR. (A, B) *TOC1:LUC* expression in WT plants treated with 25 μM ABA at the indicated circadian times (CT) during the subjective day and night. (C) *TOC1:LUC* expression in *ABAR RNAi* plants treated with 25 μM ABA at the indicated CT during the subjective day. Data are means ± s.e.m. of luminescence from 6–12 plants. (D) Northern blot analysis of *TOC1* mRNA expression in WT and *ABAR RNAi* plants. *TOC1* expression was compared in the absence or in the presence of ABA.

The transient induction of *TOC1* is suggestive of a finetuned switch mechanism that regulates the ABA-mediated effects on *TOC1* expression. As feedback loops are common mechanisms for precise regulation of gene expression, we explored the possible function of ABAR in *TOC1* induction by ABA. We therefore examined the regulation of *TOC1:LUC* expression in *ABAR RNAi* plants, lacking a functional expression of the *ABAR* gene. Our results showed that in the absence of ABA, *TOC1:LUC* expression in *ABAR RNAi* plants was similar to the one observed in WT plants, although we found a slightly advanced phase of *TOC1:LUC* expression that was not reproducibly observed in all *ABAR RNAi* lines (Figure 14). After treatment with ABA, the acute induction of *TOC1:LUC* in WT plants was completely abolished in *ABAR*

RNAi plants (Figure 13C) with a slight delay in the oscillatory phase of *TOC1:LUC* expression and with bioluminescence signals lower than those of untreated *ABAR RNAi* samples (Figure 13C). Furthermore, upregulation of *TOC1:LUC* expression by ABA was not observed at any time during the circadian cycle. The role of ABAR in *TOC1* induction was also verified by northern blot analysis. Our results showed that in *ABAR RNAi* samples, *TOC1* mRNA steady state was not upregulated after ABA treatment as opposed to the gated induction observed in WT plants (Figure 13D; Figure 15). Together, these results indicate that the ABA-mediated acute induction of *TOC1* requires the presence of a functional ABAR, which positively regulates the expression of *TOC1*. Noticeably, this regulation seems to have an important function only in the presence of ABA. In the absence of exogenous hormone, the waveform of *TOC1* expression is not clearly affected, with the possible exception of a slightly advanced phase. These results suggest that ABAR is important for regulation of *TOC1* expression within the ABA signalling pathway but not within the circadian pathway. Our results also indicate the existence of a regulatory feedback loop involving the reciprocal regulation of *ABAR* and *TOC1*.

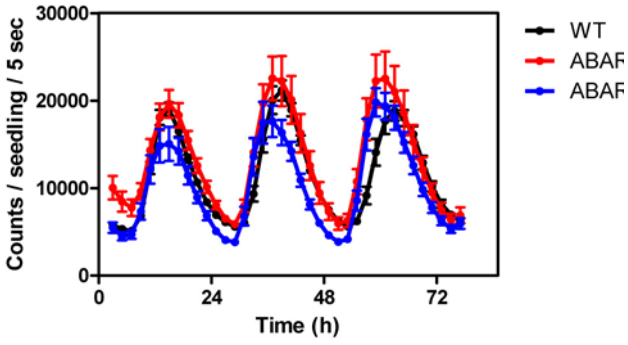

Figure 14: Analysis of *TOC1:LUC* expression examined by luminescence assays in WT plants and several *ABAR RNAi* lines in the absence of ABA. Data are means ± s.e.m of luminescence from 6-12 plants.

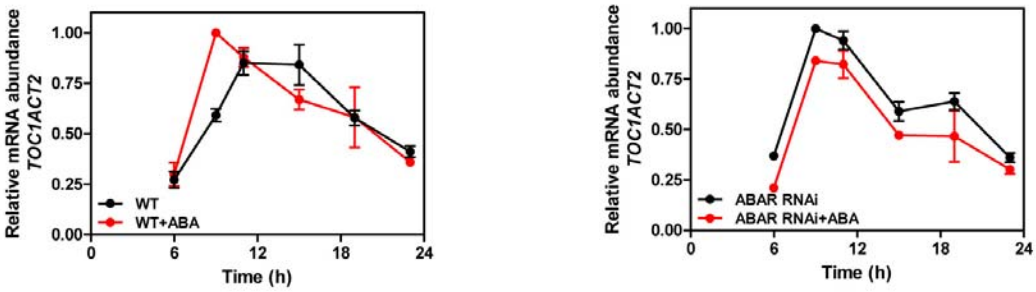

Figure 15: Quantification of *TOC1* mRNA expression by Northern blot analysis in WT and *ABAR RNAi* plants. Analyses were performed with RNA from twelve day-old seedlings and *TOC1* expression examined in the presence or in the absence of 100 μM (±) ABA. Data are plotted relative to the maximum value.

Biological relevance of ABAR and TOC1 interaction

To further investigate whether the reciprocal regulation between *TOC1* and *ABAR* expression is important for ABA signalling, we conducted a genetic study in which the TOC1-ox and the *TOC1 RNAi* plants were transformed with the *ABAR RNAi* construct (Shen et al, 2006). Several lines for each genotype were selected and the decreased *ABAR* expression was verified by Q-PCR (Figure 16).

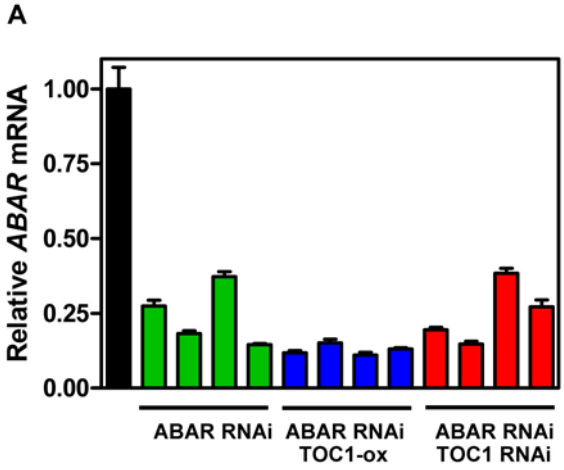

Figure 16: Comparison of relative *ABAR* mRNA abundance in different lines. Quantitative-PCR (Q-PCR) analysis of *ABAR* abundance in *ABAR RNAi, ABAR RNAi/TOC1 RNAi* and TOC1-ox/*ABAR RNAi* lines. *ABAR* mRNA was quantified relative to *IPP2* mRNA. Data are plotted relative to the value of WT plants.

Initial studies were focused on water-loss rates, as analysis of different *ABAR RNAi* transgenic lines showed that the severity of the water-loss phenotypes correlated with the *ABAR* mRNA abundance (Figure 7). In our genetic studies, we predicted that if the *TOC1 RNAi* phenotypes were due to increased *ABAR* expression, then the significantly diminished transpiration rate of *TOC1 RNAi* plants should be overridden by the opposing phenotype of *ABAR RNAi* plants. Conversely, if the ABA-related phenotypes of TOC1-ox plants are mediated by the repressed *ABAR* expression, then the transpiration rates of *ABAR RNAi*/TOC1-ox plants should be similar to those of plants over-expressing the repressor TOC1. Consistent with these assumptions, our results showed that double *TOC1 RNAi*/*ABAR RNAi* plants displayed higher transpiration than WT plants, with similar water-loss rates to those of *ABAR RNAi* plants (Figure 17A). As predicted, the water-loss phenotype of *TOC1 RNAi*/*ABAR RNAi* clearly contrasted to the decreased transpiration of *TOC1 RNAi* (Figure 17A) indicating that the *TOC1 RNAi* phenotype is only apparent in the presence of a functional ABAR. Conversely, our results showed that the transpiration rate of TOC1-ox was not significantly affected by the reduced expression of *ABAR* in TOC1-ox/*ABAR RNAi* plants. These plants displayed an increased transpiration rate, similar to the phenotype observed for single TOC1-ox (Figure 17B). The *ABAR RNAi* plants displayed intermediate phenotypes (Figure 17B) as *ABAR* expression was overall higher in these RNAi lines than in TOC1-ox/*ABAR RNAi* (Figure 16). Similar genetic conclusions were obtained when we performed experiments of dehydration on plates. The high percentages of *TOC1 RNAi* plant survival after dehydration were completely overridden in the double RNAi plants, suggesting that *TOC1 RNAi* phenotypes are only evident in the presence of a functional ABAR (Figure 17C and D). Conversely, the reduced survival percentages of TOC1-ox plants were not clearly affected by the reduced ABAR expression in TOC1-ox/ABAR RNAi lines (Figure 17C and D).

Altogether, the genetic studies are in agreement with the molecular data and functionally connect the reciprocal regulation of *TOC1* and *ABAR* expression with plant tolerance to drought conditions (Figure 17E).

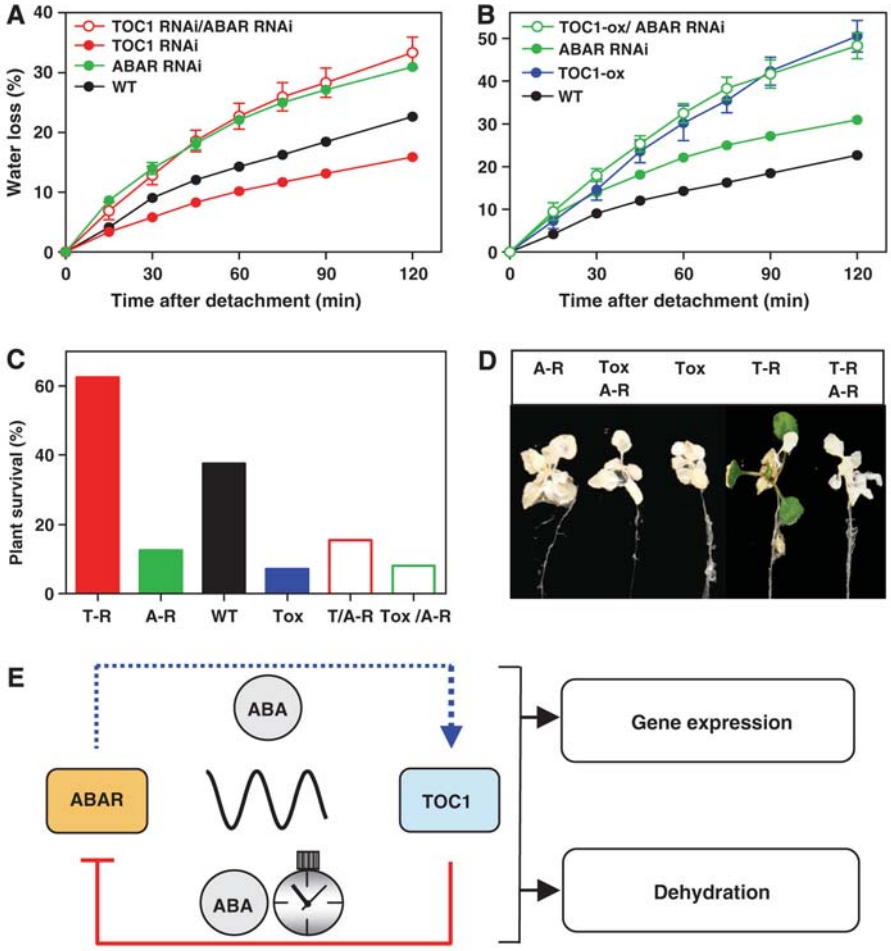

Figure 17: ABAR and TOC1 interaction in plant responses to drought. (A, B) Water-loss percentages of detached rosettes from WT, ABAR RNAi, TOC1 RNAi, double ABAR/TOC1 RNAi, TOC1-ox and TOC1-ox/ABAR RNAi plants. Data are means ± s.e.m. of triplicate measurements with at least 10 rosettes for genotype at each time point. (C) Survival percentages of WT, ABAR RNAi (A-R), TOC1 RNAi (T-R), double ABAR/TOC1 RNAi T/A-R, TOC1-ox (Tox) and TOC1-ox/ABAR RNAi (Tox/A-R) plants subjected to dehydration on agar plates. (D) Representative photographs of plants in the dehydration experiments. (E) Schematic representation depicting the reciprocal regulation between TOC1 and ABAR and the implication of ABA and the circadian clock in this regulation.

We propose that increased ABA concentrations activate *TOC1* expression and this activation is gated by the clock and determines the timing of TOC1 binding to the *ABAR* promoter and the changes in *ABAR* expression. Our

results indicate that ABAR is required for *TOC1* induction by ABA. Proper timing of this feedback loop is important for ABA-mediated changes in gene expression and plant responses to drought conditions.

Chapter 2

The Transplanta collection

The Trasplanta consortium is a project involving a group of laboratories aimed at achieving a higher functional comprehension of transcription factors in plants. The consortium has developed a network constituted by core labs, performing high throughput cloning and in plant transformation of *Arabidopsis thaliana* TF genes, and satellite labs, selecting single insertion and homozygote plant lines. The cloning covered more than 1000 Arabidopsis thaliana transcription factors that were transferred to a specific vector (pER8) designed for in plant ß-estradiol-inducible gene expression (Zuo et al, 2000a). For each transcription factor, up to three independent lines of Arabidopsis single insertion homozygote plants were generated. This battery of lines constitutes an exceptional tool for phenotypical screenings that gave us a strong advantage compared to traditional mutagen or activation tagging based screening. Firstly we already know what gene is over-expressed in each of the plants we screen. Secondly we minimize the possibility of detecting phenotypes due to the disruption of genomic elements by our transgene because we strictly use single insertion lines and we test more then one line for each gene. In addition, by using an inducible system, we reduce the influence of eventual pleiotropic effects of the over-expressed gene on the plant. Our contribution to the creation of the Transplanta collection was the selection of among hundreds of T_1 strains of those who displayed a single insertion. The selecting criterion was the 3:1 ratio of resistant to sensitive of one-week-old seedlings in the selective medium. At the following generation we chose the homozygotes among the progeny excluding the lines that still presented sensitivity to hygromycin. Once the homozygous lines were obtained we amplified them and send the fresh bulk of seeds to be redistributed among the other members of the Transplanta consortium.

The screening

The observation of leaf temperature by infrared thermal imaging has been used widely as a method for detecting impaired stomatal movements in several plant species (Dong et al, 2012; Jones, 1999; Jones et al, 2009; Merlot et al, 2002; Raskin & Ladyman, 1988; Vollsnes et al, 2009) and calculations have resolved that this approach produces highly correlated data with other type of measurements such as transpiration rates obtained by diffusion prometers (Jones, 1999). Tissues with differences in their gas exchange rate have different water contents, closed stomata favour water accumulation thus increasing leaf temperature, open stomata, in turn reduces temperature. Temperature difference reflects in a different amount of infrared irradiance of the leaf surface. A modern infrared camera, such as a FLIR A655sc coupled with FLIR Researcher PRO software, can detect temperature differences lower than 0,1ºC. Moreover, thanks to a colour-based image rendering, it allows the immediate identification of plants with altered gas exchange levels within a test of few minutes. We therefore decided to use this infrared imaging based method in order to screen for differential transpiration levels among the lines of the Transplanta collection.

β-estradiol induction

The first step of the screening was the set up of the working conditions for the inducible promoter. The Transplanta consortium provided us with two independent reporter lines for this purpose. These lines expressed a GUS-GFP fusion protein under the control of the ß-estradiol inducible promoter, and both of them were used to determine the optimal inductive conditions.

Our aim was to induce the expression of our transgenes in the guard cells with a protocol that could be coupled with a thermal imaging screening. Thermal imaging could be performed on two-weeks-old seedlings on accurately dry soil, thus, we tested two possible approaches for the application of ß-estradiol under these conditions.

Our first test was to spray seedlings with three different concentrations of ß-estradiol: 10, 50 and 100µM. We tried a simple solution and with three different surfactants separately in order to favour the absorption of the estradiol by the leaf tissue: Triton-X100 (0,01%), Tween20 (0,01%) and SilwetL77 (0,005%). Microscopic analysis for these treatments did not reveal any GFP in the guard cells or in any other part of the plant after 2, 4, 24 nor 48 hours of treatment. The absence of expression of the transgene was confirmed also by RT-PCR for the same time points. Being difficult to raise the concentration of the inducing agent, we tried with a 10-fold increase in the surfactants but we did not manage to produce any expression either.

The second approach in order to get a proper induction in soil was to add ß-estradiol directly to the watering solution of the seedlings. In order to maximize the uptake of inductor, seedlings were left for three days without watering before the treatment. Again, in this case we applied a plain estradiol solution as well as the three previously mentioned surfactants at the described concentrations without any detectable result after 4, 24 and 48h of treatment.

On the other hand we could observe a fairly strong induction of our transgene when we treated seedlings grown *in vitro*, pushing us to move our experimental conditions to this environment. In vitro induction revealed us that our promoter could produce expression in guard cells (Figure 1), suggesting that it would work as a model for perturbing stomatal movements.

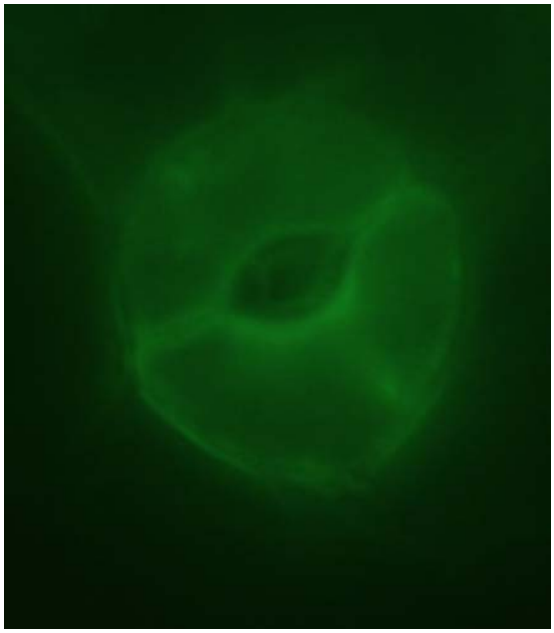

Figure 1: Representative picture of a stomate of a 12 days old seedling expressing the GUS-GFP fusion protein after 48 hours of in 20μM ß-estradiol medium (63x magnification on a fluorescence microscope).

Microscopy analysis suggested that the wider transgene expression for the leaf tissue was observed during the second day of induction. Then we tested different concentrations of inductor in order to obtain the highest transcriptional levels of our transgenes. We verified the induction of four different transgenes (USP, LOM2, RAP2.3 and GUSGFP test lines), each of them in two independent lines, by semi quantitative RT-PCR and joined the results in one graph to select the best performing concentration among 1, 5, 10, 20 and 50μM (Figure 2).

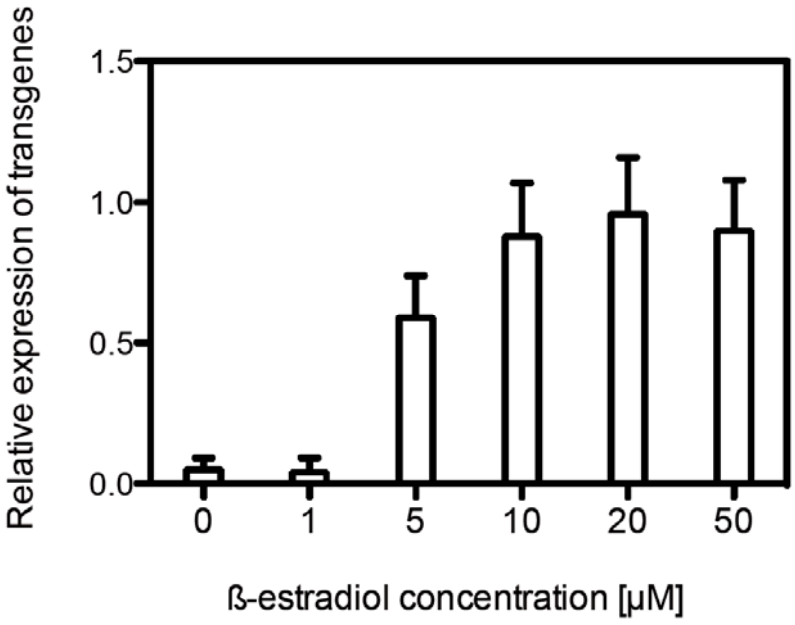

Figure 2: Average expression of the transgenes in 12 days old seedlings after 2 days of induction with different concentrations of ß-estradiol.

Based on these results we chose to perform the screening on 12-days-old seedlings grown in Petri dishes and to induce the transgenes expression through a medium complemented with 20µM ß-estradiol during 2 days. The adoption of such experimental conditions forced us to adapt the known protocols for thermal imaging to *in vitro* conditions.

The screening procedure

In the screening performed, every Petri dish included a positive control for impaired stomatal closure, the *ost1* (*open stomata 1*) mutant, and the induction control, the GUS-GFP reporter line, in addition to 4 lines to be tested. Each of the plates was produced in duplicate, in order to be able analyze the phenotype both in presence and absence of estradiol.

The final procedure adopted for the screening was to sow seeds on solid medium over a thin net, stratificate them during 3 days a 4ºC, and let them grow in long day conditions for 10 days. At this point the nets with the seedlings were

delicately removed from the growing medium and passed to freshly prepared inducing or mock medium. After another 2 days of induction the seedlings were examined with a FLIR Thermacam to determine their leaf temperature (Figure 3). The images where then quantified by the FLIR Thermacam Researcher Software. The phenotype was also tested for persistence after 24 hours from the first observation. We analyzed more then 250 lines from the Transplanta collection and found 6 genes whose over expression produced alterations of leaf temperature: three of them where “cold”, while the other half displayed a rather “warm” phenotype.

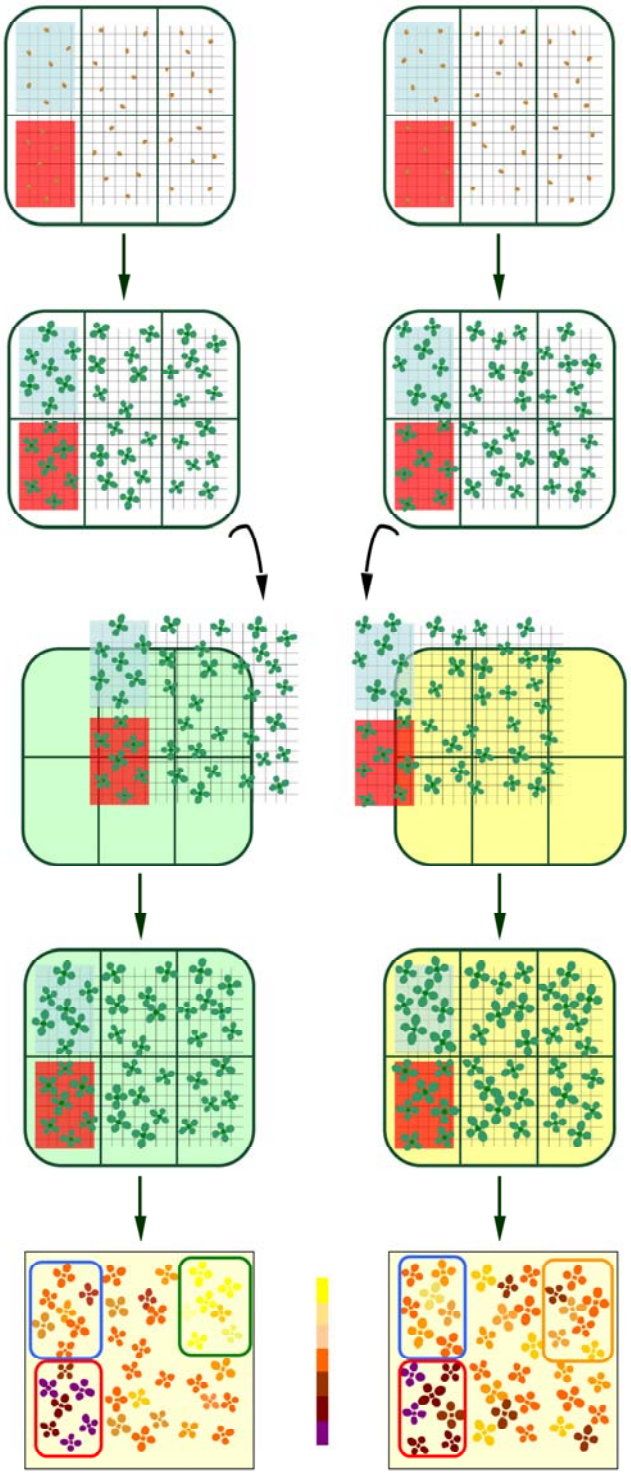

Figure 3: Scheme representing the screening procuedure. Seeds are sowed in MS1 medium plates with a sterile net. Each plate contains an induction control (GUSGFP reporter line, shown on blue background), a positive control for open stomata induced temperature shift (*ost1* mutant, shown in red background) and four of the lines to be tested. After 3 days of stratification and 10 days of growth, the nets with the seedlings are transferred to plates containing either ß-estradiol (green) supplemented MS1 medium or a mock medium (yellow). After two days of treatment, the plates are opened and observed with the Thermacam. The image rendering assigns a colour to every range of temperature, as represented by the colorimetric scale depicted between the two pictures. The induction control (blue squares), displays a *wildtype* temprature, while the positive control (red squares) is "colder". Candidate lines display altered temperature in inductive medum, in this case warm (green square), and wiltype phenotype in mock medium (brown square).

Selection criteria and composition of the batch of screened lines

The lines we managed to screen where selected on the base of the natural expression levels of the interested transcription factors in response to several abiotic stresses (Matsui et al, 2008; Nemhauser et al, 2006; Yoshida et al, 2010) o by their homology to transcription factors known for their implication in drought stress response (Fujita et al, 2011).

The list of tested lines included factors from several different families, as seen on figure 4.

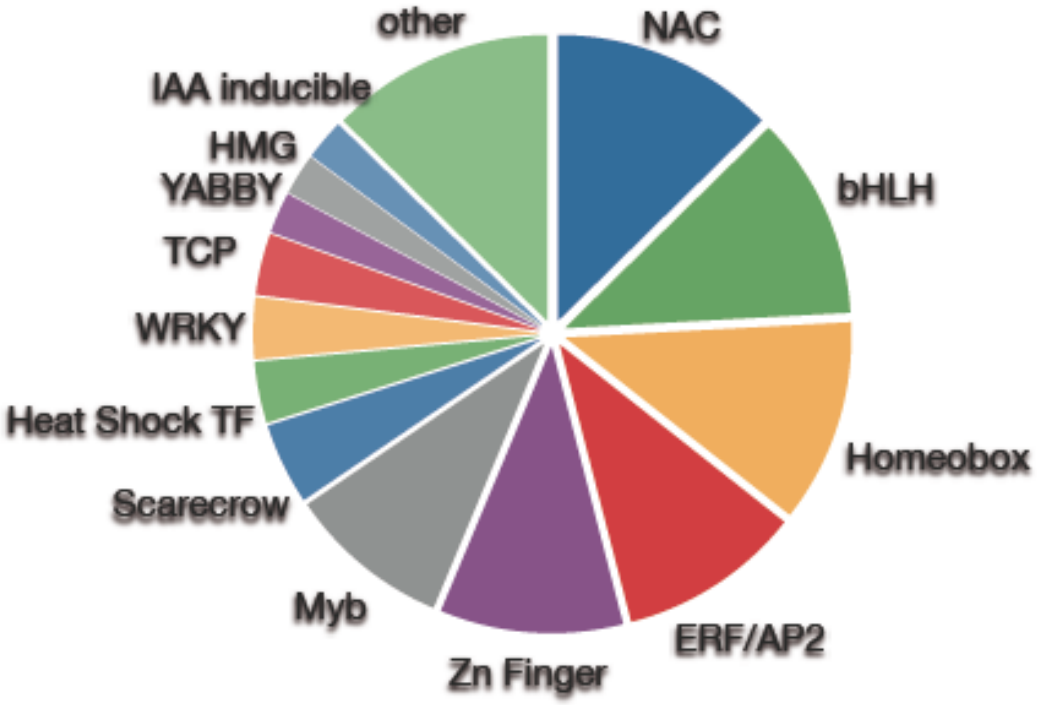

Figure 4: Families of transcription factors represented in the batch of *Arabidopsis* lines used for screening.

Screening results

The candidate genes were selected on the base of the phenotype displayed at the camera. After the first selection, a more precise imaging quantification was used to confirm the consistency of the temperature difference. All the selected genes presented their phenotype in at least two independent lines and only in presence of ß-estradiol, excluding the possibility of positional effects of the transgene.

TCP23

The first gene reporting a slight "warm" phenotype was TCP23 (At1g35560), as shown on figure 5.

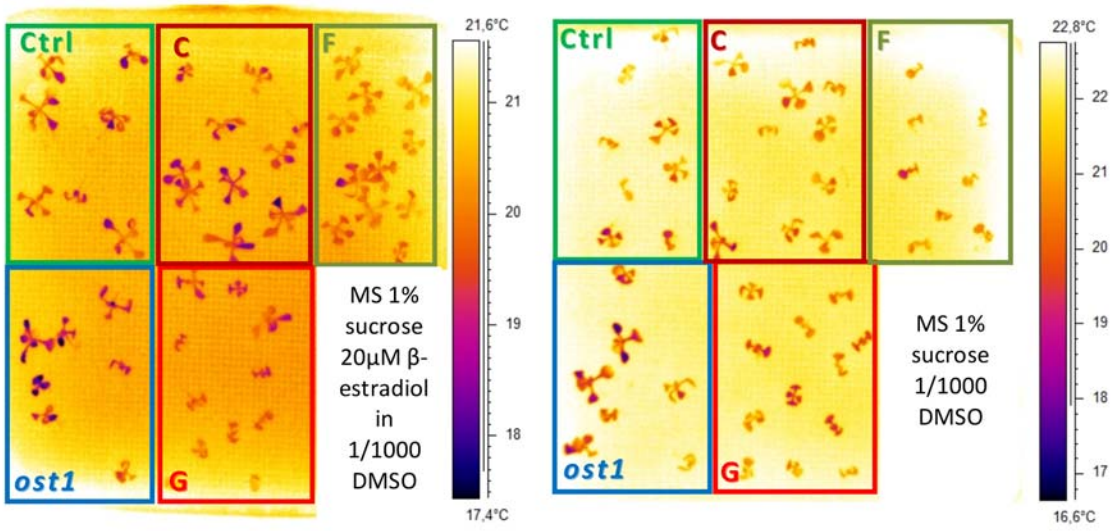

Figure 5: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include three independent lines of TCP23 conditional over-expressors (C, F and G). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

The three independent lines over-expressing TCP23 displayed different levels of phenotype, being line F the most different from the control while line C appeared to be equivalent to *wildtype*. The quantification (Figure 6) of the pictures shown in figure 5 demonstrated that both line F and G showed a significant difference from control in a two ways ANOVA with Bonferroni post-test ($P<0,05$).

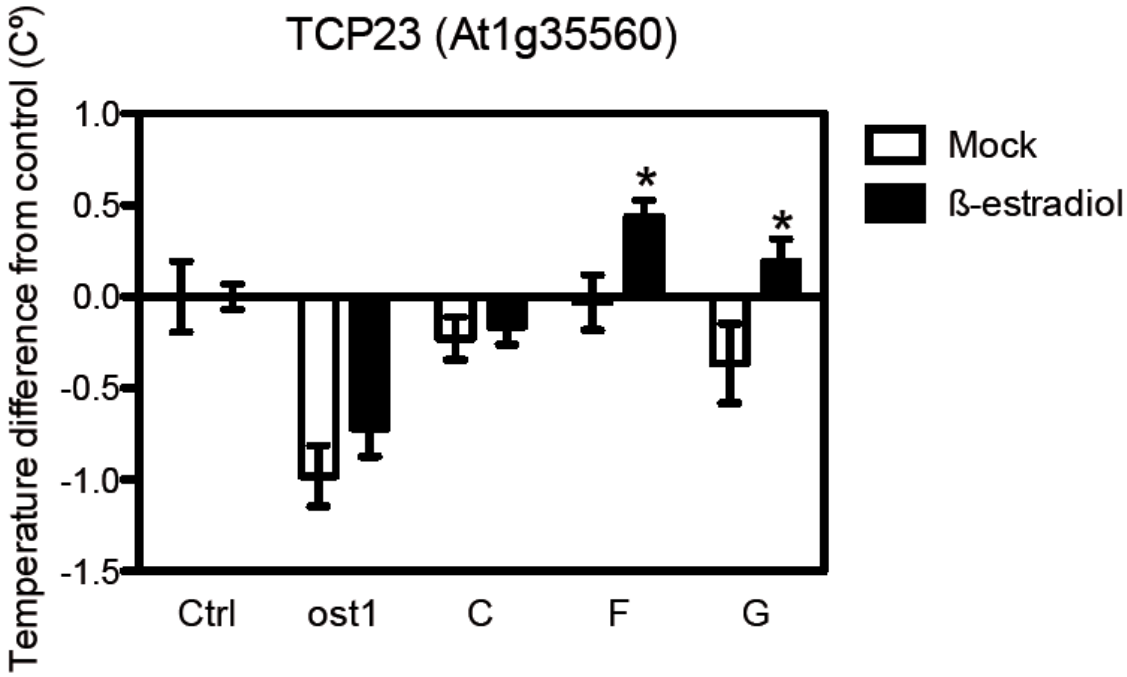

Figure 6: Quantification of the pictures shown in figure 5. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Asterisks stand for $P<0,05$ in two ways ANOVA with Bonferroni post-test.

DEAR4

A stronger example of "warm" phenotype was detected with the over-expression of DEAR4 (At4g36900). Figure 7 shows the dramatic enhancement of leaf temperature observed in two independent lines.

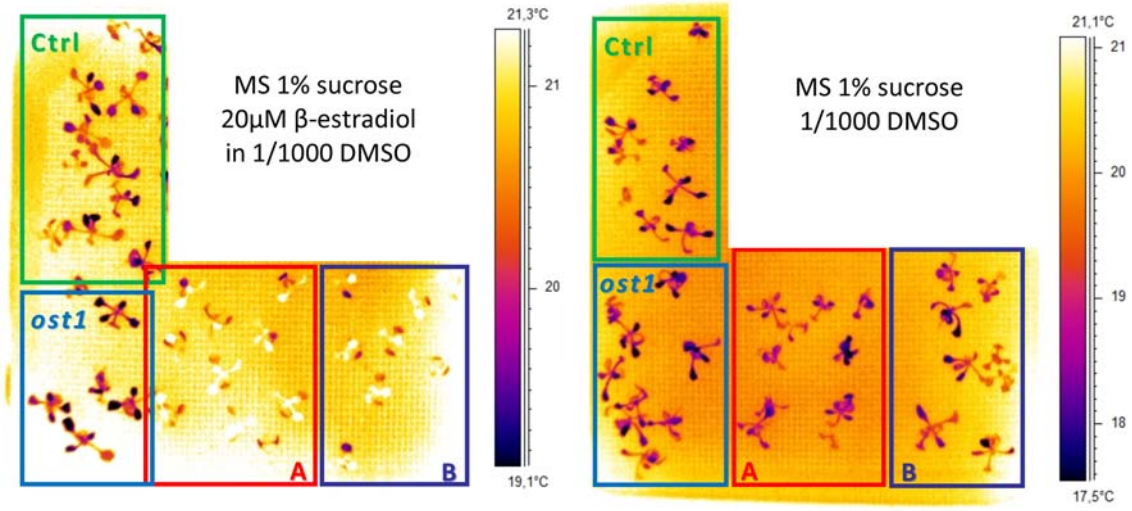

Figure 7: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include two independent lines of DEAR4 conditional over-expressors (A and B). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

The two ways ANOVA with Bonferroni post-test on the quantification data (Figure 8) confirmed that the induction of DEAR4 produced a significant increase in leaf temperature in both lines ($P<0,001$).

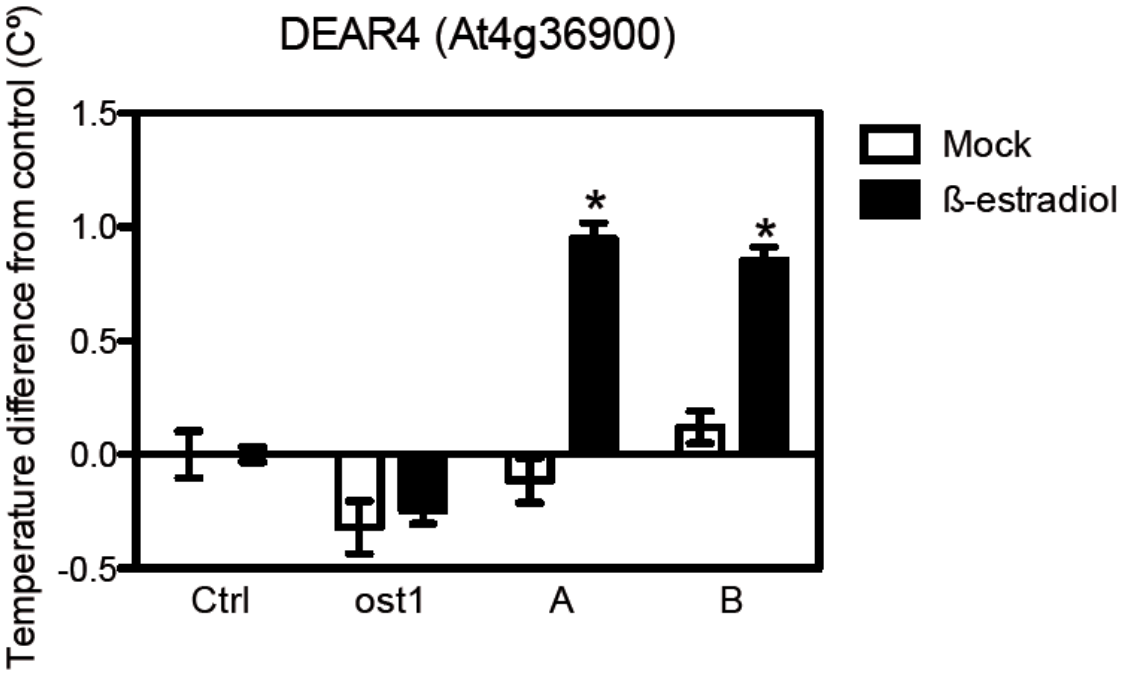

Figure 8: Quantification of the pictures shown in figure 7. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Asterisks stand for P<0,001 in two ways ANOVA with Bonferroni post-test.

NAC87

The third gene identified for its “warm” phenotype is the NAC87 (At5g18270) transcription factor, presenting a visible phenotype in three independent lines (Figure 9).

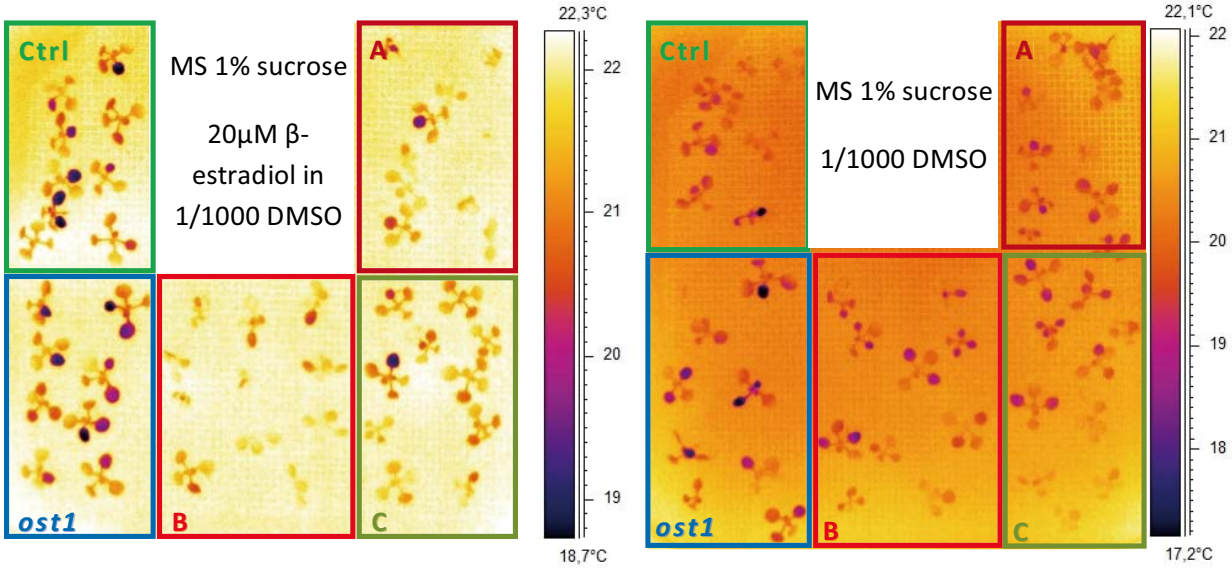

Figure 9: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include three independent lines of NAC87 conditional over-expressors (A, B and C). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

The quantification (Figure 10) revealed that the three lines over-expressing NAC87 displayed a significant phenotype ($P<0,001$).

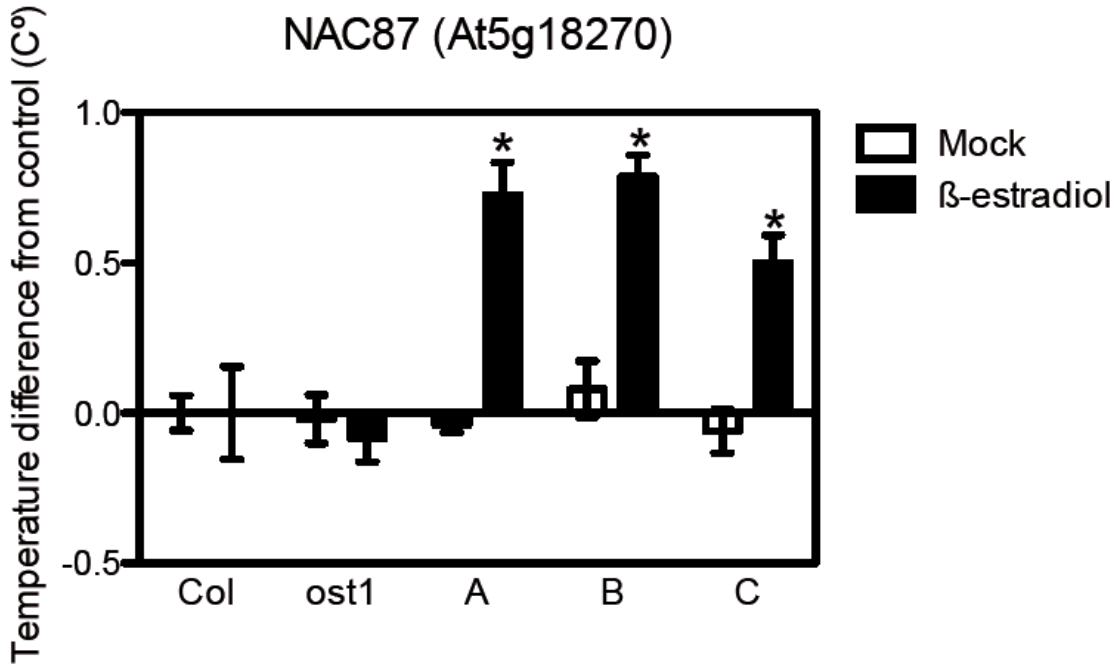

Figure 10: Quantification of the pictures shown in figure 9. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Asterisks stand for P<0,001 in two ways ANOVA with Bonferroni post-test.

HB23

The opposite phenotype ("cold") was detected with the over-expression of Homeobox23 (At1g26960), as shown in Figure 11.

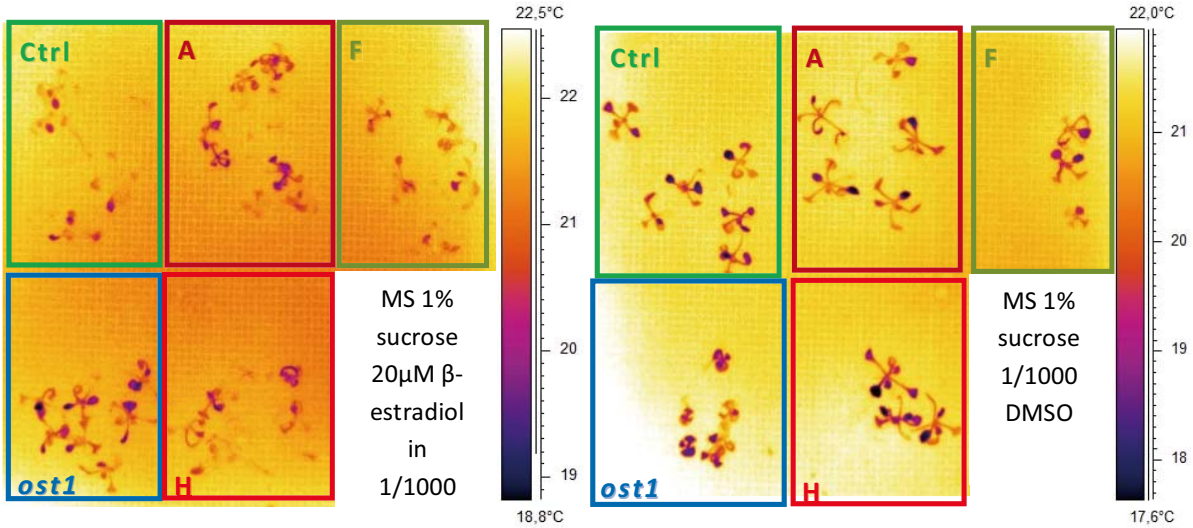

Figure 11: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include three independent lines of HB23 conditional over-expressors (A, F and H). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

In this case the quantification demonstrated that line A had a clearer phenotype ($P<0,001$), while line F had no significant difference from the control and line H has a less evident temperature shift ($P<0,05$) (Figure 12).

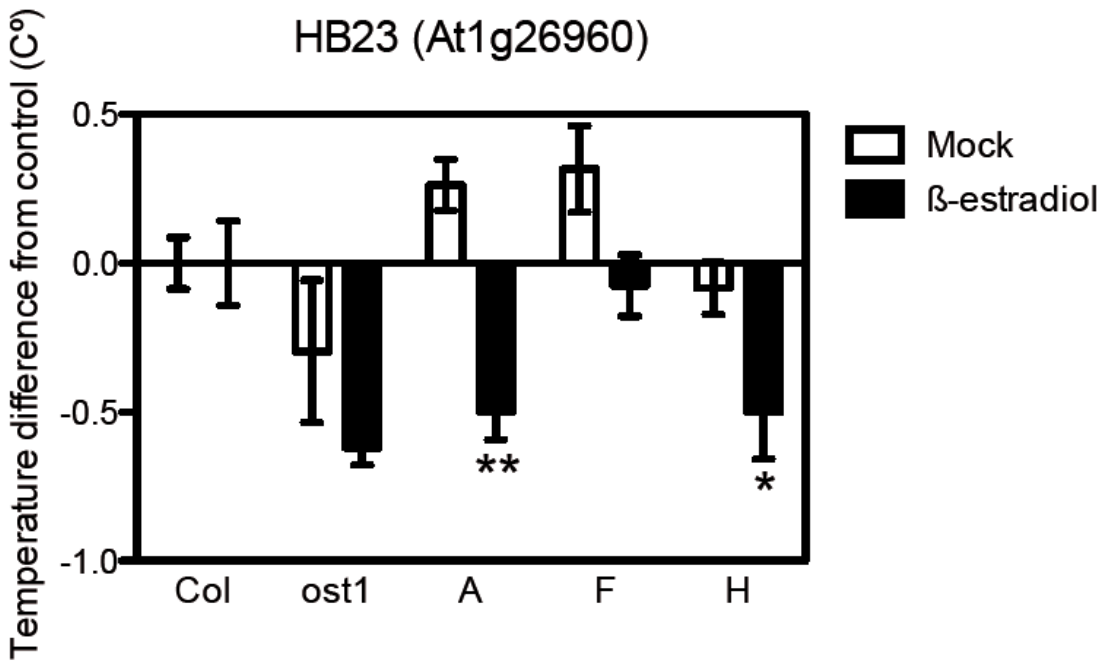

Figure 12: Quantification of the pictures shown in figure 11. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Double asterisks stand for P<0,001 and single ones for P<0,05 in two ways ANOVA with Bonferroni post-test.

HSFA8

In the case of the transcription factor HSFA8 the “cold” phenotype was observed in both lines B and C (Figure 13).

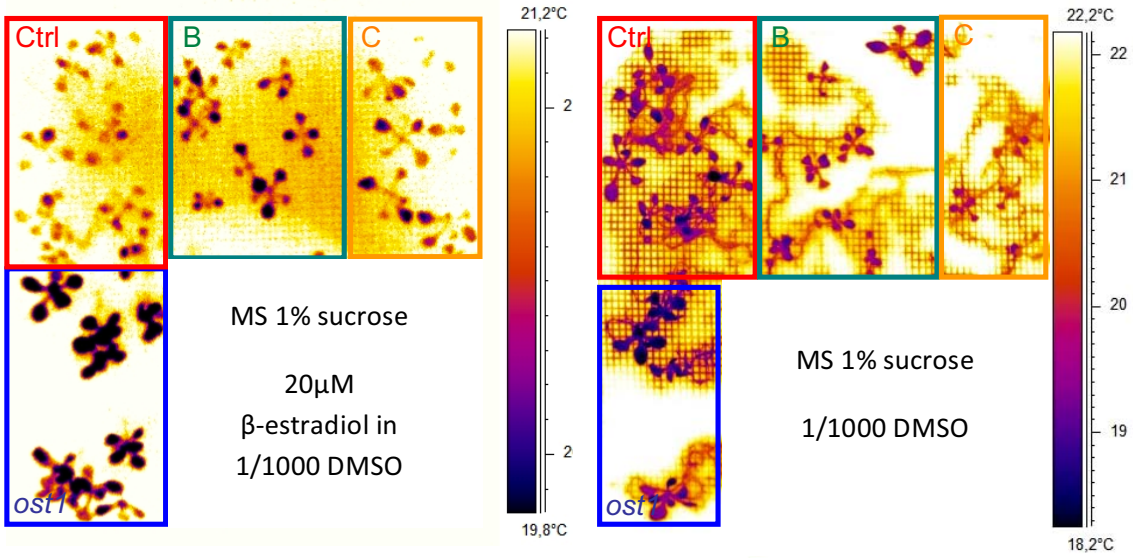

Figure 13: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include two independent lines of HSFA8 conditional over-expressors (B and C). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

The quantification (Figure 14) confirmed that the phenotype displayed by both lines was statistically relevant in a two ways ANOVA test with Bonferroni post-test. Being line B most significant ($P<0,01$) compared to line C ($P<0,05$).

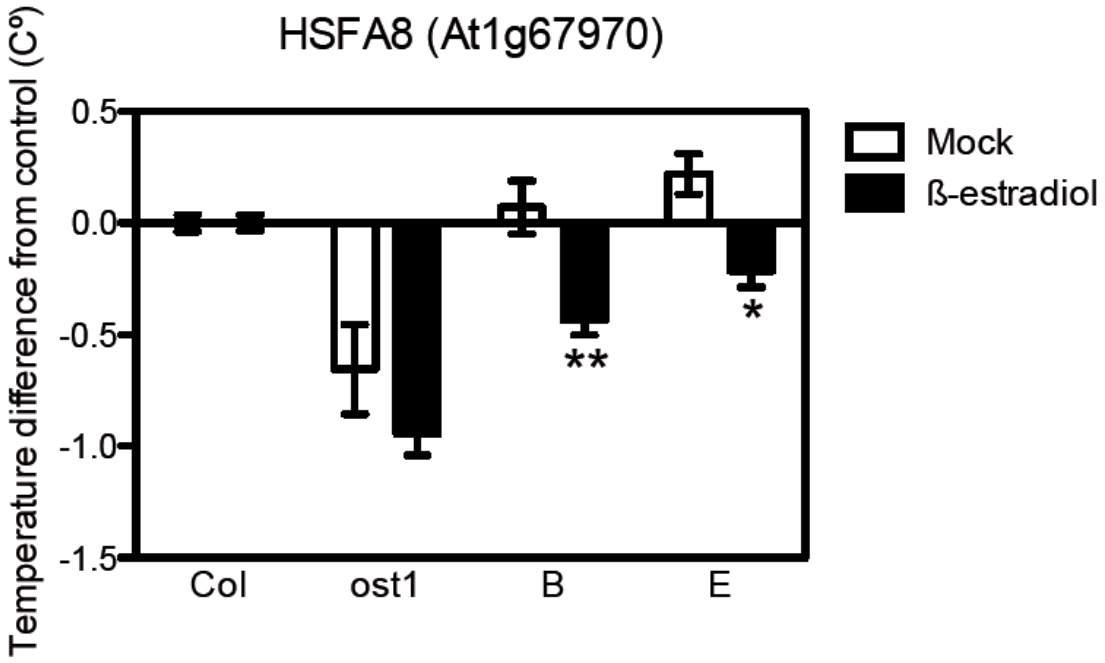

Figure 14: Quantification of the pictures shown in figure 13. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Double asterisks stand for P<0,01 and single ones for P<0,05 in two ways ANOVA with Bonferroni post-test.

At3g10670

Also a Myb transcription factor, At3g10760, was the last example of “cold” phenotype (Figure 15), with a slight reduction of leaf temperature compared to the control in two of the three available lines.

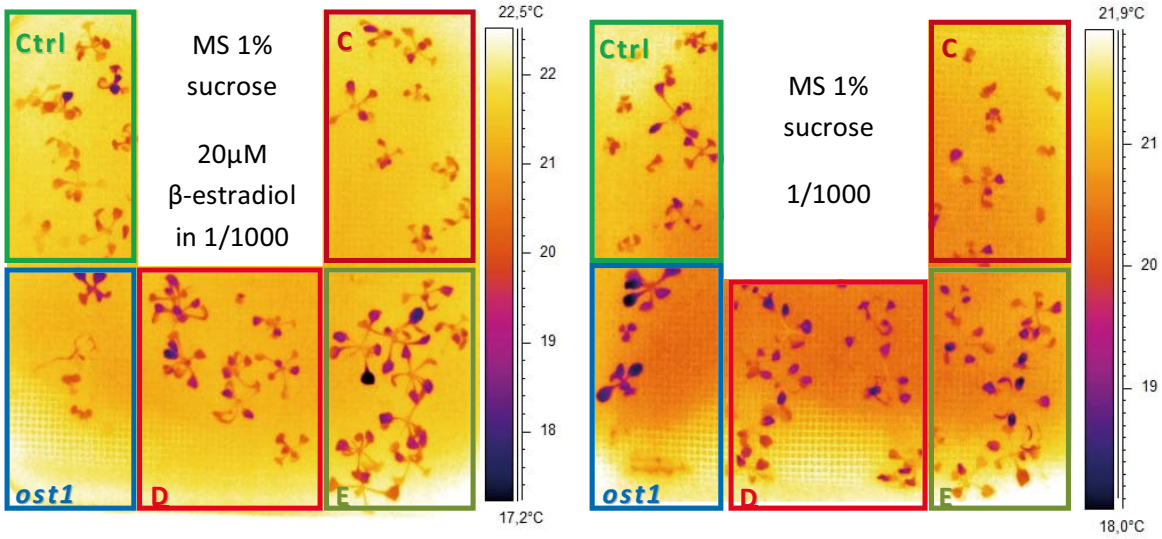

Figure 15: Pictures obtained with a FLIR Thermacam showing on the right the corresponding temperature-chromatic scale. The left picture portrays the ß-estradiol treated plants, while the right one shows the mock controls. In each plate squares of different colours mark the different lines of plants. On the top-left corners there are the negative control (Ctrl) plants and on the bottom-left ones the *ost1* mutants. The other squares include three independent lines of At3g10760 conditional over-expressors (C, D and E). The picture was taken during the subjective afternoon of the plants (ZT 8-10).

The data derived from the quantification (Figure 16) showed that the phenotype was consistent for both line D and F ($P<0,05$).

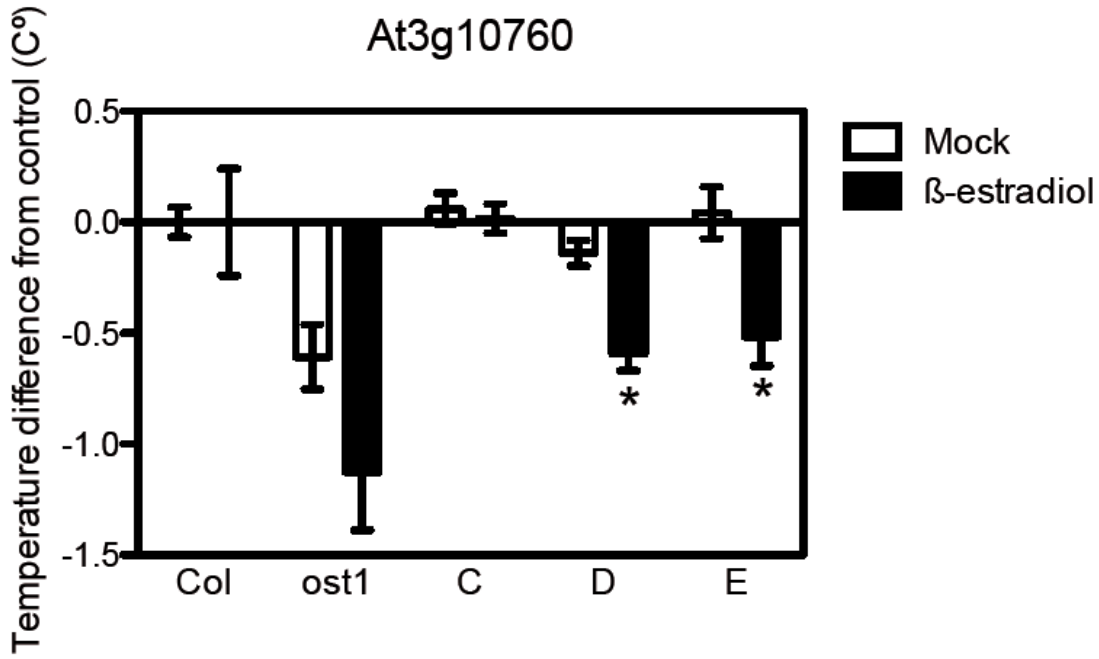

Figure 16: Quantification of the pictures shown in figure 15. The graph represents the average temperature difference with the negative control (Ctrl) of each plate and its relative standard error for each plant line. Genotypes are indicated at the bottom. The white columns represent the mock plate plants while the black ones stand for the ß-estradiol treated plants. Asterisks stand for P<0,01 in two ways ANOVA with Bonferroni post-test.

Phenotype confirmation

The following step was to proceed to a deeper verification and characterization of the observed phenotypes in the most interesting and reliable among the observed candidate genes. With this purpose we selected DEAR4, NAC87 and HSFA8 in order to demonstrate the reliability of our screening method with examples of both types of the observed phenotypes.

In order to verify the origin of our thermal imaging phenotypes we checked by quantitative RT-PCR the expression levels of the transgenes in the conditions used for the thermal imaging (Figure 17). We confirmed that the genes were over-expressed in the three cases that we examined with the exception of NAC87 C line, which presented a reduced induction compared to

the other 2 lines for the same gene. The induction in this line reched only 3-fold increase compared to the control. This data also assured us that the inducible system presented no leakage expression in absence of the inductor.

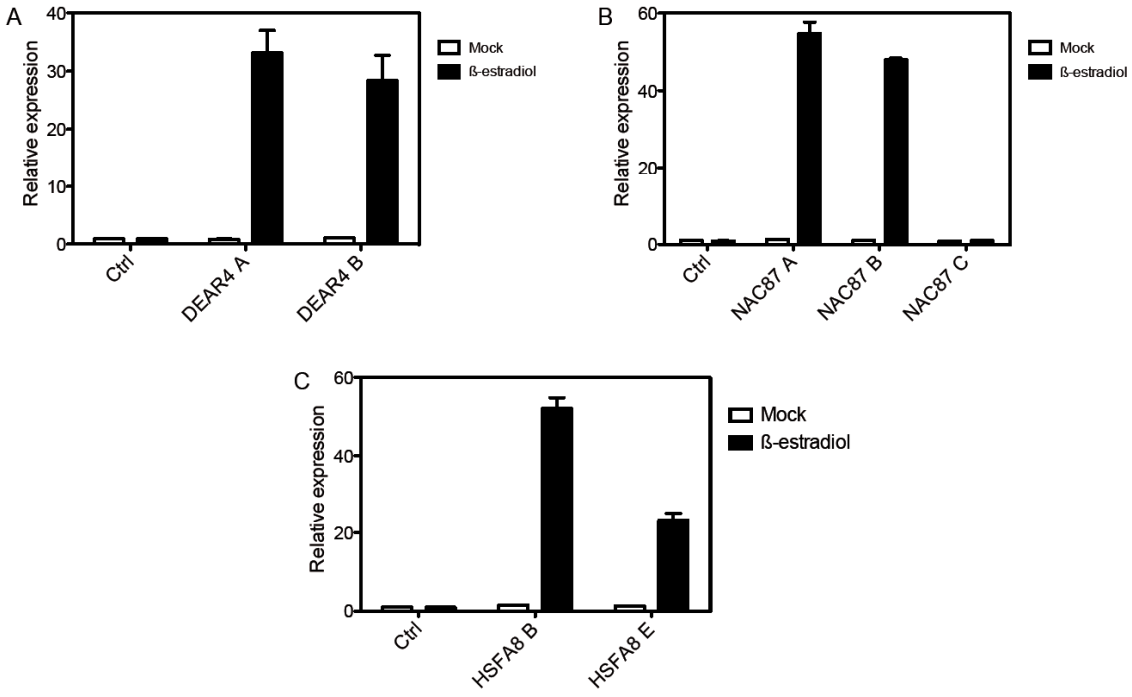

Figure 17: Transgene relative expression compared to control (Ctrl) in DEAR4 (A), NAC87 (B) and HSFA8 (C) inducible lines in presence (Black columns) or absence (White columns) of ß-estradiol (20µM). The represented values correspond to mean and standard error on three independent experiments.

The phenotypes observed by thermal imaging let us suppose that the over-expression of our candidate genes produced alterations in the proper control of the stomata. In order to verify our hypothesis we proceeded to measure the stomatal aperture of the selected lines.

The measurements of stomatal pore width/length ratio were performed on seedlings grown and treated exactly as in the thermal imaging experiments and respecting the time of the subjective day at wich the original experiments were

performed. We also tested the observed phenotypes in presence of abscisic acid.

In the case of DEAR4 we observed a significant (P<0,001) reduction in stomatal aperture under inducing conditions both in presence and absence of ABA (Figure 18).

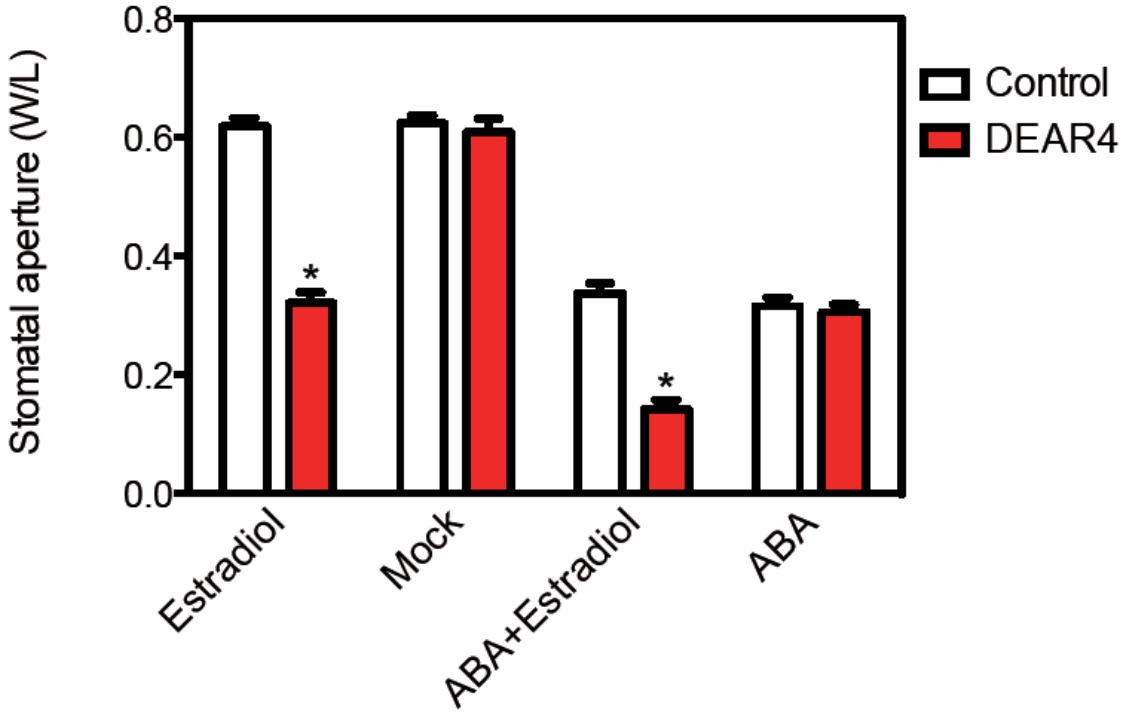

Figure 18: Average stomatal pore aperture measured by width/length ratio. Measurements were done on pictures taken by bright field microscopy with a 63x objective. Each column represents the mean and standard error of more than 200 measurements divided among three independent experiments. Treatments with ß-estradiol (20µM) continued after the canonical induction throughout every phase of sample handling, while ABA (3µM) was applied only starting from the last two hours of incubation.

Asterisks indicate P<0,001 in two ways ANOVA with Bonferroni post-test.

The effects of DEAR4 over-expression on stomatal aperture were though consistent with the leaf temperature phenotype previously described.

The stomatal measurement for NAC87 inducible lines revealed that the over-expression of this gene induces a reduction in stomatal pore aperture as suggested by its “warm” phenotype (Figure 19).

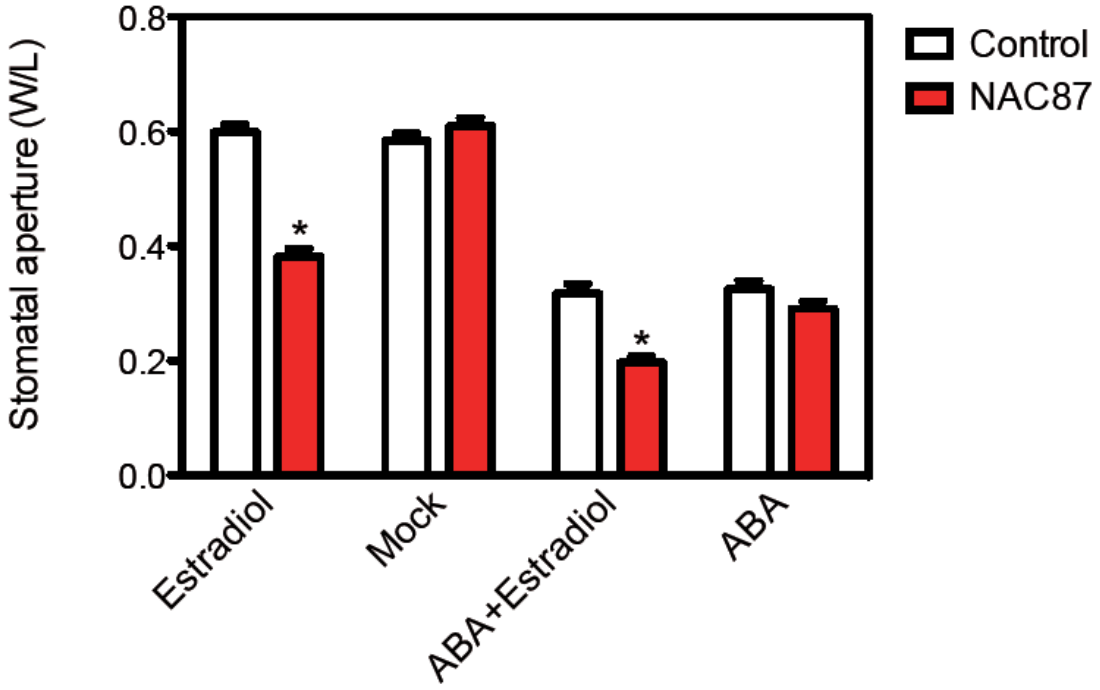

Figure 19: Average stomatal pore aperture measured by width/length ratio. Measurements were done on pictures taken by bright field microscopy with a 63x objective. Each column represents the mean and standard error of more than 200 measurements divided among three independent experiments. Treatments with ß-estradiol (20µM) continued after the canonical induction throughout every phase of sample handling, while ABA (3µM) was applied only starting from the last two hours of incubation.

Asterisks indicate P<0,001 in two ways ANOVA with Bonferroni post-test.

The “cold” phenotype produced by HSFA8 induction corresponded to a significantly wider average stomatal aperture (Figure 20).

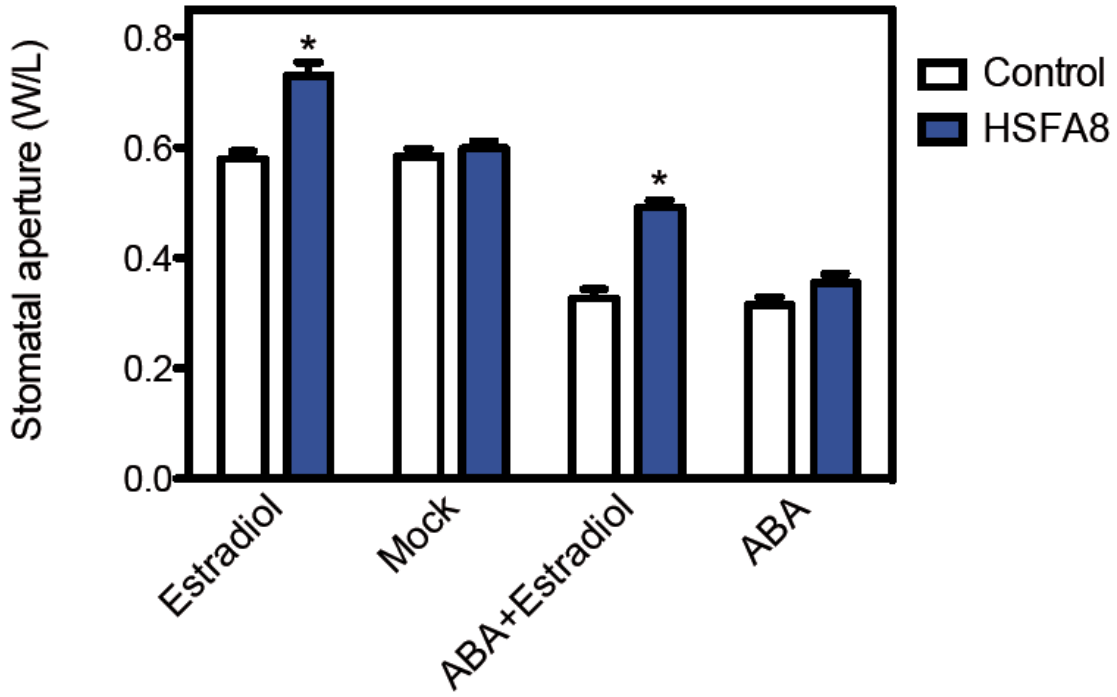

Figure 20: Average stomatal pore aperture measured by width/length ratio. Measurements were done on pictures taken by bright field microscopy with a 63x objective. Each column represents the mean and standard error of more than 200 measurements divided among three independent experiments. Treatments with ß-estradiol (20µM) continued after the canonical induction throughout every phase of sample handling, while ABA (3µM) was applied only starting from the last two hours of incubation.

Asterisks indicate P<0,001 in two ways ANOVA with Bonferroni post-test.

Expression of HSFA8 in *wildtype* plants

Analysing the promoter of HSFA8 we found some elements that respond to abiotic stress, circadian clock and pathogen response (Figure 21).

Figure 21: Scheme representing *HSFA8* promoting region with some of the known cis-elements detected by PLACE database search. The black arrow represents the transcription intiation site, the green bar represents the pathogen responsive element GT1 binding site (Place number S000395), green triangles are CCAAT boxes, known for being Heat Shock enhancers (S000030), red bars represent Evening Elements (S000385), blue pentagons are *ERD1* dehydration responsive elements (S000415) and the yellow square stands for CBF1 binding site (S000497).

The over-expression of HSFA8 produced stomatal opening and reduced sensitivity to abscisic acid so we decided to analyze the effects of abiotic stress on endogenous HSFA8 expression (Figure 22).

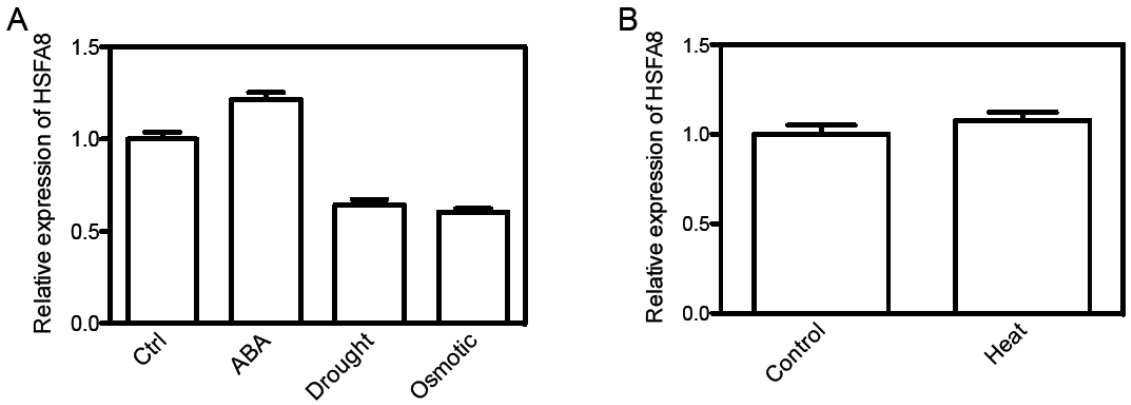

Figure 22: Relative expression HSFA8 in response to various abiotic stresses applied 12 days old seedling grown on MS1. (A) Response to ABA (50µM during 2 hours), drought (30 minutes) and osmotic stress (200mM Mannitol during 2 hours). (B) Response to heat shock (42ºC during 2 hours).

The different sources of abiotic stress did not affect dramatically the levels of transcript of HSFA8, with a slight increase in response to ABA and a reduction in response to drought or osmotic stress. The response to flagellin as

an elicitor (Flg22, 10μM) resulted in an increase in the levels of HSFA8 transcript (Figure 23).

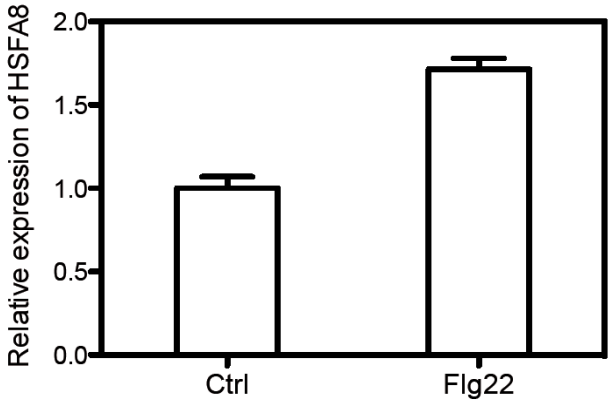

Figure 23: Relative expression of HSFA8 in Flg22 treated (10μM) and control seedlings grown for 12 days on MS1.

We observed the highest difference of these levels between subjective dawn and subjective dusk in free running conditions (Figure 24).

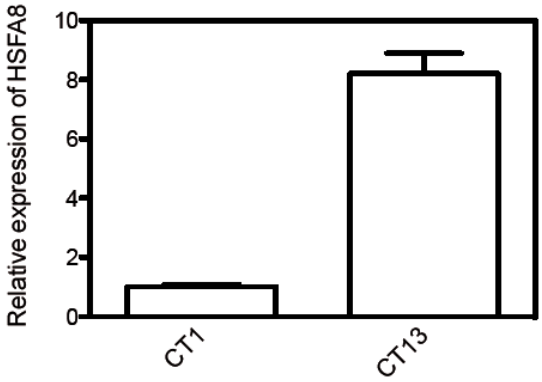

Figure 24: Relative expression HSFA8 in 12 days old seedling grown on MS1 at the subjective dawn (CT1) and dusk (CT13) of the second day of continuous light (LL).

This result suggested that the transcription of our candidate gene could be regulated by the circadian clock.

Subcellular localization of HSFA8

At the protein level we investigated the subcellular localization of HSFA8. We generated a 35S::GFP-HSFA8 construct and introduced it in *Nicotiana benthamiana* leaves by agroinfiltration. The GFP fluorescence was then detected with a confocal microscope (Figure 25).

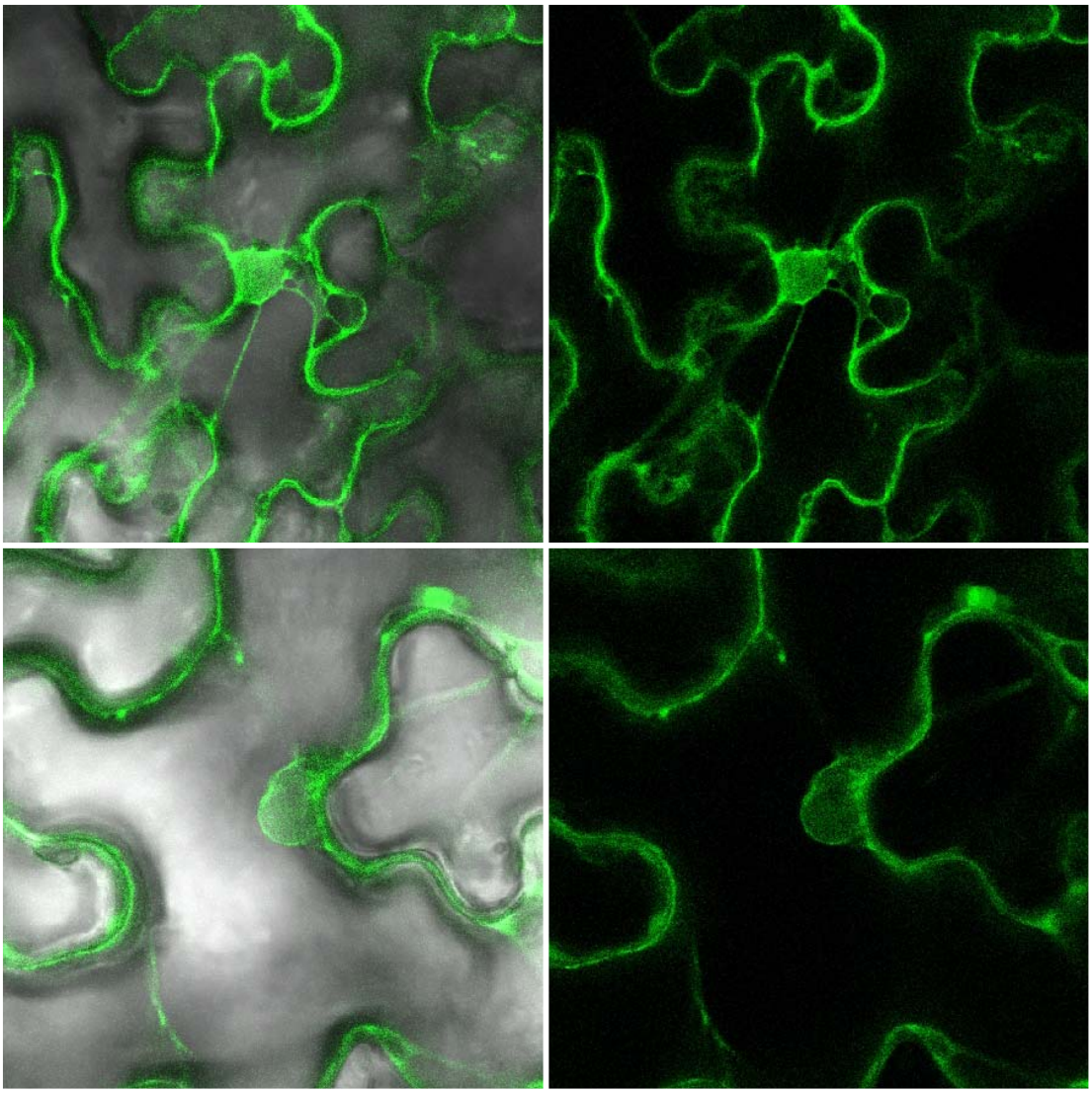

Figure 25: Subcellular localization of GFP-tagged HSFA8 expressed under the control of a 35S promoter in agroinfiltrated *Nicotiana Benthamiana* leaves.

The GFP fused protein was observed in both nucleus and cytoplasm of the cells of the infiltrated foliar tissues.

HSFA8 stomatal phenotype in response to foliar pathogens

Despite the mild transcriptional induction of *HSFA8* observed in response to FLg22, the effects of this elicitor on stomatal closure resulted to be strongly attenuated in case of *HSFA8* over-expression (Figure 26), suggesting that it reduces stomatal sensitivity to this foliar pathogen related elicitor.

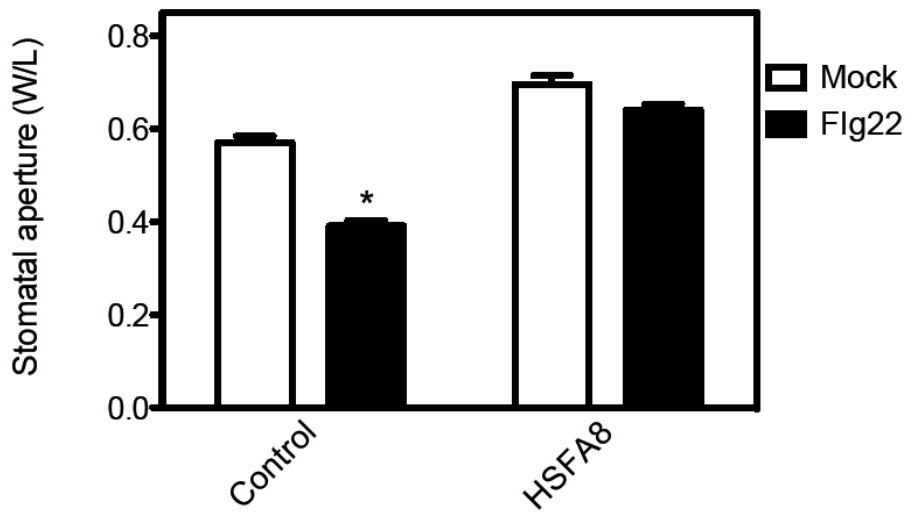

Figure 26: Average stomatal pore aperture measured by width/length ratio. Measurements were done on pictures taken by bright field microscopy with a 63x objective. Each column represents the mean and standard error of more than 200 measurements. Treatments with ß-estradiol (20µM) continued after the canonical induction throughout every phase of sample handling, while Flg22 (10µM) was applied only starting from the last two hours of incubation (Black columns).

Effects of HSFA8 over-expression on the proteome

In order to gain insights on the mechanism underlying the phenotypes observed in response to HSFA8 over-expression we recurred to the CRAG proteomics facility to observe the proteomic profile in a 2D gel electrophoresis of the seedlings with HSFA8 induced over-expression compared to the same line without the inductor and the *wildtype* in presence of ß-estradiol.

Samples were collected in duplicate at the same conditions described for the thermal phenotype and processed for protein extraction. Each extract was divided in two aliquots to have a couple of technical replicates for each biological sample. The protein extracts were then diffused on the base of their isoelectric point (p*I*) on 18 cm strips with a 3-11 pH gradient, which was subsequently loaded on a 26x20 cm 12% acrilamide gel for mass separation. The gels were then stained with colloidal Comassie and scanned. The images obtained by scanning were then analyzed with the Imagemaster Platinum 2.0 software.

The analysis showed that internal controls did not contain variability higher than 10%. The correlation among the replicates in the treated *wildtype* samples was in fact of 856 matching spots, corresponding to the 90% of the total number of observed spots; the HSFA8 uninduced samples had 871 matched spots, the 93% of the total; while the HSFA8 over-expressor samples displayed a 100% correlation with 902 matching spots.

A global correlation analysis permitted to identify the amount of matching spots among the three experimental conditions we considered, revealing that the correlation indexes were between 73 and 77% (Figure 27).

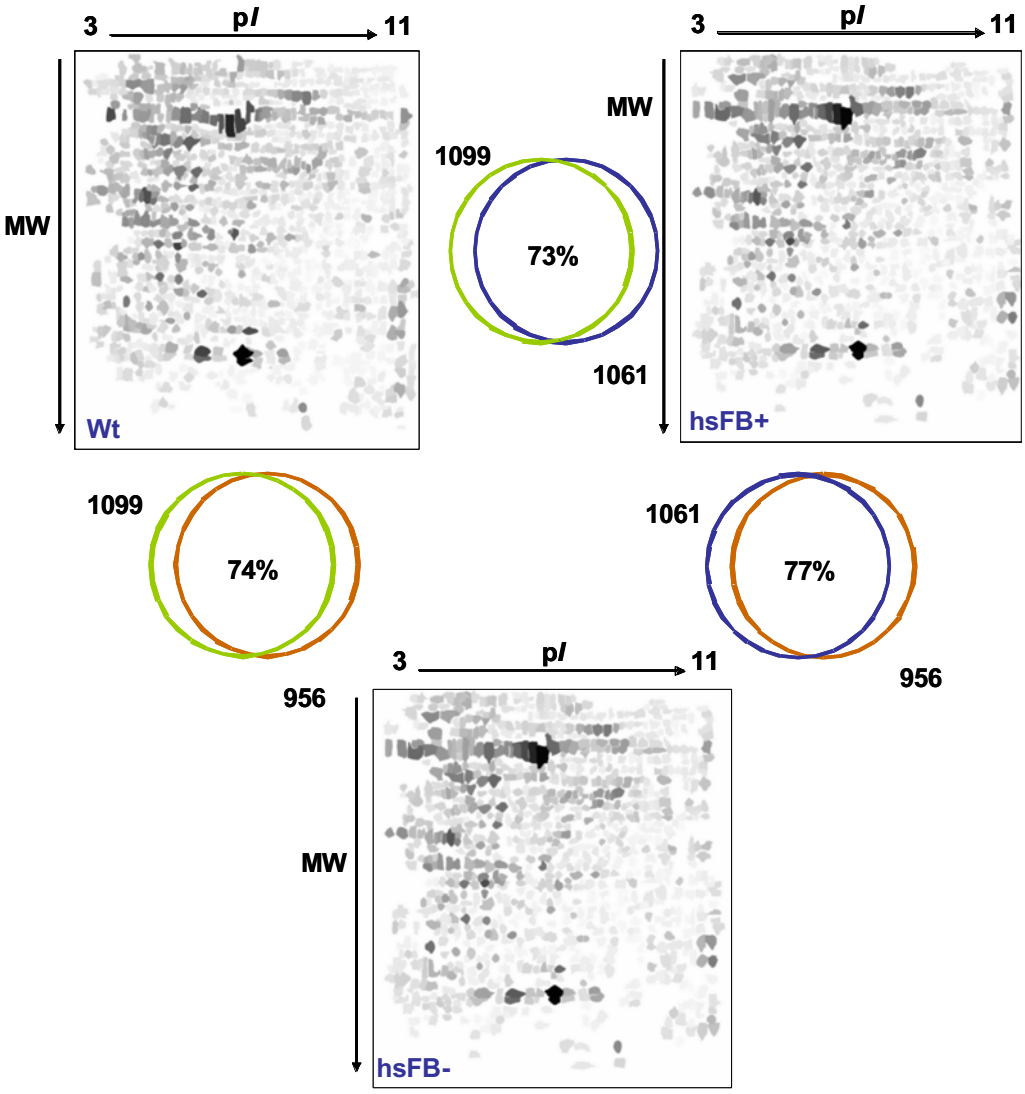

Figure 27: Images of Comassie blue stained 2D gels of treated control plants (top-left, green circles), HSFA8 induced over-expressor (top-right, blue circles) and HSFA8 uninduced plants (bottom, orange circles). The Venn diagrams show the percentage of matching spots between each couple of conditions.

The following step of the analysis was directed to the quantification of the intensity and comparison of matching spots among the different conditions. With this approach we selected 12 spots displaying the most reliable intensity increase (7) or decrease (5) in the HSFA8 over-expressing samples compared with both controls. The selected spots were sent to the proteomics platform in the Parc Científic de Barcelona in order to determine their composition. The samples were there processed by liquid chromatography and mass spectrometry (LC-MSMS) and the results were acquired with the Thermo Xcalibur software (v. 2.1.0.1140) and crossed with the MASCOT database based on NCBInr Eukaryota (v. February 2012) with the Proteome Discoverer (v. 1.3.0.339) software.

We examined the results and selected the most reliable candidate for each spot among the list of suggested peptides created by MASCOT. The selection was made on the base of MASCOT score, expected size compared to gel detection size, number of peptide sequence matches (PSMs), number of unique peptides detected and coverage of the predicted protein sequence (Table 1).

DOWN					
Accession	Description	Score	Unique Peptides	PSMs	Coverage
At3g63170	Chalcone-flavanone isomerase family protein	1003,18	13	45	50,54
At3g09200	60S acidic ribosomal protein P0-2	7127,51	6	371	82,81
At2g43090	Aconitase/3-isopropylmalate dehydratase protein	1861,74	13	70	72,91
At5g20720	Chaperonin 20	5558,72	28	409	77,08
At5g35630	Glutamine synthetase 2	5179,17	27	519	77,44

UP					
Accession	Description	Score	Unique Peptides	PSMs	Coverage
At2g44650	Chloroplast chaperonin 10	875,78	8	33	48,2
At2g33150	3-KETOACYL-COA THIOLASE 2	1408,77	23	67	67,87
At4g09650	F-type H+-transporting ATPase subunit delta	394,41	11	15	50,43
At5g09660	Malate dehydrogenase	153,84	7	7	31,53
At2g21870	Putative ATP synthase subunit	3455,66	32	192	73,75
At3g62030	Cyclophilin 20-3	2512,45	16	199	57,69
At1g13440	Glyceraldehyde 3-phosphate dehydrogenase	6781,96	5	514	87,28

Table 1: Results of the peptide sequencing on the 12 spots of differential intensity in HSFA8 over-expressing samples.

We then checked the mRNA levels of the considered genes in the same conditions in which we collected the proteomics samples by quantitative RT-PCR. The only gene displaying a significant change was the chloroplast chaperonin 10 (CPN10, At2g44650), which repeated the up-regulation observed in the proteomics (Figure 28)

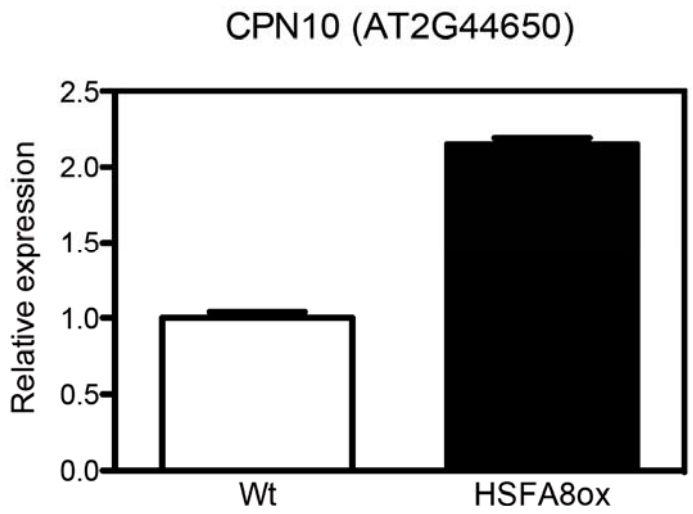

Figure 28: Relative expression of CPN10 in 12 days old seedling grown on MS1 in control plants (White column) compared to HSFA8 induced over-expression (Black column).

Effects of HSFA8 over-expression on the transcriptome

In order to find possible explanations for the phenotype observed in response to HSFA8 over-expression, we chose to analyze its effects on the transcriptome. With this purpose, we utilized samples taken in the same conditions in which we performed the thermal imaging analysis. We used three biological replicates for each genotype and treatment. The results were analyzed with the CARMAweb (https://carmaweb.genome.tugraz.at/carma/) online software suit using the Robust Multiarray Analysis (RMA) algorithm; differentially expressed genes were identified by mean of paired moderated t test statistics (limma) with BH adjusted pValues (Benjamini & Hochberg, 1995). The analysis suggested that ß-estradiol treatment did not produce any significant shift in gene expression and that 69 genes presented significantly (BH pValue<0,05) altered levels of transcripts in the HSFA8 over expressing line compared to the control, being 24 up-regulated (Table 2) and 45 down-regulated (Table 3).

Fold change	Description	Symbol	Gene ID
3,85	Encodes a ferretin protein that is targeted to the chloroplast. Member of a Ferritin gene family. Gene expression is induced in response to iron overload and by nitric oxide. Expression of the gene is downregulated in the presence of paraquat, an inducer of photoxidative stress.	ATFER1	AT5G01600
3,57	polygalacturonase, putative / pectinase, putative; similar to polygalacturonase, putative / pectinase, putative [Arabidopsis thaliana] (TAIR:AT1G60590.1); contains InterPro domain Virulence factor, pectin lyase fold; (InterPro:IPR011050); contains InterPro domain Glycoside hydrolase, family 28; (InterPro:IPR000743); contains InterPro domain Pectolytic enzyme, Pectin lyase fold; (InterPro:IPR012334); contains InterPro domain Parallel beta-helix repeat; (InterPro:IPR006626)	NA	AT5G14650
3,52	Member of Alpha-Expansin Gene Family. Involved in the formation of nematode-induced syncytia in roots of Arabidopsis thaliana.	ATEXP1; ATEXPA1 ;EXP1	AT1G69530
3,06	invertase/pectin methylesterase inhibitor family protein; similar to invertase/pectin methylesterase inhibitor family protein / DC 1.2 homolog (FL5-2I22) [Arabidopsis thaliana]; contains InterPro domain Plant invertase/pectin methylesterase inhibitor; (InterPro:IPR007186); contains InterPro domain Pectinesterase inhibitor; (InterPro:IPR006501)	NA	AT5G62360
2,88	cysteine proteinase, putative; similar to cysteine proteinase, putative [Arabidopsis thaliana] (TAIR:AT3G49340.1); similar to cysteine proteinase precursor [Ipomoea batatas] (GB:AAL14199.1); contains InterPro domain Peptidase, cysteine peptidase active site; (InterPro:IPR000169); contains InterPro domain Peptidase C1A, papain; (InterPro:IPR013128); contains InterPro domain Proteinase inhibitor I29, cathepsin propeptide; (InterPro:IPR013201); contains InterPro domain Peptidase C1A, papain C-terminal; (InterPro:IPR000668)	NA	AT2G27420
2,71	ATFER3 (FERRITIN 3); ferric iron binding; Identical to Ferritin-3, chloroplast precursor (EC 1.16.3.1) (FER3) [Arabidopsis Thaliana] (GB:Q9LYN2;GB:Q8WHW6); similar to ATFER4 (FERRITIN 4), ferric iron binding [Arabidopsis thaliana] (TAIR:AT2G40300.1); similar to ferritin [Chorispora bungeana] (GB:ABB22752.1); contains InterPro domain Ferritin-related; (InterPro:IPR012347); contains InterPro domain Ferritin; (InterPro:IPR001519); contains InterPro domain Ferritin/ribonucleotide reductase-like; (InterPro:IPR009078); contains InterPro domain Ferritin-like; (InterPro:IPR009040); contains InterPro domain Ferritin and Dps; (InterPro:IPR008331)	ATFER3	AT3G56090
2,63	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G64190.1); similar to hypothetical protein [Oryza sativa (japonica cultivar-group)] (GB:AAP46203.1)	NA	AT2G15020

Fold change	Description	Symbol	Gene ID
2,55	heavy-metal-associated domain-containing protein; similar to metal ion binding [Arabidopsis thaliana] (TAIR:AT4G16380.1); similar to unknown protein [Glycine max] (GB:AAN03471.1); contains InterPro domain Heavy metal transport/detoxification protein; (InterPro:IPR006121)	NA	AT1G51090
2,53	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G22250.1); similar to WW domain containing protein, expressed [Oryza sativa (japonica cultivar-group)] (GB:ABA96334.2); similar to Os03g0191200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001049232.1)	NA	AT1G78170
2,51	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G01430.1); similar to unknown protein Cr17 [Brassica napus] (GB:AAX51387.1); contains InterPro domain Protein of unknown function DUF231, plant; (InterPro:IPR004253)	NA	AT4G01080
2,37	unknown protein	NA	AT1G73120
2,33	Encodes a protein with high affinity, hexose-specific/H+ symporter activity. The activity of the transporter appears to be negatively regulated by phosphorylation. Importantly, microarray analysis, as well as the study of the expression of this gene in mutants involved in programmed cell death (PCD) demonstrated a tight correlation between this gene's expression and PCD.;Encodes a protein with high affinity, hexose-specific/H+ symporter activity. The activity of the transporter appears to be negatively regulated by phosphorylation. Importantly, microarray analysis, as well as the study of the expression of this gene in mutants involved in programmed cell death (PCD) demonstrated a tight correlation between this gene's expression and PCD.	MSS1;STP13	AT5G26340
2,11	member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of Alpha-Expansin Gene Family.	ATEXP11;ATEXPA11;EXP11	AT1G20190
2,10	UDP-glucoronosyl/UDP-glucosyl transferase family protein; similar to UDP-glucoronosyl/UDP-glucosyl transferase family protein [Arabidopsis thaliana] (TAIR:AT4G15280.1); similar to glucosyltransferase [Nicotiana tabacum] (GB:BAB60720.1); contains InterPro domain UDP-glucuronosyl/UDP-glucosyltransferase; (InterPro:IPR002213)	NA	AT3G21760

Fold change	Description	Symbol	Gene ID
2,09	Encodes cystine lyase which is expected to be involved in amino acid metabolism, providing the plant with cysteine and the generation of precursors of ethylene biosynthesis. mRNA levels are elevated in response to wounding.;Encodes cystine lyase which is expected to be involved in amino acid metabolism, providing the plant with cysteine and the generation of precursors of ethylene biosynthesis. mRNA levels are elevated in response to wounding.	CORI3;JR2	AT4G23600
2,08	subtilase family protein; similar to subtilase family protein [Arabidopsis thaliana] (TAIR:AT5G59120.1); similar to subtilase family protein [Arabidopsis thaliana] (TAIR:AT5G58840.1); similar to subtilase [Arabidopsis thaliana] (TAIR:AT5G59090.3); similar to Peptidase S8 and S53, subtilisin, kexin, sedolisin; Protease-associated PA; Proteinase inhibitor, propeptide [Medicago truncatula] (GB:ABE86992.1); contains InterPro domain Protease-associated PA; (InterPro:IPR003137); contains InterPro domain Peptidase S8 and S53, subtilisin, kexin, sedolisin; (InterPro:IPR000209); contains InterPro domain Proteinase inhibitor I9, subtilisin propeptide; (InterPro:IPR010259); contains InterPro domain Proteinase inhibitor, propeptide; (InterPro:IPR009020)	NA	AT5G59130
2,03	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G09950.1); similar to Os06g0523300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001057752.1); similar to unknown protein [Oryza sativa (japonica cultivar-group)] (GB:BAD45825.1)	NA	AT5G55620
2,03	a B-box zinc finger protein that interacts with COP1. contains a novel 11 amino acid motif at the C-terminus (also found at the N-terminus of HY5) that is involved in the COP1 interaction.	STH	AT2G31380
1,91	trehalose-phosphatase; similar to trehalose-6-phosphate phosphatase, putative [Arabidopsis thaliana] (TAIR:AT4G39770.1); similar to 117M18_9 [Brassica rapa] (GB:AAZ66928.1); contains InterPro domain Trehalose-phosphatase; (InterPro:IPR003337); contains InterPro domain HAD-superfamily hydrolase subfamily IIB; (InterPro:IPR006379)	TPPE	AT2G22190
1,88	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G75160.1); similar to BAC19.5 [Lycopersicon esculentum] (GB:AAG01120.1); contains InterPro domain Protein of unknown function DUF620; (InterPro:IPR006873)	NA	AT5G66740
1,88	leucine-rich repeat protein kinase, putative; similar to leucine-rich repeat protein kinase, putative [Arabidopsis thaliana] (TAIR:AT3G46340.1); similar to leucine-rich repeat family protein / protein kinase family protein [Arabidopsis thaliana] (TAIR:AT3G46420.1); similar to leucine-rich repeat protein kinase, putative [Arabidopsis thaliana] (TAIR:AT3G46400.1); similar to Protein kinase [Medicago truncatula] (GB:ABE90329.1); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Leucine-rich repeat; (InterPro:IPR001611); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Leucine-rich repeat, plant specific; (InterPro:IPR007090); contains InterPro domain Serine/threonine protein kinase, active site; (InterPro:IPR008271)	NA	AT3G46370

Fold change	Description	Symbol	Gene ID
1,88	protein kinase family protein; similar to protein kinase family protein [Arabidopsis thaliana] (TAIR:AT1G77280.1); similar to At5g63940 [Oryza sativa (japonica cultivar-group)] (GB:AAX96058.1); contains InterPro domain UspA; (InterPro:IPR006016); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Serine/threonine protein kinase, active site; (InterPro:IPR008271)	NA	AT1G21590
1,78	Encodes a chloroplastic stromal ascorbate peroxidase sAPX. Ascorbate peroxidases are enzymes that scavenge hydrogen peroxide in plant cells. Eight types of APX have been described for Arabidopsis: three cytosolic (APX1, APX2, APX6), two chloroplastic types (stromal sAPX, thylakoid tAPX), and three microsomal (APX3, APX4, APX5) isoforms.	SAPX	AT4G08390
1,77	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).	ATGSTU25	AT1G17180

Table 2: List of up-regulated transcripts detected by microarray in *HSFA8* over-expressing seedlings.

Fold change	Description	Symbol	Gene ID
0,126993906	cystathionine gamma-synthase, chloroplast, putative / O-succinylhomoserine (Thiol)-lyase, putative; similar to MTO1 (METHIONINE OVERACCUMULATION 1) [Arabidopsis thaliana] (TAIR:AT3G01120.1); similar to similar to the metB gene product of Escherichia coli; cloned by functional complementation of a metB mutant strain of Escherichia coli LE392 (GB:AAC49574.1); contains InterPro domain Cys/Met metabolism pyridoxal-phosphate-dependent enzymes.	NA	AT1G33320
0,165528848	putative cytochrome P450	CYP72A14	AT3G14680
0,185267226	member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio);member of EXPANSIN-LIKE. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio)	ATEXLA1; ATEXPL1; ATHEXP;EXPL1	AT3G45970
0,22704918	seven in absentia (SINA) family protein; similar to seven in absentia (SINA) family protein [Arabidopsis thaliana] (TAIR:AT3G58040.1); similar to Os07g0659800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001060528.1); similar to SINA2p [Vitis vinifera] (GB:CAB40578.1); contains InterPro domain TRAF-like; (InterPro:IPR008974); contains InterPro domain Seven in absentia protein; (InterPro:IPR004162); contains InterPro domain TRAF-type; (InterPro:IPR013322); contains InterPro domain SIAH-type; (InterPro:IPR013323);seven in absentia (SINA) family protein; similar to seven in absentia (SINA) family protein [Arabidopsis thaliana] (TAIR:AT3G58040.1); similar to SINA2p [Vitis vinifera] (GB:CAB40578.1); contains InterPro domain TRAF-like; (InterPro:IPR008974); contains InterPro domain Seven in absentia protein; (InterPro:IPR004162)	NA	AT3G13672
0,256374354	member of Fe(II) transporter isolog family	IRT3	AT1G60960
0,264250545	DNA binding; similar to aspartyl protease family protein [Arabidopsis thaliana] (TAIR:AT2G42980.1); similar to Os02g0314600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001046662.1); similar to Avr9/Cf-9 rapidly elicited protein 36 [Nicotiana tabacum] (GB:AAV92892.1); contains InterPro domain Peptidase A1, pepsin; (InterPro:IPR001461); contains InterPro domain Peptidase aspartic, catalytic; (InterPro:IPR009007);aspartyl protease family protein; similar to aspartyl protease family protein [Arabidopsis thaliana] (TAIR:AT2G42980.1); similar to Os02g0314600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001046662.1); similar to Avr9/Cf-9 rapidly elicited protein 36 [Nicotiana tabacum] (GB:AAV92892.1); contains InterPro domain Peptidase A1, pepsin; (InterPro:IPR001461); contains InterPro domain Peptidase aspartic, catalytic; (InterPro:IPR009007)	NA	AT3G59080

Fold change	Description	Symbol	Gene ID
0,267026309	U-box domain-containing protein; similar to U-box domain-containing protein [Arabidopsis thaliana] (TAIR:AT3G61390.2); similar to Os10g0552400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001065331.1); similar to Protein kinase; U box [Medicago truncatula] (GB:ABD32822.1); contains InterPro domain U box; (InterPro:IPR003613)	NA	AT2G45920
0,278601058	member of WRKY Transcription Factor; Group II-e;member of WRKY Transcription Factor; Group II-e	AtWRKY22; WRKY22	AT4G01250
0,299374272	transporter-related; similar to transporter-related [Arabidopsis thaliana] (TAIR:AT1G79410.1); similar to putative organic cation transporter [Oryza sativa (japonica cultivar-group)] (GB:BAC83382.1); contains InterPro domain Major facilitator superfamily; (InterPro:IPR007114); contains InterPro domain Major facilitator superfamily MFS_1; (InterPro:IPR011701);transporter-related; similar to transporter-related [Arabidopsis thaliana] (TAIR:AT1G79410.1); similar to putative organic cation transporter [Oryza sativa (japonica cultivar-group)] (GB:BAC83382.1); contains InterPro domain Major facilitator superfamily; (InterPro:IPR007114); contains InterPro domain Major facilitator superfamily MFS_1; (InterPro:IPR011701)	ATOCT6;OCT6	AT1G16370
0,307153192	Al-stress-induced gene;Al-stress-induced gene	ATBCB;BCB	AT5G20230
0,31219974	U-box domain-containing protein; similar to U-box domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G37490.1); similar to syringolide-induced protein 13-1-1 [Glycine max] (GB:BAB86896.1); contains InterPro domain Armadillo-like helical; (InterPro:IPR011989); contains InterPro domain U box; (InterPro:IPR003613);U-box domain-containing protein; similar to U-box domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G37490.1); similar to syringolide-induced protein 13-1-1 [Glycine max] (GB:BAB86896.1); contains InterPro domain U box; (InterPro:IPR003613)	NA	AT1G66160
0,316722124	peroxidase; Identical to Peroxidase 25 precursor (EC 1.11.1.7) (Atperox P25) (PER25) [Arabidopsis Thaliana] (GB:O80822;GB:Q8L728); similar to peroxidase, putative [Arabidopsis thaliana] (TAIR:AT5G64120.1); similar to putative peroxidase [Oryza sativa (japonica cultivar-group)] (GB:BAD68110.1); contains InterPro domain Haem peroxidase; (InterPro:IPR010255); contains InterPro domain Haem peroxidase, plant/fungal/bacterial; (InterPro:IPR002016); contains InterPro domain Plant peroxidase; (InterPro:IPR000823)	NA	AT2G41480

Fold change	Description	Symbol	Gene ID
0,357481142	glyoxal oxidase-related; similar to glyoxal oxidase-related [Arabidopsis thaliana] (TAIR:AT1G75620.1); similar to Os03g0258900 [Oryza sativa (japonica cultivar-group)] (GB:NP_001049609.1); similar to glyoxal oxidase [Vitis pseudoreticulata] (GB:ABA42922.1); contains InterPro domain Galactose oxidase, central; (InterPro:IPR011043); contains InterPro domain Glyoxal oxidase, N-terminal; (InterPro:IPR009880)	NA	AT1G19900
0,362922022	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G40000.1); similar to putative Hs1pro-1-like receptor [Glycine max] (GB:AAG44839.1); contains InterPro domain Hs1pro-1, C-terminal; (InterPro:IPR009743); contains InterPro domain Hs1pro-1, N-terminal; (InterPro:IPR009869)	NA	AT3G55840
0,36335965	unknown protein	NA	AT4G28085
0,364916665	Encodes a protein with invertase activity.;Encodes a protein with invertase activity.	ATBFRUCT1;ATCWINV1	AT3G13790
0,376840665	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein; similar to MD-2-related lipid recognition domain-containing protein / ML domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G23820.1); contains InterPro domain E1 protein and Def2/Der2 allergen; (InterPro:IPR003172)	MD2-related	AT5G23840
0,377939699	Member of a diversely expressed predicted peptide family showing sequence similarity to tobacco Rapid Alkalinization Factor (RALF), and is believed to play an essential role in the physiology of Arabidopsis. Consists of a single exon and is characterized by a conserved C-terminal motif and N-terminal signal peptide.	RALFL27	AT3G29780
0,381972388	unknown protn	NA	AT2G25735
0,387026649	Encodes a protein with xylosyltransferase activity, which is specific for UDP-xylose as donor substrate and for oligosaccharides with a degree of polymerization >4. Although the enzyme utilizes either cellopentaose or cellohexaose, its activity is four-fold higher with cellohexaose as an acceptor compared to cellopentaose. The enzyme is able to add several xylosyl residues to the acceptor forming mono-, di- and trixylosylated polysaccharides.;Encodes a protein with xylosyltransferase activity, which is specific for UDP-xylose as donor substrate and for oligosaccharides with a degree of polymerization >4. Although the enzyme utilizes either cellopentaose or cellohexaose, its activity is four-fold higher with cellohexaose as an acceptor compared to cellopentaose. The enzyme is able to add several xylosyl residues to the acceptor forming mono-, di- and trixylosylated polysaccharides.;Encodes a protein with xylosyltransferase activity, which is specific for UDP-xylose as donor substrate and for oligosaccharides with a degree of polymerization >4. Although the enzyme utilizes either cellopentaose or cellohexaose, its activity is four-fold higher with cellohexaose as an acceptor compared to cellopentaose. The enzyme is able to add several xylosyl residues to the acceptor forming mono-, di- and trixylosylated polysaccharides.	ATXT1;XT1;XXT1	AT3G62720

Fold change	Description	Symbol	Gene ID
0,395520204	xyloglucan endotransglycosylase-related protein (XTR6);xyloglucan endotransglycosylase-related protein	XTH23;	AT4G25810
0,396788148	protein kinase, putative; similar to protein kinase, putative [Arabidopsis thaliana] (TAIR:AT2G05940.1); similar to Os03g0364400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001050171.1); similar to Avr9/Cf-9 induced kinase 1 [Nicotiana tabacum] (GB:AAP03880.2); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Serine/threonine protein kinase, active site; (InterPro:IPR008271)	NA	AT1G61590
0,411078785	harpin-induced protein-related / HIN1-related / harpin-responsive protein-related; similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G21130.1); similar to Harpin-induced 1 [Medicago truncatula] (GB:ABE82616.1);harpin-induced protein-related / HIN1-related / harpin-responsive protein-related; similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G21130.1); similar to Harpin-induced 1 [Medicago truncatula] (GB:ABE82616.1); contains InterPro domain Harpin-induced 1; (InterPro:IPR010847)	NA	AT2G27080
0,426924871	glucosamine/galactosamine-6-phosphate isomerase family protein; similar to EMB2024 (EMBRYO DEFECTIVE 2024), catalytic [Arabidopsis thaliana] (TAIR:AT5G24400.1); similar to putative 6-phosphogluconolactonase [Oryza sativa (japonica cultivar-group)] (GB:BAC83870.1); contains InterPro domain 6-phosphogluconolactonase; (InterPro:IPR005900); contains InterPro domain Glucosamine/galactosamine-6-phosphate isomerase; (InterPro:IPR006148)	NA	AT1G13700
0,438684775	leucine-rich repeat transmembrane protein kinase, putative; similar to MRH1 (morphogenesis of root hair 1), ATP binding / protein serine/threonine kinase [Arabidopsis thaliana] (TAIR:AT4G18640.1); similar to transmembrane kinase [Oryza sativa (japonica cultivar-group)] (GB:AAQ01158.1); similar to Os01g0206800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001042346.1); contains InterPro domain Serine/threonine protein kinase; (InterPro:IPR002290); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Leucine-rich repeat; (InterPro:IPR001611); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Tyrosine protein kinase; (InterPro:IPR001245); contains InterPro domain Leucine-rich repeat, plant specific; (InterPro:IPR007090); contains InterPro domain Leucine rich repeat, N-terminal; (InterPro:IPR013210)	NA	AT5G45840

Fold change	Description	Symbol	Gene ID
0,445627567	lectin protein kinase family protein; similar to lectin protein kinase, putative [Arabidopsis thaliana] (TAIR:AT5G55830.1); similar to OSJNBa0088A01.21 [Oryza sativa (japonica cultivar-group)] (GB:CAD41381.2); similar to lectin-like protein kinase [Populus nigra] (GB:BAA82556.1); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Concanavalin A-like lectin/glucanase, subgroup; (InterPro:IPR013320); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Concanavalin A-like lectin/glucanase; (InterPro:IPR008985); contains InterPro domain Tyrosine protein kinase; (InterPro:IPR001245); contains InterPro domain Serine/threonine protein kinase, active site; (InterPro:IPR008271); contains InterPro domain Legume lectin, beta domain; (InterPro:IPR001220); contains InterPro domain Legume lectin, alpha; (InterPro:IPR000985)	NA	AT5G06740
0,446368709	polygalacturonase inhibiting protein 1 (PGIP1) mRNA,;polygalacturonase inhibiting protein 1 (PGIP1)	ATPGIP1;PGIP1	AT5G06860
0,446991245	galactosyltransferase family protein; similar to galactosyltransferase family protein [Arabidopsis thaliana] (TAIR:AT1G05170.2); similar to galactosyltransferase family protein [Arabidopsis thaliana] (TAIR:AT2G32430.1); similar to Os03g0577500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001050543.1); similar to putative Avr9 elicitor response protein [Oryza sativa (japonica cultivar-group)] (GB:BAD25162.1); contains InterPro domain Glycosyl transferase, family 31; (InterPro:IPR002659)	NA	AT1G11730
0,452782898	UDP-glucoronosyl/UDP-glucosyl transferase family protein; similar to UDP-glucoronosyl/UDP-glucosyl transferase family protein [Arabidopsis thaliana] (TAIR:AT1G07260.1); similar to UDP-glucoronosyl/UDP-glucosyl transferase family protein [Arabidopsis thaliana] (TAIR:AT2G29740.1); similar to Chain A, Crystal Structure Of Medicago Truncatula Ugt71g1 (GB:2ACV); similar to Chain A, Crystal Structure Of Medicago Truncatula Ugt71g1 Complexed With Udp-Glucose (GB:2ACW); contains InterPro domain UDP-glucuronosyl/UDP-glucosyltransferase; (InterPro:IPR002213)	UGT71C1	AT2G29750
0,460236792	Member of the R2R3 factor gene family.	AtMYB70	AT2G23290
0,460471502	similar to Os03g0170100 [Oryza sativa (japonica cultivar-group)] (GB:NP_001049105.1)	NA	AT2G35290
0,460603971	an aquaporin whose expression level is reduced by ABA, NaCl, dark, and dessication. is expressed at relatively low levels under normal conditions.;an aquaporin whose expression level is reduced by ABA, NaCl, dark, and dessication. is expressed at relatively low levels under normal conditions.;an aquaporin whose expression level is reduced by ABA, NaCl, dark, and dessication. is expressed at relatively low levels under normal conditions.	AT-NLM1;NIP1;1;NLM1	AT4G19030

Fold change	Description	Symbol	Gene ID
0,460999979	DC1 domain-containing protein; similar to DC1 domain-containing protein [Arabidopsis thaliana] (TAIR:AT1G62030.1); similar to MEE53 (maternal effect embryo arrest 53), zinc ion binding [Arabidopsis thaliana] (TAIR:AT4G10560.1); similar to DC1 domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G48320.1); similar to DC1 domain-containing protein [Brassica oleracea] (GB:ABD65623.1); contains InterPro domain Zinc finger, PHD-type; (InterPro:IPR001965); contains InterPro domain C1-like; (InterPro:IPR011424); contains InterPro domain DC1; (InterPro:IPR004146)	NA	AT4G02540
0,461183006	xyloglucan endotransglycosylase-related protein (XTR7)	XTR7	AT4G14130
0,464094731	protein kinase family protein / peptidoglycan-binding LysM domain-containing protein; similar to protein kinase family protein / peptidoglycan-binding LysM domain-containing protein [Arabidopsis thaliana] (TAIR:AT2G23770.1); similar to Os02g0193000 [Oryza sativa (japonica cultivar-group)] (GB:NP_001046166.1); similar to Os06g0625300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001058111.1); similar to Protein kinase domain containing protein, expressed [Oryza sativa (japonica cultivar-group)] (GB:ABA94286.1); contains InterPro domain Protein kinase-like; (InterPro:IPR011009); contains InterPro domain Protein kinase; (InterPro:IPR000719); contains InterPro domain Peptidoglycan-binding LysM; (InterPro:IPR002482)	NA	AT2G33580
0,488138797	binding; similar to similar to Uncharacterized conserved protein [Crocosphaera watsonii WH 8501] (GB:ZP_00518391.1); contains InterPro domain Protein prenyltransferase; (InterPro:IPR008940); contains InterPro domain Tetratricopeptide-like helical; (InterPro:IPR011990);binding; similar to similar to Uncharacterized conserved protein [Crocosphaera watsonii WH 8501] (GB:ZP_00518391.1); contains InterPro domain Tetratricopeptide-like helical; (InterPro:IPR011990)	NA	AT4G19160
0,497267613	Member of a diversely expressed predicted peptide family showing sequence similarity to tobacco Rapid Alkalinization Factor (RALF), and is believed to play an essential role in the physiology of Arabidopsis. Consists of a single exon and is characterized by a conserved C-terminal motif and N-terminal signal peptide.	RALFL22	AT3G05490
0,512460803	DC1 domain-containing protein; similar to DC1 domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G48320.1); similar to protein binding / zinc ion binding [Arabidopsis thaliana] (TAIR:AT2G28460.1); similar to DC1 domain-containing protein [Arabidopsis thaliana] (TAIR:AT1G66450.1); similar to DC1 domain-containing protein [Brassica oleracea] (GB:ABD65623.1); contains InterPro domain Zinc finger, FYVE/PHD-type; (InterPro:IPR011011); contains InterPro domain C1-like; (InterPro:IPR011424); contains InterPro domain DC1; (InterPro:IPR004146)	NA	AT1G66440

Fold change	Description	Symbol	Gene ID
0,513600374	similar to Hypothetical protein CBG24759 [Caenorhabditis briggsae] (GB:CAE56916.1); contains InterPro domain Protein of unknown function DUF1720; (InterPro:IPR013182)	NA	AT1G30750
0,519890065	Encodes a novel Cys-rich protein with a B-box like domain that acts as a negative regulator of meristem cell accumulation in inflorescence and floral meristems as loss-of-function ult1 mutations cause inflorescence meristem enlargement, the production of extra flowers and floral organs, and a decrease in floral meristem determinacy.;Encodes a novel Cys-rich protein with a B-box like domain that acts as a negative regulator of meristem cell accumulation in inflorescence and floral meristems as loss-of-function ult1 mutations cause inflorescence meristem enlargement, the production of extra flowers and floral organs, and a decrease in floral meristem determinacy.	ULT;ULT1	AT4G28190
0,520213185	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G01300.1); similar to Os01g0121500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001041876.1); similar to Os01g0121600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001041877.1)	NA	AT1G15010
0,533469874	auxin-responsive family protein; similar to auxin-responsive family protein [Arabidopsis thaliana] (TAIR:AT3G53250.1); similar to Auxin responsive SAUR protein [Medicago truncatula] (GB:ABE87816.1); contains InterPro domain Auxin responsive SAUR protein; (InterPro:IPR003676)	NA	AT2G37030
0,547022422	hydroxyproline-rich glycoprotein family protein; similar to hydroxyproline-rich glycoprotein family protein [Arabidopsis thaliana] (TAIR:AT5G57070.1); similar to conserved hypothetical protein [Medicago truncatula] (GB:ABE81528.1)	NA	AT1G72790
0,552111128	protein binding / zinc ion binding; similar to protein binding / zinc ion binding [Arabidopsis thaliana] (TAIR:AT1G10650.1); similar to Os02g0130300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001045784.1); similar to putative S-ribonuclease binding protein SBP1 [Oryza sativa (japonica cultivar-group)] (GB:BAD07738.1); contains InterPro domain Zinc finger, RING-type; (InterPro:IPR001841)	NA	AT1G60610
0,560794544	mechanosensitive ion channel domain-containing protein / MS ion channel domain-containing protein; similar to mechanosensitive ion channel domain-containing protein / MS ion channel domain-containing protein [Arabidopsis thaliana] (TAIR:AT5G12080.2); similar to putative protein T30F21.6 [Sorghum bicolor] (GB:AAL68849.1); contains InterPro domain EF-Hand type; (InterPro:IPR011992); contains InterPro domain Like-Sm ribonucleoprotein-related, core; (InterPro:IPR010920); contains InterPro domain MscS Mechanosensitive ion channel; (InterPro:IPR006685)	MSL9	AT5G19520

Table 3: List of down-regulated transcripts detected by microarray in *HSFA8* over-expressing seedlings.

We proceeded to validate the shifts observed in transcripts levels by quantitative Real Time PCR on the RTs obtained from the RNA samples previously used for the microarray analysis. We confirmed the transcriptomics predictions for all the cases we analyzed in both up (Figure 29) and down regulated genes (Figure 30).

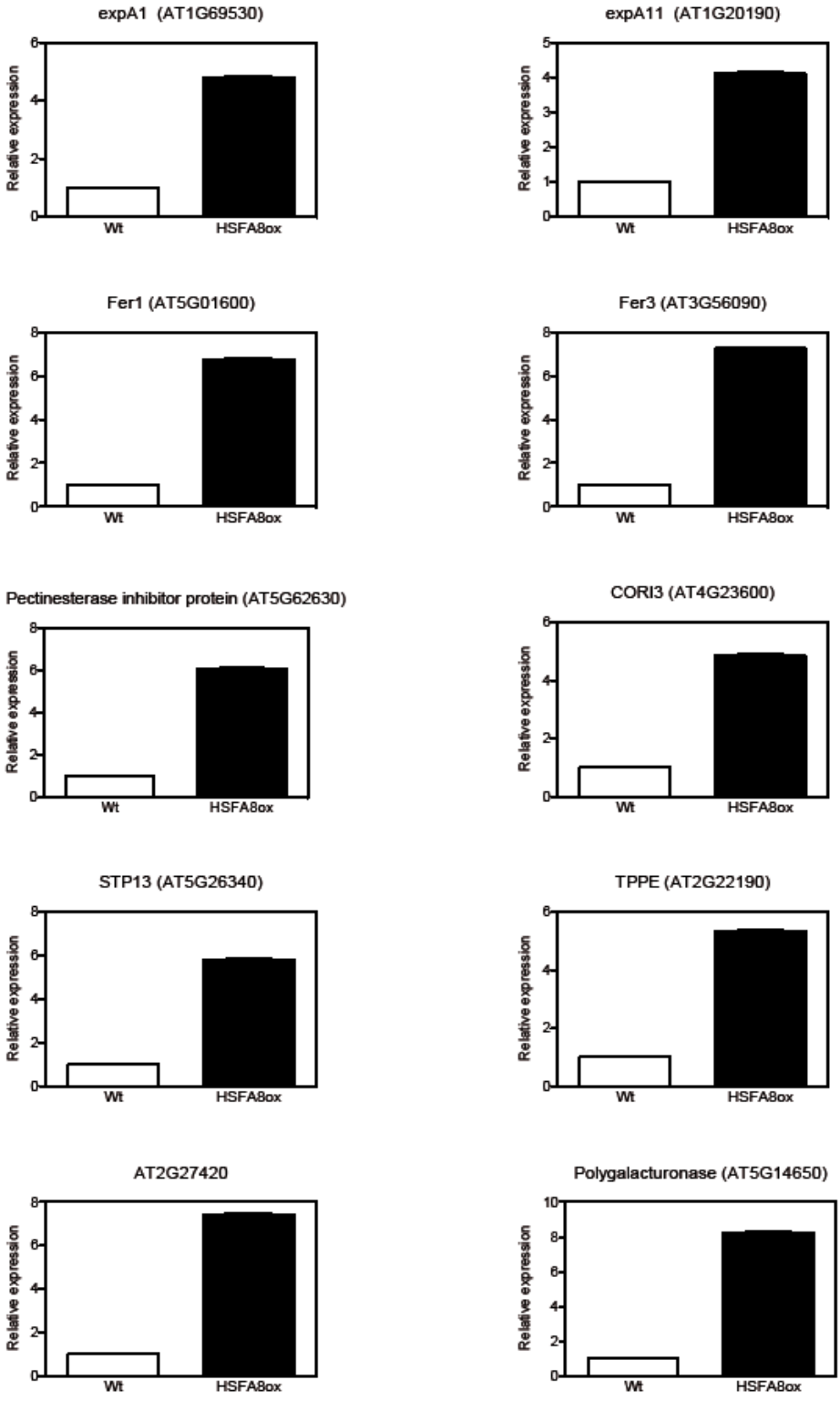

Figure 29: Q-PCR of RT obtained from microarray samples showing the relative expression of examples of up-regulated genes in HSFA8 inducible over-expressor (Black columns) compared to *wildtype* (White columns): ExpA1, ExpA11, Fer1, Fer3, Pectinesterase inhibitor, CORI3, STP13, TPPE, Polygalacturonase and AT2G27420.

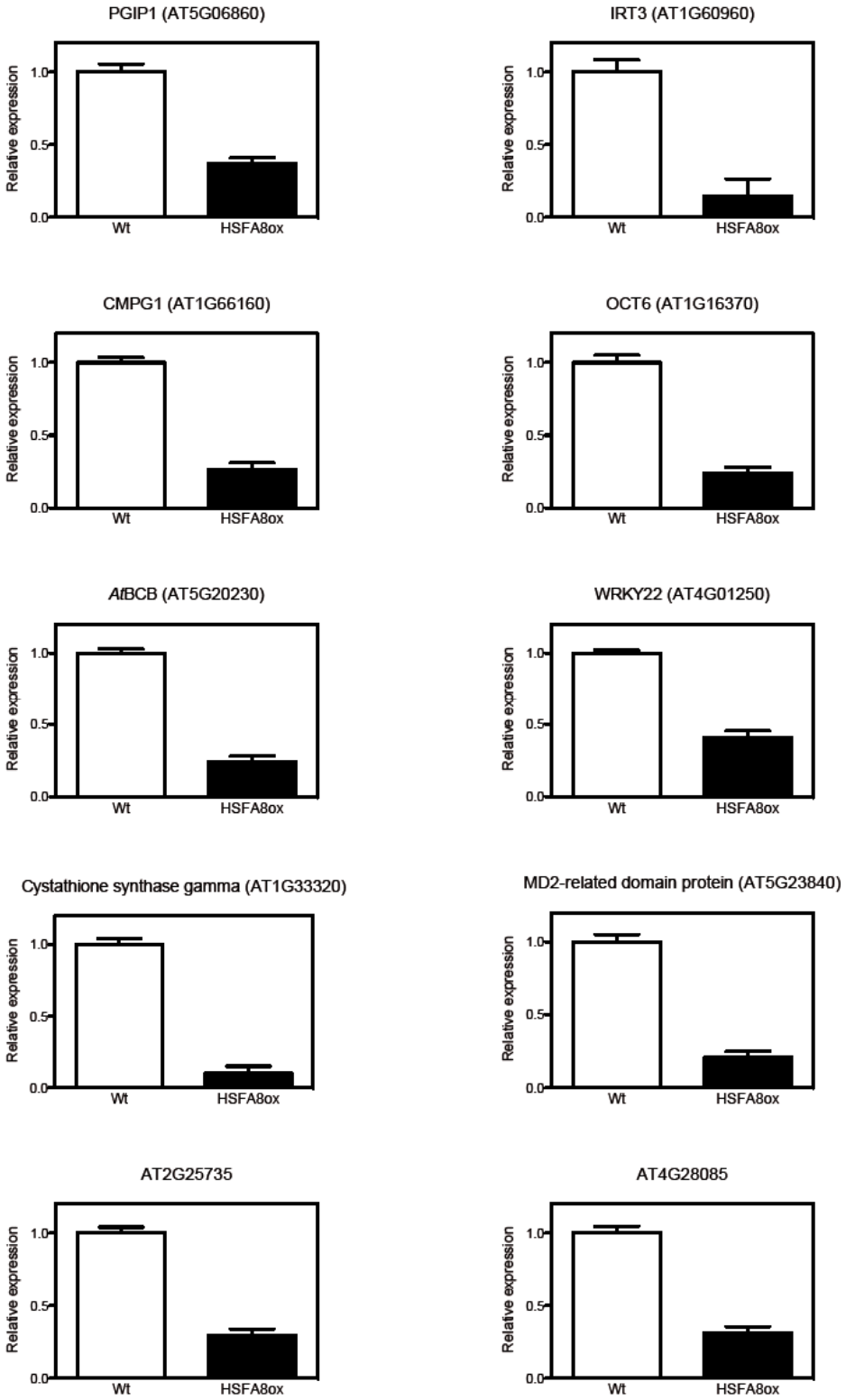

Figure 30: Q-PCR of RT obtained from microarray samples showing the relative expression of examples of down-regulated genes in HSFA8 inducible over-expressor (Black columns) compared to *wildtype* (White columns): PGIP1, IRT3, CMPG1, OCT6, Blue Copper Binding (*At*BCB), WRKY22, cystathione synthase gamma (CSG), MD2-related domain protein, AT2G25735 and AT4G28085.

We also analyzed the expression of two genes which did not accomplish the parameters previously fixed as limit (BH pValue<0,05): the down-regulated Polygalacturonase inhibiting protein 2 (PGIP2, At5g06870) and the up-regulated Cytochrome P450, family 707, subfamily A, polypeptide 2 (CYP707A2, At2g29090), an ABA catabolic enzyme. We could confirm that the tendency observed in the microarray results was correct for both of theme (Figure 31) by quantitative RT-PCR.

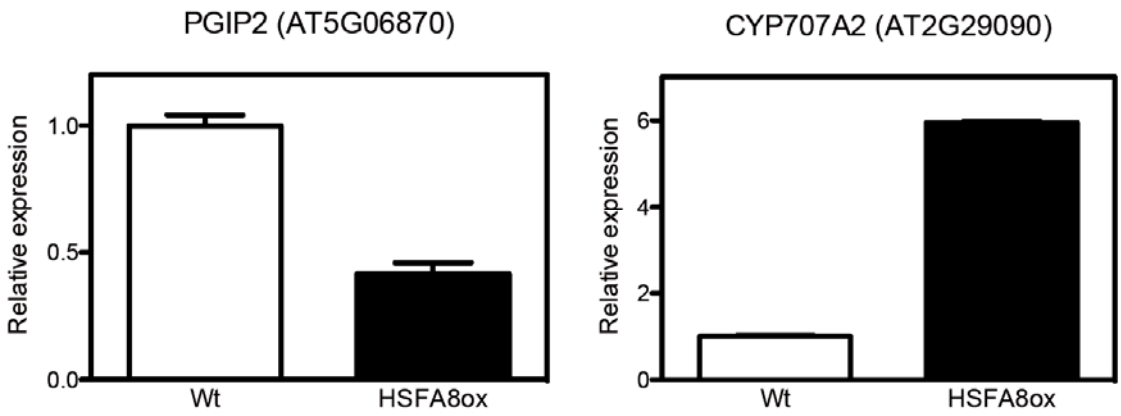

Figure 31: Relative expression of PGIP2 (left) and CYP707A2 (right) in 12 days old seedling grown on MS1 in control plants (White columns) compared to HSFA8 induced over-expression (Black columns).

A more general insight on our transcriptomics data was given by the Gene Ontology (GO) analysis we performed with the total list of mis-regulated genes (Figure 32).

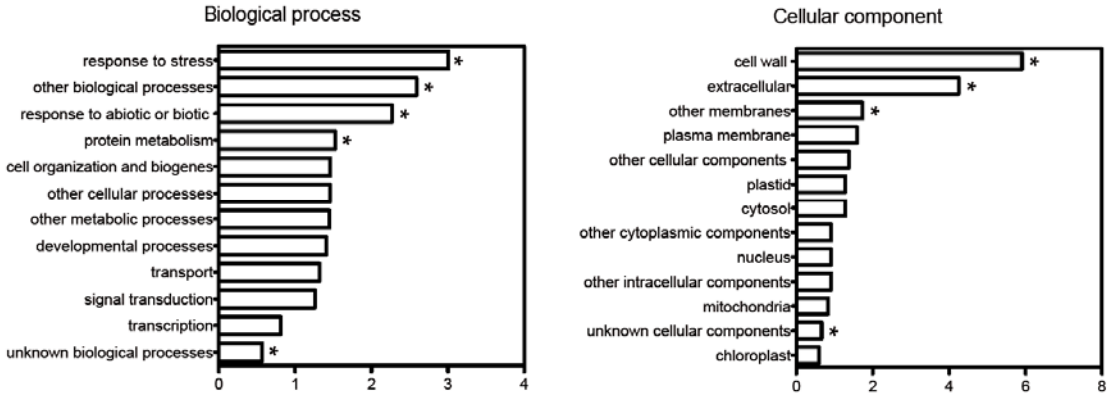

Figure 32: Gene Ontology of the 69 HSFA8 responding genes by biological process (left) and cellular component (right).

Following these data we focused on a possible biological function related to stress response in extracellular spaces and cell wall such as an involvement in the response to foliar pathogen.

Flagellin response in HSFA8-regulated genes

We therefore tested the response to Flg22 in some HSFA8 responsive genes and found that some of the HSFA8-inhibited genes are induced by Flg22 (Figure 33).

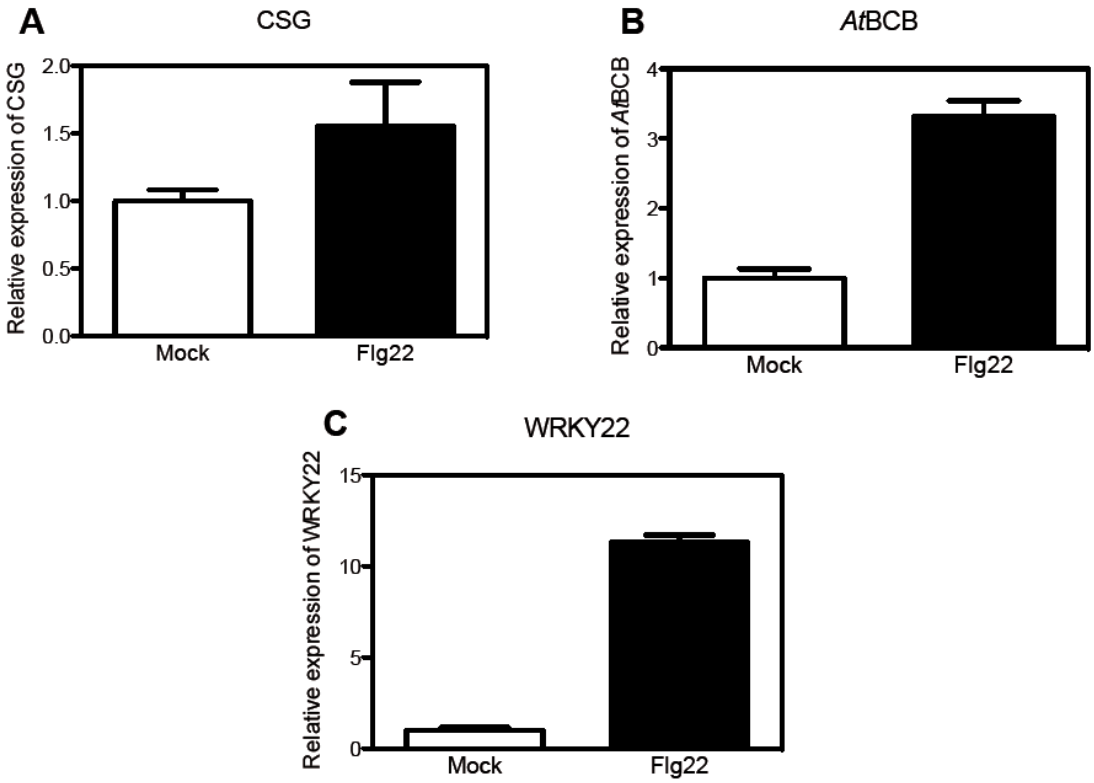

Figure 33: Relative expression of cystathione synthase gamma (CSG) (top left), Blue Copper Binding (*At*BCB) (top right) and WRKY22 (bottom) in 12-days-old seedling grown on MS1 and treated with 10µM Flg22 (Black columns) or a mock solution (White columns).

Moreover, we found that some transcripts that were up-regulated in *HSFA8* over-expressing seedlings responded negatively to Flg22 (Figure 34).

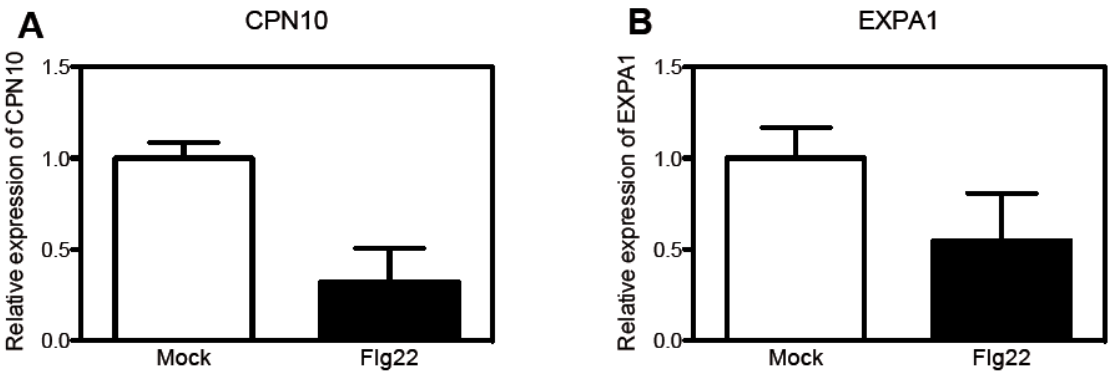

Figure 34: Relative expression of Chaperonin 10 (CPN10) (A) and Expansin 1 (EXPA1) (B) in 12-days-old seedling grown on MS1 and treated with 10µM Flg22 (Black columns) or a mock solution (White columns).

These results suggest a possible superposition of the HSFA8 regulated and Flg22 responsive transcriptomes. Together with the phenotype this crossed regulations might indicate that HSFA8 plays a role in the modulation of foliar pathogen responses.

IV. DISCUSSION

Chapter 1

Comparisons of circadian and ABA-responsive transcriptional profiles show that the genome-wide fraction of ABA-related genes controlled by the clock is around 40%, a significant percentage that is in agreement with previous studies (Covington et al, 2008) and with the notion that ABA signaling is under circadian control. The connection of the circadian clock with ABA pathways was also proven in studies showing the overlap between the ABA-related signalling molecule cyclic adenosine diphosphate ribose (cADPR) and the circadian transcriptome (Dodd et al, 2007) and in studies in which a significant proportion of ABA-responsive genes was found to display diurnal oscillation (Mizuno & Yamashino, 2008). Together, these data imply that the ABA pathway, if considered as a clock output, should be altered when the circadian clock is not properly functioning. This is consistent with our studies showing a significant overlap among TOC1-regulated genes and ABA-related transcriptional networks.

Among the multiple roles of ABA along the plant life cycle, the hormone implications in plant responses to stressful environments are well established (Finkelstein et al, 2002; Leung & Giraudat, 1998; Zhu, 2002). Our studies show ABA-mediated drought phenotypes in TOC1-ox, *TOC1 RNAi* and *toc1-2* mutant plants. The stomata phenotypes in *toc1-2* and *TOC1 RNAi* plants were less severe than in TOC1-ox, suggesting a possible functional redundancy with other genes. This would be in consonance with a recent report showing that other members of the TOC1 family, the PRR9, 7 and 5 negatively regulate the biosynthetic pathways of ABA, chlorophyll, carotenoid and alpha-tocopherol (Fukushima et al, 2009). The physiological phenotypes of *TOC1* mis-expressing plants establish a direct or indirect connection of TOC1 with plant responses to drought. In this sense, our results were in agreement with previous studies showing that proper rhythmic oscillation of stomatal opening is impaired in *toc1-1* (Dodd et al, 2005; Somers et al, 1998). In these studies, it was proposed that the clock might allow stomata to anticipate dusk, contributing to increased water-

use efficiency. The differential plant responses to drought, stomatal aperture and water-loss rates of TOC1 over-expressing and mutant plants were thereby consistent with previous studies and validated our microarray data.

Our studies show that *ABAR* is mis-regulated in TOC1-ox and *TOC1 RNAi* plants. A recent study has questioned the function of ABAR as a receptor, reporting that in barley, mutant plants do not display ABA phenotypes (Muller & Hansson, 2009). However, this issue was convincingly addressed in a recent study (Wu et al, 2009a). Furthermore, our own studies with *ABAR RNAi* plants were consistent with the phenotypes reported in *Arabidopsis*, which are also in agreement with the presence of ABA-related motifs in the *ABAR* promoter and with the inclusion of *ABAR* as a gene regulated by ABA (Matsui et al, 2008). We found a significant hyposensitivity in *ABAR RNAi* plants to the ABA-mediated stomata closure, altered water-loss rates and decreased plant survival after dehydration. Furthermore, we observed an inverse correlation between *ABAR* mRNA abundance and water-loss rates, which highlights the important function of ABAR in the regulation of this plant response. ABAR function was also reinforced by our genetic studies in which the *TOC1 RNAi* phenotypes were completely reverted by the *ABAR RNAi* construct, which in addition to provide clues about TOC1 and ABAR genetic interaction, also assign a function for ABAR in the regulation of plant responses to drought.

Our ChIP results suggest that regulation of *ABAR* expression by TOC1 occurs through direct binding of TOC1 to the *ABAR* promoter. A recent study has reported the physical association of TOC1 with chromatin, most likely through interaction with a TCP clock-associated factor-denominated CHE (Pruneda-Paz et al, 2009). It would be interesting to examine whether TOC1 forms protein complexes with CHE or other factors at the *ABAR* promoter. The mechanisms linking ABA with the circadian clock may also involve the ABA signalling factor ABI3, as it was shown earlier that ABI3 physically interacts with TOC1 (Kurup et al, 2000). In our studies, we found TOC1 binding in the absence of ABA, suggesting a role for TOC1 in the control of *ABAR* circadian expression. The binding itself is controlled by the clock, judging by the rhythmic oscillation found in TMG plants. Interestingly, treatment with ABA advanced the binding of

TOC1 to the *ABAR* promoter and acutely repressed *ABAR* expression. Both advanced binding and *ABAR* repression occurred specifically during the subjective day, which is a highly relevant time in ABA-regulated processes. Indeed, the peak of ABA diurnal fluctuation, the maximal leaf transpiration rate and maximal effectiveness of ABA at closing stomata all occur at this specific time window (Robertson et al, 2009). Furthermore, *TOC1* expression is acutely induced by ABA, and this induction is gated by the clock at this particular time. A plausible explanation for all these results is that plants use the circadian clock to coordinate *TOC1* induction by ABA at a highly sensible phase. This induction rapidly shifts TOC1 binding to the *ABAR* promoter, which acutely advances *ABAR* repression. The effects of ABA treatment on the circadian expression of other clock-associated genes were examined earlier (Hanano et al, 2006). The study showed that ABA lengthens periodicity of bioluminescence in seedlings expressing the promoter of *CHLOROPHYLL A/B-BINDING PROTEIN* (*CAB2*/*LHCB1-1*), *COLD- AND CIRCADIANREGULATED 2* (*CCR2*/*AtGRP7*) and CCA1 (Hanano et al, 2006) fused to the luciferase. The acute effects of ABA, which we observe on *TOC1:LUC* expression, might not affect the other genes examined in Hanano's study. It is also possible that due to the different experimental designs, the acute changes were missed. In agreement with this notion, we also found that after the acute induction of *TOC1*, the bioluminescence signals decreased in amplitude with a slight delay in the phase of *TOC1:LUC* expression. It cannot be ruled out, however, a possible saturation of the response because of the extended time with the high concentration of the hormone. Interlocked feedback loops are common mechanisms for precise regulation of gene expression within the circadian oscillator (Rand et al, 2004). In the case of other metabolic or physiologic processes, feedback loops might also exert an important regulatory role. For instance, the ABA-insensitive 1(ABI1) and 2 (ABI2) phosphatases were found to function in a negative feedback regulatory loop within the ABA signaling pathway (Merlot et al, 2001). It was suggested that this loop might be responsible for resetting the ABA signalling cascade, thereby allowing the cell to continuously monitor the presence or absence of ABA (Merlot et al, 2001). A similar mechanistic explanation may underlie the reciprocal regulation between TOC1 and ABAR. It

would be interesting to examine the mechanisms and identify other components implicated in this regulation. The existence of a feedback loop connecting the clock and ABA signalling pathways is also in line with previous studies showing that cADPR, which has an important function in molecular and physiological ABA responses (Sánchez et al, 2004), forms a feedback loop within the plant circadian clock (Dodd et al, 2007).

Our genetic studies are in agreement with the molecular data and functionally connect the reciprocal regulation of *TOC1* and *ABAR* expression with plant tolerance to drought conditions. Although we could not obtain double ABAR-ox/ TOC1-ox or ABAR-ox/*TOC1 RNAi* plants, the results showing that the phenotypes of *TOC1 RNAi* plants were reverted in the double *ABAR*/*TOC1 RNAi* plants indicating that *TOC1 RNAi* phenotypes are only evident in the presence of a functional ABAR. Conversely, the drought-related phenotypes of TOC1- ox plants were not significantly affected by decreasing ABAR abundance, reinforcing the negative role of TOC1 in the regulation of *ABAR* expression. Although our studies do not exclude the interaction of TOC1 with other ABA signaling components, the feedback loop between TOC1 and ABAR links a key oscillator component with drought-related responses. This mechanism might contribute to the clockcontrolled gating of ABA activity at midday, a time when transpiration rate is higher and plants need a more precise control of hormone function if water supply is limited. Thus, the circadian gating of stomatal closure would ensure proper adjustments of guard cell closure in the heat of the afternoon, when this regulation is more needed. We propose that the reciprocal regulation between TOC1 and ABAR might function as a fine-tuned switch that helps to modulate the plant sensitivity to ABA, which in turn favours the temporal regulation of plant responses to dry environments (Figure 17E). The direct involvement of an essential clock component in these responses also provides a plausible explanation on how the circadian clock confers an adaptive advantage to plants (Dodd et al, 2005). Detailed characterization of the molecular components involved in clock interaction with other hormones such as auxin (Covington & Harmer, 2007) or cytokinins (Hanano et al, 2006; Salome et al, 2006; Zheng et al, 2006) might be useful to get further insights into the gating

mechanisms synchronizing hormone signalling with plant growth and metabolism.

Chapter 2

With the availability of hundreds of *Arabidopsis* transgenic lines generated in the frame of the TRANPLANTA network our goal was to find TFs whose over-expression produces an impaired stomatal response to water deficit. Therefore,we develop and optimize a fast and sensitive screening method based on the detection of cotyledon temperature by infrared imaging (adapted from Merlot et al. 2002). Seedlings with differences in their gas exchange rate will display different temperatures of the aqueous phase of their cotyledons, which will reflect in a different amount of infrared irradiance of the leaf surface This system is sensitive to temperature differences lower than 0,5ºC and, thanks to a colour-based image rendering, it allows the immediate identification of plants with altered gas exchange levels within a test of few minutes.

In the original screening developed by from Merlot et al. 2002 plant were grown in soil and subjected to progressive drought stress before to be analyzed by IR imaging. In our case, due to the fact that the preliminary results obtained with the β-estradiol-mediated transgene induction in soil was not highly reproducible and with low level of transgene induction, we develop a fair over-expression in several different lines *in vitro*. Although a good induction level was observed in seedlings with 5μM β-estradiol, the highest levels were observed at 20μM, which was therefore the chosen concentration. We could also observe β-estradiol induced expression of GFP in guard cells after 48h, assuring us that we would have the chance to affect directly the stomatal behaviour in our screening.

The screening allowed us to identify 6 putative candidates, 3 “warm” (with stomata closer than wildtype) and 3 “cold” (with open stomata), among 264 *Arabidopsis* lines of conditional over-expressors. All of them displayed the phenotype in at least two independent insertions suggesting that the observed phenotypes are not due to the insertion site of the transgenes. The temperature

differences, that can be appreciated through the colorimetric scale of the image rendering, were quantified and tested for statistical significance. The further analysis of the candidates revealed that all of the lines showing a phenotype actually over-expressed the expected transgene. The following measurement of stomatal aperture confirmed that our phenotypes are due to altered stomatal behaviour. These data suggest that the overall experimental scheme used for the screening produced qualitatively good candidates for both kinds of phenotypical alterations: “cold” and “warm”.

One the most interesting candidate genes selected corresponds to *ANAC087 (*At5g18270), that induces stomatal closure when over-expressed in presence of β-estradiol. Several studies have evidenced the importance of NAC (NAM, ATAF1/2 and CUC2) transcription factors in response to abiotic stress in both rice and *Arabidopsis* (Nakashima et al, 2012; Nuruzzaman et al, 2010). Based on the sequence of the NAC domains, a phylogenetically consistent group of transcription factors was named SNAC (Stress Responsive NACs). This group includes most of the known NAC transcription factors implied in abiotic stress response in *Arabidopsis thaliana* and rice (Nakashima et al, 2012). Three highly related *Arabidopsis* SNACs, ANAC019, ANAC055 and ANAC072/RD26 are positive regulators of ABA signalling. The over-expression of either of them display ABA-sensitive and drought-resistant phenotype (Tran et al, 2004). They are also transcriptional activators of the gene *early response to dehydration stress 1,* cooperatively with the transcription factor ZINC FINGER HOMEODOMAIN 1 (ZFHD1) (Tran et al, 2007). The expression of ANAC072/RD26 is induced by drought and ABA through SnRK2-mediated signalling (Fujii et al, 2011). Another important SNAC in *Arabidopsis* is *ATAF1*, which is transcribed in response to ABA and contributes positively to its signalling pathway (Wu et al, 2009b).

Even though *ANAC087* doesn’t belong to SNAC subfamily, it has been reported to be transcribed in response to H_2O_2 treatments and localize in both nucleus and cytoplasm (Inzé et al, 2012). These results, together with our

phenotypic characterisation, suggest that ANAC87 may play a role in ROS-dependent signalling that leads to stomatal closure.

The strongest phenotypic alteration was displayed by *DEAR4/RAP2.10* (*DREB AND EAR MOTIF 4*/*RELATED TO APETALA 2.10*) (At4g36900) over-expressing lines. These plants showed a marked increase in leaf temperature, which was confirmed to be related to a closed-stomata phenotype. The members of AP2/ERF family transcription factors play various roles in plants responses to abiotic stress (Lata & Prasad, 2011; Mizoi et al, 2012). A particularly relevant contribution to dehydration response is given by the members of the DREB group of transcription factors. The DREB1 subgroup (subfamily A1) includes the CBF transcription factors and has been mostly related to cold stress responses, although the over-expression of either *DREB1A*/*CBF3*, *DREB1B*/*CBF2*, *DREB1C*/*CBF1* or *CBF4* produce both cold and drought resistant phenotype in *Arabidopsis thaliana*, being the last one transcribed in response to drought (Haake et al, 2002; Liu et al, 1998; Mizoi et al, 2012). The DREB2 subgroup (subfamily A2) is, in turn, mostly related to dehydration and heat shock responses, being DREB2A and 2B involved in gene expression through DRE/CRT *cis*-elements (Liu et al, 1998; Mizoi et al, 2012; Nakashima et al, 2000; Sakuma et al, 2006). Interestingly, DREB1A, DREB2A and DREB2C were shown to interact in with AREB1/ABF2 and AREB2/ABF4, suggesting that they may be regulators of the main ABA-responsive transcriptional regulation mechanism (Lee et al, 2010).

DEAR4/RAP2.10 is a member of subfamily A5 of the AP2/ERF family of transcription factors since it presents both DREB (DRE BINDING PROTEIN) and EAR (ERF ASSOCIATED AMPHIPHILIC REPRESSION) motifs in its sequence. Members of this family are up-regulated by the transcriptional activity of DREB1A/CBF3 and DREB2A (Maruyama et al, 2004; Sakuma et al, 2006). At least one of theme, *RAP2.1*, seems to be involved in the modulation of drought and freezing responses, given the increased stress tolerance displayed by its knock-out mutant (Dong & Liu, 2010). Another member of A5 subfamily, DEAR1,

inhibits cold response while favouring defence mechanisms against the foliar pathogen *Pseudomonas syringae* (Tsutsui et al, 2009). In the case of our selected gene DEAR4/RAP2.10, it does not seem to be implied in cold response through DRE/CRT-mediated transcription activation like the CBFs and is not transcribed in response to *Pseudomonas* (Haake et al, 2002; Tsutsui et al, 2009). Taken together, these data suggest that the stomatal closure mediated by DEAR4 might rely on pathways separated from those of cold response or pathogen resistance related mechanisms.

The β-estradiol induced expression of HSFA8 (At1g67970) enhances stomatal aperture resulting in the observed reduction of foliar temperature. The study of Heat Stress transcription Factors (HSF) in plants has revealed that they participate in a wide range of processes in addition to the heat stress response (Kotak et al, 2004; Nover et al, 2001; Scharf et al, 2012). These transcription factors were divided into three classes based on the characteristics of their oligomerisation domains (OD) (Nover et al, 2001). Plants HSF commonly display also a DNA BINDING DOMAIN (DBD) and a NUCLEAR LOCALIZATION SIGNAL (NLS) peptide (Nover et al, 2001; Scharf et al, 2012). Interestingly class A HSF also display in their C-terminal domain one or two short activator peptide motif (AHA), implied in transcriptional activation of target genes (Scharf et al, 2012). The transcriptional regulation activity of *Arabidopsis* class A HSF varies significantly among the members of the family. Assays of promoter activation performed in yeast and tobacco protoplasts revealed that HSFA8, A9 and A4c do not activate transcription in yeast nor bind to the transcription machinery but are capable of activating transcription in tobacco protoplasts (Kotak et al, 2004). The peculiar pattern of transcriptional activation described for *Arabidopsis* HSFA8 might depend on the fact that the first AHA motif is located in the central region of the protein, before the NLS, while the second one, though located in the C-terminal domain do not display any acidic residue in the active site (Kotak et al, 2004; Scharf et al, 2012). Conversely, HSFA5, though presenting a complete AHA motif displayed the opposite results, being capable of inducing transcription only in yeast (Kotak et al, 2004; Nover et al, 2001; Scharf et al,

2012). Other peculiar cases are: HSFA3, which, though presenting two AHA motifs (Nover et al, 2001), activates transcription through a characteristic pattern of tryptophane residues (Scharf et al, 2012), and HSFA9, which activates transcription even lacking AHA motifs (Nover et al, 2001; Scharf et al, 2012). The B and C class HSFs, in turn, do not produce any transcriptional activation in the assays described above (Kotak et al, 2004). Moreover, HSFBs present a transcriptional repressor motif in the C-terminal domain (Scharf et al, 2012). Nevertheless, it has been shown that HSFB1 can act as a co-activator in combination with HSFA1 (Scharf et al, 2012). Such a various pattern in the transcriptional regulation functions is, however, gated by a reduced variability of the DNA binding domain among the HSF family members (Scharf et al, 2012). The binding sequence of HSF-mediated activation of transcription in response to heat is generally considered to be the HEAT STRESS PROMOTER ELEMENT (HSE) (AGAAnnTTCT), which is required in repeats upstream of the TATA box (Sakurai & Enoki, 2010), although other binding sites with weaker affinity might be targeted in correspondence to the adjacent binding of partner transcription factors (Bharti et al, 2004; Guo et al, 2008).

An important feature of most of class A HSF is the NUCLEAR EXPORT SIGNAL (NES) peptide (Nover et al, 2001). The NES constitutes an important regulatory element for class A HSF, since *Arabidopsis* HSFA1 and A2 localize in both nucleus and cytoplasm in tobacco protoplasts. These localizations become nuclear in presence of nuclear export inhibitors or mutation in the NES sequence, suggesting that there is a control mechanism based on shuttling of these transcription factors to cytoplasm (Kotak et al, 2004). The importance of this regulation would be even more extreme for HSFA8, which has been reported by Kotak and colleagues to localize exclusively in the cytoplasm unless co-expressed with a nuclear-localized HSF (Kotak et al, 2004). In spite of this, our data over-expressing HSFA8 fused to GFP in tobacco leaves suggest that the accumulation of HSFA8 protein also in the nucleus. Another evidence of the presence of HSFA8 in the nucleus is given by the same work of Kotak and co-workers, in which it can be appreciated that this transcription factor can activate the transcription of a reporter gene in tobacco protoplasts (Kotak et al, 2004).

At a functional level, the main activators of heat shock response are HSFA1 subclass members in cooperation with HSFA2s or HSFB1 (Scharf et al, 2012). The B class members 1 and 2 are also transcriptional inhibitors that negatively regulate pathogen defence mechanisms (Kumar et al, 2009). *HSFA3*, in turn, participates in heat and drought response and is transcribed through the action of DREB2A and DREB2C (Chen et al, 2010; Qin et al, 2007; Yoshida et al, 2008). Finally, HSFA9 seems to be implied in ABA signalling in seeds downstream of ABI3 and in cooperation with DREB2 transcription factors (Almoguera et al, 2009; Kotak et al, 2007; Tejedor-Cano et al, 2010). Conversely, HSFA5 seems to have a function independent of direct transcription regulation. This heat stress factor is, in fact, an inhibitor of HSFA4 activity. This inhibition is mediated by a direct interaction with its target thus impeding its binding to DNA (Baniwal et al, 2007). The action of the latter has been related to oxidative stress response and ROS reduction since its inactivation reduces ASCORBATE PEROXIDASE 1 (APX1) levels (Davletova et al, 2005).

HSFA8 is among the less studied HSF of *Arabidopsis thaliana*, nevertheless, based on our results and previous works we can suppose it is a transcription factors whose localization, distributed between nucleus and cytoplasm, is probably regulated by transcriptional partners such as other HSFs. At the same time, we may speculate that HSFA8 acts as a transcriptional activator, as observed by Kotak and collaborators in tobacco protoplasts (Kotak et al, 2004), though it might also act as an indirect inhibitor as observed for HSFA5. Based on our data we know that *HSFA8* transcript levels are slightly up-regulated by ABA and reduced in response to drought or osmotic stress. Heat stress, in turn, at least in the conditions tested, seems not produce significant effects on its mRNA levels. A subtle increase in HSFA8 transcript was, in turn, observed in response to treatment with *flg22,* the biologically active epitope of the bacterial PAMP flagellin. The most significant variation of *HSFA8* transcript levels was, although, observed between different moments of the subjective day in free running conditions. All together, these results suggest that our candidate gene is regulated independently in response to ABA and osmotic stress. The effects of ABA were, in turn, consistent with those observed in response to

Flg22. Nevertheless, these regulations do not produce as strong alterations of the transcript levels as the time of the day. The fact that *HSFA8* mRNA levels oscillate during the second day of free running conditions suggests that the accumulation of this transcript is under control of the circadian clock. Another interesting result is the stomatal insensitivity to flagellin observed in *HSFA8* over-expressing seedlings. This phenotype suggests that HSFA8-mediated stomatal aperture may overlap with the flagellin response pathway. The transcriptional enhancement observed in *HSFA8* in response to Flg22 suggests that this factor may operate as a modulator of defence response.

As a matter of fact, the circadian clock has been shown to modulate the response to pathogens throughout the day, given that foliar pathogen virulence is enhanced by light and humidity, this mechanism permits to anticipate and be prepared for predictable increases in pathogen virulence (Bhardwaj et al, 2011; Roden & Ingle, 2009; Wang et al, 2011b). In fact, it has been shown that CCA1 enhances the expression of several defence genes implied in both R-mediated programmed cell death and basal defence. This mechanism aims to prime the defence responses around dawn (Wang et al, 2011b). Another example of morning specific priming of defence is given by *Pseudomonas syringae* pv. *tomato* DC3000 response in *Arabidopsis* (Bhardwaj et al, 2011). The mRNA levels of several effectors of *Pseudomonas* response such as Flagellin receptor FLS2, kinases involved in flagellin signalling and the transcription factor WRKY22, were shown to oscillate diurnally. This oscillation resulted in a differential sensitivity to the foliar pathogen during the subjective day in *free running* conditions; being the plants less sensitive around subjective dawn and more at dusk (Bhardwaj et al, 2011). An important target of flagelling response pathway is the control of stomatal aperture, which responds to necessity of reducing the possibilities of pathogen penetration below the cuticle (Kumar et al, 2012; Melotto et al, 2006). Therefore, it has been suggested that the circadian gating of defence responses to foliar pathogens partially relies on the clock modulation stomatal aperture (Roden & Ingle, 2009). An important contact point of circadian modulation of biotic and abiotic stress responses in guard cells is given by GRP7/CCR2 (Roden & Ingle, 2009). This RNA binding protein

accumulation is regulated by the clock (Heintzen et al, 1997); moreover, GRP7 localizes in guard cells and induces stomatal closure through the nuclear export of mRNAs (Kim et al, 2008). Consistently, *GRP7* mutation in *Arabidopsis* was shown to increase plant sensitivity to *Pseudomonas*. Interestingly, the nuclear export activity of this protein was shown to be the target of a pathogenic strategy: GRP7 binding to its target transcripts is, in fact, blocked by a *Pseudomonas* effector through ADP-ribosylation of its active site (Fu et al, 2007). These studies, together with our results, led us to speculate whether HSFA8 might be a modulator of stomatal closure acting in the last hours of the subjective day, thus optimizing water use efficiency in a temporal window presenting reduced dehydration risk and foliar pathogen virulence.

The next matter addressed by our work is the mechanism of action responsible for stomatal opening observed in *HSFA8* over-expressing seedlings. The proteomic approach revealed alterations in the amounts of several proteins, though only one of theme seems to be due to changes in mRNA levels. The case of chaperonins is particularly interesting, being chaperonin 10 up-regulated both at a transcriptional and protein level, while chaperonin 20, which is a doubled form of chaperonin 10, is down regulated only at protein level. Chaperonin 20 has been described as regulator of SUPEROXIDE DISMUTASE (SOD) activity (Kuo et al, 2012). This important regulation contributes to reduce ROS levels, thus down-regulating stress signalling. The change in the relative amounts of these two isoforms of the same kind of enzyme might also be related to ROS signalling modulation. Another interesting protein increased in reponse to HSFA8 over-expression is cyclophilin 20-3: this enzyme has been related to redox signalling modulation in the chloroplast and to the diurnal oscillation of thiols (Dominguez-Solis et al, 2008). Finally, the down-regulation of glutamine synthetase 2 (GS2) has been described as a marker of senescence, which relates with high stomatal aperture (Schildhauer et al, 2008).

At a transcriptomic level, we observe that β-estradiol did not affect dramatically the amounts of any transcript detectable by Affymetrix Gene Chip ATH1. On the other hand, the over-expression of HSFA8 produced changes in 69 transcripts. We should consider that the limited number of targets is possibly

an underestimation, as suggested by the direct analysis of transcripts showing up or down-regulation that failed to reach the p-Value threshold set for the transcriptomic analysis. Moreover, the reduced size of the impacted transcriptome might depend on the nature of HSFA8 itself, whose activity might be largely regulated by partners through subcellular localization as much as target DNA recognition. Nevertheless the observed open stomata phenotype relies on HSFA8 itself and, most likely, on its ability of regulating transcription.

At a transcriptional level, we observe that *HSFA8* over-expression produces a significant up-regulation of two ferretin genes transcripts: *Ferretin 1* (At5g01600) and *3* (At3g56090). These two genes have been related with the down-regulation of oxidative stress responses in Arabidopsis (Ravet et al, 2009). In fact, ferretins function as a buffer reducing the pool of intracellular free iron. The free iron pool operates as a ROS enhancer by producing highly reactive hydroxil radicals through the Fenton reaction, therefore the Ferretin-mediated reduction of this pool is considered an action of ROS scavenging that can prevent oxidative damage (Kim et al, 2011). Interestingly *Ferretin 1* is a clock-regulated gene that is implied in the circadian regulation of iron homeostasis (Duc et al, 2009). Consistent with the hypothesis that HSFA8 would be a regulator of the pool of free iron, we observed that *IRON REGULATED TRANSPORTER 3* (*IRT3*) mRNA is down regulated in *HFA8* over-expressing seedlings. IRT3 is a divalent ion transporter that participates to iron uptake in *Arabidopsis* (Lin et al, 2009). The possible reduction of free iron pool in response to HSFA8 would, therefore, result in a down-regulation of ROS-mediated signalling that could explain the open stomata phenotype observed in the over-expressing plants. This hypothesis finds support in the observed down-regulation of the transcript of the hydrogen peroxide-induced gene At2g25735 (Inzé et al, 2012). Another possible explanation for the phenotype is the up-regulation of *CYP707A2* (At2g29090) mRNA, that codes for an enzyme catalyzing abscisic acid catabolism by 8 and 9-hydroxilation (Okamoto et al, 2011). This suggests that *HSFA8* over-expressing seedlings may present reduced levels of ABA and ROS, resulting in enhanced stomatal aperture.

Another possible explanation for the open stomata phenotype is given by the up-regulation of alpha-expansin genes *EXPA1* (At1g69530) and *EXPA11* (At1g20190). More precisely, *EXPA1* over-expression has been shown to enhance stomatal aperture by altering the structure of the guard cell wall and thus decreasing its volumetric elastic modulus (Wei et al, 2011; Zhang et al, 2011). Interestingly, among the subset of HSFA8-altered transcripts, the most relevant trait is the predominance of genes whose products are targeted extracellularly. Consistent with an enhancement in expansins transcription, we observed other clues suggesting that HSFA8 might induce a relaxation of the cell wall. It is the case of the induction of a polygalacturonase gene (At5g14650) and the down-regulation of two polygalcturonase inhibiting proteins (PGIP): PGIP1 (At5g06860) and PGIP2 (At5g06870). Interestingly these PGIPs have been described for their capacity of enhancing resistance to foliar pathogens like *Botrytis cinerea* (Ferrari et al, 2006), *Fusarium graminearum* (Ferrari et al, 2012) and *Stemphylium solani* (Di et al, 2012) in *Arabidopsis thaliana*. These observations are consistent with our stomatal phenotypic result that depicts HSFA8 as a down regulator of pathogen responses. Other evidences are given by the down regulation of *CMPG1* (Cys, Met, Pro and Gly), a gene implied in early pathogen response (Heise et al, 2002), and WRKY22 a transcription factor master transcription factor of pathogen response (Asai et al, 2002) that is regulated by the circadian oscillator (Bhardwaj et al, 2011). Conversely HSFA8 over-expression enhances the transcription of *CORONATINE INDUCED 3* (*CORI3*), a gene up-regulated by the phytotoxin coronatine, that ultimately provokes stomatal aperture (Lopukhina et al, 2001). CORI3 is also induced in senescence, a process that implies stomatal aperture (Hollander-Czytko et al, 2005). Moreover we could observe that, among the HSFA8-inhibited genes, *WRKY22*, *AtBCB* and *CSG* are induced in response to Flg22. Conversely, transcripts that are up-regulated in *HSFA8* over-expressing seedlings like *CPN10* and *EXPA1*, are inhibited by Flg22.

These results, therefore, reinforce the hypothesis of the implication of HSFA8 in a general relaxation of the cell wall accompanied by an enhanced

stomatal aperture in order to favour photosynthesis and growth in a time window of decreasing pathogen risk and increasing air humidity.

All together our results contribute to identify three novel players in the picture of transcriptional control of stomatal behaviour. Two of them, DEAR4 and NAC087, participate in stomatal closure while, in turn, HSFA8 induces stomatal aperture (Figure 1).

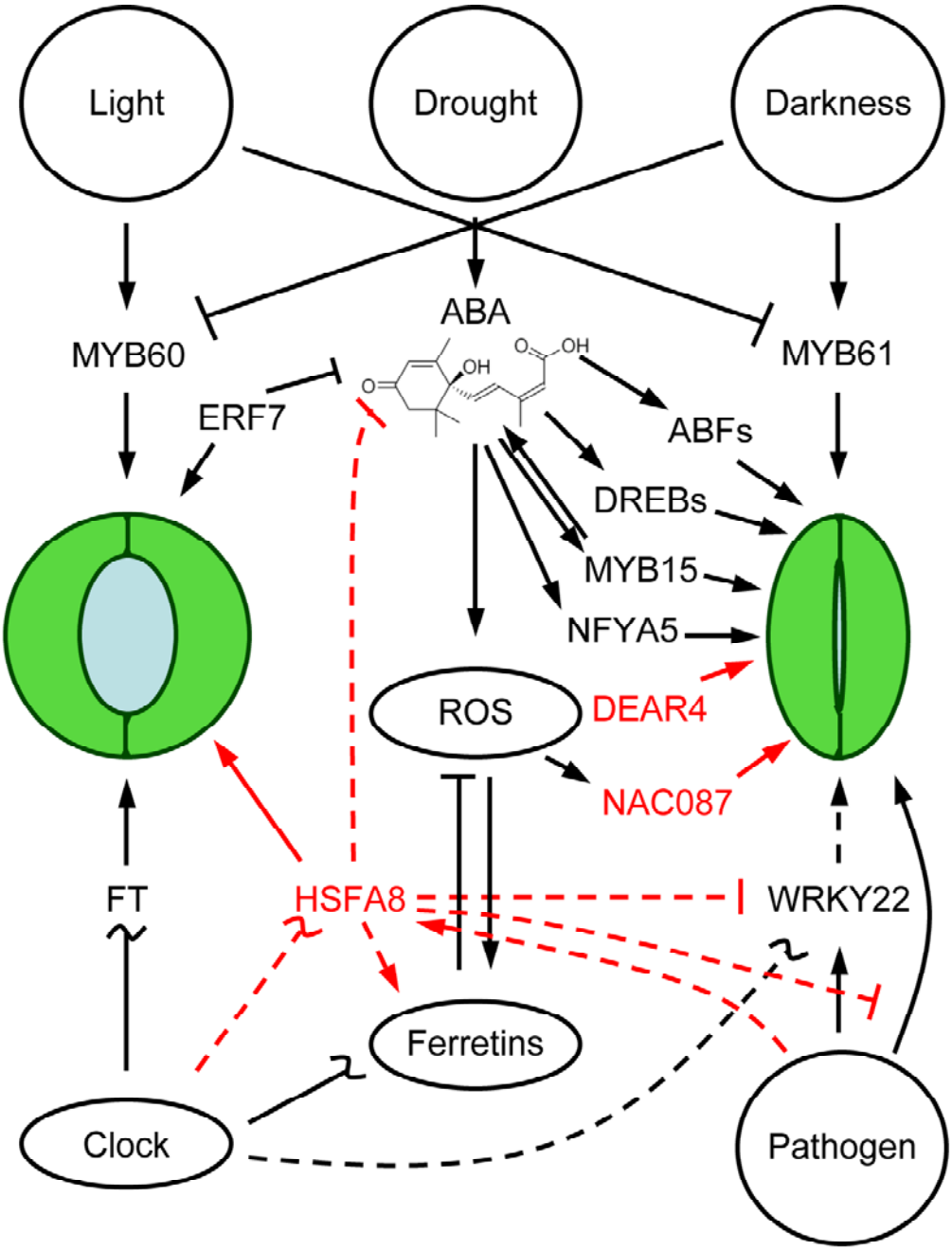

Figure 1: Scheme representing some of the most relevant mechanisms of transcriptional regulation of stomatal behaviour. The inductions are depicted as arrows, bars at the end of a line represent repression and wave-shaped lines stand for circadian regulation by the clock. Discontinuous lines represent indirect or hypothetic functional connections. The contribution of this work is shown in red.

The stomatal opening is sustained by light through MYB60, by ERF7 and by the clock regulated FT and HSFA8. The factors favouring stomatal closure are, in turn, the AREB/ABFs, DREBs, MYB15, NFYA5, DEAR4, and NAC87, possibly through a ROS mediated induction. Moreover the darkness-induced closure is mediated by MYB61.

V. CONCLUSIONS

Proper *TOC1* expression is important for stomatal function and regulation of leaf transpiration rates.

Our results verify a role of *ABAR* within the ABA signalling pathway in *Arabidopsis*.

The circadian clock controls the timing of *ABAR* expression.

TOC1 modulates the phase and amplitude of *ABAR* transcript diurnal and circadian waveform.

There is a temporal coincidence between the ABA-mediated regulation of *ABAR* expression and TOC1 binding to the ABAR promoter.

TOC1 expression is acutely induced by ABA and this regulation is gated by the clock and requires a functional ABAR.

The genetic studies are in agreement with the molecular data and functionally connect the reciprocal regulation of *TOC1* and *ABAR* expression with plant tolerance to drought conditions.

The Transplanta collection constitutes a powerful and reliable tool for phenotypical screenings of *Arabidopsis* seedlings *in vitro*.

The combination of our ß-estradiol induction protocol and the thermal imaging technique is a reliable method for screening leaf temperature phenotypes in *Arabidopsis* seedlings.

DEAR4 (At4g36900) and *NAC087* (At5g182700) over-expressions induce stomatal closure.

HSFA8 (At1g67970) over-expression opens stomata also in presence of ABA or Flg22.

The mRNA levels of *HSFA8* display light changes in response to abiotic and biotic stresses being augmented in presence of ABA and Flg22 and

reduced in response to drought. Heat stress does not affect its transcription under the tested conditions.

The circadian clock has a strong influence on the expression of *HSFA8*.

HSFA8 presents a nuclear and cytoplasmic subcellular localization in tobacco leaves.

The over-expression of *HSFA8* produces alterations in the levels of proteins involved in ROS signalling and senescence.

The over-expression of *HSFA8* affects 69 transcripts. The functional characterization of these transcripts suggest that this regulation may affect iron homeostasis, and down-regulate ROS and ABA levels. An overall cell wall relaxation pattern could also be detected from the transcriptomics data. Cell wall relaxation would be consistent with stomatal opening through a reduced volumetric elastic modulus of guard cells.

Transcritomics and direct quantification data on Flg22 responsive genes suggest that HSFA8 might play a role in antagonising the response to flagellin.

VI. MATERIALS AND METHODS

Plant material, growth conditions and bioluminescence analysis of *TOC1* and *ABAR* mis-expressing plants and relative controls

The *TOC1 RNAi*, TMG-YFP (TMG), TOC1-YFP-ox (TOC1-ox) and the *toc1-2* mutant plants (Más et al, 2003a; Strayer et al, 2000) were provided by Prof. Steve A Kay (University of California San Diego, La Jolla, CA, USA). The *ABAR RNAi* construct (Shen et al, 2006) was provided by Prof. Da-Peng Zhang (Tsinghua University, Beijing, China). The *TOC1 RNAi*/*ABAR RNAi* and TOC1-ox/*ABAR RNAi* plants were obtained after transforming the *ABAR RNAi* construct (Shen et al, 2006) by *Agrobacterium tumefaciens*-mediated DNA transfer (Clough & Bent, 1998). Seedlings were grown on Murashige and Skoog (MS) agar plates with 3% sucrose under controlled environmental conditions (50 $mmolm^{-2}s^{-1}$ of cool white fluorescent light at 22ºC). Luminescence analyses were performed as described earlier (Portolés & Más, 2007). In experiments of hormone treatments, (+/-) ABA (A4906; Sigma-Aldrich) was added to seedlings at the indicated concentration at different times during the diurnal and circadian cycle.

ChIP assays

ChIP assays were performed as described earlier (Perales & Más, 2007). Briefly, Seedlings were immersed in buffer A (0,4 M sucrose, 10 mM Tris pH 8, 1 mM EDTA, 1 mM PMSF, 1% formaldehyde, 0,05% Triton X-100) under vacuum for 10 min followed by additional 10 min incubation with glycine 0,125 M. Seedlings were ground in liquid nitrogen and resuspended in buffer B (0,4 M sucrose, 10 mM Tris pH 8, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin). Nuclei were then collected by centrifugation, resuspended in lysis buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin) and sonicated to approximately 500–1000bp fragments. After centrifugation, the supernatants were incubated in dilution buffer (15 mM Tris pH 8, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin) overnight at 4ºC with sepharose beads conjugated with a

anti-GFP antibody (A11122, Invitrogen). The immune-complexes were washed four times with washing buffer (0,1 % SDS, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin) and eluted from the beads with 1% SDS and 0,1 M $NaHCO^3$. Cross-links were reversed by incubation at 65°C for 5–6h followed by proteinase K treatment for 1h at 45ºC, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were washed with 70% ethanol and resuspended in TE. The results presented in the manuscript come from two biologically independent experiments, meaning independent time course analysis, with independent chromatin preparations. Quantifications were performed relative to the input. As negative controls, we used other ABA-related genes (see list of primers below). Samples similarly processed but without antibody in the immunoprecipitation incubation were also used as negative controls.

For hormone treatment, seedlings were sprayed 4 h before sampling with 7.5 ml of 0,001% Triton X-100 aqueous solution containing 100µM (+/-) ABA and samples were analysed at the indicated time points. Q-PCR was performed with the SYBR Premix Ex Taq (Takara) in a 96-well Lightcycler 480 system (Roche) and quantified with the Lightcycler 480 software (Version 1.5.0.39, Roche). A list of primers used for locus amplification is shown in following table.

Gene	Primer 1	Primer 2
ABAR (prom)	5' cggattaaaccggctaccc 3'	5' tgacaagtatttttatgttgctctcttga 3'
ABAR (5'UTR)	5' ttctttttagttccttggtccgaa 3'	5' agaggataggagaagagatgaatcgt 3'
ABAR (4th exon)	5' caagatggtggcggagctag 3'	5' aatatcaatgccaagcgcct 3'
RD29B	5' ctgagacgtggcaggacgaa 3'	5' tggccgaaccacctctctct 3'
ABA1	5' aggtgacacaatattattagtccacatcgtat3'	5' gctcatagaaaataacacaagcccat 3'
RD20	5' tgttcttattctgaaaatgacacaaattt 3'	5' tcattggactaaaaaacaccaaacata 3'
ABI3	5' ccagatagtttatgttgtttaagattcga 3'	5' gatagggtttgtatgtgagtaaggaggc 3'
ABI5	5' gaatagctgaacagggacaagtaactg 3'	5' accacctaaacgacaataacaattttg 3'

Analysis of gene expression by northern blot and Q-PCR

Northern blot analysis of *ABAR* and *TOC1* expression was performed with RNA from 12-day-old seedlings grown under the indicated conditions. For hormone treatment, seedlings were sprayed with 7,5 ml of 0,001% Triton X-100 aqueous solution containing 100 mM (+/-) ABA and samples analysed at the indicated time points. The northern blot of *TOC1* expression shown in Figure 13D was obtained after spraying seedlings with the hormone at CT5 (so that can be compared with the waveform of *TOC1:LUC* expression adding ABA at CT5 in Figure 13A). RNA was isolated using the Purelink Total RNA purification system (Invitrogen) and separated on 1,0% agarose/formaldehyde gels as described earlier (Perales & Más, 2007). ABAR probe was described earlier (Shen et al, 2006). *TOC1* probe was described earlier (Más et al, 2003a). Quantification analysis was performed on a Bio-Rad phosphorimager using Quantity One software, version 6.1 (http://www.biorad.com/). For Q-PCR analysis, RNA was treated with RNAse-free DNase (Ambion) and the single-strand cDNA was synthesized using Superscript III (Invitrogen) and a mixture of oligo-dT18 and random hexamers (Invitrogen) following the manufacturer recommendation. The cDNA was diluted 10-fold with nuclease-free water and Q-PCR was performed with the SYBR Premix Ex Taq (Takara) in a 96-well Lightcycler 480 system

(Roche). The *IPP2* gene (isopentenyl pyrophosphate:dimethyl-allyl pyrophosphate isomerase) was used as a control (Hazen et al, 2005). Quantification was performed using Lightcycler 480 software (Version 1.5.0.39, Roche). Primers were designed using the PrimerExpress 2.0 software (Applied Biosystems) with lengths of 18–25 nucleotides, PCR amplicon lengths of 80–180 bp, 40–60% G:C content and 60–65ºC. The list of primers used for Q-PCR assays is presented in the following table.

Gene	Primer 1	Primer 2
COR15A	5'tgtcagagtcggccagaaaac3'	5'cacctttagcggcgtagatca3'
ABI5	5'aagctatgggtgtgggtgacc3'	5'caaacacctgcctgaactggt3'
RAB18	5'atggcgtcttaccagaaccgtc3'	5'tcgtcatactgctgctggatcg3'
COR15B	5'ccacaacgtaggagcaaagca3'	5'ttcttgcgctgagcaacga3'
At3g15670	5'tcgttcccatagcttgtcca3'	5'gaaatggcatccaaccaaca3'
At5g06760	5'ttatccagtatatccccgcc3'	5'tcgcagaaccgctcataacac3'
RD29A	5'tggatacggtgaggcatcag3'	5'ccgtctttgggtctcttccc3'
At3g17520	5'tgttgtgtccaagctacgatcg3'	5'ctttgcccaatcagtccatgat3'
At5g44310	5'aacgagaacggttttcttcgg3'	5'ccgcaagagttctaggagcttg3'
RD29B	5'ggagcggtcacttcttggct3'	5'ggtggtgccaagtgattgtg3'
At3g02480	5'atcattccaccggccttct3'	5'agcaaaacgcgagctacca3'
At4g21020	5'gataaaaatggcggccatgc3'	5'agccggagctttgattccag3'

Measurements of guard cell dimensions, stomatal conductance and plant survival to drought conditions for *TOC1* and *ABAR* mis-expressing plants and relative controls

Detached rosette leaves from 3-week-old plants grown under ShD conditions (8 h light:16 h dark) were incubated for 2 h in a buffer containing

50mM KCl and 10mM MES, pH 6,15 with cool white fluorescent light (50 $mmolm^{-2}s^{-1}$). ABA was subsequently added in the same buffer solution at different concentrations (0, 1 and 5 μM) and leaves were incubated for 2 more hours with the hormone. Abaxial epidermis were carefully taken with tweezers and mounted with the same buffer solution. Epidermis strips were observed with a Zeiss AxioPhot microscope with a X63 objective. Approximately 100–200 stomata per sample were photographed using an Olympus DP70 camera attached to the microscope. Stomatal aperture was scored as width:length pore ratio using CellD software (Olympus). For stomatal conductance measurements, plants were grown under ShD for 5 weeks and 60–65% relative humidity. A Licor 6400 photosynthesis system with a 6400-15 *Arabidopsis* leaf chamber attachment (Licor, NE) was used for gas exchange measurements. Temperature was maintained at 22ºC, humidity to 62–67% and CO^2 to $400mmolmol^{-1}$. Stomatal conductance was monitored every 20 seconds until stabilization. For water-loss assays, rosette leaves of comparable size from 4-week-old plants grown under ShD were detached, placed abaxial side up on a Petri dish and weighted at different times after detachment. Assays were performed at 22ºC and 60–65% relative humidity. For dehydration experiments and plant survival assays, 2-week-old plants grown on agar plates under ShD were transferred to filter paper on Petri dishes and left un-watered for 10–12 h under LL. Survival percentages were scored 2 days after re-watering the plants. Greenish, healthy-looking plants were counted as surviving. Experiments were repeated at least twice with about 20–40 plants per treatment and genotype. Statistical significance was evaluated using one- and two-way ANOVA.

Microarray experiments of *TOC1* mis-expressing plants and data analysis

For microarray experiments, WT (C24) and *toc1-2* mutant seeds were stratified in the dark at 4ºC for 4 days on MS plates with 3% sucrose. Plates were subsequently placed on a growing chamber under 12 h light:12 h dark cycles (LD) for 10 days followed by constant light conditions (LL). Samples were

taken after 40 h in LL. RNA from four independent biological replicates was isolated using the RNeasy Mini kit (Qiagen). RNA was quantified with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies) and quality was verified with a 2100 Bioanalyzer from Agilent Technologies. RNA processing, hybridization of microarrays and scanning was performed as previously described (Adie et al, 2007). For *toc1-2* analysis, we used the two-color *Arabidopsis* Oligonucleotide Microarrays with 29.000 oligonucleotides of 70 mer (Qiagen-Operon, *Arabidopsis* Genome Array Ready Oligo Set [AROS] Version 3.0). Raw data was processed using the SOLAR software (BIOALMA). Briefly, global intensities were scaled and normalized using the LOWESS procedure (Yang et al, 2002). The absolute value of the t statistic and the local z-scores were calculated as previously described (Quackenbush, 2002; Yang et al, 2002). Selection of differentially expressed genes was performed based on three-fold criterion: p-value below 0,05, a zscore above 2 and a log ratio over 0,55.

WT (Col-0) and TOC1-ox samples were taken at Zeitgeber Time 16 (ZT-16) from plants synchronized under LD cycles for 10 days. The ATH1 *Arabidopsis* GeneChips (Affymetrix) were utilized for hybridization of WT (Col-0) and TOC1-ox samples. RNA from three independent biological replicates was isolated using the PureLink Total RNA Purification System (Invitrogen), quantified with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies) and examined with a 2100 Bioanalyzer from Agilent Technologies. Labeling, hybridization and scanning was performed following the manufacturer recommendations. Raw data were normalized with the GCRMA package (Wu et al, 2004) and the differentially-expressed genes were identified using the empirical Bayes moderated t statistics from the LIMMA package (Smyth & Speed, 2003). All data were processed using the “Comprehensive R based Microarray Analysis web frontend” (https://carmaweb.genome.tugraz.at/carma) (Rainer et al, 2006). Genes with a false discovery rate (FDR) <5% were selected as misregulated in TOC1-ox. Microarray data were deposited at GEO (www.ncbi.nlm.nih.gov/geo; GEO accessions: GSE18001 and GSE18007).

For promoter *cis*-element identification and TOC1 misexpressed genes meta-analysis only genes with FDR<5% were used. Publicly available databases were used for functional clustering of mis-expressed genes (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). Selected datasets included experiments of 3-hour treatments with the hormones ABA, gibberellic acid (GA3), 1-Aminocyclopropane-1-carboxylic Acid (ACC), indole acetic acid (IAA) and Zeatin (Zeat) (ME-00334, ME00344, ME00336, ME00333, ME00343) as well as dehydration experiments (Matsui et al, 2008). Hierarchical clustering with Euclidean distance and complete linkage was performed using the TIGR Multi experiment Viewer (The Institute for Genomic Research, TIGR) (Saeed et al, 2003). Significant enrichments of promoter elements within the 1000bp upstream from the ATG of the mis-regulated genes were identified using the Athena software (O'Connor et al, 2005) (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) and the SCOPE suite (Carlson et al, 2007) (http://genie.dartmouth.edu/scope/). Phase enrichment was analyzed using the *PHASER* plot web tool (http://phaser.cgrb.oregonstate.edu/). Meta-analysis and transcriptional crosstalk was performed by using publicly available datasets including: Circadian (Covington et al, 2008; Hazen et al, 2009), Hormone (Matsui et al, 2008; Nemhauser et al, 2006) http://www.arabidopsis.org/info/expression/ATGenExpress.jsp; https://www.genevestigator.com/gv/index.jsp) and drought related datasets (Matsui et al, 2008). The percentages of overlapping genes in different arrays were calculated taking into account the differences in the number of probes amongst the various arrays.

The Transplanta collection

The collection was built starting from 2 previous *Arabidopsis thaliana* transcription factors cloned open reading frames (ORFs) collections: REgulatory Gene Initiative in Arabidopsis (REGIA) (Paz-Ares & The REGIA Consortium, 2002) and REGULATORS (http://urgv.evry.inra.fr/projects/arabidopsis-TF/). The

combination of these two libraries was previously used succefully for screenings in yeast (Castrillo et al, 2011). The Transplanta consortium enlarged the collection reaching more then 1000 different ORFs.

The inserts were cloned by gateway in the pER8 gateway vector (Papdi et al, 2008) and the resulting vectors were used for *Arabidopsis* plant transformation. The transformation was performed by *Agrobacterium tumefaciens*-mediated DNA transfer (Clough & Bent, 1998).

The primary transformatns selection was performed as follows: sterilized and stratified seeds were sowed on Murashige and Skoog medium (MS) with 20 μgml^{-1} hygromcycin. After 6 hours of exposition to white fluorescent light (50 $mmolm^{-2}$ s^{-1}) at 21ºC, plates were covered with aluminium foil and left in the same growing chamber for 72 hours. After this etiolation period, transformed seedlings displayed longer hypocotyl. After 7-8 days under constant light, 9 selected seedlings for each construct were transplanted to soil (Harrison et al, 2006). This selection provided 9 independent *Arabidopsis* lines for each construct. The steps described above were performed by Transplanta core facilities, conversely, for the following steps of the process the lines were distributed among all the laboratories participating to the Transplanta project in order to be processed separately. In our laboratory, the screening for single insertion lines was performed by sowing 100-150 seeds on MS medium with 1% sucrose (MS1) supplemented with 50 μgml^{-1} hygromcycin. The seeds were then stratified for 3 days at 4ºC in the darkness. After stratification, the plates were transferred to growing chambers providing a constant 21ºC temperature and cycles of 16 hours of light (50 $mmolm^{-2}$ s^{-1} of cool white fluorescent light) followed by 8 hours of darkness (Long Day). After 9 days, seedlings displaying normal size and root length were scored as resistant while underdeveloped and short rooted ones were considered sensitive to hygromycin, The lines displaying a resistant/sensitive ratio of approximately 3/1 were considered to bear a single t-DNA insertion. At this step, a maximum of three single insertion lines were selected for each construct. For each of the selected lines, 6-7 resistant seedlings were transplanted to soil. At the following generation, the percentage of resistant seedlings was assessed with the same method, but at this step, only

the lines that displayed a 100% hygromycin resistant phenotype were selected. Seedlings form these homozygote lines were passed on soil in order to obtain a higher amount of seeds at the next generation. The homozygous populations of seeds finally obtained were then tested once more for 100% hygromycin resistance and distributed among the laboratories of the Transplanta consortium.

Through this process, the Transplanta consortium has generated a collection of *Arabidopsis* single insertion homozygous lines constructed for the conditional over-expression of more then 650 transcription factors.

β-estradiol induction optimization

The ß-estradiol induction was tested by semi quantitative RT-PCR. The seeds were sowed on MS1 medium plates with a sterile net and stratified for 3 days at 4ºC in the darkness. After the stratification the plates were transferred to a growing chamber providing 21ºC temperature and Long Day conditions for the next 10 days. The nets allowed a proper root growth and permitted a fast and delicate transplant of the seedling for the induction. The ß-estradiol treatment was performed preparing a mother solution of 20mM ß-estradiol dissolved in DMSO. We tested the induction with 0, 1, 5, 10, 20 and 50µM ß-estradiol on 5 lines presenting 4 different transgenes. These lines were: two control lines over-expressing a GUSGFP fusion protein and over-expressors of RAP2.3 (At3g16670), LOM2 (At3g60630) and USP (At3g62550). The transgene expression levels were assessed by semi quantitative RT-PCR. The analysis was performed on three biological replicates.

RNA extraction and quantification from transplanta lines seedlings and relative controls

The RNA was extracted from 150mg of plant tissue with AMBION PureLink ® Mini Kit. Tissue was grinded in liquid nitrogen and resuspended on

ice in lysis buffer with 1% 17ß-mercaptoethanol. This solution was cleaned by precipitating the remaining tissue by centrifuging for 5 minutes at 12000g at 4ºC and recovering supernatant twice. The cleaned extract was mixed with one isovolume of 70% ethanol and then loaded on the column. The following steps were performed as suggested by the manual of the kit. Elution was performed in 50µl and the eluted solution was passed a second time for the column to improve yield. The RNA was then quantified with a Nanodrop ND-100 spectrometer.

cDNA synthesis from transplanta lines seedlings and relative controls

The genomic DNA elimination and cDNA synthesis was QuantiTech ® Reverse Transcription Kit. Each sample was obtained using 1µg of RNA. The reactions were performed following the manual of the kit. Time of incubation for genomic DNA elimination was brought to 10 minutes and RT reaction time was prolonged to 30 minutes. For each experiment, 2 independent negative controls were generated performing the reaction on 2 different RNA samples without adding Reverse Transcriptase to the mix (RT-).

Semi quantitative PCR analysis of transplanta lines seedlings and relative controls

The PCR was performed on cDNA samples diluted 1:10. The reaction total volume was 50µl for 3µl of template cDNA. PCR were performed with Takara^{TM} ExTaq^{TM} Polymerase following manufacturer instruction.

The samples were amplified for 22-26 cycles and loaded on a 1% agarose gel with ethidium bromide. The gels were photographed under UV light and the pictures were quantified with Fiji software (http://fiji.sc/).

The Ubiquitin10 (At4g05320) transcript was used for normalization among the samples of each replicate. The primers used for these analyses are presented in the following table.

Gene ID	Gene Name	Primer name	Primer sequence
-	GFP	GFP_f	GGAAAGCTCCCTGTGCCATGGCC
		GFP_r	GGCAGACTGGGTGGACAGGTAATG
AT4G05320	UBQ10	UBQ10_f	GGCCTTGTATAATCCCTGATGAATAAG
		UBQ10_r	AAAGAGATAACAGGAACGGAAACATAGT
AT3G16670	RAP2.3	RAP2.3_f	GTTTCCCGGTGGAGTGTATAGG
		RAP2.3_r	CACAACAGACATCAGCAGTCC
AT3G60630	LOM2	LOM2_f	CGTCTTAACATTTCCAAGCTGAGAC
		LOM2_r	CGCGCTTCAGTACTACACATC
AT3G62550	USP	USP_f	GCTCGGTCTCGAACCGTGTAC
		USP_r	CGCATACGAAGATTGATTCAAGTG

The screening

The screening was performed in the laboratory of Dr. Jeffrey Leung, in the Institut des Sciences du Végétal (ISV), in Gif Sur Yvette, France. Seedling growth and induction treatments were performed as described previously.

Each plate contained an induction control (GUSGFP reporter line), a positive control for open stomata induced temperature shift (*ost1*) and four of the lines to be tested, each plate was prepared in duplicate in order observe the phenotype in presence and absence of induction. The 20mM ß-estradiol solution

was prepared fresh for each round of plates. The inducing medium was prepared adding 1/1000 mother solution to MS1, thus achieving a final concentration of 20μM ß-estradiol. Conversely, mock medium was prepared adding 1/1000 DMSO to MS1. The nets with the seedlings were then transferred to the plates containing either ß-estradiol supplemented MS1 medium or a mock medium. After two days of treatment, the plates were opened and observed with a FLIR A655sc Thermacam several times during the following 24 hours. The thermal images were then quantified with Thermacam Researcher Pro Software.

Measurements of stomatal aperture of the selected candidate lines

Seedlings were grown and treated as described for ß-estradiol induction, then incubated for 2 h in a buffer containing 50mM KCl and 10mM MES, pH 6.15 +/- 20μM ß-estradiol with cool white fluorescent light (50 $mmolm^{-2}s^{-1}$). The following incubation was performed with the same solution complemented with +/- 3μM ABA or +/- 10μM Flg22. After the second incubation non-cotyledon leaves were detached and observed with a Zeiss AxioPhot microscope with x63 objective and GFP was detected in control line (GUSGFPb) to asses transgenes induction.

Approximately 100–200 stomata per sample were photographed using an Olympus DP70 camera attached to the microscope. Stomatal aperture was scored as width:length pore ratio using Fiji software. The experiments were performed in triplicate.

Quantitative RT-PCR of samples from transplanta lines seedlings and relative controls

Part of the cDNA samples of each experiment were used to create a pool that was later diluted 4 times with 1:4 serial dilutions. The cDNAs were then diluted 1:10. The experiments were performed with a Roche Lightcycler 480 using Roche SYBRgreen® Master Mix. Primer efficiency was calculated using the serial dilutions of the cDNA pool. For each experiments at least 2 RT- and a non-template (NTC) were checked as negative controls. Ubiquitin10 or IPP2 were used as normalizing controls. All the experiments were performed on three biological replicates. The primers used for Q-PCR experiments are listed in table 4.

Gene ID	Primer name	Primer sequence
AT1G01060	lhyF	CCAAGCTAAATGCCGACTCA
	lhyR	TGCATCAGTTTCTGCGTCAC
AT1G11730	Gal_transfF	TTCGTTGGGATCTTGGTTCA
	Gal_transfR	AACGTCGGCCATCCTCTC
AT1G13440	GAPC2_F	AGGCCATCAAGGAGGAATCT
	GAPC2_R	CCCATTCGTTGTCGTACCAT
AT1G13700	gluco6PF	TTGAGGTGAAGGACGATTGG
	gluco6PR	CCTTGCAGGCAGAGACAGAG
AT1G15010	UP_AT1G15010F	TGGTTGAACGCAACTTTGAA
	UP_AT1G15010R	GAACGCTGACGAGGAGAAGA
AT1G16370	ATOCT6_F	GCTCGGCAACAATGATGTTT
	ATOCT6_R	TCCATCGAATCGCAGAGACT

Gene ID	Primer name	Primer sequence
AT1G17180	ATGSTU25_F	TTTGGAGGTGAAACATTCGG
	ATGSTU25_R	GCCACACTCTCTCTCTCCACA
AT1G19900	glioxal_F	GGTATTTCCGACGTTCACGA
	glioxal_R	CCAAACACCTTCGCTTGGTA
AT1G20190	Exp11_f	GTGGTGCTTGAAAGGAGCTT
	Exp11_r	GGGAACGATTCCTCCTCTGT
AT1G21590	PK_F	CTCAAGGACGAGAAATTGCG
	PK_R	GAAGCTTGATGAACGGCTTG
AT1G30750	UP_AT1G30750F	TCAACCAACCGATTTCTTCG
	UP_AT1G30750R	AGTACCGTGCAAAGCAAGGA
AT1G33320	Cysta_F	AGCACATCCCAAGGTGAGTC
	cysta_R	CAAACGATGCTGCAAGGTAA
AT1G51090	HMT1_F	GTCCATCGTGATCCTTGAGC
	HMT1_R	GAGCGGAAGACGAAACAAGA
AT1G60610	Zincbind_F	AGTGGCAGTGGAAGCTCAGA
	Zincbind_R	GCTGCTTCGTCATCAATCTCA
AT1G60960	IRT3_F	TCAGGTTCTGGAGCTTGGAA
	IRT3_R	TTTGTTCCTGAATTGCGCTT
AT1G61590	PKb_F	TAACGGGAAGAAGAGCCACA
	PKb_R	TAGGGTTTGGGCTCACACAT
AT1G66160	CMPG1_F	GATCAATGGCGTCCAAGAAG
	CMPG1_R	CGTTAACGCTTTCTCGCAAA
AT1G66440	DC1a_F	TTGCAAAGAGTCAAGCCGTT
	DC1a_R	GCTTCACACCAATACGCCAT
AT1G67970	HSFA8_F	TTGCTGCTGCGTTTGCCTCACC
	HSFA8_R	TACAACCGCCTCAGTTCCACCGTC
AT1G69530	ExpA1_f	TAACGTCGGAGGAGCCGGAGATG
	ExpA1_r	CCGAAAGACCAGCCTGCGTTAG
AT1G72790	hprolrich_F	AAATTCGTGAAGCGTGGAGA
	hprolrich_R	GGCTCTGAATCTTGCGATGA
AT1G73120	UP_AT1G73120F	AACACCATGTTCGTCAAGCC
	UP_AT1G73120R	ATGTGGCACCCAACTACTCG
AT2G15020	UP_AT2G15020F	GGATGGTGTTTGGAAGGGAT
	UP_AT2G15020R	CAACGGCAAATCAACCTCAT
AT2G21870	ATPSynF1F0_F	GATGTTCGAGGCTTTGGAGA
	ATPSynF1F0_R	TCAGCTCATCCAACTGTGCTT
AT2G22190	T6P_f	GCCCATCAAGTTCGATCAGT
	T6P_r	TCGTCGGTACGATCATCTCC
AT2G23290	Myb70_F	GTGACGGCGTTAAGTCCAAC
	Myb70_R	ACCCAAGGCAAAGACAACCT
AT2G25735	UP_AT2G25735F	CTTGCCCAAATGGAAGGTTT
	UP_AT2G25735R	AGCGACAGGAGAAAGACCGT
AT2G27080	HIN1_F	GGTATGAGGAAGGAGATGCGTA
	HIN1_R	TCCACGTCATGACTGCATTT
AT2G27420	cysprot_f	CGTGGAGGGATAATGTCGAA
	Cysprot_r	GCTTGTAGCAACGCTTCCTC
AT2G29090	cyp707a2F	TCTGCTCTCAGACCAACCGT
	cyp707a2R	TCGTAGTGGGCTCCTCTTTG
AT2G29750	UGT71C1_F	TGTGGATGTGCCGAAGAGTA
	UGT71C1_R	CCACATGCCCAATTACATGA

Gene ID	Primer name	Primer sequence
AT2G31380	STH_F	TCTTAGCCACTGGAATCCGA
	STH_R	GAATTCATCGGTAGCCCACA
AT2G33150	KAT2_F	GCAGGAGCGGTTCTCCTAAT
	KAT2_R	TAAACCAGCCGCCTTAACTG
AT2G33580	LysM-PK_F	TGCGGTTCTTGAGCTTCTTT
	LysM-PK_R	TAAGCCAGCTCCAACGGATA
AT2G35290	UP_AT2G35290F	GGAGTTCATGGTCGAACCG
	UP_AT2G35290R	CGAGAACATCCAAATCCGAA
AT2G37030	auxinresp_F	AATATCAACCTGCTTAAACACCCA
	auxinresp_R	GATACATATGCAGTTATTCTGATGCTG
AT2G41480	Perox25_F	AAGAACTTACGCGACGGAAA
	perox25_R	TTCCTAACCTCGCCATCAAC
AT2G43090	acon_F	CTGGTGAGGTTTATCCTTTGGA
	acon_R	CAATCACTGGTCCAGCATCA
AT2G44650	CPN10_F	TTCCACTAAATGGGAACCGA
	CPN10_R	ACCCTCTTTCCAGGTCCAAC
AT2G45920	Ubox_F	TCCGTTCACGAGGATCTGTT
	Ubox_R	TCCAAATAGGCACTTTCCCA
AT3G02780	IPP2_F	TGATGGCAAATGGGGAGAGCATGAAC
	IPP2_R	GTCCACCACCAATCTGAACCATGGTG
AT3G05490	RALFL22_F	AGCGGAAGAAGAAGAGATGGA
	RALFL22_R	TGCACCTAGTGATGGTGCTG
AT3G09200	P02_F	TCACTTCCTTGGCTCTTGCT
	P02_F	CAGACACCGCAGCTACTGAA
AT3G13672	SINA_F	TCTCGATTGTTGTGGTCGAA
	SINA_R	CCTTGCCATGTCAGCTTTCT
AT3G13790	ATBFRUC1_F	GCTCATGTGCAGTGACCAAA
	ATBFRUC1_R	TCCCTTTCCACCGAAACTCT
AT3G14680	CYP72A14_F	CCGAGCCATTCACAAAGAGA
	CYP72A14_R	ACGCAAAGGGAAAGAAGGAG
AT3G18780	Act2_F	AAATCACAGCACTTGCACCA
	Act2_R	TGTGAACGATTCCTGGACCT
AT3G21760	HYR1_f	GGATGGAACTCGACGCTAGA
	HYR1_r	CGTCATCAACTCATCATCCG
AT3G29780	RALF27_F	GTAGTCCGGCGGATTCTACA
	RALF27_R	GCACGTTTGCATCTCTGGTA
AT3G45970	ExpL1_f	CACGGAGCTGTTTGGGTAAC
	ExpL1_r	CGCAAGGATCACAACCTTCT
AT3G46370	LRRPK_F	TGAATTGGCTATGTCATGCG
	LRRPK_R	ACAAGGGACCGCCTCAGTAT
AT3G55840	HSpro1_F	GTTTCCAAACGACGCATTTT
	HSpro1_R	GATCCACCTCCACCTCAAGA
AT3G56090	Fer3_f	TCCAAGAACGATGATGTCCA
	Fer3_r	CAGATTAAGCAGCAGCACCA
AT3G59080	Asp_F	CATAACGTTCAGCTGCCAGA
	Asp_R	ATTGCCAAGCAAACCAAATC
AT3G62030	CYP203_F	GGCAAACGCTGGTCCTAATA
	CYP203_R	GCTCTCCGCAGGCATAGAT
AT3G62250	Ubi_F	GTCTCCGTGGTGGTGCTAAG
	Ubi_R	ATGACTCGCCATGAAAGTCC

Gene ID	Primer name	Primer sequence
AT3G62720	ATX1_F	AAAGTCTTGACCCGGGAACT
	ATX1_R	CCTCGTACCGGTCTACCAAA
AT3G63170	Chalcone_F	AAGTTCGGTGGCTCAGACAA
	Chalcone_R	GCTCTTCACACTCCCGACAT
AT3G63490	Ribl1p_F	GCTGGTACAGTCACAGCGAA
	Ribl1p_R	GCTTGTTTGTCTCCACCGAT
AT4G01080	UP_AT4G01080F	TGCGTGATGTTGAGCTTGAG
	UP_AT4G01080R	AGCACCAATGCAGACAATCA
AT4G01250	WRKY22_f	CTTACACGGCGGAGCATAAT
	WRKY22_r	GATCTCCCACCGCTAGATGA
AT4G02540	DC1b_F	CCGACACATCCTCATCTGCT
	DC1b_F	CGTGGACTAACGGCTCAGAA
AT4G08390	SAPX_f	GGAGGACAGTCATGGACACC
	SAPX_r	GCATGGGCTACAGCGTAATC
AT4G09650	ATPSyntD_F	GCTCAGATTGCGAAACAGGT
	ATPSyntD_R	CCAAGTTCAAGCTGAGAAGCA
AT4G14130	XTR7_F	CCCAAAGAGTCAACCCATGA
	XTR7_R	ATCCTGAAGAGGCTGTGCAA
AT4G19030	AT-NLM1_F	GGACCTGCACTGGTCTATGG
	AT-NLM1_R	GTGCTACCGATTCTCACGGT
AT4G19160	UP_AT4G19160F	CTCATAACTGGGCACTTCGC
	UP_AT4G19160R	GGTCAAGCGATCAGAACCAA
AT4G23600	CORI3_f	TGCACCTTCTTATGGACCGA
	CORI3_r	CCAATGCATCCTCCAATACC
AT4G25810	XTR6_f	GGTTCGTGGTTGTCTCAGGA
	XTR6_r	TCATGGCTTTGGATCATTTGT
AT4G28085	UP_AT4G28085F	GGGTGAGATGATCAAAGCCA
	UP_AT4G28085R	TTGAGGCTGCAGAGGATGTT
AT4G28190	ULT1_F	AGGAGGTTCAGGCTGAGGAG
	ULT1_R	GTGCAGCCTTTGCAACTAGG
AT5G01600	Fer1_f	CATCTCATGCCTCTCTCGCT
	Fer1_r	TCTCAGCATGCCCTCTCTCT
AT5G06740	Lectin_PK_F	CAGATGTGTATGCGTTCGGA
	Lectin_PK_R	CATCCGTGATGGTTCCATTT
AT5G06860	ATPGIP1_F	GATCGATTTATCCCGCAACA
	ATPGIP1_R	CCAGTGATCCCATTGTGGTT
AT5G06870	pgip2F	CGCATCGATTTATCTCGGAA
	pgip2R	GCCGGAATACTCCCTGTGAT
AT5G09660	MalDH_F	CCTCCTAGCAGCTTCACACC
	MalDH_R	AAGGCAAGCATCTGCAAACT
AT5G14650	polygact_F	GTCAGCAACGTAGCCAATCC
	polygact_R	TGCATGGCAAGCTATCACTG
AT5G19520	MSL9_F	CGGAGAATACTTGGTGGCAA
	MSL9_R	ATTCGCTTGATTGCGATGAT
AT5G20230	ATBCB_F	GTCGGTGACCATTGTCGTTT
	ATBCB_R	CAGCTGGAGTAGTGGTTCCG
AT5G20720	CPN20_F	AAACAGCTGGAGGGTTGTTG
	CPN20_R	GAACCATCTTTGCCCTTGAA
AT5G23840	MD2_F	ATATCCGCAGGAACCCTTGT
	MD2_R	CGCGCAAAGATACCGAATAC

Gene ID	Primer name	Primer sequence
AT5G26340	STP13-MSS1_f	TGATCTGCACATACGTCGCT
	STP13-MSS1_r	ATCCCAAACTTGAAATGGCA
AT5G35630	GLN2_F	AACCGTGGATGCTCTATTCG
	GLN2_R	TTCAGCCTCAAGAGTTGGCT
AT5G45840	LRRTPK_F	AACGCGAGGACGAGGATAAT
	LRRTPK_R	TCTCGGTTTACTCCCGGTTT
AT5G55620	UP_AT5G55620F	TTCCCAAGTGGATTCCAAGTC
	UP_AT5G55620R	AGCTGATCAGGCCAGAGAAA
AT5G59130	subt_F	TGACAACAGCTTGGTCGATG
	subt_r	CGCAGAGGAAGGCAAAGTAA
AT5G62360	invertase_f	GTGACACGTTGGAGGAGCTT
	invertase_r	AACCTTAACCTTCCCGTCCA
GFP	GFP_f_q	CCCACAATGTGTACATCATGGCCG
	GFP_r	AAAGAGATAACAGGAACGGAAACATAGT

Table 4: List of primers used in quantitative RT-PCR ordered by geneID.

HSFA8 expression analyses

HSFA8 promoter analysis was performed using PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al, 1999), relevant *cis*-elements were selected manually.

The expression of *HSFA8* was checked under various experimental conditions on 12-days-old Col-0 seedlings grown in Long Day conditions at 21ºC temperature. The ABA treatment was performed by passing the seedlings to 100µM ABA MS1 medium for 2 hours. The drought treatment was done removing the seedlings from the medium and leaving them on a piece of paper for 30 minutes. The osmotic stress was produced passing the seedlings to 300mM Mannitol MS1 medium for 2 hours. The Flg22 treatment was performed submerging the seedlings in 10µM Flg22 liquid MS1 solution for 1 hour. The samples for circadian oscillation experiments were taken at the second day of constant light conditions (50 $mmolm^{-2}$ s^{-1}) after 10 days of entrainment in a regime of 12 hours of light-12 hours of darkness. The morning time point was taken one hour after subjective dawn (CT1), and the evening point was taken one hour after the subjective dusk (CT13). All the experiments were performed on three biological replicates.

Agroinfiltration of *Nicotiana benthamiana*

The 35S:*GFPHSFA8* construct used for agroinfiltration experiments was produced using the *HSFA8* gene cloned into the pER8 vector supplied by the Transplanta consortium. The insert was cloned into a pDONR^{TM}221 (Life Technologies) with a BP recombination reaction using gateway® BPclonase® kit following the manufacturer recommendations. The donor vector was then used to pass the insert into a pGWB6 destination vector containing the 35S promoter and the N-terminal *GFP* gene (Nakagawa et al, 2007) through an LR recombination reaction. The recombination was performed using gateway® LRclonase® Enzyme Mix following the manual specifications.

For the agroinfiltration experiments, two *Agrobacterium* lines of the EHA105 strain containing the *35S:GFP-HSFA8* construct and a construct for the expression of the viral suppressor HCPro, were grown overnight at 28ºC with 220rpm agitation in 30ml of selective medium. The cultures were pelleted by centrifugation at 3000g for 10 minutes at room temperature and resuspended in Transformation buffer (10mM $MgCl_2$, 10mM MES pH 5,6, 150µM Acetosyringone) in order to obtain a final OD_{600} of 1. After 2 hours of incubation at room temperature, the two cultures were mixed 1/1 v/v and the resulting solution was infiltrated from the abaxial surface of leaves in 6-weeks-old *Nicotiana benthamiana* plants using a 1ml syringe without needle. Infiltrated leaves were observed 48 hours after infiltration at confocal microscope (Leica SP5) with a x63 objective.

Proteomics of *HSFA8* over-expressing seedlings and relative controls

Plant material was grown and treated as described in for ß-estradiol induction. For each sample was used 0,6g of plant tissue. Samples were ground with mortar and pestle in liquid nitrogen and the powder obtained was homogenised with 1mL of lysis buffer (8M Urea, 2M Thiourea, 4% w/v CHAPS, 40mM Tris-HCl, pH 8.0) containing protease inhibitors (1mM PMSF, 50mM leupeptin, 1 mM pepstatin, 10 mM E-64, 10 mg/mL aprotinin) and a protease-

free DNaseI-RNaseA mixture. Protein extracts were centrifuged at 10 000g for 20 minutes at 4ºC temperature until the supernatant contained no lipids and it was completely clear. Two independent protein extraction events (biological replicates) were performed for each variety. Protein concentrations were determined using the Bio-Rad Protein Assay and BSA as standard and equal loading amounts were confirmed on1-DE gels stained with CBB.

The bidimensional electrophoresis gel (2-DE) was analyses were performed in the proteomics facility of CRAG. One milligram of protein sample was diluted in rehydration solution (8M Urea, 18mM Tris-HCl, pH 8.0, 4%w/v CHAPS, 0.5% v/v IPG buffer (pH 3–11), 1.6% v/v DeStreak Reagent (GE Healthcare) and 0.002% w/v Bromophenol Blue) and loaded onto 18cm IPG strips (NL pH 3–11) (GE Healthcare). Strips were rehydrated for 6 h at room temperature, followed by 6.5 h at 30V. IEF was performed at 500 V (1 h), 1000V (1 h) and 8000 V (7 h) using the Ettan ^{TM} IPGphor ^{TM} Isoelectric Focusing System (GE Healthcare). Prior to second dimension, strips were equilibrated with 50mM Tris-HCl (pH 8.8), 6M urea, 30% v/v glycerol, 2%v/v SDS, a trace of Bromophenol Blue and 10 mg/mL DTT (15 min), followed by a second equilibration step (25 mg/mL iodoacetamide, 15 min). The Ettan DALTsix System (GE Healthcare) was used for the second dimension and proteins were separated on 12% Laemmli gels (26x20x0.1 cm) at 2.5 W/gel for 30 min, then at 100W total, until the run was completed. Gels were run in duplicate (technical replicates) and then stained with CBB R-250.

The 2-DE gels stained with CBB were scanned using the ImageScanner desktop instrument and the LabScan application (GE Healthcare). Images were saved as Tag Image File Format (TIFF) and the ImageMaster^{TM} 2-D Platinum 5.0 Software (GE Healthcare) was used for gel image analysis. After automatic spot detection, manual spot editing was carried out. Spots matching in both technical replicates for each protein extraction event were considered as reproducible spots and included in the synthetic 2-DE gel images.

The identification of proteins in the selected spots was performed at the proteomics platform of Barcelona Scientific Park. Protein samples were

digestion was performed with porcine trypsin (Promega) in a Progest robot (Genomic Solutions) with the following protocol. Bands were washed sequentially with a 25 mM ammonium bicarbonate (NH^4HCO^3) buffer and a acetonitrile (MeCN) buffer. Alter that, proteins were reduced with a treatment with 10mM DTT for 30 minutes at 56ºC followed by 55 mM Iodineacetamide for 15 minutes at room temperature. Finally proteins were digested overnight at 37ºC with trypsin (80ng). Tryptic peptides were extracted from the gel matrix with a MeCN and 10% formic acid (FA) solution in a speed vac.

The LC-MS/MS analysis for peptide identification was performed with a nanoAcquity liquid chromatographer (Waters) coupled with an Orbitrap-Velos mass spectrometer (Thermo Scientific). Samples were resuspended 100µl 1% formic acid (FA) solution and 2µl were injected for chromatography on a reverse phase column C18 (75µm internal diameter, 10cm length, nanoAcquity, 1,7µm BEH column, Waters). Separation was achieved with a 250nl/min flux using A solution 0,1% FA and B solution 0,1% FA in MeCN. The first step was a 2-40% B solution gradient in 20 minutes, followed by a 40-60% B solution gradient in 5 minutes. Eluted peptides were ionized using a metallized nanoelectrospray glass needle (PicoTip™, New Objecitve) and applying a voltage of approximately 2000V. Peptide masses (*m/z* 350-1700) were measured in Full Scan MS in the Orbitrap mass spectrometer with a resolution of 60000 FWHM at 400m/z. Up to 5 of the most abundant peptides (minimum intensity 500 counts) for each MS analysis were selected to be fragmented by ion trap Collision Induced Dissociation (CID) using nitrogen as collision gas and a normalized collision energy of 38%.

Raw data were acquired with Thermo Xcalibur software (version 2.1.0.1140). Raw datas were then crossed with the MASCOT NCBInr Eukaryota database (version February 2012) using Proteome Discoverer software (version 1.3.0.399). The parameters used for the analysis admitted up to 2 missed cleavages, assumed carbammidomethylation of cystein as fixed modification, admitted as possible the oxidation of methionines and N-terminal glutamines and restricted the peptide tolerance to 10ppm for MS and 0,6Da for MS/MS. The obtained results were then filtered using Percolator algorithm with False

Discovery Rates (FDR) of 0,01 (strict) and 0,05 (relaxed), using q-value based validation.

Microarray of *HSFA8* over-expressing seedlings and relative controls and consequent data analysis

The ATH1 Arabidopsis GeneChips (Affymetrix) were utilized for hybridization of Col-0 +ß-estradiol and *HSFA8*-inducible line +/-ß-estradiol samples. RNA from three independent biological replicates was isolated and quantified as described above. The samples were then passed to CRAG genomics facility where they were examined with a 2100 Bioanalyzer from Agilent Technologies. Labeling, hybridization and scanning was, then performed following the manufacturer recommendations. The data were then analyzed using the Comprehensive R based Microarray Analysis web frontend (CARMAweb) online software (https://carmaweb.genome.tugraz.at/carma/) (Rainer et al, 2006). The analysis was performed using the Robust Multiarray Analysis (RMA) (Irizarry et al, 2003) algorithm; differentially expressed genes were identified by mean of paired moderated t test statistics (LIMMA) (Smyth & Speed, 2003) with BH adjusted pValues (Benjamini & Hochberg, 1995). The selection criterion for altered expression was considered BH p-Value lower than 0,05.

VII. RESUMEN

Introducción

Señalización por ABA

El aumento de la eficiencia del uso del agua a fin de mantener o mejorar el rendimiento de los cultivos en tiempos de calentamiento global y reducción gradual de los recursos hídricos es uno de los mayores desafíos que enfrentan las ciencias agrícolas en las últimas décadas (Battisti & Naylor, 2009; Boyer, 1982; Cominelli et al, 2009). Abordar esta cuestión a nivel de la fisiología de plantas nos lleva a considerar cuáles son los mecanismos que estos organismos utilizan para sobrevivir y optimizar el crecimiento en condiciones ambientales restrictivas como la sequía. La mayor parte de la pérdida de agua de las plantas se debe a la transpiración asociada a la necesidad crucial de absorber dióxido de carbono de la atmósfera para la fotosíntesis. Los poros estomáticos constituyen la principal conexión entre el apoplasto y el ambiente externo y son responsables de más del 95% de las pérdidas de agua en las plantas (Schröder et al, 2001). La regulación de la apertura de los estomas es, por lo tanto, el principal mecanismo que las plantas usan para controlar el intercambio de gases con la atmósfera exterior. Dada la centralidad de su función, el comportamiento de los estomas requiere la integración de un gran número de señales fisiológicas y externas en una compleja red capaz de responder a una amplia gama de condiciones ambientales. La comprensión de esos mecanismos es un paso fundamental para lograr una mejora en la eficiencia del uso del agua por parte de las plantas (Cominelli et al, 2009; Ingram & Bartels, 1996; Schröder et al, 2001).

Se sabe que los movimientos estomáticos están regulados por un complejo sistema que responde a la fitohormona ácido abscísico (ABA), siendo el ABA la principal señal de cierre estomático. Esta molécula es detectada por receptores capaces de modificar la actividad de la proteína fosfatasa 2C (PP2C). La unión de ABA al receptor inhibe la actividad de las PP2C (Ma et al, 2009; Park et al, 2009).

Esta inhibición permite, a su vez, la activación de proteínas quinasas SnRK2 tales como OPEN STOMATA 1 (OST1) que, en ausencia de ABA, son inhibidas por desfosforilación mediada por PP2C (Umezawa et al, 2009; Vlad et al, 2009; Yoshida et al, 2006).

La activación de SnRK2 lleva a la fosforilación de AtrbohF, una subunidad catalítica de una NADPH oxidasa de membrana (Kwak et al, 2003; Sirichandra et al, 2009). Este evento de fosforilación desencadena la activación de la enzima, lo que conduce al rápido aumento citoplasmático de especies reactivas del oxígeno (ROS), comúnmente denominado *"oxidative burst"*. Después de la generación y difusión de ROS, ocurre una liberación de Ca^{2+} proveniente de compartimentos intracelulares y del apoplasto en el citoplasma (Cho et al, 2009), con la consecuente activación de proteínas quinasas dependientes de calcio (CPK). La acción de quinasas como las SnRK2 y CPK produce cambios biofísicos que eventualmente resultan en una contracción de las células de guarda y, en consecuencia, en el cierre de los estomas. (Joshi-Saha et al, 2011).

Varios estudios aportan evidencia del gran impacto que la señalización por ácido abscísico tiene sobre el transcriptoma de *Arabidopsis*; además la regulación de la transcripción génica tiene un gran impacto en la capacidad de la planta para resistir a la sequía (Fujita et al, 2011; Shinozaki et al, 2003). Una visión global de la respuesta a ABA y a estrés osmótico sugiere que alrededor del 10% de los transcriptos codificantes conocidos de *Arabidopsis thaliana* son regulados por ácido abscísico (Nemhauser et al, 2006; Seki et al, 2002). Datos de micromatrices sugieren que entre los genes inducidos por ABA en tejidos vegetativos se encuentran las proteínas *LATE EMBRYOGENESIS ABUNDANT* (*LEA*), proteínas quinasas y fosfatasas implicadas en señalización, transportadores y enzimas involucradas en el metabolismos secundarios tales como la biosíntesis de osmoprotectores y una amplia variedad de factores de transcripción. Por otro lado, los genes reprimidos corresponden a proteínas implicadas en el crecimiento y en el desarrollo, tales como componentes ribosomales, enzimas asociadas a la expansión de la pared celular, y proteínas

de la membrana plasmática y de cloroplasto (Fujita et al, 2011; Fujita et al, 2009b; Matsui et al, 2008; Nemhauser et al, 2006; Yoshida et al, 2010).

Los factores AREB1/ABF2 y ABF3 AREB2/ABF4 (ABSCISIC ACID RESPONSIVE ELEMENTS BINDING FACTORS) son los factores de transcripción que responden a la activación de la vía SnRK2 mejor caracterizados. De hecho, se ha demostrado que SnRK2s fosforila *in vivo* AREB/ABFs de forma dependiente de ABA (Kline et al, 2010). Estos factores de transcripción desempeñan un papel fundamental en la respuesta de *Arabidopsis* a la deshidratación, puesto que su sobreexpresión aumenta la resistencia a la sequía y la sensibilidad a ABA (Kang et al, 2002) y su triple mutación produce los efectos opuestos (Yoshida et al, 2010). Además de los factores AREB/ABF, un gran número de factores de transcripción de diferentes familias y especies de plantas han sido caracterizados debido a su impacto en las respuestas de las plantas al estrés osmótico y ABA, tales como deshidratación o toxicidad salina (Fujita et al, 2011)

Además, otras proteínas han sido implicadas en la percepción y la señalización por ABA en células de guarda que no han sido directamente relacionadas con la vía descrita anteriormente. Este es el caso de la proteína CHLH/GUN5/ABAR, un controvertido receptor putativo de ABA, subunidad H de la magnesio-quelatasa IX, cuya capacidad de unión a ABA es aún discutida (Shen et al, 2006; Tsuzuki et al, 2011; Wu et al, 2009a), aunque está claro que toma parte en el proceso de transducción de señales de ABA y que, en última instancia, lleva a la activación transcripcional de genes de respuesta a ABA a través de la inhibición de tres factores de transcripción WRKY (Shang et al, 2010; Tsuzuki et al, 2011).

Estímulos externos que influyen en el comportamiento estomático

Una amplia gama de información ambiental converge en las células de guarda, y se integra en los sistemas de señalización descritos anteriormente para optimizar el uso de los recursos y minimizar los riesgos.

Una de las señales que influyen en el estado de las células de guardia es la concentración de CO_2. Los estomas se cierran en respuesta a altos niveles de dióxido de carbono y se abren cuando la concentración de este compuesto básico para la fotosíntesis es baja (Hu et al, 2010; Negi et al, 2008; Xue et al, 2011).

Otra señal ambiental clave que regula el estado del estoma es la luz. De hecho, la luz es la principal fuente de energía para las plantas debido a su función esencial en la fotosíntesis. Esta es la razón por la cuál las células de guarda están preparadas para detectar y elaborar rápidamente respuestas a señales lumínicas y por la cuál estas señales constituyen un fuerte estímulo para la apertura de estomas y, por lo tanto, para la absorción de CO_2 (Chen et al, 2012; Shimazaki et al, 2007). La percepción de la luz recae en varios fotorreceptores: PHOT1 y PHOT2 participan en la apertura de los estomas en respuesta a luz azul en *Arabidopsis* (Kinoshita et al, 2001) junto con CRY1 y CRY2 (Mao et al, 2005; Talbott et al, 2003). La señalización por luz roja está mediada por el fitocromo B (PHYB) y, en su ausencia, por PHYA (Wang et al, 2010a). Los efectos de la luz sobre los estomas se deben también a reguladores transcripcionales: es el caso de MYB60, un factor de transcripción específico de células de guarda cuya transcripción aumenta en respuesta a luz azul por acción de CRY, PHYA y PHYB (Cominelli et al, 2005; Wang et al, 2010a). Mientras MYB60 ha sido descrito por su acción positiva en la apertura estomática (Cominelli et al, 2005), su homólogo MYB61, expresado principalmente en células de guarda en ausencia de luz, parece tener la función opuesta (Liang et al, 2005).

Un factor importante capaz de inducir el cierre de los estomas es la presencia de agentes patógenos foliares, que utilizan los poros estomáticos como una forma de superar la barrera física que constituye la cutícula de la planta (Grimmer et al, 2012; Zeng et al, 2010). Se descubrió que diversos patrones moleculares asociados a patógenos o microbios (MAMP-PAMP) inducen el cierre estomático (Melotto et al, 2006). Se sabe, además, que estas vías de señalización compartan componentes con las respuestas a estrés abiótico tales como la síntesis de ABA, el "*oxidative burst*", la síntesis de NO, la

señalización por proteína G y la regulación de la actividad de canales de K^+ (Melotto et al, 2006; Neill et al, 2008; Zhang et al, 2008). Por otra parte, la respuesta a MAMP- PAMPs involucra también componentes específicos tales como la hormona ácido salicílico (SA), que es epistática respecto a ABA (Melotto et al, 2006; Zeng & He, 2010). La señalización en la inmunidad innata también recae en cascadas de fosforilaciones que involucran MAP quinasas activadas por MEKK1 y MKK4 así como también MPK3 5 y 6. Estas cascadas de fosforilaciones también conducen a la regulación transcripcional a través de la acción de los factores de transcripción WRKY29 y WRKY22 (Asai et al, 2002).

El reloj circadiano

Debido a los cambios periódicos en el ambiente causados por la rotación de la tierra sobre su eje muchas especies han desarrollado y conservado mecanismo moleculares para predecir y anticiparse a esos cambios. El mecanismo subyacente a la adaptación a las fluctuaciones de los parámetros ambientales diarios se conoce comúnmente como reloj circadiano. Este mecanismo es auto-sostenible, por lo que subsiste en condiciones constantes, además de presentar la capacidad de amortiguar cambios ambientales manteniendo su ritmo (Bell-Pedersen et al, 2005). Los procesos regulados por el reloj endógeno en las plantas incluyen, entre otros: desarrollo (Dowson-Day & Millar, 1999; Nozue et al, 2007; Ruts et al, 2012; Yazdanbakhsh et al, 2011), ,movimiento de las hojas (Millar et al, 1995), la regulación fotoperiódica del tiempo de floración (de Montaigu et al, 2010; Imaizumi, 2010; Song et al, 2010; Zhao et al, 2012), la fijación de CO_2 (Warren & Wilkins, 1961), el metabolismo central y secundario (Blasing et al, 2005; Fukushima et al, 2009; Kolosova et al, 2001), la biosíntesis de hormonas y respuestas a las mismas (Covington & Harmer, 2007; Mizuno & Yamashino, 2008; Novakova et al, 2005; Thain et al, 2004), las respuestas a estrés abiótico (Bieniawska et al, 2008; Fowler et al, 2005; Kidokoro et al, 2009; Nakamichi et al, 2009), las concentraciones intracelulares de Ca^{2+} (Johnson et al, 1995; Xu et al, 2007), la homeostasis de

ROS y la respuesta a los mismos (Lai et al, 2012), la absorción de agua (Takase et al, 2011) y la defensa contra patógenos (Bhardwaj et al, 2011; Burgess & Searle, 2011; Wang et al, 2011b).

A nivel transcripcional, se ha observado que alrededor del 25% que los transcriptos de *Arabidopsis thaliana* oscilan a lo largo del día en condiciones constantes (Covington et al, 2008; Hazen et al, 2009), teniendo en cuenta la gran cantidad de cambios posibles de temperatura y la luz durante el día, el 90% de los ARNm presentan una tendencia oscilatoria (Michael et al, 2008b). Este fenómeno aporta una enorme ventaja adaptativa en términos de capacidad fotosintética, acumulación de biomasa y supervivencia (Dodd et al, 2005; Green et al, 2002) así como en la optimización de la utilización de almidón durante la noche (Graf et al, 2010). Además, un estudio de genética de poblaciones mostró que la óptima funcionalidad del reloj circadiano es un criterio de presión selectiva en *Arabidopsis* (Yerushalmi et al, 2011).

En los últimos años se han realizados grandes esfuerzos para descifrar los mecanismos moleculares y bioquímicos subyacentes a la función de reloj circadiano. En plantas, al igual que en muchos otros organismos, la ritmicidad circadiana parece basarse en múltiples bucles de retroalimentación negativa en el oscilador central (Bell-Pedersen et al, 2005; Wijnen & Young, 2006). Estudios de genética molecular en *Arabidopsis thaliana* han ayudado en la identificación de los componentes del reloj y los mecanismos de regulación del oscilador circadiano (McClung, 2011; Nagel & Kay, 2012; Nakamichi, 2011; Troncoso-Ponce & Mas, 2012). Inicialmente fue propuesto que la regulación recíproca entre dos factores de transcripción Myb, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) (Wang & Tobin, 1998) y LATE ELONGATED HYPOCOTYL (Schaffer et al, 1998), con un pseudo-regulador de respuesta TIMING OF CAB EXPRESSION 1 (TOC1 o PRR1) (Makino et al, 2000; Strayer et al, 2000) constituye un importante mecanismo de retroalimentación central para la función del reloj (Alabadí et al, 2001). Posteriormente, ha sido confirmada mediante modelos informáticos y validación experimental la existencia adicional de múltiples bucles interconectados que confieren robustez y flexibilidad para que las actividades oscilatorias (Nagel & Kay, 2012).

La regulación circadiana del comportamiento estomático

Como muchas otras importantes funciones en las plantas, el estado estomático es también influenciado por el reloj circadiano. Ha sido demostrado en muchos modelos de plantas que la conductividad y la apertura estomática fluctúan a través del día y que estas fluctuaciones persisten en condiciones constantes tanto en luz como en oscuridad (Correia et al, 1995; Holmes & Klein, 1986; Kaiser & Kappen, 1997; Kumar et al, 2012; Lebaudy et al, 2008; Martin & Meidner, 1971; Somers et al, 1998). Esta oscilación se refleja en los patrones circadianos de parámetros tales como la velocidad de fijación de CO_2 (Warren & Wilkins, 1961), absorción de agua (Takase et al, 2011) y sensibilidad a patógenos foliares (Bhardwaj et al, 2011). La regulación circadiana de la conductividad estomática podría estar parcialmente sostenida por la oscilación de las cantidades endógenas de ABA (Novakova et al, 2005) así como también por las fluctuaciones de mensajeros secundarios: concentración intracelular de Ca^{2+} (Johnson et al, 1995; Xu et al, 2007) y niveles de ROS y sensibilidad a los mismos (Lai et al, 2012). por otro lado, se ha reportado que ABA actúa como un alterador de la periodicidad en las oscilaciones de los genes circadianos (Hanano et al, 2006), sugiriendo que el reloj y la señalización por ácido abscísico se regulan entre sí. Existen numerosas evidencias de esta interacción funcional a niveles transcriptómicos. Se calcula que el 68% de los transcriptos que oscilan a ritmo circadiano están implicados en respuestas a estrés (Kreps et al, 2002). Por otro lado, una significativa proporción de genes de respuesta a ABA presentan fluctuaciones diarias (Mizuno & Yamashino, 2008). Finalmente, ha sido demostrado que la respuesta transcripcional a sequía cambia significativamente durante el día tanto en *Populus* como en *Arabidopsis* (Wilkins et al, 2010; Wilkins et al, 2009). Además, el factor de transcripción de respuesta a ABA *DRE*/*CRT BINGING PROTEIN 1C* (*DREB1C*) está implicado en respuesta a frío y sequía y es directamente inhibido por el factor PHYTOCHROME INTERCATING FACTOR 7 (PIF7), el cuál es vehicula tanto la regulación circadiana mediada por TOC1, como la respuesta a luz roja mediada por PHYA (Kidokoro et al, 2009). Además, los factores de transcripción *DREB1* también se ven aumentados por el complejo "*morning complex*" (Dong et al,

2011) y su inducción mediada por frío es canalizada por el reloj a través de la inhibición mediada por PRR5, 7 y 9 (Nakamichi et al, 2009). La importancia de la adecuada regulación circadiana de la conductancia estomática se refleja en las peculiaridades observadas en el oscilador de las células de guarda. En este tipo de células, las fluctuaciones de los transcriptos y proteínas circadianos muestran algunas diferencias al ser comparados con otros tipos de células presentes en hojas. Estas diferencias pueden ser la causa de las alteraciones en la fase y longitud de período observadas en condiciones de "*free running*" (Yakir et al, 2011). Un mecanismo crucial para la modulación circadiana de cierre de los estomas involucra al gen regulado por reloj *FLOWERING LOCUS T* (*FT*). De hecho, recientemente ha sido demostrado que el fenotipo de estoma cerrado de la planta doble mutante *phot1phot2* es revertido por una mutación nula en el gen *ELF3*. La apertura del estoma en esa situación se debe a un incremento en la actividad H^+-ATPasa. El gen diana de la inhibición transcripcional mediada por ELF3 es *FT*. Notablemente, la mutación "*knock out*" *ft-1* recupera el fenotipo *phot1phot2*, demostrando que FT es una pieza central en la modulación de la floración fotoperiódica así como también en la apertura estomática inducida por luz azul. La interacción genética descrita anteriormente prueba que estos procesos son modulados por el reloj circadiano durante las últimas horas del día (Kinoshita et al, 2011). El significado biológico de tal circuito regulador puede ser la extensión de las horas de apertura, que favorece una mayor fotosíntesis en una ventana temporal en la que la humedad generalmente disminuye, con un reducido riesgo de deshidratación (Hubbard & Webb, 2011). Otra interesante regulación circadiana del comportamiento estomático consiste en la acción de GRP7/CCR2, un componente del oscilador secundario regulado circadianamente, que actúa como un inductor del cierre estomático mediante un mecanismo que involucra la exportación de ARNm del núcleo (Kim et al, 2008). Toda esta información sugiere que los movimientos estomáticos son modulados por el reloj circadiano a través de varios mecanismos y que, en dicha modulación, la regulación transcripcional desempeña una función especialmente relevante.

Capítulo 1

A pesar del creciente conocimiento de las redes moleculares que forman parte del oscilador circadiano de *Arabidopsis*, se sabe poco sobre la función bioquímica y molecular de TOC1 en el reloj. El mutante *TOC1* y las plantas RNAi muestran un fenotipo de periodo corto por la expresión de los genes que controlan el reloj, así como el tiempo de floración insensible a la duración del día (Strayer et al, 2000) .La expresión constitutivamente alta de *TOC1* produce arritmia bajo condiciones de luz constante en una serie de fenotipos regulados por el reloj circadiano (Makino et al, 2002; Más et al, 2003a). También se propuso que TOC1 tiene un papel importante en la gestión de las señales ambientales hacia el reloj y en la regulación fotomorfogénica (Más et al, 2003a). Otros estudios han evidenciado que una precisa regulación del gen *TOC1* y la expresión rítmica de su proteína es esencial para un adecuado funcionamiento del reloj.

Diferentes mecanismos contribuyen a esta regulación incluyendo cambios en la estructura de la cromatina (Perales & Más, 2007), regulación transcripcional (Alabadí et al, 2001) y degradación de la proteína por la vía proteosómica (Más et al, 2003b). Estos procesos, juntos, controlan la oscilación rítmica de 24h en la expresión y función de TOC1.

La agrupación funcional de genes regulados por el reloj ha ayudado a comprender las vías metabólicas y fisiológicas que están bajo el control circadiano. De hecho, los genes regulados por el reloj están sobre-representados en varias vías de respuesta a hormonas vegetales y de respuestas a estrés. Esto es consistente con las variaciones diurnas de la abundancia de fitohormonas (Robertson et al, 2009) y con la regulación de la sensibilidad a hormonas vegetales controladas por el reloj (Covington & Harmer, 2007; Dodd et al, 2007).

Una de las hormonas reguladas por el reloj circadiano es el ácido abscísico (ABA). Esta fitohormona es esencial en la regulación de la mayoría de

procesos de crecimiento y desarrollo de la planta, así como el control de las respuestas de las plantas a los estreses ambientales (Finkelstein et al, 2002; Leung & Giraudat, 1998; Zhu, 2002). El importante número de genes relacionados con ABA que son controlados por el reloj ha sido observado en varios estudios (Covington & Harmer, 2007; Dodd et al, 2007; Mizuno & Yamashino, 2008). Por otra parte, la regulación entre ABA y las vías de señalización de reloj es bidireccional, dado que el tratamiento con ABA afecta la actividad de los promotores de los genes del reloj (Hanano et al, 2006).

El análisis transcriptómico de plantas con alterada expresión de *TOC1* mostró un incremento significativo en 245 mensajeros y una disminución en otros 160 en TOC1-ox en comparación con el WT. También analizamos los datos del transcriptoma de plantas WT y mutantes *toc1-2* (Strayer et al, 2000), sincronizadas bajo condiciones de día largo (LD) seguidos de 2 días bajo luz constante (LL) y encontramos que 75 genes estaban regulados negativamente y 105 regulados positivamente en las muestras de *toc1-2*. La agrupación funcional utilizando conjuntos de datos específicos relacionados con ABA (Matsui et al, 2008), mostró un aumento significativo del número de genes de respuesta a ABA en TOC1-ox y *toc1-2*. Además una proporción significativa de estos genes relacionados con ABA muestran una oscilación circadiana. Nuestro análisis de datos también mostró que alrededor del 38% de los genes regulados por TOC1 están implicados en la respuesta de las plantas a la deshidratación, dato significativo, comparado con el 18% observado en la totalidad del genoma.

Análisis por PCR cuantitativa (Q-PCR) confirman la alteración de la expresión de algunos genes seleccionados en plantas mutantes *toc1-2* y TOC-ox, así como de otros genes relevantes relacionados con deshidratación. Por lo tanto analizamos las respuestas al estrés por seguía y encontramos que la sobre-expresión de *TOC1* reduce significativamente la tolerancia a la sequía de la planta, mientras que plantas *toc1-2* y *TOC1 RNAi* responden mejor que el WT a condiciones de deficiencia de agua.

A continuación examinamos el movimiento de las células de guarda del estoma, un proceso controlado tanto por ABA como por el reloj circadiano (Robertson et al, 2009). Como se esperaba el tratamiento con ABA en hojas WT causa un cierre estomático significativo. Sin embargo, este efecto no fue tan pronunciado en TOC1-ox, mientras que las hojas de mutantes *toc1-2* mostraron un cierre estomático más efectivo. Acorde con estos resultados, la conductancia estomática de plantas TOC1-ox no resultó tan sensible al tratamiento con ABA como en las plantas WT, mientras que las plantas mutantes *toc1-2* mostraron una mayor respuesta. También se midieron las tasas de pérdida de agua de la roseta en plantas con expresión de TOC1 afectada. Nuestros estudios mostraron que 3h después del corte de la roseta, las hojas de las plantas *TOC1 RNAi* mostraron un 40% de pérdida de agua en contraste con el 54% y el 75% de las plantas WT y TOC1-ox, respectivamente.

Uno de los genes desregulados en respuesta cambios en la expresión de *TOC1* en los datos obtenidos con micromatrices fue *ABAR/CHLH/GUN5* (Shen et al, 2006; Wu et al, 2009). Aunque un estudio en cebada ha puesto en duda la función de ABAR en la señalización de ABA (Muller & Hansson, 2009), nuestros análisis fenotípicos hechos utilizando diferentes líneas *ABAR RNAi* de *Arabidopsis*, con unos patrones disminuidos de la expresión de *ABAR*, fueron acordes con los artículos anteriores (Shen et al, 2006; Wu et al, 2009). De hecho, comparando con el efecto de ABA en las plantas WT, los estomas en las diferentes líneas *ABAR RNAi* se mostraron casi insensibles al tratamiento con la hormona. De acuerdo con esto y los fenotipos descritos previamente (Shen et al, 2006; Wu et al, 2009), nuestros estudios mostraron también que la pérdida relativa de agua de las plantas *ABAR RNAi* fue significativamente mayor que la del WT.

A continuación se exploró la posible relación entre el reloj circadiano y la expresión de *ABAR*. Nuestros resultados muestran que la abundancia del transcrito de *ABAR* oscila rítmicamente con un pico de expresión cerca del amanecer en condiciones de día largo, día corto y luz continua. Además la forma de la oscilación del mRNA de *ABAR* está alterada en plantas *TOC1 RNAi* y TOC1-ox bajo luz continua. La fase de expresión de *ABAR* en *TOC1*

RNAi está avanzada, conduciendo a una cantidad superior de ARNm al mismo tiempo que se observa un mínimo de expresión de *ABAR* en plantas WT. En las plantas TOC1-ox, a su vez, la expresión de *ABAR* presenta una abundancia reducida durante el ciclo circadiano. En conjunto, estos datos indican que el reloj circadiano controla la temporización de expresión de *ABAR.* Nuestros estudios también asignan una función clave para TOC1 en la modulación de la fase y la amplitud en la forma de la oscilación diurna y circadiana de la expresión ABAR.

Se realizaron ensayos de inmunoprecipitación de cromatina (CHIP) con plantas TOC1-ox y TOC1-minigen (TMG, que expresa *TOC1* bajo el control de su propio promotor). Experimentos de seguimiento en función del tiempo durante el ciclo de 24h mostraron una amplificación significativa, coherente con la unión in vivo de TOC1 al promotor de *ABAR*. Nuestros resultados mostraron, que en las plantas TMG, la unión de TOC1 es antifásica a la expresión del ARNm de *ABAR* y esto es consistente con la correlación inversa entre la abundancia de ARNm y los fenotipos de *TOC1* y *ABAR*. La unión los ensayos de ChIP con las plantas TOC1-ox indica que la proteína TOC1 permanece unida durante todo el ciclo.

Relevante para nuestros estudios fue la observación que *TOC1* estaba incluido en los datos de micromatrices de los genes regulados por ABA (Matsui et al, 2008). Para examinar este nuevo mecanismo de regulación, se monitorizaron los ritmos de bioluminscencia de la planta expresando el gen de la luciferina (*LUC*) bajo el control del promotor de *TOC1* (*TOC1:LUC*) en presencia de ABA. Nuestros resultados muestran que los tratamientos con ABA administrada exógenamente inducen la expresión de *TOC1:LUC*. La magnitud de la inducción no resultó ser constante, más bien aumentaba progresivamente a lo largo del día, alcanzando un valor máximo al cabo de 5-10h después del alba. La administración de ABA durante la misma noche, no tuvo claras consecuencias inmediatas en la amplitud de fase de *TOC1:LUC*. En los siguientes días, el tiempo prolongado de exposición a la hormona, condujo a una disminución de la amplitud y ligero retraso en la fase en la expresión de *TOC1:LUC*. La expresión de *TOC1* es regulada por ABA, la intensidad de esta

regulación depende del momento del día en el que se administre la fitohormona, lo cual indica que se trata de una respuesta restringida por el reloj a una precisa ventana temporal. El momento de alta sensibilidad correlaciona temporalmente con la unión de TOC1 al promotor de *ABAR* y los cambios de la expresión de *ABAR* mediados por ABA.

Siendo los bucles de retroalimentación mecanismos comunes para una regulación precisa de la expresión génica, hemos explorado la posible función de ABAR en la inducción de *TOC1* mediada por ABA. Para ello se ha examinado la regulación de la expresión de *TOC1:LUC* en plantas *ABAR RNAi*, que carecen de una expresión funcional del gen ABAR. Nuestros resultados muestran que en ausencia de ABA la expresión de *TOC1:LUC* en plantas *ABAR RNAi* es similar a la observada en las plantas WT. La aguda inducción de *TOC1:LUC* observada en plantas WT después del tratamiento con ABA, es completamente abolida en plantas *ABAR RNAi*. El papel de ABAR en la inducción de *TOC1* también fue verificado por "*Northern blot*". Estos resultados indican que la inducción de *TOC1* mediada por ABA, requiere la presencia funcional de ABAR, la cual regula positivamente la expresión de *TOC1*. Notablemente, ésta regulación parece tener una función importante sólo en presencia de ABA. En ausencia de hormona exógena, la forma de onda de expresión de *TOC1* no se ve claramente afectada, con la posible excepción de un ligero adelanto de fase. Estos resultados sugieren que ABAR es importante para la regulación de la expresión *TOC1* dentro de la vía de señalización de ABA, pero no en la regulación circadiana. Nuestros resultados también indican la existencia de un bucle de retroalimentación relativo a la regulación recíproca de *ABAR* y *TOC1*. Para investigar más a fondo si la regulación recíproca entre la expresión de *TOC1* y de *ABAR* es importante para la señalización de ABA, se realizó un estudio genético en el que se transformaron las plantas TOC1-ox y las *TOC1 RNAi* con la construcción *ABAR RNAi* (Shen et al, 2006). En primer lugar se analizaron los fenotipos de pérdida de agua y se encontró que el fenotipo de las plantas *TOC1 RNAi/ABAR RNAi* contrasta claramente con la disminución de transpiración observada en las líneas *TOC1 RNAi*, lo que indica que el fenotipo de *TOC1 RNAi* es observable solo en presencia de un ABAR

funcional. Por el contrario, nuestros resultados muestran que la tasa de transpiración de TOC1-ox no se ve afectada significativamente por la reducción de expresión de *ABAR* en plantas *TOC1-ox/ABAR RNAi*. Similares conclusiones genéticas se obtuvieron cuando realizamos experimentos de deshidratación en las placas. En conjunto, los estudios genéticos están de acuerdo con los datos moleculares y funcionalmente conectan la regulación recíproca de la expresión de *TOC1* y *ABAR* con la tolerancia de las plantas a la sequía.

Capítulo 2

Esta parte de nuestro trabajo está dedicada a la identificación de factores de transcripción capaces de influir en el comportamiento de los estomas. La estrategia utilizada se basa en el uso de una batería de líneas de *Arabidopsis thaliana* que sobre-expresan factores de transcripción bajo el control de un promotor inducible por ß-estradiol (Zuo et al, 2000a).

No se pudo conseguir la inducción del transgén con ß-estradiol en plantas en suelo descrita en estudios anteriores con un vector similar (Borghi, 2010; Curtis et al, 2005; Zuo et al, 2000b) Sin embargo, en este trabajo conseguimos inducir la sobre-expresión en diversas líneas *in vitro*. Gracias a esto, finalmente pudimos acoplar la inducción por ß-estradiol con un sistema térmico de captura de imágenes para realizar un *screening*. La determinación de la temperatura de la hoja por imagen térmica infrarroja se ha usado ampliamente como un método para detectar los movimientos estomáticos alterados en varias especies de plantas. (Dong et al, 2012; Jones, 1999; Jones et al, 2009; Merlot et al, 2002; Raskin & Ladyman, 1988; Vollsnes et al, 2009). Los tejidos que presentan diferencias en la velocidad de intercambio de gas tienen diferentes contenidos de agua. El cierre estomático favorece la acumulación de agua, lo que conduce a un aumento de la temperatura foliar, por el contrario, si los estomas se abren, se reduce la temperatura. Las

diferencia en temperatura se reflejan, entonces, en una diferencia en la radiación infrarroja de la superficie de la hoja.

El *screening* realizado ha permitido identificar seis putativos candidatos, 3 de los cuales presentan un fenotipo "caliente" (con estado estomático cercano al salvaje) y los otros 3 un fenotipo "frío" (con estomas abiertos), entre las 264 líneas de *Arabidopsis* sobreexpresoras condicionales analizadas. Los 6 factores de transcripción seleccionados presentan el mismo fenotipo en al menos dos inserciones independientes lo que sugiere que los fenotipos observados no se deben a la región de inserción del transgen. Las diferencias de temperatura, que pueden ser apreciadas a través de la escala colorimétrica de la imagen, se cuantificaron y se analizaron estadísticamente.

Un análisis más profundo de los candidatos reveló que todas las líneas que muestran un fenotipo realmente sobre-expresan el transgen esperado. Posteriormente, se cuantificó la apertura estomática confirmando que los fenotipos observados son debidos al comportamiento alterado de los estomas. Estos datos sugieren que el sistema experimental utilizado produjo c candidatos cualitativamente buenos para ambos fenotipos de alteraciones en el comportamiento estomático: "frío" y "caliente".

Uno de los genes candidatos más interesantes entre los seleccionados corresponde a ANAC087 (At5g18270), que induce el cierre de estomas cuando es sobre-expresado en presencia de ß-estradiol. Varios estudios han puesto de manifiesto la importancia de los factores de transcripción NAC (NAM, ATAF1/2 y CUC2) en las respuestas a estrés abiótico tanto en arroz como en *Arabidopsis* (Nakashima et al, 2012; Nuruzzaman et al, 2010). En base a la secuencia de los dominios NAC, se nombró SNAC (*Stress Responsive NACs*) a un grupo filogenéticamente consistente de factores de transcripción. Este grupo incluye a la mayoría de los factores de transcripción NAC conocidos implicados en las respuestas a estrés abiótico en *Arabidopsis thaliana* y en arroz (Nakashima et al, 2012). En *Arabidopsis*, tres factores SNACs altamente relacionados, ANAC019, ANAC055 y ANAC072/RD26 son reguladores positivos de la señalización por ABA. La sobre-expresión de cualquiera de ellos produce un

fenotipo sensible a ABA y resistente a sequía (Tran et al, 2004). También son activadores transcripcionales del gen *early response to dehydration stress 1,* cooperativamente con el factor de transcripción ZINC FINGER HOMEODOMAIN 1 (ZFHD1) (Tran et al, 2007). La expresión de ANAC072/RD26 es inducida por sequía y ABA a través de la vía de señalización mediada por SnRK2 (Fujii et al, 2011). Otro importante factor SNAC en Arabidopsis es ATAF1, que se transcribe en respuesta a ABA y contribuye positivamente a su vía de señalización (Wu et al, 2009b).

Aunque *ANAC087* no pertenezca al grupo SNAC se ha reportado que su transcripción es inducida en respuesta a H_2O_2 y su proteína se localiza en ambos núcleo y citoplasma (Inzé et al, 2012). Estos resultados, junto a nuestra caracterización fenotípica, sugieren que ANAC087 podría tener un papel en el cierre estomático mediado por especies reactivas del oxigeno.

La alteración fenotípica más fuerte se observa en las plantas sobreexpresoras de *DEAR4/RAP2.10* (*DREB AND EAR MOTIF 4*/*RELATED TO APETALA 2.10*) (At4g36900). Dichas plantas presentan un marcado incremento en su temperatura foliar, que está relacionada con el fenotipo observado a nivel estomático. Los miembros de la familia de factores de transcripción AP2/ERF desempeñan varios papeles en las respuestas de las plantas a estrés abiótico.

El subgrupo DREB (subfamilia A1) incluye los factores CBF y se ha relacionado primariamente con frío, aunque la sobre-expresión de *DREB1A*/*CBF3*, *DREB1B*/*CBF2*, *DREB1C*/*CBF1* o *CBF4* produce fenotipos resistentes tanto a frío como a sequía en Arabidopsis, siendo el ultimo inducido por deshidratación (Haake et al, 2002; Liu et al, 1998; Mizoi et al, 2012). El subgrupo DREB2 (subfamilia A2) ha sido relacionado con sequía y respuesta a calor: en concreto DREB2A y B están involucrados en la expresión génica mediada por motivos DRE/CRT (Liu et al, 1998; Mizoi et al, 2012; Nakashima et al, 2000; Sakuma et al, 2006). Además, DREB1A, DREB2A y DREB2C interactúan con factores de transcripción de tipo AREB/ABF, confirmando su rol de peso en la respuesta a ABA (Lee et al, 2010). DEAR4/RAP2.10 pertenece a la subfamilia A5 de factores AP2/ERF ya que presenta motivos DREB (DRE

BINDING PROTEIN) y EAR (ERF ASSOCIATED AMPHIPHILIC REPRESSION). Algunos miembros de esta subfamilia son inducidos por DREB1A/CBF3 y DREB2A (Maruyama et al, 2004; Sakuma et al, 2006). Al menos uno de ellos, RAP2.1, parece estar involucrado en la modulación de la respuesta a congelación, dado el aumento de la tolerancia a congelación observado en el mutante *knock out* (Dong & Liu, 2010). Otro miembro de la subfamilia A5, DEAR1, inhibe la respuesta a frío a favor de la respuesta contra patógenos. (Tsutsui et al, 2009). En el caso de DEAR4, no parece haber implicación en la respuesta a frío mediada por elementos DRE/CRT y tampoco en la respuesta a *Pseudomonas* (Haake et al, 2002; Tsutsui et al, 2009). Estos resultados sugieren que DEAR4 participa en el cierre estomático por medio de mecanismos separados de los de respuesta a frío o a *Pseudomonas*.

La inducción por ß-estradiol de HSFA8 (At1g67970) abre los estomas, como sugerido por la baja temperatura foliar. HSFA8 está entre los menos estudiados factores respuesta a calor de *Arabidopsis*, sin embargo, considerando nuestros resultados y la literatura disponible al respecto, podemos suponer que HSFA8 es un factor de transcripción cuya localización es tanto nuclear como citoplásmica y es regulada por interactores cuales otros HSFs. Se puede también plantear la hipótesis de que se trata de un activador transcripcional, dada su capacidad activadora observada en protoplastos de tabaco (Kotak et al, 2004), aunque, *in vivo*, podría igualmente tener funciones de un inhibidor, como observado por HSFA5. Nuestros datos nos sugieren que la transcripción de *HSFA8* es ligeramente inducida por ABA y reducida por deshidratación. El calor, en cambio, no produjo cambios observables en los niveles de ARNm en las condiciones utilizadas. Se pudo observar una ligera inducción en respuesta al elicitor Flg22, el epítopo biológicamente activo del PAMP flagelina. Sin embargo, las variaciones más consistentes se observaron al analizar los niveles de mensajero en diferentes momentos del día subjetivo en condiciones constantes. En el conjunto podemos deducir que este gen está regulado por ABA y deshidratación de forma independiente. Los efectos en respuesta ABA correlacionan con los observados en Flg22. Sin embargo estas regulaciones producen efectos reducidos comparadas con la regulación

observada a lo largo del día. El hecho que dicha oscilación se produzca en condiciones constantes nos sugiere que este fenómeno esté regulado por el reloj circadiano.

Otro resultado interesante en la insensibilidad estomática a flagelina, observada en las plántulas que sobre-expresan *HSFA8*. Considerada la inducción observada en respuesta Flg22, podemos suponer que este gen opere como modulador de la respuesta a patógeno. De hecho ha sido demostrado que el reloj circadiano modula la respuesta a patógenos foliares a lo largo del día, para anticipar los momentos de mayor virulencia que se acompañan a altas irradiaciones lumínicas y humedad elevada (Bhardwaj et al, 2011; Roden & Ingle, 2009; Wang et al, 2011b).

El siguiente punto de nuestro trabajo fue determinar los posibles mecanismos de acción por medio de los cuales HSFA8 produce la abertura del estoma. Un análisis proteómico ha revelado cambios en la acumulación de varias proteínas aunque solo una se pudo relacionar con alterados niveles de ARNm. El caso de las chaperoninas resulta de interés siendo la chaperonina 10 (CPN10) inducida y la 20 (CPN20) reducida a nivel proteico. CPN10 es, además, inducida también a nivel de mensajero. CPN20 es constituida por dos unidades altamente similares a CPN10 y se ha descrito como regulador de la actividad de la superóxido dismutasa (SOD) (Kuo et al, 2012). Esta regulación contribuye a rebajar los niveles de ROS y, consecuentemente, la señalización de estrés. Los cambios en las concentraciones relativas de estos dos enzimas podrías estar relacionado con la modulación de la señalización por ROS. Otro dato interesante es el aumento de ciclofilina 20-3: esta enzima también ha sido relacionada con la modulación de la señalización por ROS y con la oscilación diaria de los thioles (Dominguez-Solis et al, 2008). Finalmente la disminución de glutamina sintetasa 2 (GS2) se ha descrito como marcador de procesos de senescencia, cosa que la relaciona con un aumento de abertura estomática.

A nivel transcriptómico, observamos que el tratamiento con ß-estradiol no afecta de forma significativa a ningún mensajero detectable con un Gene Chip ATH1 de Affymetrix. Por otro lado, la sobre-expresión de *HSFA8*, ha afectado

significativamente los niveles de 69 ARNm. Se podría considerar que, el número limitado de dianas de regulación sea una subestimación, como sugerido por el análisis directo de mensajeros que presentaban alteraciones que no llegaban a cumplir los requisitos de P-Value impuestos en el análisis de los datos de micromatrices. Además el tamaño reducido del impacto transcriptómico de HSFA8 podría deberse en la naturaleza de dicho factor, que parece ser regulado en parte de *partner* transcripcionales que afectan su localización subcelular y, posiblemente sus dianas en el ADN. Sin embargo, el fenotipo observado depende de la sola sobreexpresión de este gen y probablemente de su función como regulador transcripcional.

A nivel transcripcional, observamos la inducción de dos ferretinas: *Ferretin 1* (At5g01600) y *3* (At3g56090). Estos dos genes han sido relacionados con la inhibición de respuestas a estrés oxidativo en *Arabidopsis* (Ravet et al, 2009). De hecho, estas proteínas actúan como tampón reduciendo la cantidad de hierro libre en las células. El hierro libre es un amplificador de ROS gracias a su capacidad de generar radicales hidroxilos por medio de la reacción de Fenton. Consecuentemente, la reducción en los niveles de hierro libre mediada por la ferretinas se considera como un mecanismo para rebajar los ROS y prevenir daño oxidativo (Kim et al, 2011). Es interesante destacar que la *ferretina 1* es un gen regulado por el reloj circadiano (Duc et al, 2009). Acorde con la hipótesis que ve HSFA8 como regulador de los niveles de hierro libre, observamos que el mensajero de *IRON REGULATED TRANSPORTER 3* (*IRT3*) presenta niveles reducidos en las plántulas sobreexpresoras de *HSFA8*. IRT3 es un transportador de iones divalentes que participa en la absorción de hierro en *Arabidopsis* (Lin et al, 2009). La reducción en la cantidad de hierro libre mediada por HSFA8 se reflejaría en los niveles ROS, determinado así la mayor abertura estomática. Esta hipótesis es avalada por la inhibición observada en los niveles de mensajero del At2g25735, conocido como marcador de la respuesta a peróxido de hidrogeno (Inzé et al, 2012). Otra posible explicación del fenotipo es la inducción observada en *CYP707A2* (At2g29090), que codifica por una enzima que cataliza la degradación de ABA

(Okamoto et al, 2011). Estos datos sugieren que las plántulas sobreexpresoras de *HSFA8* podrían presentar niveles reducidos de ABA y ROS.

Otro dato interesante es la inducción observada en los genes de dos alfa-expansinas *EXPA1* (At1g69530) y *EXPA11* (At1g20190). Concretamente EXPA1, se ha descrito como facilitador de la abertura estomática que actúa alterando la estructura de la pared celular de las células de guarda y aumentando así su elasticidad (Wei et al, 2011; Zhang et al, 2011). Curiosamente, el rasgo más claro del transcriptoma modificado por HSFA8 es la alta presencia de productos dirigidos al exterior de la célula. De hecho, como en el caso de las expansinas, pudimos observar una tendencia general en favor de los mecanismos de relajación mecánica de la pared celular. Es el caso de la inducción de una poligalacturonasa (At5g14650) y la reducción de dos proteínas inhibidoras de poligalacturonasas (PGIP): PGIP1 (At5g06860) and PGIP2 (At5g06870). Cabe destacar que estas PGIP se caracterizaron por su capacidad de generar resistencia a varios patógenos *Botrytis cinerea* (Ferrari et al, 2006), *Fusarium graminearum* (Ferrari et al, 2012) y *Stemphylium solani* (Di et al, 2012) en *Arabidopsis thaliana*. Estas observaciones correlacionan los resultados de reducida respuesta estómatica a flagelina. Otra evidencia del posible papel de HSFA8 como antagonista de la respuesta a patógenos, es la inhibición observada en genes de respuesta a estrés biótico cuales *CMPG1* (Cys, Met, Pro and Gly) (Heise et al, 2002) y *WRKY22* (Asai et al, 2002), factor de transcripción que es también regulado por el reloj circadiano (Bhardwaj et al, 2011). Por otra parte, se observó la inducción de *CORONATINE INDUCED 3* (*CORI3*), un gen inducido por la fitotoxina coronatina, que induce la abertura de los estoma (Lopukhina et al, 2001). Este gen está también inducido en procesos de senescencia (Hollander-Czytko et al, 2005).

Además se pudo observar que, entre los genes inhibidos por HSFA8, WRKY22, AtBCB y CSG se inducen en respuesta a Flg22. Por el contrario, los transcriptos que se inducen en las plántulas sobreexpresoras de HSFA8, tales como CPN10 y EXPA1, son inhibidos por Flg22.

Por lo tanto, estos resultados refuerzan la hipótesis de la implicación de HSFA8 en la relajación general de la pared celular acompañada por una mejoría en la apertura estomática, con el fin de favorecer la fotosíntesis y el crecimiento en una ventana de tiempo en la que disminuye el riesgo de patógenos y aumenta la humedad en el aire.

Todos estos resultados contribuyen a la identificación de tres nuevos componentes en el control transcripcional del comportamiento estomático. Dos de ellos, DEAR4 y NAC087, participan en el cierre de estomas, mientras que, HSFA8 induce apertura estomática.

VIII. REFERENCES

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IX. ANNEX

Definitions and details of labels in annexed tables

The **SOLAR** package analyses microarray data by scaling the global intensities and normalizing by the *Lowess* curve to correct the deviation of the M values. From each gene, normalized M (log Ratios) and A (log gene signals) values are averaged over all correct spot measures.

Mean M: mean of the M values obtained from the replicates. The M values are the log differential expression ratios obtained directly by the detected intensities:

$M=\log_2(R/G)$

Where R and G are the red and green intensities corresponding to the expression level in *toc1-2* and control, respectively.

SD M: standard deviation of M values obtained from the replicates.

z-score: the number of standard deviations that the gene is far away from the mean of the spots cloud. This value is calculated locally for each intensity range as:

$Zi=(Mi/sn)$

Where Zi is the local Z-score for the gene i, Mi is the M value for the gene i and sn is the standard deviation of M calculated among the n genes with A values nearest to i. A is the log-intensity of a spot, which means a measure of the expression level of the corresponding gene. This parameter is calculated as:

$A=\log_2((R \times G)1/2)$

p-value: the probability that the gene is not differentially expressed, calculated on the basis of the absolute value of the t statistic. Where t is:

t=mean M/(SD M/(n)1/2)

Where M and SD M correspond to the definitions previously given and n is the number of replicates. p-values are calculated as two-tailed tests from t-distribution under the null hypothesis M=0. The number of freedom degrees is the number of replicates (n) –1.

Annotation: brief information about the locus function or putative function. Obtained from BAR (Bio-Array Resource for *Arabidopsis* functional genomics) (http://bar.utoronto.ca/) and derived from TAIR9_pep_20090619 merged with gene_aliases.20090313 from TAIR (The *Arabidopsis* Information resource: http://www.arabidopsis.org/index.jsp).

The **GCRMA** package adjusts for background intensities in Affymetrix data, which include optical noise and non-specific binding (NSB) (Wu et al, 2004). GCRMA converts background adjusted probe intensities to expression measures using the same normalization and summarization as “Robust Multiarray Average”.

The **LIMMA** is a package used for differential expression analysis of microarray data. The package analyzes complex experiments involving comparisons between many RNA targets simultaneously while remaining easy to use also for simple experiments. The basic statistical used for significance analysis is the moderated t-statistic, which is computed for each probe and for each contrast.

BH: The Benjamini and Hochberg´s method to control the false discovery rate (FDR).

If all the genes with p-value below a threshold 0,05 are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value.

Mean M: The mean of the M values obtained from the replicates. The M values represent the $\log_2$-fold change observed between the samples.

Mean A: The average log_2-expression for a gene across the arrays and channels in the experiment.

Affy ID: Probe identifying the code for the ATH1 gene Chip by Affymetrix.

CEL Files: contain the measured intensities and locations of the hybridized array.