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PLANT TOLERANCE TO DROUGHT: MODULATION OF STOMATAL MOVEMENTS

Alessandra Albertini

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Scientific tutor: Prof. C. Tonelli

Co-tutor: Dr. E. Cominelli

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Abstract

Plants are continuously exposed to several types of abiotic and biotic stresses. As sessile organisms, the mechanisms developed by plants to respond to those stresses are extremely vital for the plant growth and survival. Drought is one of the most common environmental stresses to which plants are exposed and the major cause of losses in crop production throughout the world. One of the primary plant response against water loss and dehydration is reducing the transpiration from the leaves by the closure of stomatal pores, guard cells in fact sense and respond to the environmental changes by the activation of complex intracellular signalling cascades followed by a variation of their turgor pressure and volume. Thus, understanding the complex mechanisms that control the opening and closure of stomata represents an attractive goal.

In this PhD thesis the results obtained by the analysis of two Arabidopsis genes involved in stomatal movements, *AtMYB60* and *γVPE*, are presented.

The approach performed to characterise putative targets of AtMYB60, a transcription factor specifically expressed in guard cells, was described. Then, the *AtMYB60* promoter was characterised through serial deletion and mutagenesis analysis and some DOF-binding sites were identified as fundamental *cis*-elements for the specific activity of this promoter in guard cells. Finally, the improved tolerance to water stress of *γvpe* plant was reported and the phenotypic alteration of the mutant in stomatal aperture was described.

Introduction

With the new global climate changes, water scarcity has become one of the most common environmental stresses to which plants are exposed and the factor mostly affecting plant productivity [1]. As sessile organism, plants have had to evolve several mechanisms to enable them to adapt to ever-changing environmental conditions. Drought avoidance in one of the strategies developed by plant to resist to drought [2]; it refers to a plant's ability to maintain high water status even when water is scarce, for example by growing long roots to reach deep soil moisture, or reducing water loss by restricting the aperture of the stomata on leaf surfaces. With changes in the turgor pressure of the two surrounding guard cells, stomata in fact mediate the water vapor exchanges between the plant and the atmosphere, playing a major role in plant adaptation to stress [3]. Thus, the study of the genes that regulate the stomatal opening and closure represents a useful tool to better understand one of the complex mechanisms that plants evolved to respond to water stress.

In this PhD thesis research results on two genes involved in stomatal movements, *AtMYB60* and *γVPE,* will be presented. In the first case the dissection of a guard cell-specific promoter will be discussed and analysis of mutants in *AtMYB60* putative target will be presented, while in the second case evidence of the involvement of the vacuolar processing enzyme *γVPE* in stomatal opening and closure will be provided.

AtMYB60 codes for a R2R3MYB transcription factors from *Arabidopsis thaliana*, the model plant for plant genetics and molecular biology studies. Only in the last years it has been demonstrated the importance of the role

of transcription factors in controlling the stomatal opening and closure (as reviewed by Cominelli et al., 2010) [4]. MYB proteins are a class of transcription factors that are present in all eukaryotes, and share a common DNA-binding domain. In plants, the most highly represented MYB protein group is the R2R3 subfamily, members of which contain two MYB repeats in their DNA-binding domains [5]. In *Arabidopsis thaliana*, 126 R2R3-MYB genes have been identified [6], and involvement in the regulation of plant-specific processes has been reported for some of them, such as the regulation of phenylpropanoid metabolism, the control of specialized cell morphology, and the regulation of plant responses to biotic and abiotic stresses, hormones and light [5-8].

AtMYB60 gene has been previously characterised [9, 10] The expression of the *AtMYB60* gene is specifically localised in guard cells [11], specialised epidermal cells surrounding stomata. Stomata are small pores distributed on the epidermis of plant leaves. They mediate the exchanges between the plant and the atmosphere: through stomata CO_2 enters as carbon source for photosynthesis, while water vapour is released by transpiration. Stomata are surrounded by a pair of highly specialised cells, called guard cells. *AtMYB60* expression is up-regulated by signals that induce stomatal opening, like white and blue light, and negatively down-regulated by dark, desiccation and abscisic acid treatment, signals that promote stomatal closure [9]. Leaves from the *atmyb60-1* knock-out mutant displayed a reduction in the light-induced aperture of stomatal pores of approximately 30% compared to wild-type leaves. These data indicate that this transcription factor represents a positive regulator of stomatal opening that is silenced in stress conditions. It was clearly shown that the constitutive reduced stomatal opening in these plants helps to limit water loss during drought, thus enhancing plant tolerance [9].

Microarray analysis showed a differential expression of genes involved in the response to abiotic stresses and to pathogens between wild-type and *atmyb60-1*. Based on these data it is intriguing to suggest a model in which AtMYB60 is the only one transcriptional regulator known to be involved in stomatal movements that could integrate multiple signal transduction processes, by modulating the expression of genes involved in guard cell responses to light, to biotic and abiotic stresses. In this scenario we put the study of the promoter as well as of the putative targets of this gene as a very interesting tool to understand the complex network of gene regulation in guard cells.

On the other hand, *γVPE* codes for a vacuolar processing enzyme with a caspase-1 like activity and substrate specificity towards asparagine residues. It belongs to the *VPE*s family, found in various eukaryotic organism including higher plants and mammals [12]. In plant, VPEs are separated into two subfamilies by their homology and expression pattern, seed-type and vegetative-type. VPEs were originally identified as proteases responsible for the maturation of seed storage proteins but further studies showed their involvement also during senescence and hypersensitive cell death. VPEs are synthesized as large precursors and then self-catalytically converted into the mature form under acidic condition, this implies that the VPE precursor is transported to vacuoles and there converted into the mature form [12].

The Arabidopsis genome has four VPE genes separated into the vegetative-type and seed-type: *αVPE* and *γVPE* are expressed in vegetative organs, whereas *βVPE* and *δVPE* are expressed in seeds [13-15]. The *βVPE* is the main responsible for the proper processing of seed storage proteins [16], and *δVPE* specifically expressed in the seed coat is

associated with cell death [17]. By contrast, the vegetative *αVPE* and *γVPE* are upregulated during wounding, senescence and pathogen infection, and may play vital roles in various types of cell death in plants [13, 18]. Recently, VPEs have been identified as plant-specific caspases and a VPE-mediated vacuolar system has been considered as a cellular suicide strategy in plant development and cell death programs [19, 20]. Several works demonstrated the role of γVPE in developing programmed cell death; in wild type the processing and activation of hydrolytic enzymes by γVPE lead to the collapse of the lytic vacuoles and consequently, with the release of such enzymes in the cytosol, cause the cell death, while *γVPE* silenced plant prevented the typical characteristics of cell death, such as cell shrinkage, vacuolar collapse, cytoplasmic condensation and nuclear DNA fragmentation [12, 19, 21]. Previous research into plant VPEs has mostly focused on plant senescence, terminal differentiation and pathogen-induced hypersensitive cell death. By contrast, the molecular mechanisms underlying the roles of VPEs in response to abiotic stresses are poorly understood, only recently the involvement of γVPE in the Arabidopsis response to heat shock stress (HS) has been demonstrated [22]. Thus, we analysed the role of γVPE in water stress condition to definitely prove the involvement of VPEs in processes of both biotic and abiotic stress response.

A brief description of the content of the different chapters follows below.

In **Chapter 1** the paper "DOF-binding sites [A/T]AAAG modulate expression of the *AtMYB60* gene in *Arabidopsis* guard cells", published in *BMC Plant Biology* (Cominelli, Albertini et al., 2011) [23] will be illustrated; we described the characterisation of the *AtMYB60* promoter through serial deletion and mutagenesis analysis. We isolated some DOF-

binding sites as fundamental *cis*-elements for the specific activity of this promoter in guard cells. We also confirmed that the activity of the promoter fused to *GUS* reporter gene is negatively modulated by ABA. We started a systematic search for DOF proteins regulating *AtMYB60* expression, starting from the subfamily of CDF proteins known to be expressed in guard cells. Unfortunately we did not observe any difference in the accumulation of *AtMYB60* transcript in single and multiple mutants in these genes, concluding that they are not the regulators of *AtMYB60* gene expression.

Chapter 2 is titled "Searching for DOF proteins regulating *AtMYB60* expression", in which data on the isolation and analysis of other *dof* mutants are reported; single and double mutations in two *DOF* selected genes, *At5g65590* and *At4g38000*, caused a strong reduction of the AtMYB60 expression, make the two genes the major candidates to be *AtMYB60* regulators.

Chapter 3 is focused on the "Analysis of mutants in putative targets of AtMYB60, an Arabidopsis guard cell-specific transcription factor " in which the approach performed to characterise some of the AtMYB60 putative targets is described. The microarray results obtained from the knock-out mutant *atmyb60-1* and wild-type plants [24] were the starting point to select among the genes showing different expression level in the *atmyb60-1* mutant, some candidates for more detailed analysis and then we investigated the effects of the mutations in the selected genes on water loss and transpiration rate during desiccation. We found that mutations in *At4g29780, At5g59820* and *At3g16240* genes affected the plant response to desiccation stress, supporting the hypothesis they could be putative AtMYB60 targets acting together to control stomatal movements. In the

section 3.1, the identification of new AtMYB60 putative targets through RNA-Seq analysis is presented.

In **Chapter 4** the paper in preparation "Role of the Arabidopsis vacuolar processing enzyme γVPE in the response to water stress" will be presented; we provide evidence of the *γVPE* new role in water stress response. *γVPE* is a vacuole-localised enzyme with a caspase-1 like activity involved in proteins activation and maturation. We demonstrated that within the four Arabidopsis *VPE* homologs genes, *γVPE* is the most expressed in guard cells and that gene mutation strongly affected the plant response to water dehydration, increasing the plant tolerance to water stress. Our hypothesis is that *γVPE* could be a new player in the complex mechanism that regulates stomatal opening and closure, processing some proteins involved in the stomatal movements.

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Chapter 1

Eleonora Cominelli[1], Massimo Galbiati[1], Alessandra Albertini[1], Fabio Fornara[2], George Coupland[2] and Chiara Tonelli[1,*]

DOF-binding sites [A/T]AAAG modulate expression of the *AtMYB60* gene in *Arabidopsis* guard cells

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[1]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

[2]Max Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, D-50829 Cologne, Germany

*corresponding author

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RESEARCH ARTICLE Open Access

DOF-binding sites additively contribute to guard cell-specificity of *AtMYB60* promoter

Eleonora Cominelli[1,4*], Massimo Galbiati[1,2], Alessandra Albertini[1], Fabio Fornara[3,5], Lucio Conti[1,2], George Coupland[3] and Chiara Tonelli[1*]

Abstract

Background: We previously demonstrated that the *Arabidopsis thaliana* AtMYB60 protein is an R2R3MYB transcription factor required for stomatal opening. *AtMYB60* is specifically expressed in guard cells and down-regulated at the transcriptional levels by the phytohormone ABA.

Results: To investigate the molecular mechanisms governing *AtMYB60* expression, its promoter was dissected through deletion and mutagenesis analyses. By studying different versions of *AtMYB60* promoter::GUS reporter fusions in transgenic plants we were able to demonstrate a modular organization for the *AtMYB60* promoter. Particularly we defined: a minimal promoter sufficient to confer guard cell-specific activity to the reporter gene; the distinct roles of different DOF-binding sites organised in a cluster in the minimal promoter in determining guard cell-specific expression; the promoter regions responsible for the enhancement of activity in guard cells; a promoter region responsible for the negative transcriptional regulation by ABA. Moreover from the analysis of single and multiple mutants we could rule out the involvement of a group of DOF proteins, known as CDFs, already characterised for their involvement in flowering time, in the regulation of *AtMYB60* expression.

Conclusions: These findings shed light on the regulation of gene expression in guard cells and provide new promoter modules as useful tools for manipulating gene expression in guard cells, both for physiological studies and future biotechnological applications.

Background

Land plants uptake carbon dioxide for photosynthesis and lose water vapour by transpiration through stomatal pores, present on the surface of leaves and stems. The opening and closure of the pore is mediated by turgor-driven volume changes of two surrounding guard cells, whose pressure is dynamically adjusted according to environmental and hormonal cues. In response to abiotic stresses, such as drought or high salinity, one of the most rapid responses of plants is the closure of stomata, mediated by the hormone abscisic acid (ABA), to prevent excessive water loss by transpiration (reviewed in [1]).

The genetic manipulation of stomatal activity is emerging as a promising approach to reduce the water requirement of crops, and to enhance productivity under stress conditions [2]. Proper engineering of stomatal responses requires the use of guard cell-specific promoters, or the identification of guard cell-specific mutants, to avoid undesirable side effects on plant growth and productivity.

Several promoters that confer guard cell-specific gene expression or enhanced gene expression in guard cells have been isolated through different methods: functional characterization of single genes [3-9]; large scale gene- or enhancer-trap screens [10-12]. Moreover transcriptomic and proteomic studies have identified additional candidates [13-16]. Nevertheless the majority of these promoters are not guard cell-specific, as they drive the expression of reporter genes in other cell types, including the vascular tissues [6,10,17,18], flower organs [8,9] or starch containing cells [5], significantly reducing the number of true guard cell-specific full size promoters [3,10,14,19,20]. Most importantly, a detailed experimental analysis of guard cell-specific promoters has been performed only in very few cases [11,12,14].

* Correspondence: cominelli@ibba.cnr.it; chiara.tonelli@unimi.it
[1]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Milano, Italy
Full list of author information is available at the end of the article

A true guard cell-specific promoter is driving expression of the Arabidopsis *AtMYB60* (*At1g08810*) gene [10,19,21,22]. We have previously shown that *AtMYB60* is expressed in guard cells [10], and the complete 5' and 3' intergenic genomic regions of this gene, cloned respectively upstream and downstream to reporter genes, were able to drive specific expression in guard cells [10,19]. Guard cell specificity of the *AtMYB60* promoter has been also demonstrated by Nagy *et al.* (2009) and by Meyer et al (2010), who used this promoter to complement the *mrp5-1* mutant phenotype exclusively in guard cells, and to specifically express the AtLMT12 protein at high levels in guard cells, respectively.

Very little information is available concerning promoter *cis*-elements regulating guard cell-specific expression [8,10-12,14,16]. DOF-binding sites have been suggested to have a role in such a regulation [8,10-12]. DOF (DNA binding with One Finger) proteins are plant specific transcription factors involved in light, phytohormones and pathogen signalling and responses as well as seed development (reviewed by [23]). A role for [T/A]AAAG DOF-binding sites in mediating gene expression in guard cells has been experimentally defined only for the potato *KST1* gene [8]. However, in Arabidopsis the role of DOF-motifs in controlling guard cell expression is still controversial [10-12]. The study performed on the potato *KST1* promoter [8] and the bioinformatic analysis performed on several guard-cell specific Arabidopsis promoters [10] suggest that the presence of clusters of DOF *cis*-elements, rather than their absolute number, is important to confer guard cell-specificity to a promoter region [10]. Yet, the role of DOF-binding sites in driving guard cell expression in Arabidopsis and the hypothesis of cluster organization remains to be experimentally investigated.

The guard-cell specific *AtMYB60* promoter presents several DOF clusters, making it an ideal model to test the hypothesis that DOF clusters are important for guard cell-specific expression. Moreover the *AtMYB60* expression is modulated by different environmental cues such as light, dark and drought stress [19], suggesting the presence of different *cis*-elements controlling these transcriptional responses. In this report we aimed to isolate the *cis*-elements responsible for the *AtMYB60* guard cell specific expression. We generated Arabidopsis transgenic lines carrying truncated or mutagenised *AtMYB60* promoter versions fused to the *GUS* reporter gene. Using a combination of histochemical and expression analysis we were able to identify a minimal promoter necessary and sufficient to drive guard cell specific expression. Using the same tools, we were also able to map a region required for ABA-mediated repression.

Results

In-silico analysis of the *AtMYB60* promoter

In a previous study, we demonstrated that the complete 5' and 3' *AtMYB60* intergenic genomic regions - cloned upstream and downstream of the *β-glucoronidase* (*GUS*) reporter gene, respectively - could specifically drive strong GUS activity in stomata of Arabidopsis seedlings and adult plants [19]. No GUS signals were detected in any other cell type or in tissues devoid of stomata [19].

To investigate the possible *cis*-acting elements that regulate *AtMYB60* expression, we surveyed the genomic region upstream of the *AtMYB60* translational start codon for the presence of known transcription factor binding sites using the PLACE software [24]. Our analysis produced a significant enrichment in the [A/T]AAAG motifs in the *AtMYB60* promoter compared to the average distribution of [A/T]AAAG oligos in intergenic regions throughout the Arabidopsis genome ($P < 0.01$) (Figure 1). Interestingly, these [A/T]AAAG motifs, have been shown to be involved in the regulation of guard cell expression of the potato potassium channel *KST1* gene [8]. Also, clusters of [A/T]AAAG motifs, required for the binding of DOF-type transcription factors [25], were over represented in different guard cells-specific promoters [6,10,12]. In particular, Galbiati and colleagues suggested, as guard cell-specific *cis*-element, a cluster of at least three [A/T]AAAG motifs located on the same strand within a region of 100 bp [10]. Using the criteria previously described by Galbiati and collaborators (2008), we found three of these guard cell-specific clusters in the 5' intergenic region of the *AtMYB60* gene (Figure 1), suggesting a conserved mechanism for guard cell specific expression.

Identification of the *AtMYB60* minimal promoter

To gain more insights into the *cis*-elements that regulate the *AtMYB60* expression in guard cells, we produced a set of Arabidopsis transgenic lines carrying the complete 1,307 bp 5' intergenic region upstream of the translational start codon fused to the reporter *GUS* (construct -1,307::*GUS*, Figure 2A). GUS staining analysis of 15 independent T2 lines revealed that this region contains all the *cis*-acting elements required for expression of the reporter in stomata (Figure 2B), while no GUS signals were detected in any other cell type or in tissues devoid of stomata (Additional file 1).

Next, we made a series of 5' deletions of the -1,307 bp genomic region to define the minimum sequence length required for the expression in guard cells (Figure 2A). These truncated promoters (fused to the *GUS* gene) were stably transferred to Arabidopsis and 10 to 15 independent T2 transgenic lines were analysed in detail. Deletions of the distal part of the 1,307 bp region to

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-1307 CACAAGGACACAAGGACATATGGTATGATGATATGCTTTGTTTCTCTGCTTCTCTTACTAATTTGA
-1241 AGCTGTTGGATTGATTTGTCTCTTCTTACGTTCCCTTCTTTTTTTTTTCGTTTTCTTTTGTCGTAT
-1175 AGACCAGGCAGGGGCTAGGGCCTAGTGATGGGTATTGGCCCAATACTATTGGGTTATTTGCCTGGT
-1109 TTATTATTTCGATTTTAGGTTAATTCAATTTTAAGAATACGTAGATTTGTTTGGTTTAGTTTGGTT
-1043 TGGTTGCACTAAGTTCGGTTTTACATAAATAGAATCTAACACTACTAATTGTTATACGTAAAATAC
 -977 AACAACAATAACAGATTTTTCGTTTCAATTTTCGTTTAAGAGGGTAGACATTTTGGTTTGGTTTGG
 -911 TTCATTTTTTTTTTCCCTTTCAAATTCACATCCTTCACGTAGATGACAAAATAAAGAAAAACATGA
 -845 ATGAAAGTTGTAACTTGTAAGCATCAACATGGAAATCATATCACAAAGAACACAAATCTAACTAAT
 -779 GGGTCTTTTCACATATTGGTATAATTATAAGTTGTAAGAATATTAGTTAAACAGAGGCAACGAGAG
 -713 ATGCGTGATATATGAAAAGTTGAAAACAAAAGACATGGATCTAAAGAGTCAAGCAAAATGTAATAT
 -647 CTTTTTTTCTTCTAAACTTGAGGATGTCCAAGTTGCAGTGAATGATTCCCTTTAATCATGGAGAAA
 -581 TTCAATGAAATAATTGTGTTTCTTCCCACACTTTATCTTTATTTATTTTCTTACCACAATTACAAC
 -515 TATTATCACAAAAATGTAAGTAACATAGCTTGTGACTCTTCTTCCATTTATGAGTTGATTATCACT
 -449 ATATTTATAAGTAATTACCAACGAATGTTCCAAATTAAGCAAAATATTGTAATCGATACACTATGT
 -382 ATTCATCTACAATATGTTAACGAGCTCCTTTTATGGAAATATTTCGATTGAAAAAACATTTGATGG
 -317 ATCGTTCACTAAATAAATAATCCAGTAACGTTTTCTTAAGGGAGATATACATATTCGTGTGGAGAT
 -251 CAACATATCTTCGTTAATTGACTACGCAAAATAGTTAATGGAAAAGGCAGAGTGACTCGTGAGCTT
 -185 GGCAGATCCAAAAGAGGTTGTCAAGAAAAAGCAGATTTAAAAGTTCTTCCCTCTTCTTTAAGTCAC
 -119 CCATTAATTTCACATATATGTACATACATGTTGCATTTAACTCATATACATACatattctcacatc
  -53 tataaagagagcataagactcagagagatctagaggaagagagagagagaaagATG
```

Figure 1 Nucleotide sequence of the 5'-region of the *AtMYB60* gene. Nucleotides are numbered on the left with the translational start site designated as +1. The ATG is in bold. The 5' UTR is in lower case letters. The DOF-binding sites are grey boxed, the W-box, considered in the text, is white boxed. Clusters of DOF-binding sites, as defined by Galbiati and colleagues (2008), are underlined. The CAAGTTG motif described as a putative *cis*-element for ABA repression ([16]) is dotted underlined.

position -619 (construct -619::*GUS*), -472 (-472::*GUS*), or -366 (-366::*GUS*) from the ATG codon, did not alter expression of the reporter in guard cells located on both vegetative and floral organs (Figure 2B). Further deletions (to position -262) indicated that the 262 bp proximal region was sufficient to drive expression of the reporter in stomata (Figure 2B). However, the removal of the region between -262 bp and -205 bp (construct -205::*GUS*) completely abolished GUS activity in guard cell (Figure 2B). Transgenic lines carrying the -205::*GUS* fusion did not show GUS staining in any other cell type, even after prolonged staining (up to 48 h, Figure 2B). This finding suggests that the 57 bp region located between positions -262 and -205 contains *cis*-elements essential for expression in stomatal guard cells. Based on these results, we defined the -262 bp region upstream of the ATG codon as the minimal promoter of the *AtMYB60* gene.

To thoroughly investigate quantitative differences in *GUS* expression among lines carrying different deletion: reporter constructs, we determined the relative amount of *GUS* transcript by quantitative RT-PCR (qRT-PCR). mRNA samples derived from two representative independent lines (A and B) were analysed for each construct (Figure 2C). Lines harbouring the 1,307 bp 5' intergenic region or the -619 deletion fused to the reporter, did not show any significant differences in their *GUS* transcript accumulation. Conversely, deletions to position -472 and -366 resulted in a two-fold decrease in *GUS* expression compared to the -1,307::*GUS* line, while deletion to position -262 resulted in a five-fold decrease (Figure 2C, $p < 0.01$). These results indicate that one or more sequences with function of enhancer are present in the genomic region between -619 bp and -472 bp and between -472 and -262 from the ATG of *AtMYB60*. In accordance with the results obtained from the histochemical analysis, qRT-PCR experiments did not detect significant *GUS* transcripts accumulation in lines carrying the -205::*GUS* fusion.

Site-directed mutagenesis of the *AtMYB60* minimal promoter

Promoter deletion experiments indicate that the *AtMYB60* minimal promoter region (construct -262::*GUS*) contains all the *cis*-acting elements required to sustain expression of a reporter gene in guard cells. This region encompasses the [A/T]AAAG cluster proximal to the ATG codon, which consists of four AAAAG DOF-binding sites (Figures 1 and 3A). In addition, the PLACE software identified in this region a single W-box, corresponding to the binding site of WRKY transcription factors [26], located upstream of the [A/T]

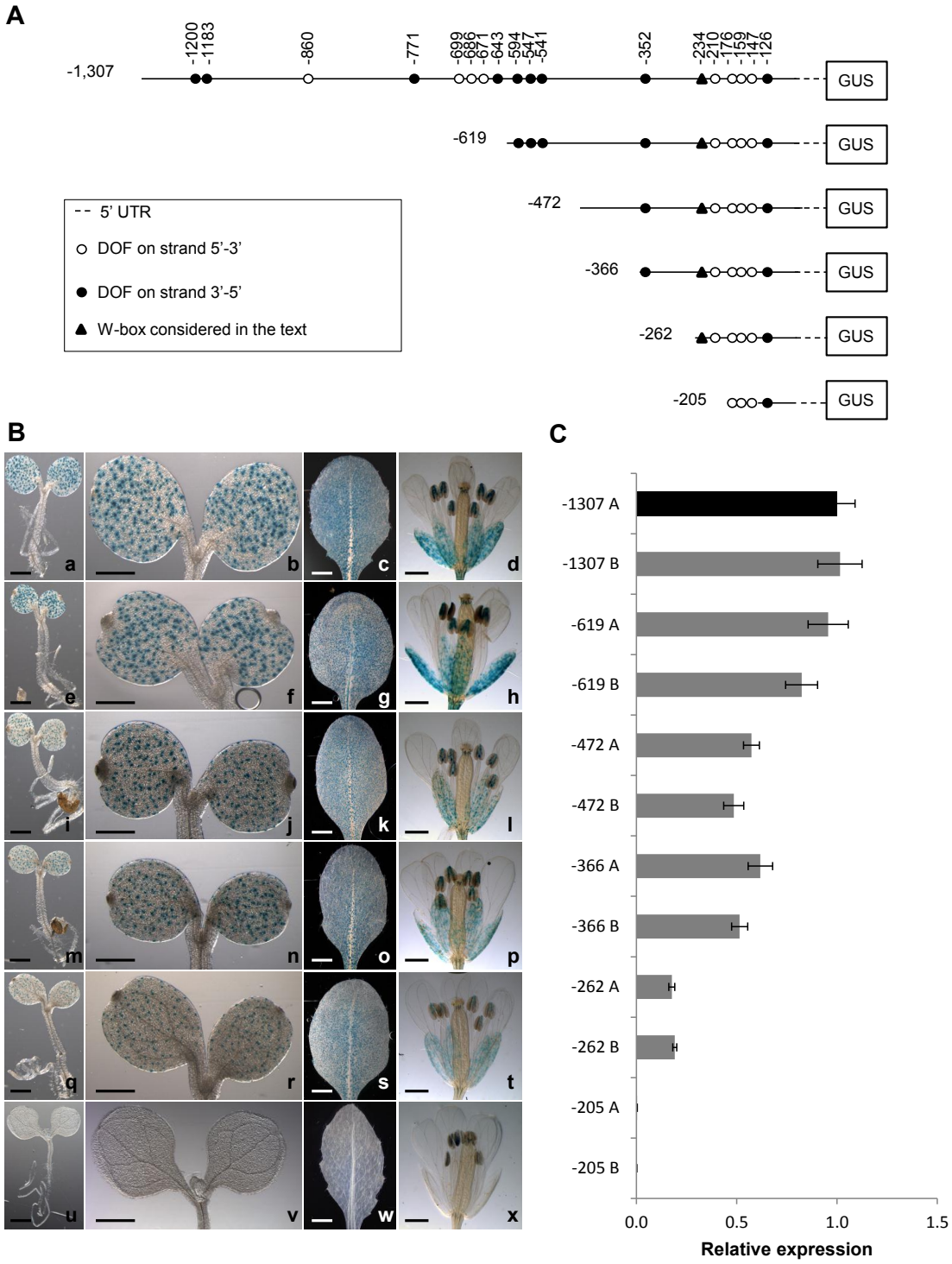

Figure 2 Deletion analysis of the *AtMYB60* upstream region. A, Schematic diagrams of different deletions of *AtMYB60* upstream region fused to the *GUS* reporter gene. The positions of the different DOF-binding sites and of the W-box, described in the text, are shown. B, Histochemical assay for GUS activity in seedlings, rosette leaves and flowers of plants transformed with -1,307::*GUS* (a-d), -619::*GUS* (e-h), -472::*GUS* (i-l), -366::*GUS* (m-p), -262::*GUS* (q-t) and -205::*GUS* (u-x) constructs. The analysis of independent lines harbouring the same construct showed identical patterns of GUS staining. Samples were incubated in the staining solution for 16 hours for all the lines, with the exception of line -205::*GUS*, for which the staining was prolonged to 48 hours. Scale bars represent 1 mm. C, Relative expression level of the *GUS* reporter gene in the different transgenic lines harbouring the -1,307::*GUS* (-1,307 A and B), -619::*GUS* (-619 A and B), -472::*GUS*, -366::*GUS* (-366 A and B), -262::*GUS* (-262 A and B) or -205::*GUS* (-205 A and B) constructs. Two lines for each construct were analysed by Real Time RT-PCR. The transcript amount in the line -1,307 A was arbitrarily set to 1 (black column) and used to normalize the relative expression levels in each line. The *ACTIN2* gene (At3g18780) was used as a control.

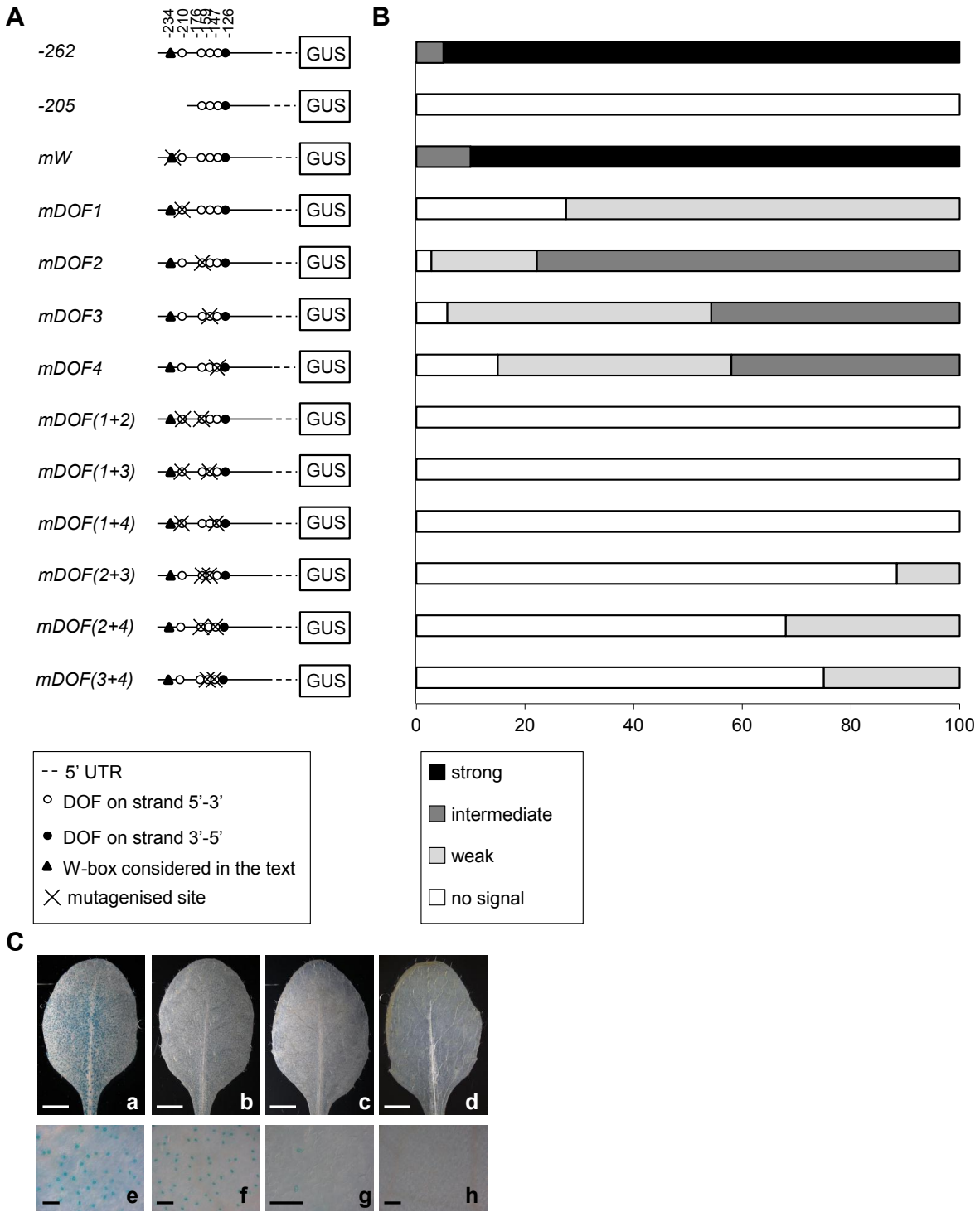

Figure 3 Role of DOF-binding sites in the minimal promoter in driving GUS activity in guard cells. A, Schematic diagrams of constructs -262::*GUS*, -205::*GUS* and of constructs containing mutagenised version of the minimal promoter in different DOF-binding sites and in the W-box at position -234. B, Percentage of lines for each construct showing strong (column segment in black), intermediate (in dark grey), weak (light grey) or no signal (white). C, A leaf from a line harbouring the -262::*GUS* construct (a and a particular in e), shown as an example of strong GUS activity. In the following pictures examples of different lines harbouring the *mDOF3*::*GUS* construct showing respectively an intermediate (b and f), a weak (c and g) and no GUS activity (d and h). Scale bars represent 1 mm (a-d) or 0.1 mm (e-h).

AAAG cluster (Figure 3A). To address the functional significance of the individual *cis*-elements present in the *AtMYB60* minimal promoter, we evaluated the effects of targeted nucleotide substitutions on *GUS* expression (Figure 3A). Mutated versions of the minimal promoter were generated by PCR and fused to *GUS* and at least 30 T2 independent transgenic lines for each mutated promoter::*GUS* combination were visually scored and classified to reflect their relative guard-cell specific GUS staining. A representative example of each category is provided in Figure 3C.

We initially tested the role of the single W-box *cis*-element, by replacing the consensus sequence TTGAC, with the non-functional TTGAA motif [27]. Lines carrying the mutated W-box (*mW::GUS*) showed similar levels of *GUS* expression to the wild-type promoter, indicating that W-box does not contribute to mediate gene expression in guard cells (Figure 3B). Next, we produced mutant promoters in which single DOF motifs within the [A/T]AAAG cluster were converted to the unrelated CGCGA sequence. Inactivation of the most distal AAAAG site relative to the ATG (hereinafter referred to as *DOF1*) resulted in a dramatic decrease of *GUS* expression (*mDOF1*::*GUS* construct, Figure 4B). 30% of the lines carrying the *mDOF1*::*GUS* construct did not show *GUS* expression, whereas the remaining 70% only showed weak staining, thus indicating a crucial role for *DOF1* in regulating *AtMYB60* expression in guard cells (Figure 3B). Mutations of the second, third or fourth most proximal AAAAG site (hereinafter referred to as *DOF2*, *DOF3* and *DOF4*, respectively), resulted in a reduced *GUS* expression, although to a lesser extent than the one in the *DOF1* (Figure 4B, m*DOF2::GUS*, *mDOF3::GUS* and *mDOF4::GUS* plants). In particular, none of the 30 *mDOF2::GUS* transgenic lines displayed strong expression of the reporter, nearly 70% showed intermediate expression, 25% showed weak expression and the remaining 5% did not show any GUS staining (Figure 3B). A comparable distribution among strong, intermediate and weak lines was obtained from the analysis of the *mDOF3::GUS* and *mDOF4::GUS* plants (Figure 3B).

To establish whether DOF-binding sites could exert additive roles in mediating gene expression in stomata we produced a second series of promoters, in which two AAAAG motifs were mutated simultaneously. Mutations of *DOF1* and *DOF2* (*mDOF(1+2)::GUS*), *DOF1* and *DOF3* (*mDOF(1+3)::GUS*) or *DOF1* and *DOF4* (*mDOF(1+4)::GUS*) completely inactivated the minimal promoter, as *GUS* expression was abolished in all the *mDOF(1+2)::GUS*, *mDOF(1+3)::GUS* and *mDOF(1+4)::GUS* lines analysed (Figure 3B). Interestingly, the concurrent mutation of *DOF2* and *DOF3* (*mDOF(2+3)::GUS*) resulted in a strong, but yet not complete, inactivation of the promoter activity in guard cells, as 15% of the *mDOF(2+3)::GUS* lines displayed weak expression of the reporter in stomata. Likewise, concomitant inactivation of either *DOF2* and *DOF4*, or *DOF3* and *DOF4* did not completely eliminate *GUS* expression in guard cell (Figure 3B). Taken together, these results indicate that the putative [A/T]AAAG DOF-binding sites located in the *AtMYB60* promoter are necessary to mediate its expression in guard cells.

A single DOF cluster is sufficient to drive low expression in guard cell

Our deletion analysis of the *AtMYB60* promoter indicates that the 57 bp region between positions -262 and -205 is essential for gene expression in stomatal guard cells (Figure 2). This region contains the *DOF1 cis*-element required for guard cell expression as shown by mutagenesis analysis results (Figure 3). To establish whether this 57 bp region was sufficient to activate expression in guard cells, we fused one (1x::*GUS* construct), two (2x::*GUS*) and four tandem copies (4x::*GUS*) of the 57 bp fragment to the minimal *CaMV35S* promoter [28] upstream of the *GUS* reporter gene (Figure 4A), effectively reconstructing an artificial DOF cluster containing one, two or four copies of the *DOF1* element. However, we did not observe GUS activity in any of the 30 independent stable transformants produced for each construct, even after prolonged staining (data not shown). These data were confirmed by qRT-PCR analysis of independent lines carrying the 4x::*GUS* fusion (Figure 4B), indicating that the multimerisation of the *DOF1* site *per se* is not sufficient to drive gene expression in guard cell. This might derive from an inappropriate organization and/or spatial distribution of the different DOF elements in the context of the minimal promoter. To test this hypothesis we made two 3' deletions of the *AtMYB60* minimal promoter: the -148-3'::*GUS* and -137-3'::*GUS* constructs containing the first three and four DOF-binding sites respectively of the most proximal cluster fused upstream of the minimal *CaMV35S* promoter (Figure 4B). Our initial histochemical analysis did not reveal any GUS positive lines (data not shown). To substantiate this result we also performed a qRT-PCR analysis on fifteen independent lines for each construct. Interestingly, eight lines out of fifteen showed a low but significant *GUS* transcript accumulation compared to the full length minimal promoter (Figure 4B). These results suggest that the presence of the cluster containing three or four DOF-binding sites is sufficient to drive GUS activity in guard cells, even though at a very low level. This finding implies that other *cis*-elements present downstream of position -137 are required for the full functionality of the minimal promoter.

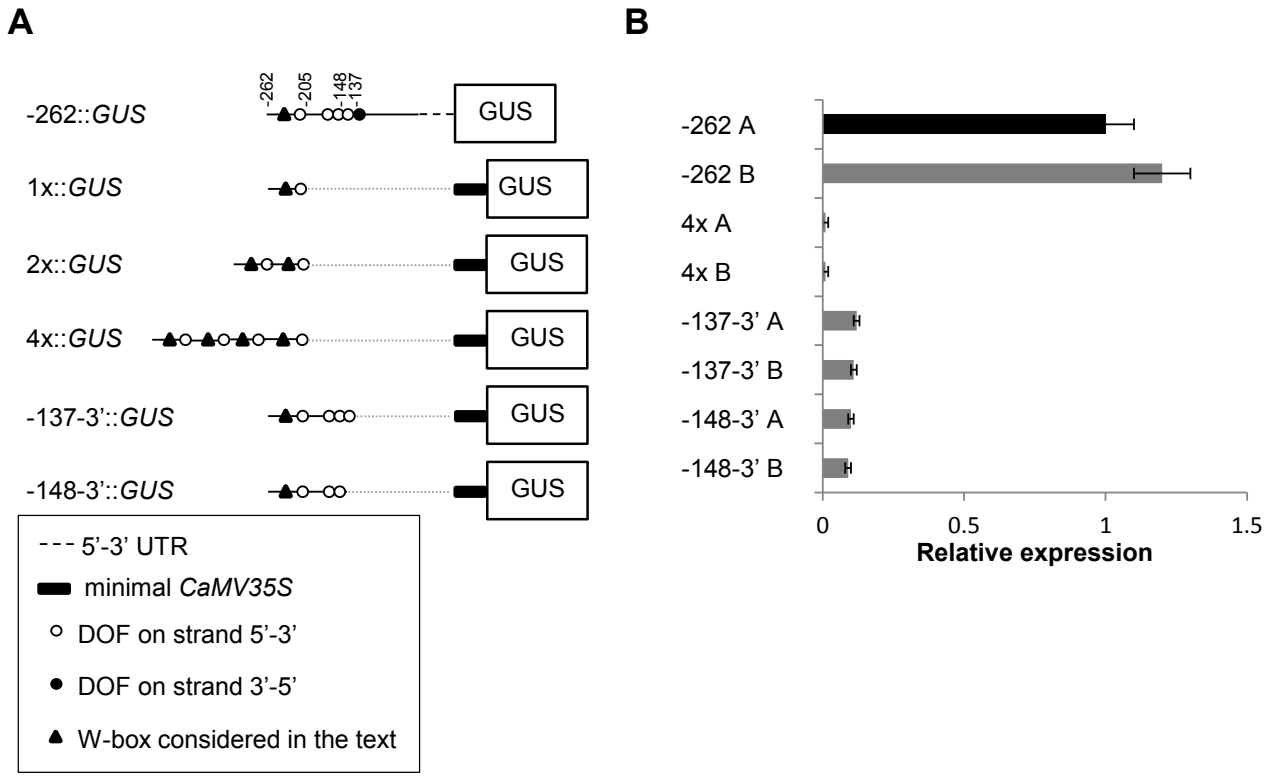

Figure 4 Oligomerisation of the 57 bp sequence and 3' deletions of the *AtMYB60* minimal promoter. A, Schematic diagrams of the constructs. In the 4x::*GUS* construct the fragment of 57 bp between -262 and -205 in tandem array of four copies was fused to the minimal promoter CaMV 35S (min 35S in the scheme, portion between -46 and +1) upstream of the *GUS* reporter gene. In the constructs -137-3'::*GUS* and -148-3'::*GUS*, 3' deleted versions of the *AtMYB60* minimal promoter were fused to the same portion of the CaMV 35S. (B) Relative expression level of the *GUS* reporter gene in the different transgenic lines harbouring the constructs. Two lines for each construct were analysed by Real Time RT-PCR. The transcript amount in the line -1307 A was arbitrarily set to 1 (black column) and used to normalize the relative expression levels in each line. The *ACTIN2* gene was used as a control. The symbols are the same described in Figure 2. The dotted lines indicate the regions deleted in *AtMYB60* minimal promoter sequences.

The guard cell-related CDF1, CDF2, CDF3 and CDF5 DOF-type transcription factors do not regulate *AtMYB60* expression in stomata

Target mutagenesis experiments of the *AtMYB60* promoter demonstrated that [A/T]AAAG DNA consensus motifs are essential *cis*-acting elements in the regulation of *AtMYB60* expression in guard cells. Consequently, their cognate DOF proteins represent the most likely candidates as *trans*-acting factors. As the Arabidopsis genome contains 36 DOF-coding genes [23], candidate DOF transcription factors involved in the regulation of *AtMYB60* expression should fulfil two criteria: they should be expressed in guard cells and the loss of their gene function should abolish or significantly down-regulate the expression of *AtMYB60* in this cell type.

The *CYCLING DOF FACTOR 1* (*CDF1*, *At5g62430*) gene, involved in the regulation of photoperiodic flowering, has been shown to be highly expressed in the vascular tissue and guard cells [29]. We thus investigated the expression of the *AtMYB60* gene in the loss-of-function *cdf1-R* allele. As shown in Additional file 2 we did not detect significant differences in the accumulation of *AtMYB60* transcripts in homozygous *cdf1-R* plants compared with the wild type.

It is important to note that in photoperiodic flowering, CDF1 acts redundantly with three other DOF proteins, namely CDF2 (At5g39660), CDF3 (At3g47500) and CDF5 (At1g69570) [30], belonging to the same phylogenetic group II [31]. Similarly to *CDF1*, promoter::*GUS* analyses revealed that *CDF2*, *CDF3* and *CDF5* are strongly expressed in guard cells.

We thus analysed the expression of *AtMYB60* in single, double, triple and quadruple *cdf* mutants to determine the possible role of these additional candidate CDF proteins. As for *cdf1-R* mutant, the level of expression of *AtMYB60* was not significantly reduced in the *cdf2-1*, *cdf3-1* and *cdf5-1* single mutants (Additional file 2). Likewise, *AtMYB60* expression was not altered in any of the double, triple or quadruple mutant combinations, indicating that, despite their expression in guard

cells, these four CDF proteins are not *trans*-regulators of *AtMYB60* expression in stomata (Additional file 2).

Identification of a promoter region that negatively responds to ABA

We previously reported that transcript accumulation of the *AtMYB60* gene is rapidly down-regulated by exogenous applications of the hormone ABA, which plays a fundamental role in regulating gene expression in response to drought stress [19]. To identify the promoter region responsible for the ABA-mediated *AtMYB60* down-regulation, we applied ABA to the previously described transgenic lines harbouring serial deletions of the *AtMYB60* promoter (Figure 2). Quantitative RT-PCR analysis revealed a similar decrease in *GUS* transcript levels in transgenic lines carrying the full length as well as the -619, -472 and -366::*GUS* fusions (Figure 5). The kinetic of down-regulation of the *GUS* transcript was comparable to the one observed for the endogenous gene *AtMYB60* [19], indicating that -619, -472 and -366 promoters maintain the sequences responsible for transcriptional down-regulation by ABA. Also, these results suggest that the CAAGTTG motif, present in the *AtMYB60* promoter between -619 and -613 (dotted underlined in Figure 1), and recently described as over-represented in ABA-repressed genes [16], does not play a significant role in the ABA-dependent repression of *AtMYB60* expression. Rather, qRT-PCR experiments performed on different independent lines carrying the -246::*GUS* construct showed that the minimal promoter sequence lacks the region responsible for negative regulation by ABA, as these lines did not show changes in *GUS* expression in response to the hormone as shown in Figure 5.

Taken together these data indicate that, although the minimal promoter maintains the *cis*-elements necessary for guard cell expression, it lacks the motifs that mediate the negative regulation by ABA, becoming ABA-insensitive. We can thus conclude that the region between -366 and -262 contains elements necessary for ABA down-regulation.

Discussion

Very few guard cell-specific promoters have been described to date [3,10,14,19,20]. Independent studies demonstrated that the *AtMYB60* promoter can be considered guard cell-specific, being sufficient to drive expression of reporter genes specifically in guard cells [19,21]. Moreover this promoter has also been used to complement a mutant phenotype specifically in guard cells [21], and to investigate subcellular localization exclusively in guard cells [22]. In this study we identified the *AtMYB60* minimal promoter that is necessary and sufficient to drive guard cell-specific expression.

DOF-binding sites are required for *AtMYB60* guard-cells expression

Our *in silico* analysis identified three DOF site clusters (Figure 1). Initial deletion studies revealed a prominent role for the most proximal DOF cluster (relative to the

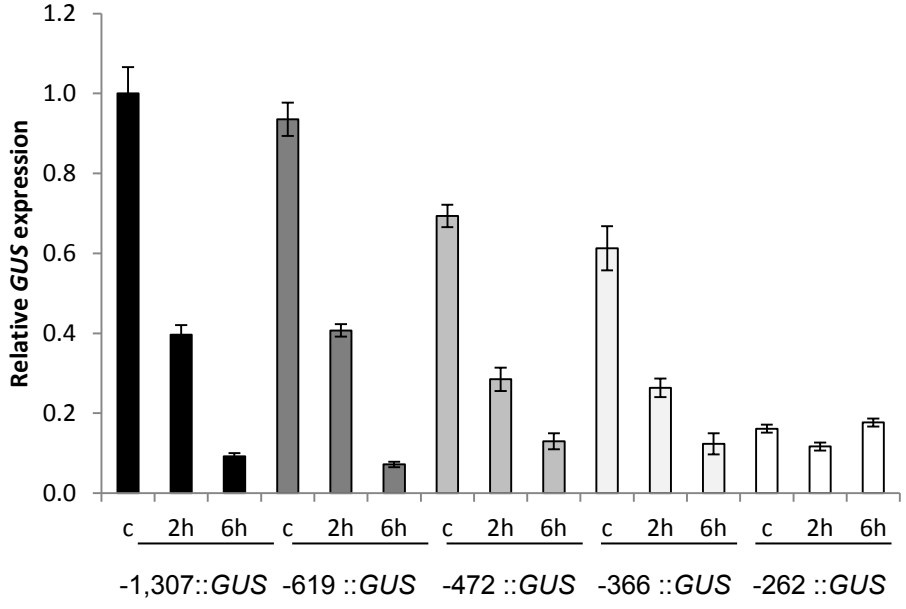

Figure 5 Expression of the *GUS* gene in different transgenic lines in response to ABA treatment. Two lines for each construct shown in Figure 2 were analysed by Real Time RT-PCR. c represent the control samples. The transcript amount in the sample -1307 A control was arbitrarily set to 1 and used to normalize the relative expression levels in each line. The *ACTIN2* gene was used as a control.

ATG start codon). Site-directed mutagenesis showed that the distal most DOF-binding site (*DOF1* at position -210, Figure 3) plays a major role in driving guard cell expression compared to other DOF motifs of the same cluster (*DOF2* at position -176, *DOF3* at -159 and *DOF4* at -147, Figure 3). These other DOF elements play partially additive roles, as clearly demonstrated by the combined mutagenesis of these sites and *DOF1* site which resulted in a drastically reduced GUS activity (Figure 3). DOF-binding sites are thus key determinants in mediating guard cell expression, in accordance with the DOF cluster hypothesis we previously formulated [10]. A suggestion for a similar involvement of DOF *cis*-elements in Arabidopsis derives from the work of Gardner and colleagues (2009) that identified DOF motifs in a region controlling guard cell expression. Other authors identified a region enriched in DOF-binding sites in the guard cell-specific pGC1 promoter, although the mutation of a single DOF site did not impair promoter activity [14]. Interestingly, a DOF cluster organization is present in the promoter of the grape *VvMYB60* gene, a putative ortholog of *AtMYB60*, indicating a conservation of the cluster structure during the evolution among *AtMYB60* orthologs [32]. The results reported by Plesch and colleagues (2001) on the DOF motif organisation in the potato *KST1* promoter highlight a more general evolutionary conservation of this module in the control of guard cell-specific activity of promoters.

Although we cannot rule out the possibility that other unknown transcription factors might interact with those same *cis*-elements, DOF factors represent likely candidates as *AtMYB60* regulators. The most parsimonious hypothesis resulting from combining our results indicates that DOF proteins act as positive regulators of *AtMYB60*. The potato StDOF1 protein has been shown to bind *in vitro* to the guard cell specific promoter of *KST1* [8], while no data are available for any Arabidopsis DOF proteins. Among the Arabidopsis *DOF* genes, *CDF1*, *CDF2*, *CDF3*, and *CDF5* (CDFs) are expressed in guard cells [29]. However, singles and multiple *cdf* mutants show a wild-type pattern of *AtMYB60* expression, ruling out their involvement in *AtMYB60* regulation (Additional file 2). The majority of Arabidopsis *DOF* genes are expressed in guard cells [33,34] and may thus act redundantly, as already demonstrated among members of this family [30]. All these aspects do not facilitate the identification of obvious candidates as *AtMYB60* regulators. We are trying to identify the *DOF* genes involved in the regulation of *AtMYB60* by analysis of its expression in mutants of genes preferentially expressed in the guard cells (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi[33]).

Multiple *cis*-elements participate to enhance *AtMYB60* guard-cells expression

Transcriptional *GUS* fusions, harbouring different deletions of the 5' intergenic region to position -262 from the ATG, conferred GUS activity exclusively in guard cells (Figures 2 and Additional file 1). The activity of these promoter regions is in apparent discrepancy with the detection of *AtMYB60* gene expression in seeds, as revealed by available microarray analysis data [33,34] and in roots, as recently reported [35]. One hypothesis to explain this incongruity could be the presence of other regulatory regions present outside the complete 5' and 3' intergenic regions flanking the *AtMYB60* coding sequence. Intron sequences, for example, may be involved in such a regulation, as previously demonstrated for different plant genes ([36] and references herein).

While guard-cell specific expression was invariably maintained by functional *AtMYB60* promoter variants, the levels of expression varied considerably. In addition to DOF-binding sites, other *cis*-elements are required to boost the *AtMYB60* expression. Indeed, an artificial *DOF1* binding site repeated in single or multiple copies could not drive guard cell expression (Figure 4A). The incorporation of the entire proximal DOF cluster (e.g. -137-3'::*GUS*) resulted in a small but significant guard cell transcriptional activity. Thus, other *cis*-elements downstream of position -137 are required for full activity of the minimal promoter. It is known that *cis*-elements other than DOF-binding sites are involved in the regulation of guard cell expression. In the case of the guard cell-specific *AtPDR3* gene no [A/T]AAAG clusters were identified in a 1000-bp region upstream of the ATG codon, suggesting the presence of other regulatory units [10].

Modular organization of the *AtMYB60* promoter

In this study we also investigated the regulation of the *AtMYB60* promoter activity in response to ABA. ABA treatments induce global changes in gene expression in Arabidopsis [16,37-40]. Transcriptomic analyses revealed extensive regulation of gene expression by ABA also in guard cells [13,14,16]. While *cis*-elements that positively regulate the response to ABA have been functionally characterised (for a review, see [41]), those that negatively regulate the response to ABA are largely unknown. A CAA[G/C]TTG motif has been shown to be over-represented in ABA-repressed gene promoters and thus proposed for such a role [16,39]. The *AtMYB60* promoter contains one CAAGTTG motif between -619 and -613 from the ATG, yet our results do not support its proposed role as negative regulator of ABA response. Conversely, a region between positions -366 to -262 contained the entire requirement for the

ABA-mediated repression Figures 5 and 6. It has been proposed that evolution may have favoured the differentiation of mechanisms for ABA down-regulation rather than up-regulation, rendering more difficult for any ABA-repression motif to achieve statistical significance [16]. Our data may provide a valuable model system to clarify the mechanism mediating ABA repression.

Our data suggests a modular organization for the *AtMYB60* promoter as summarised in Figure 6. Through a serial deletion analysis, we defined the *AtMYB60* minimal promoter, sufficient to induce guard cell-specific activity (construct -262::*GUS*, Figure 2). A 57 bp region, located between position -262 and position -205, is necessary to confer GUS activity in guard cells (Figure 2A). We also identified two regions that enhance the expression of the *GUS* gene between -619 bp and -472 bp and between -472 and -262 (Figure 2B and 2C). Besides providing pieces of evidence for such modular organization, our work indicates that the different portions of the *AtMYB60* promoter may prove useful for manipulating gene expression in guard cells, with the possibility to obtain different level of expression. Moreover, the minimal promoter (whose activity is not influenced by ABA) can be used for ABA-independent expression of target genes in guard cells

Interestingly, both the full length and the minimal promoters maintain their guard cell-specific activity in heterologous systems, such as the crop species tomato and tobacco (Francia, personal communication), thus indicating the conservation of this cell-specific regulatory mechanism among different plant species. Moreover, preliminary results suggest that the *AtMYB60* minimal promoter can be combined with other *cis*-regulatory modules to produce functional guard cell-specific chimeric promoters (Francia, personal communication). As a whole our data demonstrate that both the full length and the minimal *AtMYB60* promoters provide a valuable tool to manipulate gene expression specifically in guard cells, both for physiological studies and downstream biotechnological applications.

Conclusions

Our work provides strong evidence for the involvement of [A/T]AAAG elements in the regulation of the *AtMYB60* expression, illustrating their functional cluster organization. Future work will concentrate on the analysis of candidate DOF transcription factors that control this mechanism. Finally we identify a region of the *AtMYB60* promoter required for the negative regulation by ABA, offering the possibility to discover novel *cis*-elements for this kind of regulation.

Methods

Plant Material

All plant material described was in the Col-0 accession. The *cdf1-R* line (35S::CDF1-RNAi #23) was kindly provided by Takato Imaizumi [29]. The *cdf2-1*, *cdf3-1* and *cdf5-1* null alleles are T-DNA insertion line. Single, double, triple and quadruple *cdf* mutants have been previously described [30].

Construction of *AtMYB60* promoter::*GUS* fusions

5'-deletions of the 5' intergenic genomic region upstream of the *AtMYB60* gene were generated by PCR amplification from plasmid p1.3-2.2::*GUS*, previously described [19], using different forward primers and a single reverse primer. Forward and reverse primers incorporated a *Hind*III and a *Bam*HI, respectively. The PCR fragments were cloned into the pCR4-TOPO vector (Invitrogen Corporation, Carlsbad, CA), cut with *Hin*dIII and *Bam*HI and ligated upstream of the *uidA* coding sequence in the pBI101.3 binary vector (Clontech, Palo Alto, CA, USA). The resulting plasmids were renamed -1307::*GUS*, -619::*GUS*, -472::*GUS*, -366::*GUS*, -262::*GUS* and -205::*GUS* (Figure 2).

Chimeric promoters containing different 3'-deleted fragments of the *AtMYB60* minimal promoter and 46-

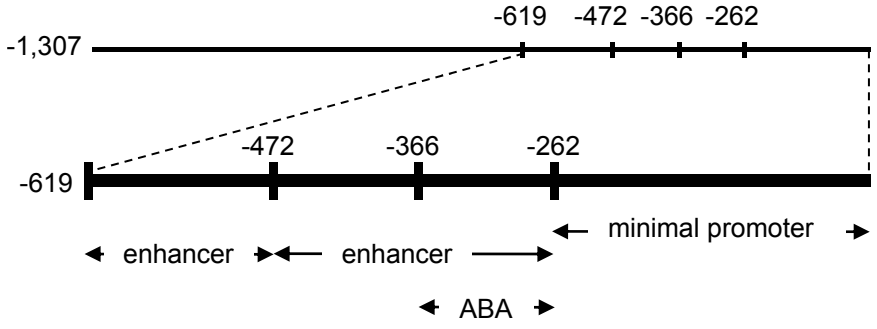

Figure 6 Modular organization of the *AtMYB60* promoter. Different portions of the *AtMYB60* promoter defined through deletion analysis are shown. ABA indicates the region responsible for the negative regulation by ABA treatment.

bp CaMV 35S promoter were produced by amplifying the sequence of the CaMV 35S promoter from -46 to +1 [28] from plasmid pBI121 (Clontech, Palo Alto, CA, USA), using the forward primer 35SXba containing a *Xba*I site and the reverse primer 35SBam with a *Bam*HI site. The PCR product was cloned into the pCR4-TOPO vector and the *Xba*I-*Bam*HI fragment was cloned into the pBI101.3 vector (renamed 35Smin-pBI101.3). The regions from -262 to -137 and from -262 to -148 of the *AtMYB60* minimal promoter were amplified by PCR from plasmid p1.3-2.2::*GUS*, using the reverse primers p60R6 and p60R7 incorporating a *Xba*I site and a single forward primer p60F3 with the *Hin*dIII site. The corresponding PCR products were cloned into the pCR4-TOPO vector and the *Hin*dIII-*Xba*I fragments were cloned into the 35Smin-pBI101.3 vector to give the -137-3'::*GUS* and -148-3'::*GUS* vectors, respectively (Figure 3).

Chimeric promoters containing different copies of the region between -262 and -205 of the *AtMYB60* promoter were obtained by synthesising one copy of this sequence, using the forward primer p60F3 with a *Hin*dIII site and the reverse primer p60R3 with an *Xba*I site. The resulting PCR product was cloned into the pCR4-TOPO vector and the *Hin*dIII-*Xba*I fragment was ligated into the 35Smin-pBI101.3 vector (construct 1x::*GUS*). A second copy of this region was generated using the primers p60F3 and p60R5b, both incorporating a *Hin*dIII site; the fragment *Hin*dIII-*Hin*dIII was cloned into the construct 1x::*GUS*, generating the construct 2x::*GUS*. This plasmid was used as a template to generate two other copies of the sequence from -262 to -205 using the primers p60F11 and p60R3 incorporating an *Xba*I site. The fragment *Xba*I-*Xba*I was cloned into the plasmid 2x::*GUS*, to generate the construct 4x::*GUS*. All the oligonucleotide sequences are reported in Table 1. PCR products were sequenced and the correct orientation of the fragment into the final vector was verified by restriction.

Table 1 Sequence of oligonucleotides used in this study

Name	Sequence
p60F1	*AAGCTT*CACAAGGACACAAGGACA
p60 F2b	*AAGCTT*CAAGTTGCAGTGAATGA
p60F8b	*AAGCTT*TAACGAGCTCCTTTTATGG
p60F9	*AAGCTT*CCATTTATGAGTTGATTATCA
p60F3	*AAGCTT*CGTGTGGAGATCAACAT
p60F5	*AAGCTT*GCAGAGTGACTCGTGA
p60R5	TCTC*GGATCC*TCTAGATCTCTCTG
p60R6	*TCTAGA*GAAGAACTTTTAAATCTGC
p60R7	*TCTAGA*AAATCTGCTTTTTCTTGAC
p60R5b	*AAGCTT*CTTTTCCATTAACTATTTTG
p60F11	*TCTAGA*CGTGTGGAGATCAACAT
p60R3	*TCTAGA*CTTTTCCATTAACTATTTTG
35SXba	*TCTAGA*CAAGACCCTTCCTC
35SBam	*GGATCC*TCCTCTCCAAATGA
mp60DOF1F1	AGTTAATGGcgcgaGCAGAGTGACTCGTGA
mp60DOF2F1	TGGCAGATCCcgcgaAGGTTGTCAAGAAAA
mp60DOF3F2	TGTCAAGAcgcgaCAGATTTAAAAGTTCTT
mp60DOF4F2	CAAGAAAAAGCAGATTTcgcgaTTCTTC
mp60WRKYF1	*AAGCTT*CGTGTGGAGATCAACATATCTTCGTTAATTGAaTAC GCAAAATA
GUSRTF1	TACGGCAAAGTGTGGGTCAATAATCA
GUSRTR1	CAGGTGTTCGGCGTGGTGTAGAG
ATACT2F	TGCTTCTCCATTTGTTTGTTTC
ATACT2R	GGCATCAATTCGATCACTCA
qRT-MYB60-F	CATGAAGATGGTGATCATGAGG
qRT-MYB60-R	TTCCATTTGACCCCCAGTAG
PP2a-F	CAGCAACGAATTGTGTTTGG
PP2a-R	AAATACGCCCAACGAACAAA

Italic and lower case letters indicate restriction and mutagenised sites, respectively

Site-directed mutagenesis analysis

Base mutations of the different DOF sites were generated using the megaprimer method [42]. For the mutagenised versions of the *AtMYB60* minimal promoter different megaprimers were PCR amplified from plasmid p1.3-2.2::*GUS*, using as forward primers mp60DOF1F1, mp60DOF2F1, mp60DOF3F2 and mp60DOF4F2 and the single reverse primer p60R5. The megaprimers were gel purified and used in a second PCR reaction on plasmid p1.3-2.2::*GUS* with the primer p60F3. The PCR products were cloned into pCR4-TOPO and sequenced before cloning into pBI101.3 vector using the restriction sites *Hin*dIII and *Bam*HI to generate the following constructs: *mDOF1*::*GUS*, *mDOF2*::*GUS*, *mDOF3*::*GUS*, *mDOF4*::*GUS*. To generate multiple mutagenised sites the templates for the second PCR amplification were plasmids already carrying a first mutagenised DOF site. In the case of the preparation of the construct *mW*::*GUS* the megaprimer method was not necessary, as the site to mutagenise is in a position 5' terminal into the minimal promoter and a single PCR reaction was performed with primers mp60WRKYF1 and p60R5, the PCR product was then cloned with the procedure already described.

All the oligonucleotide sequences are reported in Table 1.

Arabidopsis transformation and growth conditions

Wild-type Columbia (Col-0) plants were transformed using the *Agrobacterium tumefaciens* strain GV3101 carrying the constructs described above with the floral dip method [43]. Transformed lines were selected on kanamycin and single-insertion lines were selected for further analyses. Analyses of transgenic lines were

performed on T2 or on homozygous T3 plants grown under long-day conditions (16 hr light; 8 hr darkat 100 μmol m^{-2} sec^{-1}) at 22°C in a growth chamber. Seeds were germinated in Petri dishes containing Murashige and Skoog medium, 1% w/v sucrose and 0.8% w/v agar for seedling analysis or directly on soil for adult plant organ analysis. The ABA treatment was performed as previously described [19].

GUS activity assays and histochemical staining

For detection of GUS activity, tissues were fixed for 2 h in 90% (v/v) acetone at -20°C, incubated for 16-48 hours, at 37°C, in 0.05% (w/v) X-glucoronic acid, 0.1% (v/v) Triton X-100, and 0.5mM ferrocyanidine in 50 mM phosphate buffer (pH 7) and subsequently cleared in 70% (v/v) ethanol. Seedlings and flowers were cleared with a chloral hydrate:glycerol:water solution (8:1:2, v/v). Samples were examined using a Leica M205 FA stereomicroscope (Leica Microsystems GmbH Wetzlar, Germany) and a Zeiss Axiophot D1 microscope (Carl Zeiss MicroImaging, LLC Thornwood, New York, USA). Stereomicroscope images were recorded using the Leica LAS software version 2.8.1. Microscope images were recorded with an AxioCam MRc5 camera (Zeiss) using the AxioVision program (version 5.0).

Quantification of mRNA expression

RNA isolation, reverse transcription, qRT-PCR reactions and data analysis were performed as previously described [30]. *GUS* expression was analysed using primers GUSRT-F1 and GUSRT-R1, *ACTIN2* gene (primers ATACT2F, ATACT2R) was used as a reference for normalization. *AtMYB60* expression in different *cdf* mutants was analysed using primers qRT-MYB60-F and qRT-MYB60-R. *PP2A* gene, corresponding to *At1g13320* (primers PP2a-F and PP2a-R) was used as a reference for normalization [44]. All primer sequences are reported in Table 1.

Additional material

Additional file 1: Analysis of GUS activity in seeds at different developmental stages in 1,307::*GUS* line. A: open silique showing signal only in stomata and not in developing seeds. B: mature-green-stage seed (13 DAP). C: a 24 h imbibed seed. D: embryo isolated from a 24 h imbibed seed. The same results were obtained in all transgenic lines described in Figure 2. Scale bars represent 0.1 mm.

Additional file 2: Relative expression of the *AtMYB60* gene in the different *cdf* mutants. *cdf1-R* is an RNAi line ([29]). The other single and multiple mutants have been previously described ([30]). The *PP2a* (*At1g13320*) gene was used as a control [44].

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Author details
[1]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Milano, Italy. [2]Fondazione Filarete, Milano, Italy. [3]Max Planck Institute for Plant Breeding Research, Cologne, Germany. [4]Istituto di Biologia e Biotecnologia Agraria, CNR, Milano, Italy. [5]Dipartimento di Biologia, Università degli Studi di Milano, Milano, Italy.

Authors' contributions
EC carried out the construction of promoter-reporter plasmids, plant transformation and drafted the manuscript. EC, AA and LC did transgenic Arabidopsis analysis. FF carried out *cdf* mutant analysis. CT, MG, and GC conceived the study, participated in its design and coordination. MG, LC and FF helped to draft the manuscript. All authors read and approved the final manuscript.

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Chapter 2

Searching for DOF proteins regulating *AtMYB60* expression

Introduction

In Chapter 1 a detailed characterisation of the *AtMYB60* promoter has been reported. Through serial deletion and mutagenesis analysis the minimal promoter sufficient to confer guard cell-specific expression to *AtMYB60* gene was isolated. Bioinformatic analysis performed on the promoter region disclosed a significant enrichment in the presence of [A/T]AAAG DOF-binding sites organised in three clusters, one of them in the minimal promoter (see Figure 1 in Chapter 1). As recently reported, these motifs have been demonstrated to play a role in regulating the guard cells-specific gene expression in potato [1]. Site-directed mutagenesis of the DOF motifs identified in the most proximal cluster (relative to the ATG start codon) in the *AtMYB60* promoter revealed the same involvement in Arabidopsis as well: four DOF motifs have been identified in this specific cluster, targeted mutagenesis of each of them affected the role of the promoter in driving guard cell expression (see Figure 3 in Chapter 1). Within the four motifs, the most distal DOF1 has been resulted to play the major role as its mutagenesis drastically reduced the promoter activity. Mutagenesis of the other DOF elements (DOF2, DOF3 and DOF4 respectively) did not have such a drastic effect, obtained only with the contemporary mutagenesis of at least two of these binding sites, suggesting the hypothesis that the DOF motifs have an additive role in regulating the *AtMYB60* expression in guard cells. The DOF factors have been therefore indicated as likely candidates to be *AtMYB60* regulators; a single DOF protein could act alone or interact with other DOF proteins, as well with other transcription factors, to bind one or more DOF *cis*-elements contemporaneously to control the *AtMYB60* expression (Chapter 1).

DOF proteins are members of a major family of plant transcription factors with a highly conserved Zn finger DNA-binding domain and very divergent amino acid sequences outside the DOF domain, coinciding with the different functions played by the proteins: they are involved in light, stress and pathogen responses as well as in seed development and tissue-specific expression (reviewed by [2, 3]) [4, 5]. In Arabidopsis 36 *DOF* genes have been discovered. A phylogenetic tree categorised the encoded proteins into seven subgroups, based on common small structural motifs among different members of the family (Figure 1) [2].

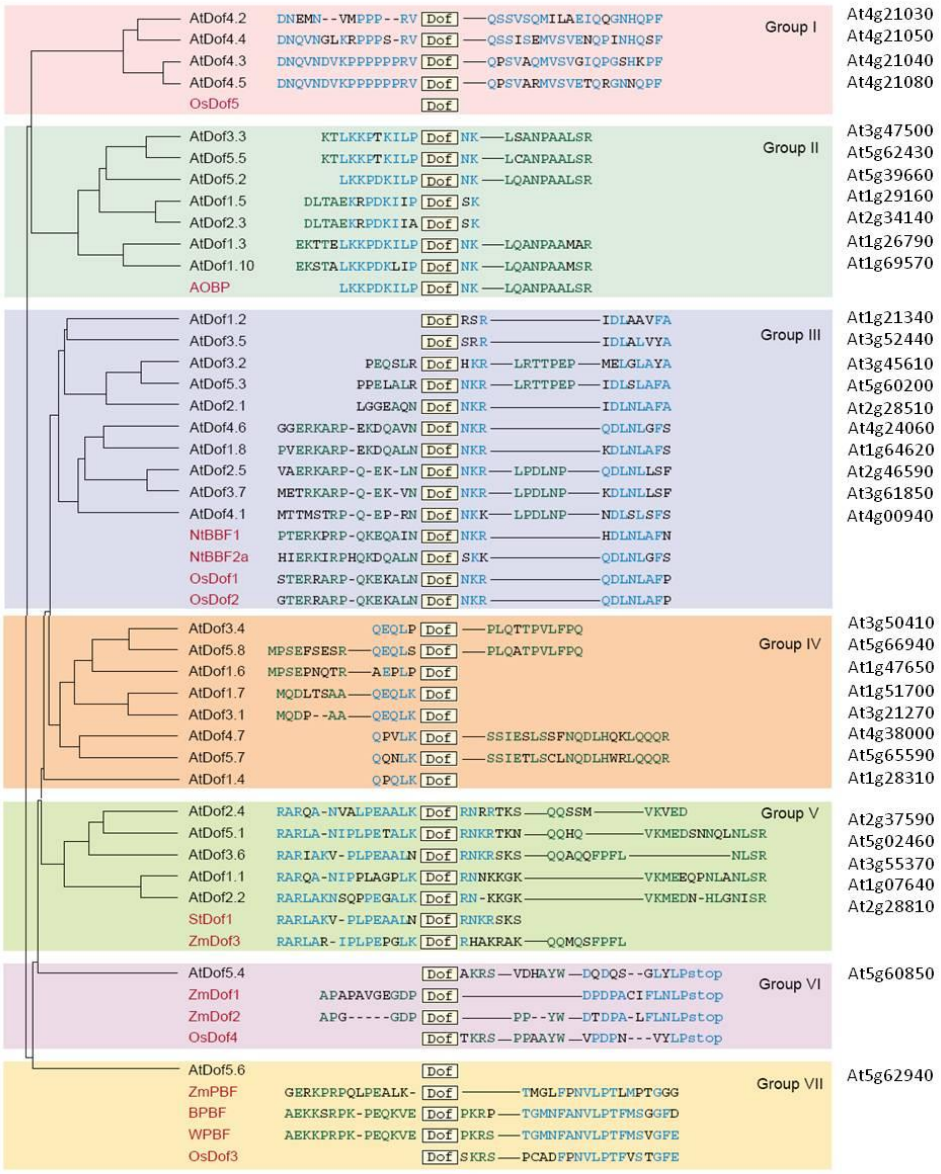

Figure 1 Dof protein relationships. The phylogenetic tree was created using full sequences of Arabidopsis Dof proteins, using the ClustalW program. *At, Arabidopsis thaliana*; B, barley; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; St, *Solanum tuberosum*; W, wheat, Zm, *Zea mays*. The signature motifs conserved among members of the same group are shown in blue. Examples of motifs conserved in only some members are shown in green. Non-homologous sequences between motifs are indicated by bars [2].

In Cominelli, 2011 [6], the effort to search for DOF proteins regulating *AtMYB60* expression has been described. *AtMYB60* is specifically expressed in guard cells and its expression is regulated by the abscisic acid (ABA), involved in the regulation of stomatal closure [7]. Therefore *DOF* genes preferentially expressed in guard cells than in mesophyll cells under normal conditions or in response to the phytohormone ABA were selected and mutants in these genes were isolated (Figure 2 and Table 1) [6].

As previously described in Chapter 1 the *CDF* genes, belonging to the group II (Figure 2), already characterised for their role in the regulation of photoperiodic flowering and highly expressed in guard cells [8], are not involved in *AtMYB60* regulation in stomata; expression analysis performed on the single and multiple *cdf* mutants did not reveal significant alterations in the *AtMYB60* expression compare to the wild-type.

A brief description of other *DOF* genes selected is presented below.

Two *DOF* genes preferentially expressed in guard cells were selected from the group IV: *At5g65590* and *At4g38000*. It has been published that the promoter sequence of the *At5g65590* gene drove GUS activity in guard cells, epidermal cells, mesophyll cells and in vascular tissues in the hypocotyl, petiole and young leaves in transgenic lines [5]. *At4g38000* when overexpressed in Arabidopsis induced floral organ abscission deficiency, as recently reported [9].

At3g55370, *At1g07640* and *At2g28810* were selected from group V. Also the previously described StDOF1 proteins from potato that *in vitro* binds to DOF motifs in the promoter of the guard cell expressed KST1 gene [1] belongs to this group (Figure 2). *At3g55370* gene is also known as *OBP3*

and transgenic lines over-expressing this gene showed a severe growth defect with altered growth development and yellowish leaves [10]. No information is available on *At2g28810* gene. Also the *At1g07640* gene, that is not preferentially expressed in guard cells, but whose induction by ABA is specific for guard cells, belongs to group V. It corresponds to the *OBP2* gene that has been implicated in the control of biosynthesis of indole glucosinolates [11], a group of secondary metabolites that function as defence substances against herbivores and micro-organisms. Constitutive and inducible overexpression of *OBP2* activates expression of *CYP83B1*, coding for a key enzyme in the biosynthesis of indole glucosinolates, while RNA interference-mediated OBP2 loss of activity leads to reduced expression of the same gene [11].

Null mutations in one or more *DOF* genes involved in the regulation of *AtMYB60* expression are expected to affect its transcript level. *AtMYB60* expression in plants harbouring homozygous mutations in the selected *DOF* genes has been investigated, unfortunately in none of them the *AtMYB60* expression level was significantly reduced compared to the wild-type (Figure 3) [6]. Concerning *At4g38000* and *At5g65590* genes, single mutants have been isolated but not yet tested.

These results support the hypothesis of an additive role of the *cis*-elements identified in the *AtMYB60* minimal promoter: different DOF proteins could have to bind more DOF motifs simultaneously to regulate the gene expression in the guard cells (Chapter 1). To test this hypothesis the different mutants previously obtained have been crossed.

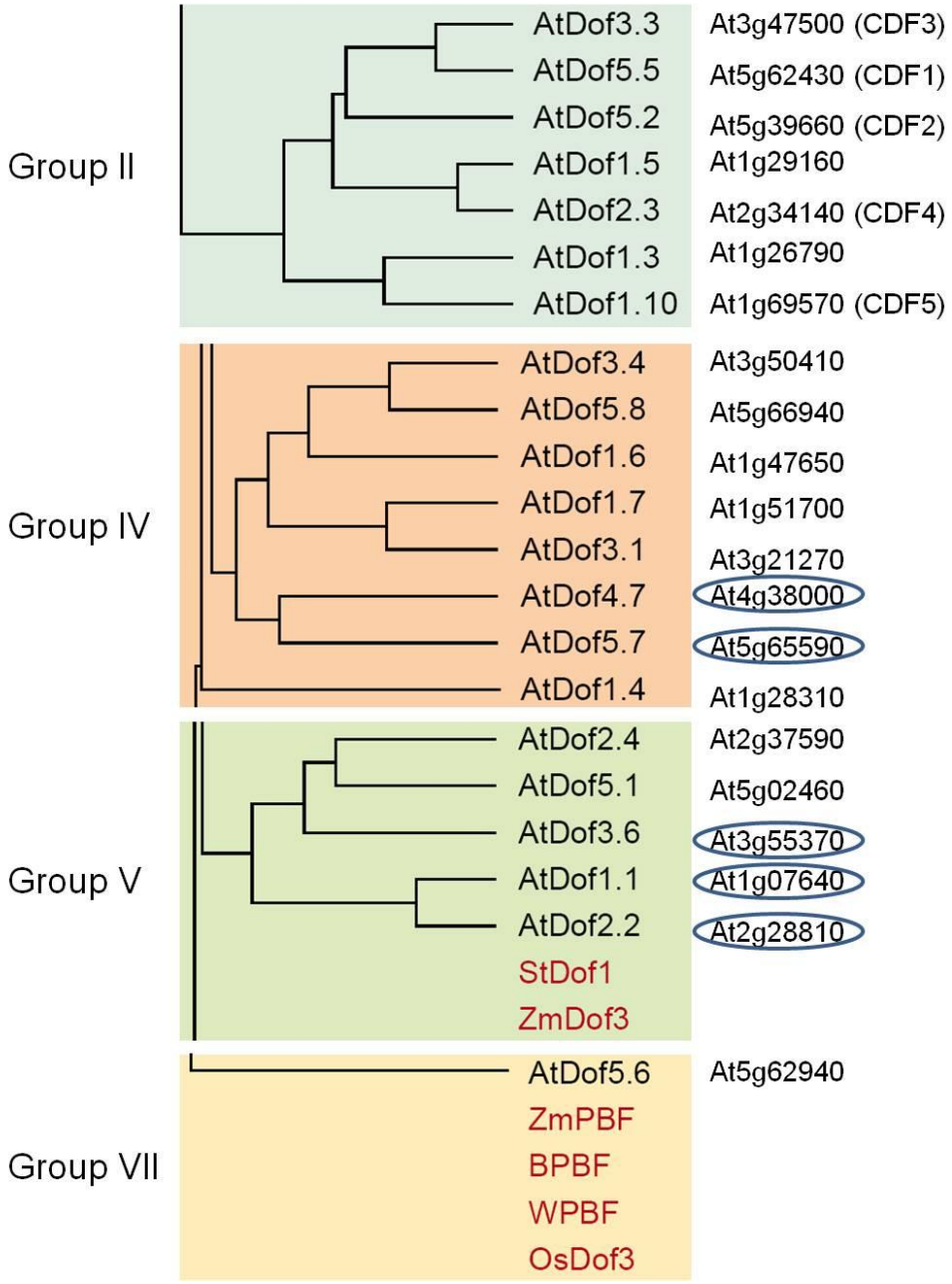

Figure 2 Position of DOF proteins considered in this study (circled) in the Arabidopsis DOF phylogenetic tree from Yanagisawa, 2002 [2].

	GC no ABA	MC no ABA	GC/MC no ABA	GC ABA	MC ABA	GC/MC ABA
At5g65590	563,8	6,0	94,8	398,8	6,0	66,9
At4g38000	241,4	115,9	2,1	239,2	98,6	2,4
At3g55370	22,8	9,0	2,5	9,7	15,4	0,6
At1g07640	56,4	47,1	1,2	82,6	42,6	1,9
At2g28810	75,6	32,3	2,3	58,3	59,0	1,0

Table 1 Expression level of selected *DOF* genes in guard cells (GC) and in mesophyll cells (MC) with (ABA) or without ABA (no ABA) and ratio between expression in guard cells and in mesophyll cells (GC/MC) with and without ABA. Members of group IV are in green while those of group V in violet. The expression patterns were generated using the Arabidopsis e-FP browser at http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi [12].

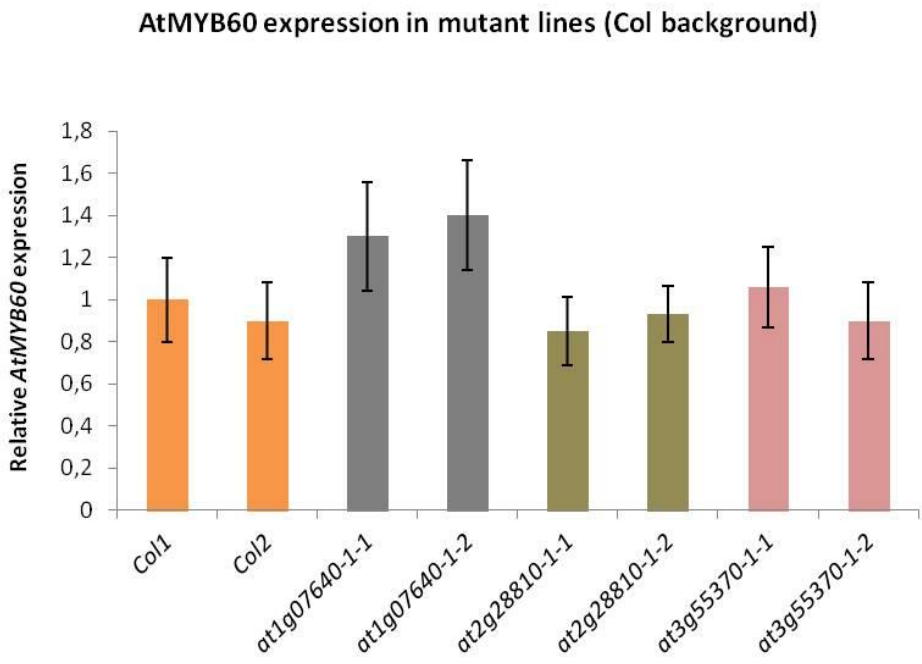

Figure 3 Relative expression level of the *AtMYB60* gene in the different mutant lines in the Columbia background. Two lines for each mutant lines were analysed by qRT-PCR. The transcript amount in the sample Col1 was arbitrarily set to 1 and served as calibrator for the relative expression levels in each line. The *ACTIN2* gene was used as a control [6]

Results and discussion

Production of *dof* double mutants

As the *AtMYB60* expression analysis was not altered in *dof* single mutants previously isolated compared to the wild–type, lines in which multiple *DOF* genes, belonging to the same phylogenetic group, are knocked-out have been produced. It could be possible in fact that the *DOF* genes under analysis encode for *AtMYB60* regulators, but they may act in a redundant way, then the mutation of only one of them does not affect the *AtMYB60* expression. For different classes of transcription factors functional redundancy of highly similar members of the family has been demonstrated, as for example into the MYB, MADS, WRKY, NAC families [13-16] but also in the DOF family as in the case of CDFs in the control of flowering time, as previously reported [17]. Another hypothesis could be also that different DOF proteins act simultaneously to regulate *AtMYB60* expression, binding more *cis*-elements at the same time.

The *DOF* genes selected are:

- *At4g38000* and *At5g65590*, belonging to the group IV (Figure 2). The insertional mutants for the two genes used for the cross are RIKEN 11-5202-1 and RIKEN 54-3788-1 respectively, from the RIKEN Arabidopsis Transposon mutants collection (Nössen ecotype) [18].
- *At3g55370*, *At1g07640* and *At2g28810*, belonging to the group V (Figure 2). The insertional mutants used for the crosses are WiscDsLox377-380K7 and WiscDsLox391E07 for *At3g55370* and *At2g28810* genes respectively, from the WiscDsLox collection (Columbia ecotype) [19]; SALK_143145 for *At1g07640* gene,

from the SIGnAL (Salk Institute Genomic Analysis Laboratory) collection (Columbia ecotype) [20].

Concerning the group IV, we crossed *at5g65590-1* with *at4g38000-1*. *at4g38000-1* is a knock out mutant whereas *at5g65590-1* line accumulates transcripts of the corresponding mutagenised gene. However in this line the transposon element is in the coding region causing a premature stop codon (data not shown). It gives origin to a truncated protein, lacking the transactivation domain, then unfunctional.

Concerning the group V, we crossed *at1g07640-1* with *at2g28810-1* and with *at3g55370-1* respectively. Finally we crossed *at2g28810-1* with *at3g55370-1*. *at1g07640-1* and *at3g55370-1* are knock out mutants whereas *at2g28810-1* line accumulates transcripts. However, also in this mutant the insertional element is in the coding region (data not shown) giving origin to a truncated unfunctional protein.

The F1 plants were then selfed and F2 population was subjected to PCR genotyping to identify homozygous double mutant plants as described in the Material and methods section (data not shown).

Analysis of *AtMYB60* gene expression in dof double mutants

The *AtMYB60* expression was analysed by qRT-PCR in different double homozygous mutants lines as well as in the corresponding single mutants used for the crosses, already isolated by Cominelli, 2011 [6]. The RNA was extracted from three plants for each mutant line and the expression of the *AtMYB60* gene in these lines was compared with the Columbia or Nössen (for the RIKEN mutants) wild-type, depending on the mutant collection background. In Figure 4A and 4B the expression data of one

sample from each line is shown. Two other biological replicates and qRT-PCR analysis gave similar results (data not shown).

Concerning the *dof* double mutants *at1g07640-1 at2g28810-1*, *at1g07640-1 at3g55370-1* and *at2g28810-1 at3g55370-1*, in the Columbia ecotype (Figure 4A), no significant differences in the *AtMYB60* gene expression level were detected compare to the wild-type. Similar results were obtained analysing the *AtMYB60* expression in the corresponding *dof* single mutants *at1g07640-1*, *at2g28810-1* and *at3g55370-1* (Figure 4A), as reported also in Cominelli, 2011 [6]. The simplest explanation of these results is that the genes under analysis encode for DOF proteins that do not act as *AtMYB60* regulators. The obtained results exclude also the possibility that these DOF proteins have an additive role to regulate *AtMYB60* expression, binding simultaneously two or more DOF motifs localised in the *AtMYB60* promoter, since also the double homozygous mutants do not show significant differences in the *AtMYB60* expression level compared to the wild-type. Finally, in order to understand if all the three DOF proteins are concurrently necessary to regulate *AtMYB60* expression, *at1g07640-1 at2g28810-1 at3g55370-1* triple mutant could be produced.

The last hypothesis to explain the results is that one of these DOF proteins interacts with another transcription factor to regulate *AtMYB60*. It has been recently published a similar situation for the RBPF rice DOF transcription factor interacts with the RISBZI basic leucine zipper [21] in the regulation of seed storage protein (SSP) genes. A protein purification assay would be helpful to reveal possible protein-protein interactions.

Surprisingly, a significant reduction in the *AtMYB60* expression has been observed in the *at5g65590-1* and *at4g38000-1* homozygous mutants, as

shown in Figure 4B. *At5g65590* and *At4g38000* are the most highly expressed selected DOF genes in the guard cells (Table 1). Moreover, according to the microarray data, *At5g65590* is the most poorly expressed in the mesophyll cells among all the DOF genes and its expression is strongly down-regulated by ABA, as the *AtMYB60* one [7]. These data, together with the results obtained by the *AtMYB60* expression analysis in the *at5g65590-1* and *at4g38000-1* mutants, make the *At5g65590* and *At4g38000* genes the major candidates to be *AtMYB60* regulators. It is interesting to note that the most significant reduction has been obtained in the double *at5g65590-1 at4g38000-1* homozygous mutant (Figure 4B) supporting this time the hypothesis of an additive role of the DOF proteins in binding the DOF *cis*-elements of the *AtMYB60* promoter. However it has to be underlined that the *AtMYB60* expression is completely abolished neither in the single nor in the double mutants analysed, suggesting the hypothesis of an involvement of other DOF proteins, or different transcription factors, in the *AtMYB60* expression control.

Further experiments will be necessary to better explain their role in controlling the *AtMYB60* expression; it will be interesting to identify which DOF *cis*-elements they bind and to clarify their additive role in binding those elements.

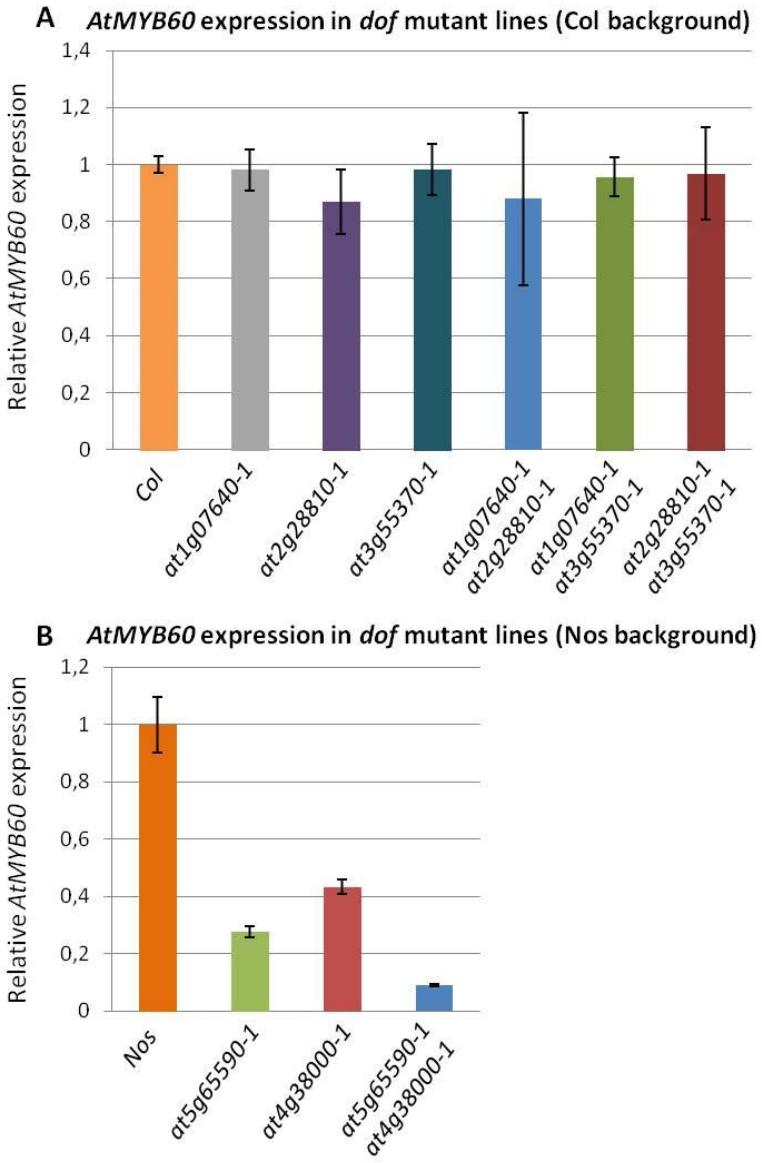

Figure 4 Relative expression level of the AtMYB60 gene in different mutant lines in the Columbia background (**A**) and in the Nössen background (**B**). Three plants for each mutant line were analysed by qRT-PCR. Here we report the results of one sample for each line. Similar results were obtained from the other two biological replicates. The transcript amount in the sample Col and Nos was arbitrarily set to 1 and served as calibrator for the relative expression levels in each line. The *ACTIN2* and *PP2AA3* genes were used as the controls.

Materials and methods

Plant material and growth conditions

The SALK_143145 (*At1g07640*), WiscDsLox377-380K7 (*At3g55370*) and WiscDsLox391E07 (*At2g28810*), obtained from the NASC European Arabidopsis Stock Center (Nottingham, UK), are in the Columbia ecotype, while the RIKEN 54-3788-1 (*At5g65590*) and RIKEN 11-5202-1 (*At4g38000*) lines, obtained from the RIKEN Stock Center (Japan), are in the Nössen ecotype.

The Arabidopsis seedlings were grown in soil at 22°C in a greenhouse with a 16-h-light/8-h-dark cycle at a fluorescent illumination of 100 μmol m^{-2} s^{-1} and 60% humidity.

Production of double mutants

Immature flowers of homozygous single mutants were emasculated and manually cross-pollinated. The F1 plants heterozygous for both alleles were selfed, and the homozygous double mutants were identified by genotyping in the F2 populations.

Double mutant genotyping

DNA was extracted as previously reported [22]. Plants of the F2 populations obtained from the crosses were genotyped using for each gene two PCR reactions: one for the mutant allele with the T-DNA/transposon insertion and one for the wild-type allele. The primers used to amplify the wild-type alleles are indicated in Table 2. One of these primers for each gene were used coupled to LBa1, pEX241 and Ds5-2a primers (Table 2), designed on the insertional elements, for mutants belonging to SIGnAL, WiscDsLox and RIKEN collections, respectively.

qRT-PCR analysis

Total RNA was extracted from 3-week-old rosette leaves as previously reported [7]. For each sample, cDNA synthesis was performed using 5 μg of DNase-treated RNA with RT Superscript™ II (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time RT-PCR analysis was performed using the fluorescent intercalating dye SYBR-Green with the Cfx96TM BioRad® Real Time system according to the manufacturer's protocol, in a final volume of 20 μL, containing cDNA, 0.3-0.4 μM of each primer and 10 μL of 2X SOS Fast™ EVA-Green® Supermix (BioRad Laboratories, Hercules, CA, USA). The *AtMYB60* expression was analysed using primers 60RTF1 and 60RTR1. Primers specific for *ACTIN2* and *PP2AA3* gene were used as references for normalization. All primer sequences are reported in Table 2. Relative quantification was performed using iCycler™ iQ Optical System Software version 3.0a (BioRad Laboratories, Inc., Hercules, CA). The PCR experiment has been repeated at least three times. The protocol used was as follows: 95 °C for 2 min, followed by 55 cycles of 95 °C for 15 s, 60 °C for 30 s. A melt curve analysis was performed following each run to ensure a single amplified product for each reaction (from 55°C to 95°C, increment 0,5°C for 10 sec). Data analysis was performed as previously described [23].

Table 2. Sequence of oligonucleotides used in this study.

Name	Sequence
At5g65590F1	ATGTCCTCCCATACCAATCTCC
At5g65590R1	TCAAGGCAATGCATTATAATGAT
At4g38000F1	CCCATATTGCACCATCTCCT
At4g38000R1	TGCTAAAAATTACAAAGGGGAAA
At3g55370F1	CATGTCGTCGCTATTGGACA
At3g55370R1	ACACCCCAACCAACAAAAGA
At2g28810F1	AAGAAACGTCCCTCCACTCA
At2g28810R2	TGTGAGAAACATAAGCTTTTTGG
At1g07640F1	CACCAGATCGGTTCCAGTTT
At1g07640R1	CGTAAGTATGCTTCAATCCATGCAGAAAG
LBa1	TGGTTCACGTAGTGGGCCATCG
pEX241	AACGTCCGCAATGTGTTATTAAGTTGTC
Ds5-2a	TCCGTTCCGTTTTCGTTTTTTAC
ATACT2F	TGCTTCTCCATTTGTTTGTTTC
ATACT2R	GGCATCAATTCGATCACTCA
PP2a-F	CAGCAACGAATTGTGTTTGG
PP2a-R	AAATACGCCCAACGAACAAA
60RTF1	GGGGATGATGACCAGGGTATAAAG
60RTR1	CATCAAGTGGCTCATCTCTTCCAT

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Chapter 3

Analysis of mutants in putative targets of AtMYB60, an Arabidopsis guard cell-specific transcription factor

Introduction

Plants are sessile organisms and therefore they have developed unique mechanism that allow them to respond to several environmental stresses [1]. Drought is one of the most common environmental stresses to which plants are exposed and the major cause of losses in crop production throughout the world [2]. Consequently, genetic engineering of important factors for drought tolerance has become very popular in research laboratories as a useful tool to produce plant more tolerant to stresses and with a major water use efficiency [3]. As plants lose over 95% of their water via transpiration through stomatal pores, understanding the mechanisms that control the opening and closure of stomata is an attractive goal; the engineering of stomatal activity is in fact a valuable tool to produce transgenic plants with a reduced water requirement and enhanced productivity under drought condition [4].

In the last years it has been demonstrated the importance of the role of transcription factors in controlling the stomatal opening and closure (as reviewed by Cominelli et al., 2010)) [5]. In *Arabidopsis thaliana* some MYBR2R3 transcription factors (AtMYB60, AtMYB61, AtMYB44 and AtMYB15) involved in stomatal movements have been characterized [6-9].

AtMYB60 has been previously reported as a transcription factor specifically expressed in guard cells [6]. *AtMYB60* gene expression is environmentally regulated. Stimuli which usually cause reductions in stomatal aperture, including drought and treatment with ABA (a phytohormone synthesised in response to water deficit [1]), strongly down-regulate the expression of this gene, whereas light treatments, which usually cause increase in stomatal apertures, induce *AtMYB60* gene expression. Analysis on the *atmyb60-1* null mutant revealed a reduction in

the stomatal aperture and a decreased sensitivity to drought stress compared to the wild-type as the unique phenotypical alterations shown by the mutant [6], making *AtMYB60* a very interesting candidate for a genetic manipulation in order to improve plant performance during drought condition. It is still unclear however how AtMYB60 can interfere with the opening and closure of stomata. A microarray hybridization experiment has been performed with RNA from wild-type and *atmyb60-1* plants to investigate the effects of the *AtMYB60* gene disruption on more than 6000 genes expression. Thirty six genes showed an altered expression profile in *atmyb60-1* mutant suggesting a role for AtMYB60 in controlling their expression. Among these, 6 genes were up-regulated in the mutant whereas the other 30 showed a down regulation in their expression level (Figure 1) [6].

In this chapter the approach performed to characterise some of the AtMYB60 putative targets will be described.

Clone ID	Gene ID	Description	Fold change	p value
205171	At2g20670	Expressed protein, unknown function	+ 2.55	2.72 e-05
204478	At3g52780	Purple acid phosphatase (PAP20)	+ 2.47	8.59 e-09
202808	At3g16240	Delta tonoplast integral protein (δ-TIP)	+ 2.39	3.87 e-07
207793	At1g33860	Hypothetical protein	+ 2.27	2.07 e-09
201818	At2g06950	Copia-like retrotransposon family	+ 2.06	4.82 e-09
207032	At5g19120	Expressed protein, unknown function	+ 2.00	3.50 e-05
200755	At4g22710	Cytochrome P450-like protein (CYP706A2)	−2.03	4.03 e-06
205228	At4g32020	Expressed protein, unknown function	−2.04	3.40 e-06
201462	At1g20450	Dehydrin ERD10	−2.12	4.24 e-06
204765	At1g73600	Related to phosphoethanolamine N-methyltransferase	−2.14	3.77 e-06
201493	At4g38550	Putative phospholipase	−2.15	6.67 e-05
205791	At5g61600	AP2-domain transcription factor, similar to AtERF5	−2.16	6.59 e-08
200958	At5g59820	Putative zinc-finger protein (C2H2 type) ZAT12	−2.18	2.20 e-05
201362	At1g14880	Expressed protein, unknown function	−2.21	1.72 e-06
204798	At4g31500	Cytochrome P450 83B1 (CYP83B1)	−2.33	6.47 e-05
205931	At1g21130	O-methyltransferase 1 putative	−2.33	1.74 e-08
200505	At2g30870	Glutathione S-transferase (ERD13)	−2.39	2.82 e-07
200131	At5g06320	NDR1/HIN1-like protein 3 (NHL3)	−2.41	6.20 e-07
202775	At1g27020	Expressed protein, unknown function	−2.43	1.52 e-07
204094	At1g73500	Mitogen-activated protein kinase kinase, putative MKK9	−2.46	1.96 e-09
205066	At2g26560	Similar to patatin-like latex allergen	−2.62	2.09 e-09
203761	At2g40100	Lhcb4:3 protein (light-harvesting chlorophyll-binding)	−2.68	3.03 e-10
203600	At3g46620	Similar to RING-H2 finger protein RHC2a	−2.68	5.52 e-12
240366	At3g15210	Ethylene-responsive element-binding factor 4 (AtERF-4)	−2.72	9.57 e-07
205714	At2g40000	Expressed protein, unknown function	−2.83	2.86 e-08
200912	At4g27280	Calcium-binding EF hand family protein	−2.83	7.72 e-08
201356	At1g57990	Purine permease-related	−2.86	6.77 e-05
206997	At4g17490	Ethylene-responsive element-binding factor 6 (AtERF-6)	−2.94	2.75 e-05
206894	At4g02380	Late embryogenesis abundant protein homolog (SAG21)	−2.98	8.21 e-05
208064	At4g24570	Mitochondrial substrate carrier family protein	−3.00	3.94 e-07
205814	At4g32940	Putative vacuolar processing enzyme gamma-VPE	−3.21	2.24 e-06
202443	At5g47220	Ethylene-responsive element-binding factor 2 (AtERF-2)	−4.02	9.72 e-05
204738	At3g52400	Syntaxin, putative (SYP122)	−4.29	2.55 e-05
203517	At1g07135	Glycine-rich protein, unknown function	−4.47	2.66 e-07
240892	At1g27730	TFIIIA-type zinc finger protein (ZAT10)	−5.10	1.60 e-08
204291	At4g29780	Expressed protein, unknown function	−5.28	6.59 e-06

Clone ID refers to the unique ID for the array feature; gene ID and description correspond to gene designation and annotation obtained from TAIR (www.arabidopsis.org). Asterisks indicate genes for which microarray expression data have been independently confirmed by RT-PCR (Figure S2). A complete dataset is available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/query/entry) under accession number E-MEXP-225.

Figure 1 Microarray gene expression analysis of wild-type and *atmyb60-1* leaves [6].

Results and discussion

Selection of AtMYB60 putative target genes

Among the genes showing different expression level in the *atmyb60-1* mutant [6], we selected some candidates for further analysis. Selected genes have to be expressed in guard cells, being the expression of *AtMYB60* specific in this particular cell type [6]. Their expression profile in guard cells has been analysed using the Arabidopsis e-FP browser at http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi [10] (Figure 2).

Particular attention was pointed out also on: genes more expressed in the guard cells than in the mesophyll ones (because this could suggest a major role in this cell type); genes showing the biggest difference in their expression level in *atmyb60-1* mutant compared to the wild type; genes known in literature to play a role in some mechanisms that could be correlated with stomatal movements; finally, on unknown genes.

Selected genes showing one or more of these features are the following ones:

- *At3g16240*: it is the only selected gene up-regulated in *atmyb60-1*. Although it is expressed at low level in guard cells it is an interesting candidate to be AtMYB60 putative target gene, because it encodes for a functional δ-TIP tonoplast-located water channel [11] that belongs to the aquaporin family. Members of this family are known to be water selective channels involved in both cell expansion and vascular water permeability [11-13]. Moreover the expression of some aquaporin genes is affected by osmotic stress strengthening the hypothesis that they play a significant role in the control of water movement [14-17]; it is known that the opening and closure of stomata pores are mediated by turgor-driven volume changes of the two surrounding guard cells [18].

Although the presence of tonoplast aquaporin in Arabidopsis guard cells has not yet been demonstrated, Sarda and colleagues (1997) identified two *TIP-like* genes, *SunTIP7* and *SunTIP20*, expressed in sunflower guard cells, showing 74% and 75% of sequence identity to Arabidopsis *δ-TIP* cDNA, respectively. They proposed that SunTIP7 is involved in stomatal closure: its transcript level increased when the stomata were closed, and assuming that this protein is located in the tonoplast, it might play role in stomatal movements accelerating water exit from guard cell vacuole [19].

- *At5g59820*: it encodes for ZAT12, a transcription factor, belonging to the Cys2/His2-type Zinc-finger protein family [20]. Its expression is induced in response to a very large number of different biotic and abiotic stresses such as oxidative, heat shock, salt, cold, wounding, pathogen and high light [21-26]. Analysis on transgenic plants overexpressing *ZAT12* suggests a role for this protein as a transcriptional repressor under various stress conditions: its overexpression leads to growth inhibition but also to higher tolerance to drought stress than wild-type [20], whereas *zat12* knock-out transgenic plants are more sensitive than wild-type plants to salinity and osmotic stress [27].
- *At1g57990*: it encodes for a member of a family of proteins related to PUP1, a purine transporter. It may be involved in the transport of purine and purine derivatives such as cytokinins, across the plasma membrane (TAIR, www.arabidopsis.org).
- *At4g32940*: it encodes for the vacuolar processing enzyme, *γ-VPE*, belonging to the small gene family of VPEs [28], responsible for the maturation of vacuolar proteins [29]. Analysis of this gene is presented in the Chapter 4.

- *At1g07135*: it encodes for a glycine-rich protein anchored to membrane, but its biological and molecular functions are still unknown. The glycine-rich RNA-binding proteins (GRPs) belong to this family and they have been implicated in the responses of plants to environmental stresses [30]. In Arabidopsis eight members of this family have been found. Two of them, GRP2 and GRP4 are involved in response to cold, salt and dehydration stress in Arabidopsis [31, 32]. GRP7, another GRP family member, is highly expressed in guard cells suggesting its role in opening and closure of stomata in response to stresses [30].
- *At4g29780*: it encodes for an unknown protein. It shows 50% of identity to *At5g12010* gene involved in response to salt stress (TAIR, www.arabidopsis.org).

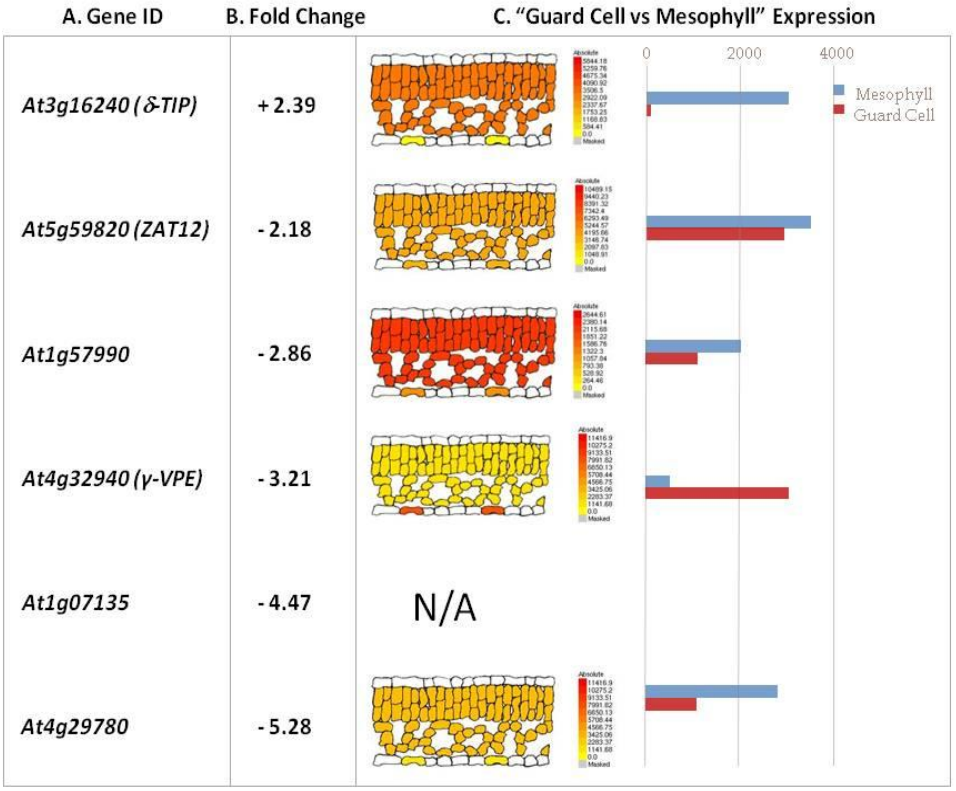

Figure 2 A Candidate gene ID and description obtained from TAIR (www.arabidopsis.org); **B** Ratio between gene expression in *atmyb60-1* and in wild-type leaves, based on microarray data [6]; **C** Expression patterns of the selected genes in guard cells and mesophyll cells. The expression patterns were generated using the Arabidopsis e-FP browser at http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi [10]. The color scale indicates the absolute expression levels of each individual gene: red means higher while yellow indicates lower expression levels. On the right side, the gene expression level in guard cells (in red) and in mesophyll cells (in blu), generated using the same browser.

Isolation of putative AtMYB60 target gene mutants

Insertional mutants for *At3g16240 (δ-TIP), At5g59820 (ZAT12), At1g57990, At1g07135* and *At4g29780* genes have been searched by BLASTN in different publicly available insertional mutant collections.

The following lines with predicted insertional mutations in the genes of interest were ordered from NASC and RIKEN stock centres:

- SALK_088755 (mutation in *At4g29780*), SM_3_1820 (*At3g16240*) from the SIGnAL (Salk Institute Genomic Analysis Laboratory) collection [33];
- RIKEN 53-2565-1 (*At5g59820*), RIKEN 15-2479-1 (*At1g57990*) and RIKEN 11-4656-1 (*At1g07135*) from the RIKEN Arabidopsis Transposon mutants collection [34];

In the SIGnAL collection the different lines (Columbia ecotype) are characterized by the insertion of a T-DNA, present on the pROK2 binary vector, introduced by transformation with *Agrobacterium tumefaciens*. The T-DNA element is 4393 bp long and contains the *nptII* gene conferring kanamycin resistance [33].

In the RIKEN collection the different lines (Nössen ecotype) are characterized by the insertion of a Ds transposable element, present on the pROK2 binary vector, introduced by transformation with *Agrobacterium tumefaciens*. The Ds element contains the *GUS* gene and the *hyg* gene conferring hygromycin resistance [34].

PCR screening was performed on about fifteen plants for each mutant to isolate plants homozygote for the insertional elements as described in the Material and Methods section (data not shown).

In Figures 3-7 gene sequences, positions of oligonucleotides used for genotyping and gene expression analysis and positions of insertional sites determined by sequencing of fragments flanking the insertion are shown.
For all the mutants the position of the insertional elements is also schematically represented in Figure 8.
In the case of SM_3_1820 line (*at3g16240-1* mutant) the insertion is in the third exon at position +993 from the ATG (Figure 3); in the RIKEN 53-2565-1 line (*at5g59820-1* mutant) it is in the exon at position +177 (Figure 4); in the RIKEN 15-2479-1 line (*at1g57990-1* mutant) it is in the putative promoter at position -570 (Figure 5); in the RIKEN 11-4656-1 line (*at1g07135-1* mutant) it is in the exon at position +1132 (Figure 6); in the SALK_088755 line (*at4g29780-1* mutant) it is in the exon at position +1081 (Figure 7).

At3g16240

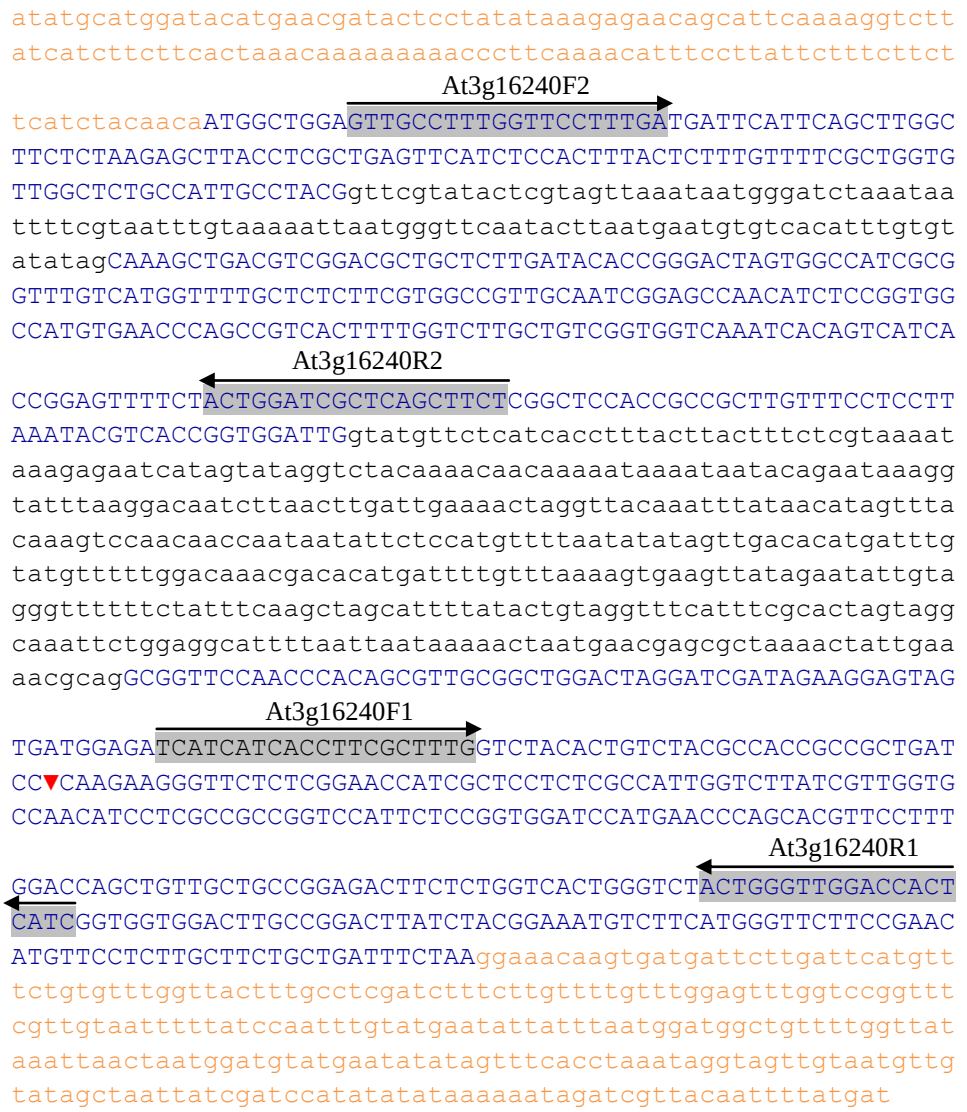

Figure 3 *At3g16240* gene sequence. The exons are in blue capital letters, the introns in black small letters and the 5' and 3' UTR are in orange. The primer sequences are highlighted in grey, their directions are shown with arrows. Oligonucleotides At3g16240F1 and At3g16240R1 were used for genotyping, while At3g1624F2 and At3g16240R2 were used for gene expression analysis. The position of the insertion element in the SM_3_1820 mutant line (from the SIGnAL collection), determined by sequence analysis is shown with a red triangle.

At5g59820

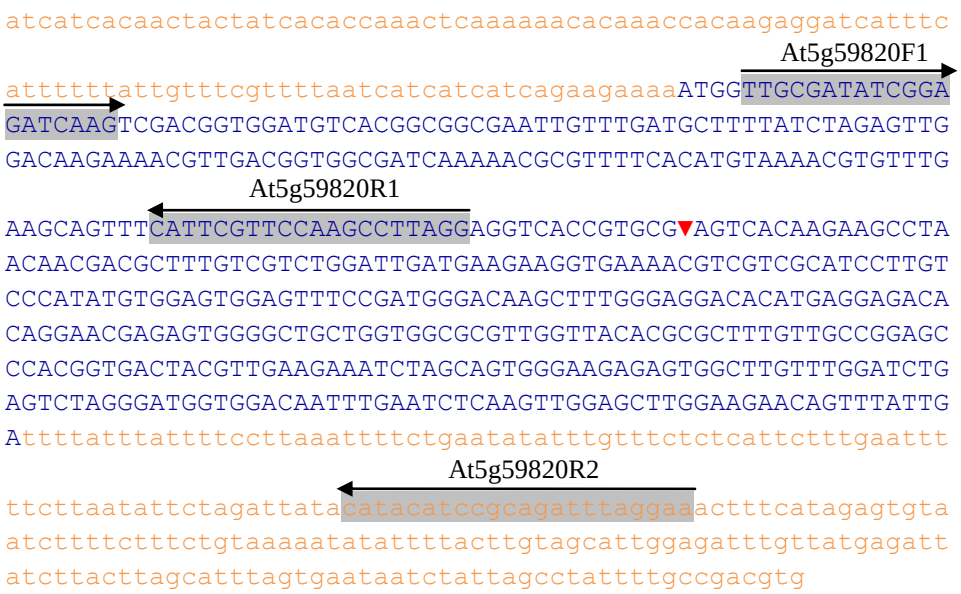

Figure 4 *At5g59820* gene sequence. The coding sequence is in blue, the 5' and 3' UTR are in orange. The primer sequences are highlighted in grey, their directions are shown with arrows. Oligonucleotides At5g59820F1 and At5g59820R2 were used for genotyping, while At5g59820F1 and At5g59820R1 were used for gene expression analysis. The position of the insertion element in the RIKEN 53-2565-1 mutant line (from the RIKEN collection), determined by sequence analysis is shown with a red triangle.

At1g57990

gaagacatcttattcttagttgtctgaatcatatccatgtattgtataagcgtgttcga
cgttgttttccccgaatataaaaatgcgaagttcgcaagacttgaccgttgagttgact
gtctccattacaacttgttcctctaaagtaggaacgagacgtataggtaactttctttg
aaagtctttgttgttgtccgaaaatttgttag▼cagtcaacttacatccaacttttttg
tttgtttatttggttttcgaaattaactttctagttaaaatcagatttttcactgatc
aggtgtgatcagtctcatccgtgtaagatttgtaatttttttttgctacatcattaaaat
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TCCGCGACTCAGTTGATATTTACCGCTATTTTCGCAGCCATTATTAACCGTTTTAAGTT
CACCAGATGGATTATCTTATCGATA
ATCGGCAGCATTTTGATTTATGTTTTCGGTAGTCCTGAATTTGGAGGAGAGCCTGATGA
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CATTATCTCTCTGTTTATTCCAACTTTGTTTCGAGAAAGTGTTGGTAAAGACAAAGAGA
TATGGTAACAAGAAAGTGTTTAGAATGGTCATAGAGATGCAAATTTGTGTCTCTTTTGT
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Figure 5 *At1g57990* gene sequence. The coding sequence is in blue, the 5' and 3' UTR are in orange. The intergenic region is in black. The primer sequences are highlighted in grey, their directions are shown with arrows. Oligonucleotides At1g57990F2 and At1g57990R1 were used for genotyping, while At1g57990F1 and At1g57990R1 were used for gene expression analysis. The position of the insertion element in the RIKEN 15-2479-1 mutant line (from the RIKEN collection), determined by sequence analysis is shown with a red triangle.

At1g07135

Figure 6 *At1g07135* gene sequence. The coding sequence is in blue, the 5' and 3' UTR are in orange. The primer sequences are highlighted in grey, their directions are shown with arrows. Oligonucleotides At1g07135Fnew and At1g07135R1 were used for genotyping, while At1g07135Fnew and At1g07135Rnew were used for gene expression analysis. The position of the insertion element in the RIKEN 11-4656-1 mutant line (from the RIKEN collection), determined by sequence analysis is shown with a red triangle.

At4g29780

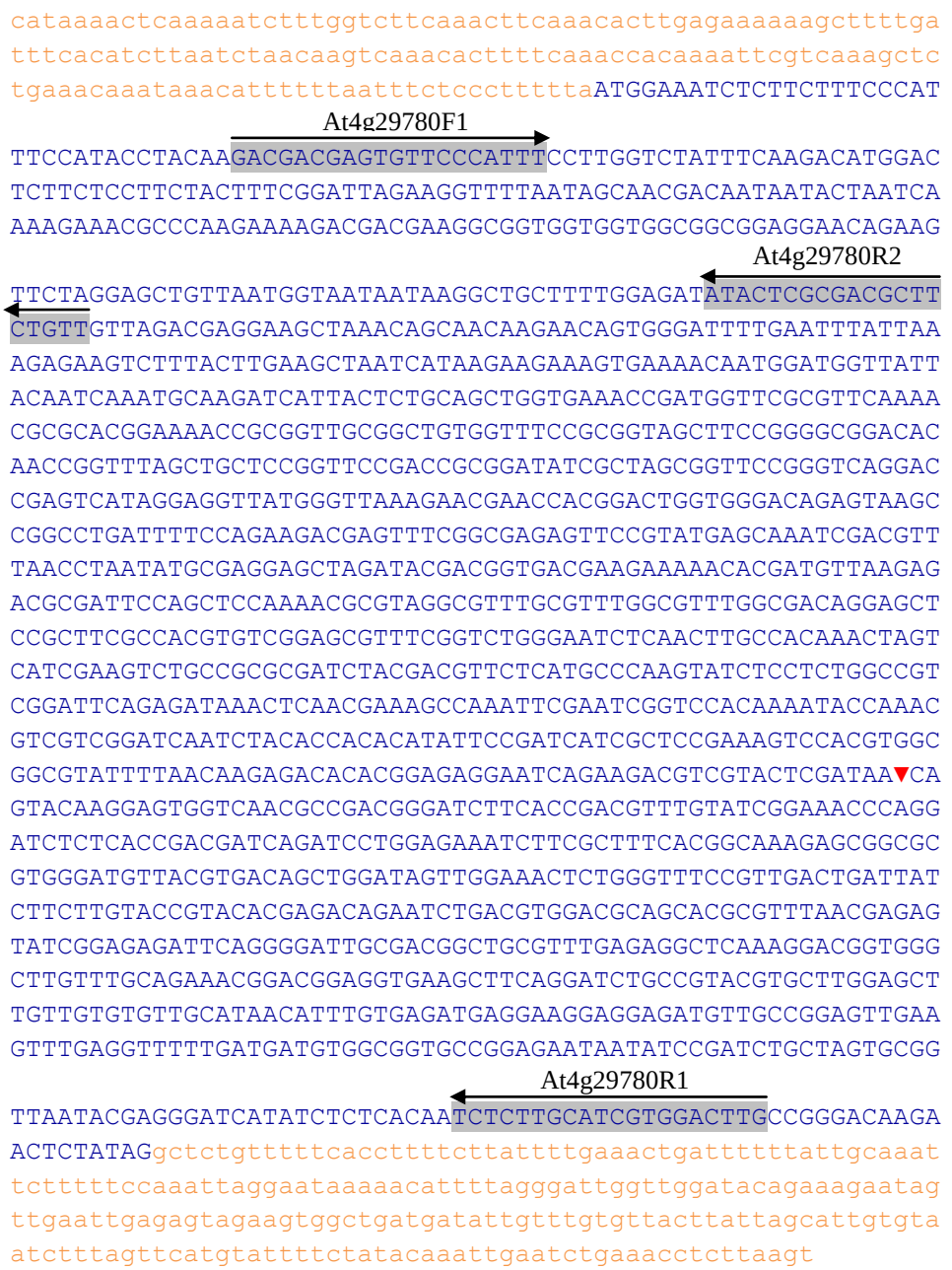

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Figure 7 At4g29780 gene sequence. The coding sequence is in blue, the 5' and 3' UTR are in orange. The primer sequences are highlighted in grey, their directions are shown with arrows. Oligonucleotides At4g29780F1 and At4g29780R1 were used for genotyping, while At4g29780F1 and At4g29780R2 were used for gene expression analysis. The position of the insertion element in the SALK_088755 mutant line (from the SIGnAL collection), determined by sequence analysis is shown with a red triangle.

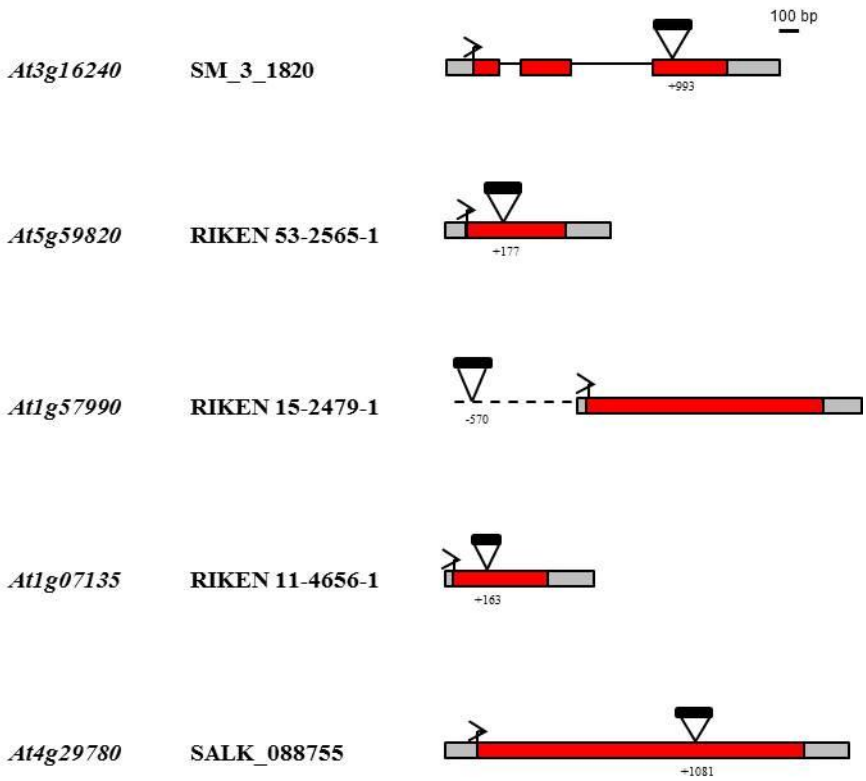

Figure 8 Schematic representation of the insertion lines used in this study. Red boxes indicate exons, black lines indicate introns and grey boxes represent 5' and 3' UTR regions. The translational start of each gene is represented by an arrow. The T-DNA/transposon inserts are represented as inverted triangles and their position from the ATG is shown.

Molecular analysis of mutants in the selected genes

To assess if the *at3g16240-1, at5g59820-1, at1g57990-1, at1g07135-1* and *at4g29780-1* lines were knock-out mutants, expression analysis of the different genes was performed by semi-quantitative RT-PCR, using as control Columbia or Nössen wild type, depending on the background of the corresponding mutant collection and primers upstream of the insertional elements (as represented in Figures 3-7).

As shown in Figure 9 all the genes were expressed in the wild-type backgrounds.

In the case of *at1g07135-1* mutant there was no mRNA detectable level of the corresponding mutated gene, indicating that this is a knock-out mutant.

Conversely, in the case of *at3g16240-1, at5g59820-1* and *at4g29780-1* mutants there was transcript accumulation of the corresponding mutated genes, at similar level to that observed in the wild-type background. However, in these lines the insertional elements are in the coding sequences causing premature stop codons (data not shown) that likely abolish the function of the corresponding genes. In fact if these transcripts were translated they would give origin to truncated proteins. In the case of *at5g59820-1* mutant, the truncated protein would lack both the two C2H2-type Zinc-finger domains, as the T-DNA is located in the middle of the sequence coding for the first one, then unfunctional protein. Also in the case of *at3g16240-1* mutant the transposon is in the middle of the sequence coding for the functional domain. In the case of *at4g29780-1* mutant, the mutated gene encodes for an unknown protein. The only domain identified by the NCBI BLAST software is nevertheless downstream the insertional element, then we can assume that the truncated protein would be unfunctional.

Finally, in *at1g57990-1* line the Ds transposable element is in the putative promoter region, consequently there is a significant reduction in the transcript accumulation in comparison with the wild-type background. Although this line is not a knock-out mutant, a significant reduction of the corresponding protein level should be hypothesised.

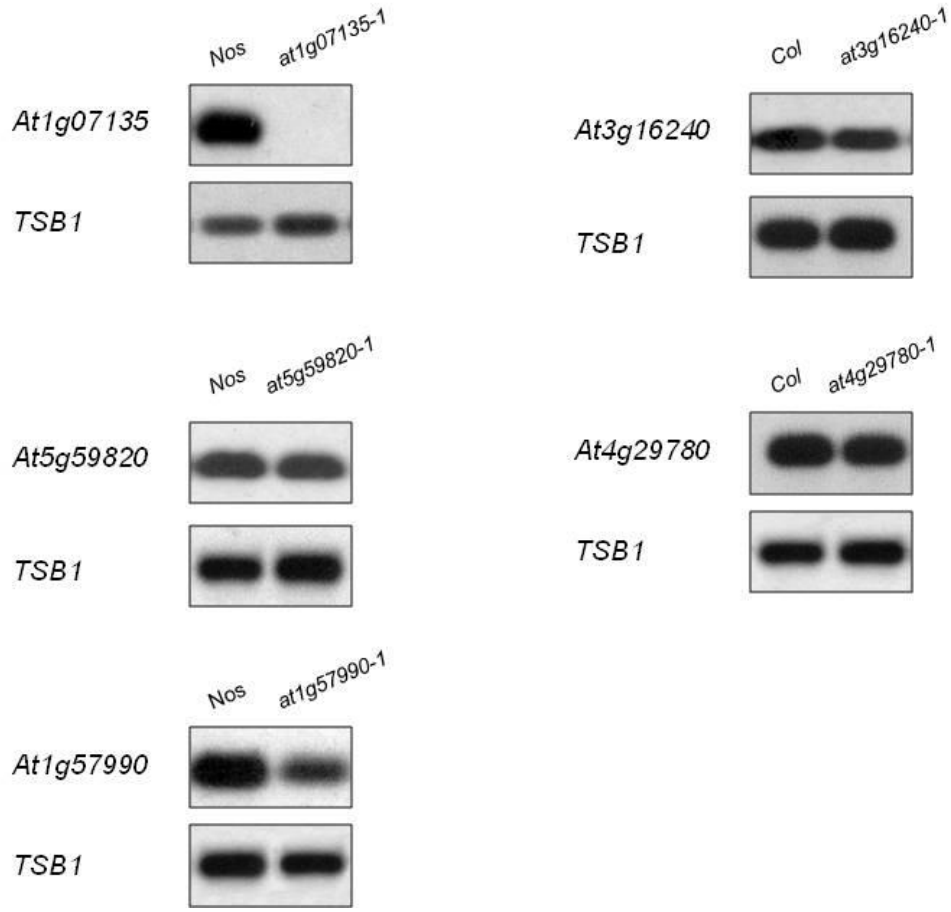

Figure 9 Expression analysis of selected AtMYB60 putative target genes in mutant lines by semi-quantitative RT-PCR analysis. Columbia and Nössen ecotype were used as controls. TSB1 gene was used as internal control.

Phenotypic analysis of mutants in the selected genes

As the selected genes are putative targets of AtMYB60, characterised for its involvement in stomatal movements, we investigated the effects of the mutations in the different genes on water loss and transpiration rate during desiccation. We measured water loss from detached wild-type and mutant rosette leaves. As shown in Figure 10A and 10B throughout the duration of the desiccation treatment, *at4g29780-1* and *at5g59820-1* leaves lost less water than wild-type leaves of about 9% and 5% respectively ($p < 0.01$), similarly to the *atmyb60-1* mutant.

Microarray analysis performed on total RNA from wild-type and *atmyb60-1* mutant plants suggest that these genes are positively regulated by AtMYB60 as they showed a decrease in their expression level in *atmyb60-1* mutant [6] (Figure 2). The similarity of response to desiccation between the different mutant lines and the *atmyb60-1* one correlates with the hypothesis formulated on the basis of microarray data. It is possible to speculate that these genes are among all the AtMYB60 targets that play the major role in response to desiccation, as the water loss during desiccation of the corresponding mutants is very similar to the *atmyb60-1* one.

Oppositely, *at3g16240-1* leaves lost almost 6% more water than the wild-type ones ($p < 0.01$) (Figure 10C). *At3g16240* gene results over-expressed in *atmyb60-1* as shown by the microarray data [6] (Figure 2) suggesting a negative regulation of this gene by AtMYB60.

Finally, we did not detect a significant difference in water amount lost by *at1g07135-1* and *at1g57990-1* leaves compared to the wild-type (Figure 10D and 10E). The *at1g57990-1* mutant is not a complete knock-out; the Ds insertion is in the putative promoter region and causes only a decrease in the gene expression level (Figure 9). Therefore, the produced proteins

could be enough to carry out their normal functions without affecting the response to desiccation. For *at1g07135* instead, this result indicates that the lack of At1g07135 protein does not lead to a detectable alteration of the phenotype in response to desiccation.

It is important to consider that multiple and complex pathways are involved in controlling the desiccation-stress response. The mutation of a single gene, even if encoding a stress responsive protein, does not always affect the response to the treatment [35]. Moreover, the modification of a single enzyme in a biochemical pathway is usually contrasted by a tendency of plant cells to restore homeostasis [36]. The modification of genes expression encoding stress-inducible transcription factors causes a more detectable phenotype alteration; in fact this class of proteins acts as master regulator of different cellular processes, controlling at the same time the expression of several genes [37]. In *atmyb60-1* for instance, the mutant phenotype is the consequence of the lack of a single transcription factor that results in the alteration of at least 36 genes expression [6].

According to all these results, we propose a really simple and speculative model to explain the AtMYB60 role to control stomatal movements, shown in Figure 11. As argued by the work of Cominelli and colleagues, AtMYB60 is a transcriptional regulator that could integrate multiple signal transduction processes by modulating the expression of genes involved in guard cell response to different stimuli and stresses [5]. The activity of AtMYB60 induces the expression of *At4g29780* and *At5g59820* genes and represses *At3g16240* gene expression. However it is still unclear whether AtMYB60 acts as a transcriptional activator or repressor. Based on microarray results reported in Figure 1 it is possible to speculate that AtMYB60 is a positive transcriptional regulator, as the majority of the genes are down-regulated in the *atmyb60-1* mutant. In this

scenario it would directly activate the expression of genes involved in stomatal opening and indirectly repress, through the activation of one or more negative regulators, genes involved in stomatal closure. *At4g29780* and *At5g59820* could belong to the first group of genes and *At3g16240* to the second one.

Further analyses will be helpful to explain better their role in the stomatal movements and to define their direct or indirect regulation by AtMYB60.

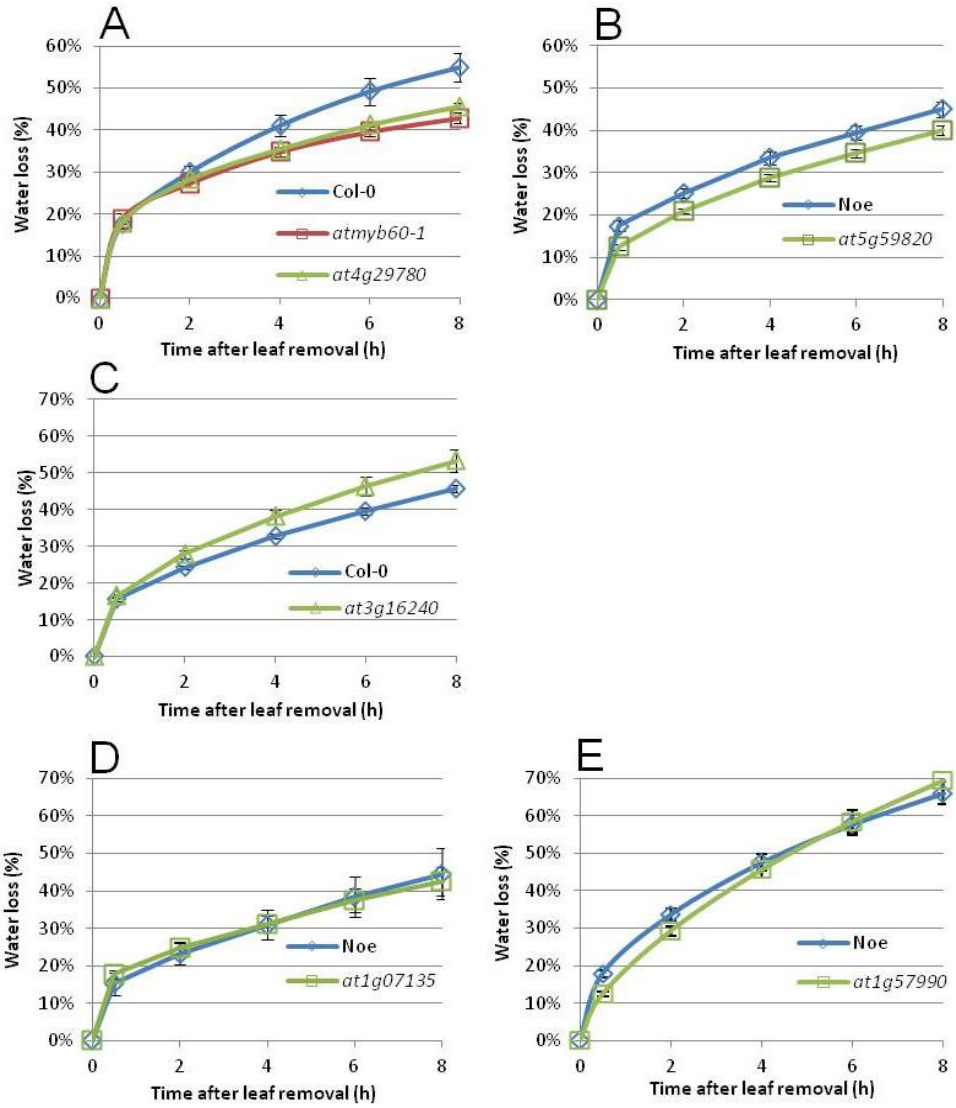

Figure 10 Time course of water loss from excised leaves of *at4g29780-1* (**A**), *at5g59820-1* (**B**), *at3g16240-1* (**C**), *at1g07135-1* (**D**) and *at1g57990-1* (**E**) mutants, expressed as a percentage of the initial fresh weight at indicated intervals. Each point indicates the mean of 12 measurements with standard errors. The wild-typeis in the Columbia (Col-0) or in the Nössen ecotype (Noe), depending on the ecotype of mutants. In the Columbia ecotype the *atmyb60-1* mutant was used as an additional control.

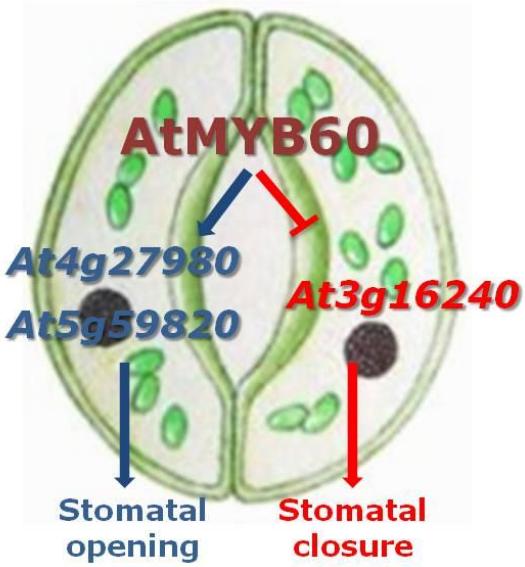

Figure 11 Hypothetical model proposed to explain the AtMYB60 action in modulating the stomatal opening/closure through its putative targets.

Materials and methods

Plant material and growth conditions

The SALK_088755 and the SM_3_1820 mutant lines, obtained from the NASC European Arabidopsis Stock Center (Nottingham, UK) are in the Columbia ecotype, while the RIKEN 53-2565-1, RIKEN 11-4656-1 and RIKEN 15-2479-1 lines, obtained from the RIKEN Stock Center (Japan), are in the Nössen ecotype.

The *atmyb60-1* mutant allele, obtained from the Yale Arabidopsis T-DNA Lines (YATDLs), is in the Columbia ecotype [6].
The Arabidopsis seedlings were grown in soil at 22°C in a greenhouse with a 16-h-light/8-h-dark cycle at a fluorescent illumination of 100 μmol m^{-2} s^{-1} and 60% humidity.

Mutant genotyping

DNA was extracted as previously reported [38]. Plants of each mutant line were genotyped using two PCR reactions, one for the mutant allele with the T-DNA/transposon insertion and one for the wild-type allele. The primers used to amplify the wild-type alleles are indicated in Figures 3-7 and in Table 1. One of these primers for each gene were used coupled to LBa1 or Ds5-2a primers, designed on the insertional elements, for mutants belonging to SIGnAL and RIKEN collections, respectively.

Semi-quantitative RT-PCR

Total RNA was extracted from 3-week-old rosette leaves as previously reported [6].

For each sample, cDNA synthesis was performed by retrotranscription of 5 μg of DNase-treated RNA with RT Superscript™ II (Invitrogen) according to the manufacturer's instructions.

For specific amplification of mutant gene transcripts primers reported in Figures 3-7 and in Table 1 were used.

The expression level of *TSB1* gene, which encodes for the β subunit of tryptophan synthase [39] was used as a quantitative control and the specific primers TSB1F1 and TSB1R1 were used. PCR reactions were carried out for 25 cycles to provide semi-quantitative data. The amplification products were separated on 1% agarose gel, blotted on positively charged nylon membrane (Roche), hybridised with specific DIG-labelled probes and the signal was detected according to the manufacturer's instructions (DIG-High Prime Labelling and Detection Kit, Roche). The reverse transcription polymerase reaction (RT-PCR) analysis was repeated at least three times for each sample with similar results.

Water loss experiment

4-week-old wild-type and mutant plants were used. For the desiccation treatment, 12 rosette leaves for each line were detached and dehydrated on Whatman paper at 21°C in the light for up to 8 hr. At different time points (30', 2hr, 4hr, 6hr, 8hr after the beginning of the experiment) the leaves weight was measured by an electronic balance. Water loss was expressed as the percentage of initial fresh weight.

Table 1. Sequence of oligonucleotides used in this study.

Name	Sequence
At3g16240F1	TCATCATCACCTTCGCTTTG
At3g16240R1	GATGAGTGGTCCAACCCAGT
At3g16240F2	GTTGCCTTTGGTTCCTTTGA
At3g16240R2	ACTGGATCGCTCAGCTTCTC
At5g59820F1	TTGCGATATCGGAGATCAAG
At5g59820R1	CCTAAGGCTTGGAACGAATG
At5g59820R2	TTCCTAAATCTGCGGATGTATG
At1g57990F1	TTTTCTTAGTCTTGCTCGGAG
At1g57990F2	TCCATGTATTGTATAAGCGT
At1g57990R1	CGAAAACATAAATCAAAATGCTG
At1g07135Fnew	ATGGGTTTGAGAAGAACATG
At1g07135Rnew	CAACAACGTCTGGCTTTGAA
At1g07135R1	TTAGCCGGTTTAGTCTTGGTT
At4g29780F1	GACGACGAGTGTTCCCATTT
At4g29780R1	CAAGTCCACGATGCAAGAGA
At4g29780R2	AACAGAAGCGTCGCGAGTAT
LBa1	TGGTTCACGTAGTGGGCCATCG
Ds5-2a	TCCGTTCCGTTTTCGTTTTTTAC
TSB1F1	CTCATGGCCGCCGGATCTTGA
TSB1R1	CTTGTCTCTCCATATCTTGAGCA

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3.1

Identification of putative target genes of AtMYB60 through RNA-Seq analysis

Introduction

As previously described, microarray analysis was performed on RNA extracted from *atmyb60-1* and wild type whole rosette leaves to identify AtMYB60 putative target genes [1]. Only almost 7000 genes were represented on the cDNA array used for the microarray hybridization experiment and 36 genes resulted differentially expressed among the two genotypes. Based on results of that analysis we selected candidate genes for the characterization described before in the chapter. Here we describe preliminary analysis of results obtained from RNA-Seq, performed on *atmyb60-1* and wild type purified guard cells. RNA-Seq provides more accurate measurement, greater sensitivity and higher resolution compared to microarrays and does not require previously genomic DNA sequence information [2]. In the last years different applications of this technique have been performed in studying gene expression also in plants [3-6].

As *AtMYB60* is specifically expressed in guard cells, RNA for RNA-Seq analysis was extracted from *atmyb60-1* and wild type purified guard cells, instead from whole rosette leaves, as previously performed for microarray analysis. For guard cell purification "the blender method" was used. As described in Figure 12, leaves from 7-week-old Arabidopsis plants were harvested and major veins were removed; leaf blades were blended for 1 to 2 min in ice-cold deionized water with additional crushed ice and filtered through a 210-mm nylon mash. After two further rounds of

blending, the light green epidermal fraction was collected. By vital staining, ≥90% of the living cells were identified as guard cells [7].

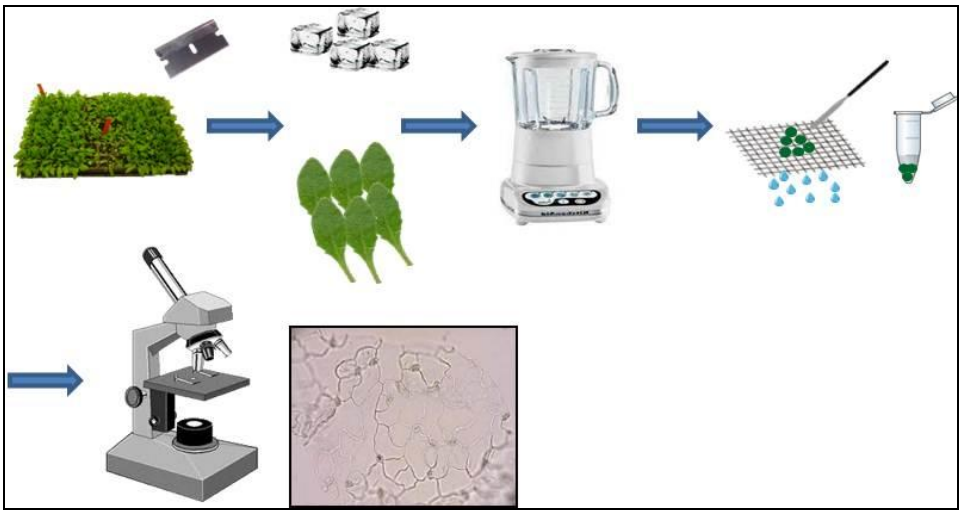

Figure 12 Schematic representation of "the blender method" used for the guard cell purification.

A powerful tool to investigate the interaction between proteins and DNA in vivo is Chromatin Immunoprecipitation (ChIP) assay. To use this technique it is necessary to have a specific antibody against the protein of interest or to use an antibody against a tag fused to the protein of interest. Homozygous *atmyb60-1* plants, harboring *AtMYB60* sequence fused to *GFP* reporter gene under the control of the *AtMYB60* promoter

(*p60::AtMYB60::GFP atmyb60-1* plants) were generated. Anti-GFP antibody will be used for the ChIP assay.

Results and discussion

RNA-Seq analysis

RNA-Seq analysis was performed on wild type and *atmyb60-1* purified guard cells.

The sequencing step yielded more than 34 million paired end 51 base pair sequence reads for the two genotypes, with an estimated insert size of 50 base pairs.

The comparison between wild-type and *atmyb60-1* transcriptome revealed the presence of 978 genes with a significant difference in transcript accumulation. Among these genes, 364 were up-regulated whereas 614 were down-regulated in *atmyb60-1* guard cells. These results are very different from those obtained through microarray analysis that identified only 36 genes with a different expression profile in the mutant (Figure 1) [1].

We took advantage of the "Database for Annotation, Visualization, and Integrated Discovery" (DAVID) web-based application (http://david.abcc.ncifcrf.gov/) for the functional annotation of differentially expressed genes in the two genotypes.

In Figure 13, representative images of the up- and down-regulated genes in *atmyb60-1* are reported.

The main results obtained from these analyses are summarized below.

- The majority of the genes were down-regulated in the *atmyb60-1* mutant, as obtained also by the microarray experiment (Figure 1, [1]), suggesting that AtMYB60 could be a positive transcriptional regulator.
- Among the 978 genes we found almost 60 genes coding for DNA-binding proteins, most of all transcription factors. It means that most of the genes with an altered transcription profile in *atmyb60-1* would be targets of these DNA-binding proteins and not direct targets of AtMYB60. Among these DNA-binding proteins, we found factors belonging to families known to be involved in the abiotic stress response, as DOF (described in Chapter 1 and 2) and DREB, that regulate the expression of many stress-inducible genes in plants and play a critical role in improving abiotic stress tolerance of plants by interacting with specific *cis*-acting elements named DRE/CRT, which are present in the promoter region of several abiotic stress-related genes [8, 9]; we found genes belonging to families having a role also in the plant pathogen defense, like MYB and WRKY. Interestingly, the genes coding for WRKY proteins are present exclusively among the transcripts down-regulated in *atmyb60-1*.

The WRKY proteins are key players in plant immune responses, participating in the control of defense-related genes either as positive or as negative regulators (as fully reviewed in Pandey, 2009 [10]). They are induced by various stimuli, including pathogen infection or endogenous signal molecule salicylic acid (SA) [11]. Several works have demonstrated their role in the plant defense response: eight *WRKY*s (*AtWRKY18, AtWRKY38, AtWRKY53, AtWRKY54, AtWRKY58, AtWRKY70*, all present among the down-regulated genes, and *AtWRKY59*

and *AtWRKY66*) were identified as targets of *AtNPR1*, a key regulator of SA signaling [12], and genetic analysis showed that these WRKYs play important roles in the regulation of SA-dependent immune responses downstream of *AtNPR1* [10, 12]; however, certain WRKY proteins act also upstream of *NPR1* and positively regulate its expression during the activation of the plant defense response, as the presence of several W-box sequences in the promoter of *AtNPR1*, that are recognized specifically by pathogen- and SA-induced WRKY proteins, has been demonstrated [13]; AtWRKY52 confers resistance toward bacterial infection [14] and AtWRKY33, present among the down-regulated genes too, functions as a positive regulator of resistance toward necrotrophic fungal pathogens [15].

Also members of MYB family play a role in the defense response, e.g. AtMYB44 that positively regulates the expression of *WRKY70* leading to enhanced resistance to the pathogen infection [16], AtMYB30 that acts as a positive regulator of the hypersensitive cell death in response to pathogen attack [17], or AtMYB28, AtMYB29 (present among the up-regulated genes) and AtMYB76 that are regulators of the biosynthesis of glucosinolates (GS) [18], a group of amino acid-derived secondary metabolites that play an antimicrobial function in controlling plant resistance against insects and pathogens [19, 20].

- Among the 978 genes almost 200 are genes involved in the defense response: *PLANT DEFENSIN 1.3* (*PDF1.3*), *PATHOGENESIS-RELATED (PR) GENE 1*, *PR4*, *PR5*, *PR-1-like* and *PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1)* exhibit a strong reduction of their transcript level in *atmyb60-1* and different genes involved in the GS

biosynthesis, SA-mediated response and jasmonic acid (JA) signaling pathway show an altered transcription profile. It is interesting to notice that the genes involved in the biosynthesis of glucosinolates are exclusively present among the transcripts up-regulated in the mutant. As reported above, several members of the MYB family regulate the transcription of genes related to the glucosinolates biosynthesis pathway [18, 19], supporting the idea that AtMYB60 could be a negative regulator of these genes.

Moreover, genes involved in the JA signaling pathway are mainly present among the up-regulated genes whereas the SA-dependent genes are mainly present among the transcripts down-regulated in the mutant. JA and SA are the major signaling molecules for the plant defense response, playing a role in defense signaling against necrotrophic and biotrophic pathogens, respectively [16]. In some cases, different defense signaling pathways act synergistically to enhance resistance against pathogen attack [21]. In other cases, antagonistic interactions between defense signaling pathways provide focused resistance against pathogens [22]. The most well documented antagonistic interaction involves just cross-talk between JA and SA; in tomato, exogenous SA suppressed JA-induced wound responses [23], while in Arabidopsis it suppresses JA-dependent gene expression [24]. JA also suppresses SA signaling: treatment with exogenous JA inhibits the expression of SA-dependent genes [25]. A large set of transcription factors is involved in the regulation of plant defense [26, 27] and antagonistic interaction between SA and JA involves transcriptional reprogramming by a subset of these transcription factors, hence AtMYB60 could be one of these, on one hand activating the

expression of genes involved in the SA-mediated response, on the other repressing the ones involved in the JA signaling.

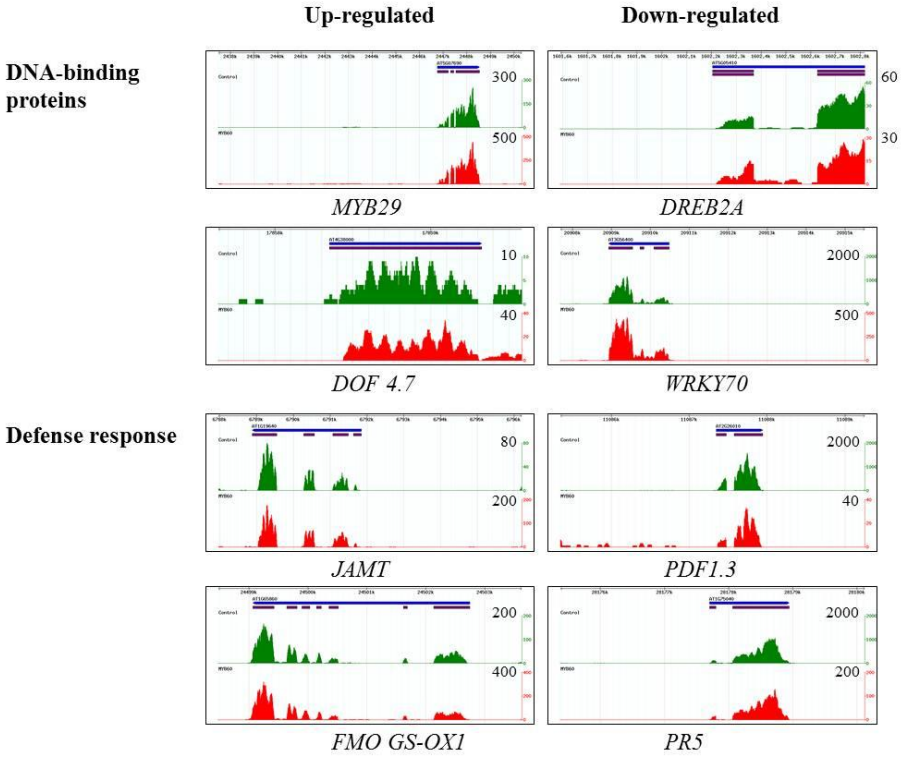

Figure 13 Representative images of up- and down-regulated genes in *atmyb60-1* mutant (in red) compared to wild type (in green) from RNA-Seq analysis..

We had preliminary results supporting the hypothesis of an involvement of AtMYB60 in response to pathogen attack: as reported in Figure 14, *atmyb60-1* plants showed a significant reduction of the leaf bacterial number compared to the wild-type, after 3 days of inoculation with the bacteria *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Prof. Jonathan Jones, personal communication).

In conclusion, among the 978 transcripts with an altered expression profile in *atmyb60-1* we found several genes involved in different processes, as the plant response to dehydration (e.g. *ERD* and *DREB*) or to pathogens attack (e.g. *PR* and *PDF*), as well as genes coding for transcription factors known to modulate gene expression during both abiotic and biotic stress (e.g. *MYB* and *WRKY*). Plants are continuously exposed to several types of abiotic and biotic stresses and stomatal movements represent a perfect example of the complex crosstalk of biotic and abiotic stress responses [28]. Based on RNA-Seq data it is intriguing to suggest a model in which AtMYB60 is a master regulator that controls stomatal movements by integrating multiple signal transduction processes and modulating the expression of different genes involved in both biotic and abiotic stresses (Figure 15) [29].

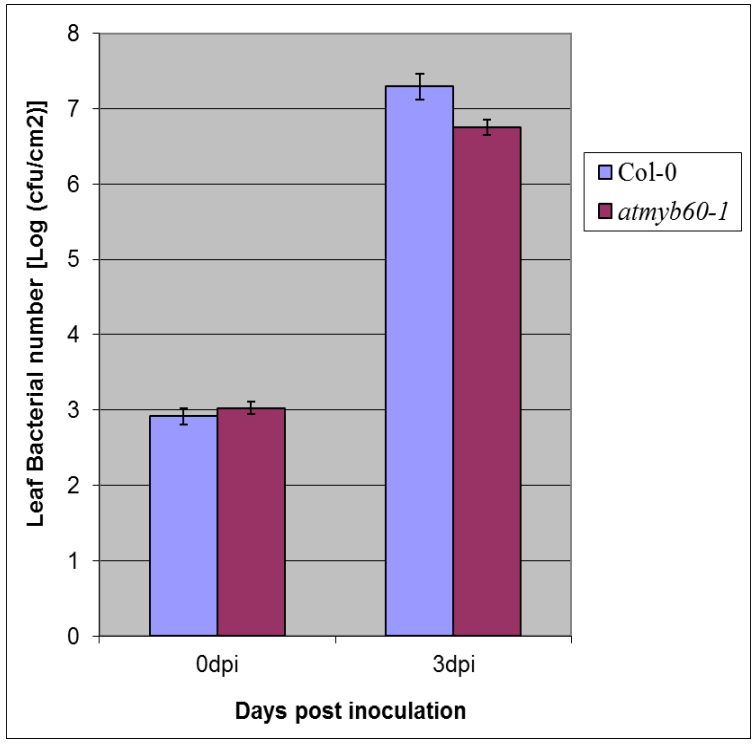

Figure 14 Leaf bacterial number was assessed 3 days after inoculation with the bacteria *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000.

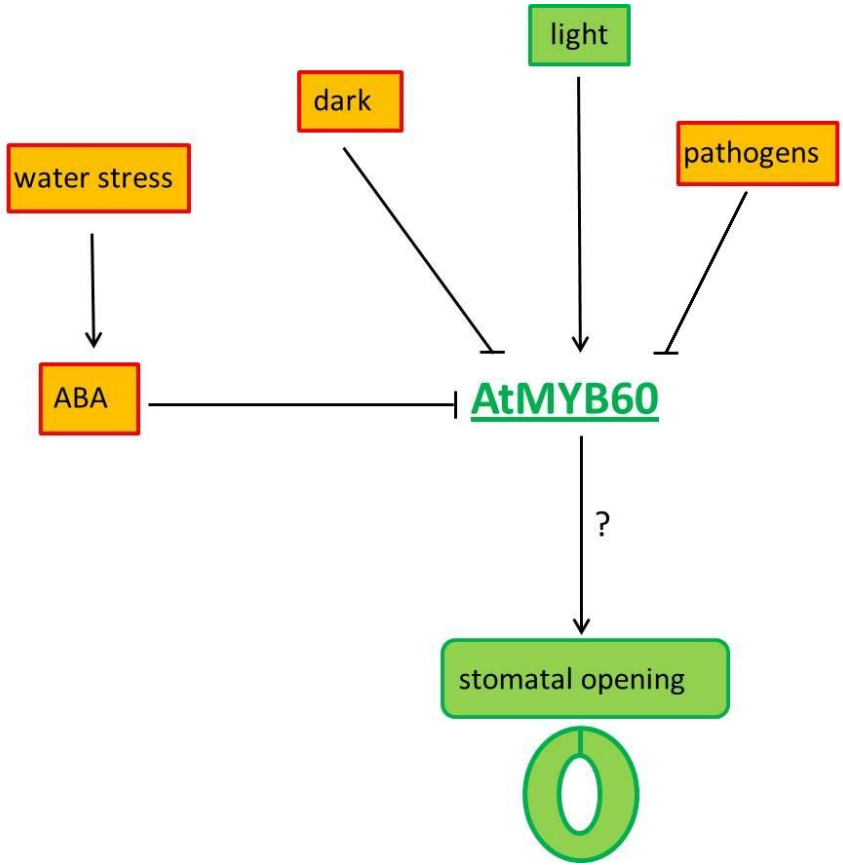

Figure 15 A simplified model representing the role of AtMYB60 to control the stomatal movements by the integration of multiple signal transduction processes [29].

Generation of *p60::AtMYB60::GFP atmyb60-1* plants

Transgenic lines harboring *AtMYB60* sequence fused to *GFP* reporter gene under the control of the *AtMYB60* promoter (*p60::AtMYB60::GFP* plants) were previously generated (unpublished results). In Figure 16A a schematic representation of the *p60::AtMYB60::GFP* construct is shown. In Figure 16B confocal images show that GFP fluorescence is present only in very localized areas of guard cells of *p60::AtMYB60::GFP* leaves, suggesting that the AtMYB60::GFP fusion protein is correctly transported to nuclei of cells in which AtMYB60 is expressed. As described in Materials and methods section and summarized in Figure 17 transgenic plants carrying the construct *p60::AtMYB60::GFP* were crossed with *atmyb60-1* mutant, then plants homozygote for the *atmyb60-1* mutation and for the transgene (*p60::AtMYB60::GFP atmyb60-1* plants) were isolated.

A

B

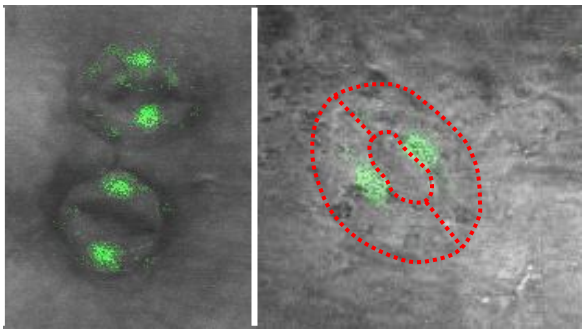

Figure 16 A Schematic representation of the p60::AtMYB60::GFP construct used in this study. *AtMYB60* cDNA, lacking of the last 15 bp, was fused upstream the *GFP* reporter gene, under the control of the *AtMYB60* putative promoter region. The *NPTII* gene confers resistance to the Kanamycin; **B** Confocal images of GFP expression in guard cells demonstrating the nuclear localization of the AtMYB60::GFP fusion protein.

To use these plants for ChIP assay it was necessary to demonstrate that the *p60::AtMYB60::GFP* construct can rescue the phenotype of *atmyb60-1* plants and thus to verify that the AtMYB60::GFP protein is biologically active.

A stomatal opening assay was then performed to verify the rescue of the *atmyb60-1* phenotype by the AtMYB60::GFP fusion protein. As reported in Figure 18, *atmyb60-1* shows stomata closer than the wild-type ($p<0.01$), as previously reported [1], while there is no significant difference in the stomatal aperture level between wild-type and homozygous *p60::AtMYB60::GFP atmyb60-1* plants.

These data prove the rescue of the *atmyb60-1* phenotype by the AtMYB60::GFP protein, demonstrating that the fusion protein is biologically active.

Identification of putative AtMYB60-binding sites in the promoter sequences of genes coding for the AtMYB60 putative targets, identified through RNA-Seq, is in progress to define fragments that could be analyzed after ChIP assay performed on guard cells purified from *p60::AtMYB60::GFP atmyb60-1* plants.

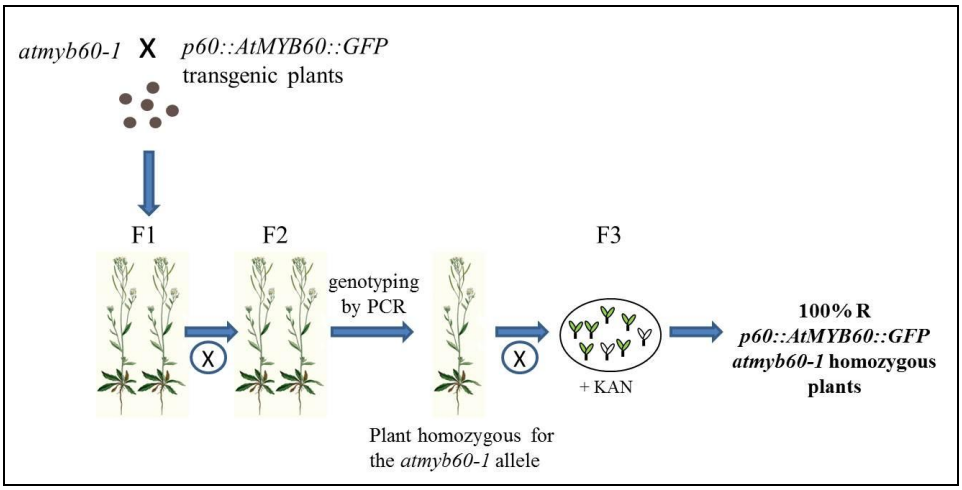

Figure 17 Schematic representation of the crosses between the *p60::AtMYB60::GFP* and *atmyb60-1* mutant plants.

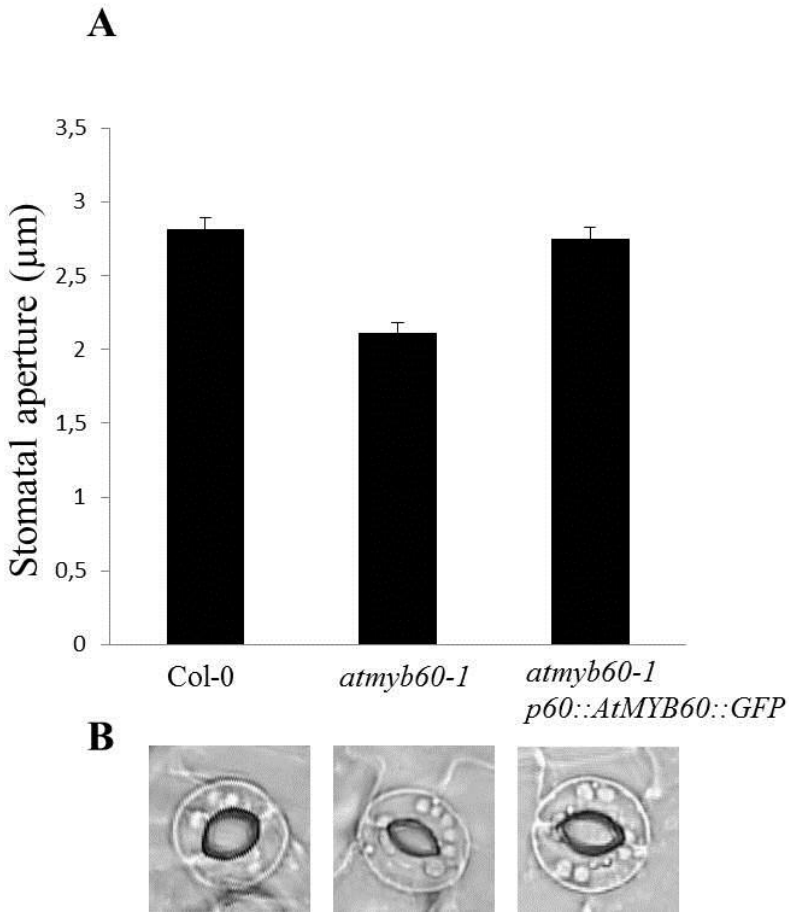

Figure 18 A Stomatal aperture in epidermal peels of Col-0, *atmyb60-1* and *p60::AtMYB60::GFP atmyb60-1* plants in light conditions. Each bar represents the mean of n=~100 stomata measurements, with standard errors. Shown results are representative of three independent experiments; **B** Representative images of Col-0 (left), *atmyb60-1* (middle) and *p60::AtMYB60::GFP atmyb60-1* (right) stomata.

Materials and methods

Plant material and growth conditions

The Columbia ecotype of Arabidopsis thaliana was used in all experiments.

The *atmyb60-1* mutant allele was obtained from the Yale Arabidopsis T-DNA Lines (YATDLs) [1].

For the isolation of *p60::AtMYB60::GFP atmyb60-1* plants immature flowers of homozygous *p60::AtMYB60::GFP* transgenic plants were emasculated and manually cross-pollinated with atmyb60-1 mutants. The F1 plants heterozygous for the atmyb60-1 allele and for the construct were selfed, and the homozygous mutants were identified. Plants of the F2 population were genotyped using a PCR reaction. The DNA was extracted as previously reported [30]. The primers used to amplify the *AtMYB60* wild-type allele are indicated in Table 2. To identify homozygous plants for the *p60::AtMYB60::GFP* construct, F2 homozygous plants for the *atmyb60-1* allele were selfed and F3 population was grown on selective media added with the antibiotic kanamycin, as the *p60::AtMYB60::GFP* construct harbours the gene that confers resistance to the antibiotic (Figure 17).

For guard cell purification, wild-type plants were grown in growth chambers under short-day conditions (8 h light/16 h darkness, 22°C/16°C) with illumination by 100 μmol m^{-2} sec^{-1} white light (25 W, 230 V, Osram TL70 F32T8/TL 741, Philips, http://www.philips.com). Enriched guard

cell fractions were isolated from leaves of 7 week old plants by the "blender method" as described in Geiger et al. 2011 [7] (Figure 12).

For the generation of *p60::AtMYB60::GFP atmyb60-1* plants the Arabidopsis seedlings were grown in soil at 22°C in a greenhouse under long-day conditions (16 h light/8 h darkness, 22°C/16°C). Transgenic seedlings were selected on MS agar plates containing 50 mg/ml kanamycin, then transplanted to soil.

Stomatal aperture measurements

Stomatal assays were performed as previously described [31] with minor changes. The Petri dishes containing the epidermis peels were kept under light (200 μE/m2/s) in a growth chamber at 22°C for 1 hr to assure that most stomata were open before beginning experiments. All the results reported were from blind experiments, the experimenter who measured stomatal responses did not know the genotypes until the end of experiments.

RNA Sequencing

RNA sequencing was performed at the Genomics Research Laboratory, The Wellcome Trust Center for Human Genetics (www.well.ox.ac.uk). The cDNA fragments were prepared for sequencing on the Illumina Genome Analyzer II using the Genomic DNA sequencing Sample Prep Kit (Illumina). Samples were quantified using Nanodrop (Invitrogen) and quality controlled on a 6000 Nano Bioanalyzer chip (Agilent). mRNA enrichment was achieved by processing 6μg of total RNA using the

Magnetic mRNA Isolation Kit from NEB (S1550S) with minor modifications.

Generation of double stranded cDNA and library construction were performed using NEBNext® mRNA Sample Prep Reagent Set 1 (E6100L) according to manufacturer specifications.

Upon ligation of Illumina Adapters (Multiplexing Sample Preparation Oliogonucleotide Kit) each library was size selected using a 2% E-Gel® EX (Invitrogen).

The following custom primers (25 µM each) were used for the PCR enrichment step:

Multiplex PCR primer 1.0

5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC GACGCTCTTCCGATCT-3'

Index primer

5'CAAGCAGAAGACGGCATACGAGAT[INDEX]CAGTGACTGGAG TTCAGACGTGTGCTCTTCCGATCT-3'

Indices were according to the six bases tags developed by Illumina.

The amplified library was purified using Ampure beads (Beckman/Agencourt) and size distribution determined using a Tapestation D1200 system (Agilent/Lab901). Libraries were quantified by realtime using Agilent QPCR Library Quantification Kit and a MX3005P instrument (Agilent) and pooled accordingly. Finally, a second realtime was performed to measure the relative concentration of the pool compared to a previously sequenced mRNA library in order to determine the volume to use for sequencing on a GAIIx.

Table 2. Sequence of oligonucleotides used in this study.

Name	Sequence
P60F3	AAGCTTCGTGTGGAGATCAACAT
2H60R	GATCCCCGGGAATTGCCATGTTAAAGCATATTAGAG

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Chapter 4

Alessandra Albertini[1], Eleonora Cominelli[2], Massimo Galbiati[1,3], Fabio Simeoni[3], Hubert Bauer[4], Rainer Hedrich[4], Chiara Tonelli[1,]*.

Role of the Arabidopsis vacuolar processing enzyme γVPE in response to water stress

Manuscript in preparation

[1]Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

[2]Istituto di Biologia e Biotecnologia Agraria, CNR, Via Bassini 15, 20133 Milano, Italy

[3]Fondazione Filarete, Viale Ortles 22/4, 20139 Milano, Italy

[4]Universität Würzburg, Biozentrum, Julius-von-Sachs-Institut für Biowissenschaften, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany

*corresponding author

Abstract

Plant vacuoles play several roles in controlling plant development, pathogen defence and stress response. *γVPE* is a vacuolar-localised cysteine protein with a caspase-1 like activity involved in the activation and maturation of downstream vacuolar hydrolytic enzymes that triggers to hypersensitive cell death and tissue senescence. This work provide evidence that *γVPE* is strongly expressed in Arabidopsis guard cells and involved in water stress response: the *γvpe* knock-out mutants showed a reduced stomatal opening and an increased resistance to desiccation suggesting a new role of *γVPE* in stomatal movements control.

Key words

vacuolar processing enzyme

guard cells

drought

Introduction

Vacuolar processing enzymes (VPEs) were originally related to the maturation of seed storage proteins [1-6] and subsequently characterised for their role in programmed cell death [7, 8]. VPEs are endopeptidases with a caspase-1-like activity and substrate specificity towards asparagine residues [7]; they are synthesised as inactive pro-protein precursors and self-catalytically activated by sequential removal of the N-terminal and C-terminal peptides at acidic conditions [9]. The Arabidopsis genome has four *VPE* homologs distributed into seed-type, *βVPE* and *δVPE*, and vegetative-type, *αVPE* and *γVPE* [1, 2, 4, 10, 11]. The seed-type *β*VPE, accumulated in the protein storage vacuoles, is the main responsible for the maturation of seed proteins in this cellular compartment [8]. VPE-mediated processing has been demonstrated to be the key machinery for proper processing of storage proteins in Arabidopsis seeds, as the lack of *βVPE* gene causes the accumulation of the precursor storage proteins in mutant seeds [8]. On the contrary, *δVPE* despite being abundantly expressed in the seed coat, has no detectable impact on seed protein processing [11], whereas it is a key player in the developmental programmed cell death of cell layers of the inner integument in the seed coat [7]. The vegetative-type *VPE*s are associated with cell death process as well; they are localised in the lytic vacuoles of vegetative tissues [2] in which senescence is occurring and their expression is up-regulated by wounding and by treatments with ethylene and salicylic acid [8, 12]. Lytic vacuoles have been in fact proposed to be responsible for several type of plant programmed cell death, including hypersensitive cell death, tissue senescence and differentiation of tracheary elements [13-15]. Different results have demonstrated the role of vegetative-type *VPEs* in the vacuole-mediated hypersensitive cell death [9, 16-18], through the

activation of hydrolytic enzymes that firstly cause the collapse of the lytic vacuoles and, when released into the cytosol, attack various organelles and nuclear DNA, crucial event in plant cell death [7, 8, 17]. So far, the research into plant VPEs has mainly focused on terminal differentiation, plant senescence and pathogen-induced hypersensitive cell death.

By contrast, the role of VPEs in response to abiotic stress is poorly understood [9]. Only recently Li and colleagues demonstrated the involvement of γVPE in the Arabidopsis response to heat shock stress (HS) [9], proving the role played by γVPE to trigger the HS-induced programmed cell death by vacuolar disruption [9]. Hence, the vacuole and thereby VPEs are involved in response to abiotic as well as to biotic stress [9].

The complex interaction between biotic and abiotic stresses has been recently fully reviewed [19, 20]. In the natural environment, plants are constantly challenged with biotic and abiotic stresses and they activate a specific stress response when subjected to a combination of multiple stress [19]. Biotic and abiotic stress response is controlled by a range of molecular mechanisms that act together in a complex regulatory network [19]. Stomatal movements represent an example of the complex crosstalk of biotic and abiotic stress responses, in fact the opening and closure of stomata not only regulate water loss during abiotic stress, such as drought, but also serve as a defence mechanism in preventing pathogen invasions [20]. Although in the past years stomata has been considered as passive portal for the plant pathogens entrance [21], Melotto and colleagues demonstrated stomata active role in the plant defence response, as they closed in response to the presence of different bacteria, restricting the pathogenic invasion [22].

In this paper, we demonstrated that the vegetative-type *γVPE* is highly expressed in guard cells and is involved in water stress response. The knock-out mutant *γvpe* shows a reduced stomatal opening and an increased resistance to desiccation. These results support the idea of the involvement of VPEs in processes of both biotic and abiotic stress response [9], demonstrating the presence of a complex regulatory network in plant vacuoles.

Results

γVPE is the most highly expressed gene in guard cells among *VPE*s and is upregulated in response to ABA and desiccation

Previous analysis showed the expression profile of the four *VPE*s genes in Arabidopsis plants [2]; in particular, GUS staining assay revealed a massive expression of *β-VPE* and *δ-VPE* genes in seeds whereas *α-VPE* and *γ-VPE* were highly expressed in vegetative tissues [1, 2, 4, 10, 11]. Transcriptomic analysis of Arabidopsis guard cells, purified from wild-type rosette leaves by the "blender method" [26], revealed that within the four *VPE* genes, *γVPE* is the most highly expressed in this cell-type (our unpublished results and [28]). We confirmed by qRT-PCR analysis that only *βVPE* and *γVPE* are expressed in entire leaves (Figure 1A). Moreover *βVPE* and *γVPE* are expressed in guard cells, with *γVPE* being the most highly expressed in this cell type, while *αVPE* and *δVPE* transcript was not even detected. These data suggest a specific role for γVPE in this cell-type.

Guard cells play a significant role in plant response to abiotic stresses as drought, regulating the quantity of water lost by transpiration [29] and ABA is a well-known stress-response hormone which induces the closure of the stomatal pores and regulates drought tolerance [30]. As shown in

Figure 1, *γVPE* transcript levels were highly increased between 3 and 7 hours of desiccation treatment on mature rosette (Figure 1B) and up to 8 hours of ABA treatment (Figure 1C).

γvpe mutants are more tolerant to water stress

To investigate the potential role of γVPE in response to drought, we isolated two *γVPE* allele mutants, *γvpe::T-DNA1*, previously described [11] and *γvpe::T-DNA2* carrying a T-DNA insertion within exon 5 and exon 6, respectively. The T-DNA insertion sites were confirmed by sequencing the T-DNA/gene junctions as shown in Figure S1A. In *γvpe::T-DNA2* mutant there was a deletion of the sequence comprised between position +1482 bp and +1525 bp from the start codon, probably due to a rearrangement caused by the insertional element (Figure S1A). As reported in Figure S1B, no *γVPE* transcript was detected in the two mutants indicating that they are true knock out alleles. No morphological or developmental abnormalities were observed in homozygous mutant plant compared to the wild-type when grown under standard conditions (data not shown). All the experiments described hereinafter were performed using both alleles.

To test the role of γVPE in drought stress we measured the water loss in detached *γvpe* mutant rosette leaves. Throughout the duration of the experiment, mutant leaves lost significantly less water than the wild-type ($p<0.05$) (Figure 2A). To estimate whole-plant transpiration rate under water scarcity we determined the relative water content (RWC) of both *γvpe::T-DNA1* and *γvpe::T-DNA2* plants after suspension of irrigation. The RWC represents a useful indicator of the water balance of the plant as it considers the interactions among all the factors involved in maintaining the flow of water through the plant [31]. Plants were properly

irrigated for 24 days and then subjected to drought stress by complete termination of irrigation. After 10 days of absence of irrigation, the relative water content was significantly reduced in the wild-type plants compared to the *γvpe* ones ($p<0.001$) (Figure 2B).

Since the water status in leaves is mainly controlled by stomatal opening and closure and the *γVPE* gene is highly expressed in guard cells (Figure 1A), we examined the stomatal movements in the *γvpe* mutants. Under light condition, the *γvpe* plants disclosed a significant reduction of stomatal opening ($p<0.005$) (Figure 3A), while no significant differences in the stomatal closure level were detected between mutants and wild-type after being moved to the darkness for 1 hour (Figure 3A). Stomatal movements are mainly controlled by ABA [32] and we previously demonstrated the *γVPE* expression is induced by the phytohormone (Figure 1C). However, the ABA-induced stomatal closure is not affected in *γvpe::T-DNA1* and *γvpe::T-DNA2* plants (Figure 3B), indicating that guard cell response to ABA is intact in *γvpe* mutants.

Discussion

This study is an attempt to understand the possible role of the *γVPE* gene in the response to abiotic stress. *γVPE* encodes for a vacuolar processing enzyme with a caspase-1 like activity already characterised for its involvement in tissues senescence and pathogen-induced hypersensitive cell death [8]. Through a transcriptome analysis performed on Arabidopsis guard cells we observed a strong expression level of the vegetative-type *γVPE*, compared to the other three *VPE* genes. We confirmed this result through qRT-PCR analysis and found that *γVPE* expression in this cell-type is higher than in entire leaves (Figure 1), suggesting it could play a fundamental role in guard cells.

In Arabidopsis, the VPEs functions in processes like maturation of seed storage proteins, leaf senescence and plant cell death have been intensively reported [7, 17, 33], but their possible role and molecular mechanism of their activity in regulating stomatal movements have not been described so far. There are only indications of a *VPE* role in guard cells in *Nicotiana tabacum* where it has been shown that the suppression of *NbVPE1a* and *NbVPE1b*, the most abundant *VPE*s in tobacco, affected the stomatal closure triggered by elicitors [34]. Phenotypic analysis on the Arabidopsis *γvpe* mutant plants showed a reduced sensitivity to water stress compared to the wild-type plants: the suppression of the *γVPE* gene significantly reduced the water loss from the plant (Figure 2) and surprisingly impaired stomatal aperture under light conditions (Figure 3). *γVPE* is induced by the abscisic acid (Figure 1), a key endogenous messenger involved in stomatal closure by triggering K^+ efflux and release of anions with a consequent reduction in turgor pressure [35] however the *γvpe* mutants did not disclose alterations in the ABA-induced stomatal closure (Figure 3), suggesting that the *γVPE* induction by the abscisic acid and the ABA-induced stomatal closure lie on different signal transduction pathways. All these results prompted us to hypothesize *γVPE* as a new player of the complex mechanism regulating the stomatal movements in response to water stress. *γVPE* is located in the vacuole where it exhibits a proteolytic activity towards the carbonyl terminal of asparagine or aspartic acid residues [7]. Its activity triggers the processing and the activity of downstream vacuolar hydrolytic enzymes such as nucleases, lipases and proteases. Plant vacuoles play a universal role in the generation of Ca^{2+} signals throughout the plant, as they are the major calcium storage in the cell, and can store large amount of solutes affecting the turgor pressure [36]. Guard cell vacuoles have fundamental roles in

controlling stomatal movements as these mechanisms are driven by changes of Ca^{2+} concentration and by the uptake or release of ions and organic metabolites that lead to changes in the osmotic salt content and turgor [37].We can assume that *γVPE*, as a vacuolar proteolytic enzyme, could activate some proteins involved in the Ca^{2+} storage or in solute content changes, acting on the signal cascades that triggers the stomatal opening. By now, only few γVPE targets have been identified: the vacuolar carboxypeptidase AtCPY, a protease enzyme directly activated by γVPE, and the vacuolar invertase AtFruct4, a sucrose cleavage enzyme, although not a γVPE direct target as it was suggested that γVPE may not directly degrade AtFruct4, but rather that it activates other proteases that in turn degrade AtFruct4 [38]. Stomatal opening and closure are directly regulated by changes in the solute content of guard cells [35] and the invertase enzymes irreversibly hydrolyze sucrose to fructose and glucose affecting this solute content [39]. So it is tempting to speculate that γVPE could directly or indirectly activate an invertase enzyme involved in the stomatal movements and that this activation could cause changes in solute content and turgor pressure leading to the stomatal opening. Further analysis will be necessary to demonstrate and clarify this interaction and to identify other γVPE targets to better understand the complex mechanism that regulates stomatal opening and closure; though, this work provide evidence of a new role of γVPE in stomatal movements and consequently in water stress response.

Materials and methods

Plant material and growth conditions

All plant material described was in the Col-0 background. Two mutant alleles of the Arabidopsis *γVPE* gene were used: the *γvpe::T-DNA1* (SALK_010372), previously described by Gruis and colleagues [11], and the *γvpe::T-DNA2* (SALK_024036), both provided by the NASC European Arabidopsis Stock Center (Nottingham, UK). Plants were grown at 22°C in a growth chamber under long-day conditions (16h light/8 h dark) with a light intensity of 100 μE/m^2/s. Homozygous *γvpe::T-DNA1* and *γvpe::T-DNA2* mutants were isolated by PCR genotyping (data not shown). The position of the T-DNA insertion was determined by sequence analysis of the T-DNA/gene junctions.

Water loss measurements

3-week-old wild-type and mutant plants were used. 3 rosette leaves were detached from 4 individual plants for each genotype and dehydrated on Whatman paper in the light at 21°C for up to 8 hr. At different time points the leaves were weighted and the water loss was expressed as the percentage of initial fresh weight, as previously described [23].

Relative water content (RWC)

Wild-type, *γvpe::T-DNA1* and *γvpe::T-DNA2* mutant plants were grown in pots and well irrigated for 24 days. Before starting the drought stress, plants were covered with Saran wrap to avoid water loss by the soil. The RWC was measured after 10 days from the beginning of the treatment, as previously reported [24].

Stomatal aperture measurements

Stomatal assays were performed as previously described [25] with minor changes. The Petri dishes containing the epidermis peels were kept under light (200 μE/m^2/s) in a growth chamber at 22°C for 1 hr to assure that most stomata were open before beginning experiments. To test the stomatal closure in dark conditions the Petri dishes were moved to the darkness for 1hr. For the ABA treatment the epidermis peels were incubate for 2 hr with 10 μM ABA added to the MES buffer. All the results reported were from blind experiments, the experimenter who measured stomatal responses did not know the genotypes until the end of experiments.

qRT-PCR analysis

Total RNA was extracted from 3-week-old wild-type, *γvpe::T-DNA1* and *γvpe::T-DNA2* rosette leaves with Trizol (Invitrogen), following the manufacturer's instructions. For the ABA and desiccation treatments, wild-type plants were treated as previously described [23]. Wild-type guard cells were purified by the "blender method" [26] and total RNA was extracted using the E.Z.N.A. Plant RNA Kit (Omega Bio-tek; http://www.omegabiotek.com). For all the RNA samples, reverse transcription was performed using 5 μg of DNase-treated RNA with RT Superscript™ II (Invitrogen), according to the manufacturer's instructions. qRT-PCR reactions and data analysis were performed as previously reported [27]. *VPEs* expression was analysed using primers RTγVPE-F and RTγVPE-R for *γVPE*, RTαVPE-F and RTαVPE-R for *αVPE*, RTβVPE-F and RTβVPE-R for *βVPE* and RTδVPE-F and RTδVPE-R for *δVPE*. *ACTIN2* and *PP2AA3* gene, corresponding to

At1g13320, were used as references for normalisation. All primer sequences are reported in Table S1.

Table S1

Table S1. Sequence of oligonucleotides used in this study.

Name	Sequence
RTγVPE-F	CGTCTTCGCATTTGTTTATACCAC
RTγVPE-R	TGAGATTGACAACTCCCAAGTTTAC
RTαVPE-F	ATCTTCGTCTCCGCCCTATATTA
RTαVPE-R	CACTGTTTCTTTTGTCTGTGACATC
RTβVPE-F	TACAATACCTACTCAGGTGGCTCTC
RTβVPE-R	GATACATATGCCAAAGGAAGAGAAG
RTδVPE-F	TGTACCAGAGACTTCTCATGTATGC
RTδVPE-R	AGAATGTCTGTAATGCTCTGATCG
ATACT2F	TGCTTCTCCATTTGTTTGTTTC
ATACT2R	GGCATCAATTCGATCACTCA
PP2a-F	CAGCAACGAATTGTGTTTGG
PP2a-R	AAATACGCCCAACGAACAAA

Figure legends

Figure 1 qRT-PCR expression analysis of *VPE* genes in guard cells and leaves (**A**), in response to desiccation (**B**) and ABA 100 μM (**C**). Total RNA was extracted from wild-type Arabidopsis guard cells and whole leaves at indicated time points, expressed in hours (hr). The *βVPE* expression level in leaves (**A**) and the sample collected at time 0 hr (**B** and **C**) were used as calibrators for the relative expression levels in each line. The *ACTIN2* and *PP2AA3* genes were used as controls. Error bars represent standard deviation.

Figure 2 (**A**) Water loss measurements in *γvpe::T-DNA1, γvpe::T-DNA2* and wild-type detached leaves, expressed as a percentage of the initial fresh weight at indicated intervals. Each point indicates the mean of 12 measurements (n=3 leaves from 4 individual plants) with standard errors. Shown results are representative of three independent experiments. (**B**) Relative water content of wild-type, *γvpe::T-DNA1* and *γvpe::T-DNA2* plants during drought. Plants were irrigated for 24 days and then withheld from water for 10 days. Each bar indicates the mean of 16 measurements with standard errors.

Figure 3 Stomatal aperture in epidermal peels of Col-0, *γvpe::T-DNA1* and *γvpe::T-DNA2* plants in dark and light conditions (**A**) and after 2 hr of treatment with ABA 10 μM (**B**). Each bar represents the mean of n=~100 stomata measurements, with standard errors. Shown results are representative of three independent experiments.

Figure S1 (**A**) Schematic representation of *γVPE* gene. Boxes indicate exons (grey) and the 5' and 3' UTR (black), black lines indicate introns. The translational start of the gene is represented by a black arrow. The

positions of the T-DNAs insertions in the *γvpe::T-DNA1* and *γvpe::T-DNA2* mutants are represented as inverted triangles and their orientations and position from the ATG are shown. Diagonal lines indicate the lacked DNA sequence in the *γvpe::T-DNA2* mutant. (**B**) qRT-PCR expression analysis of *γVPE* in wild-type, *γvpe::T-DNA1* and *γvpe::T-DNA2* plants. Col-0 wild-type sample was used as calibrator for the relative expression levels in each line. The *ACTIN2* and *PP2AA3* genes were used as controls. Error bars represent standard deviation.

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Figure 1

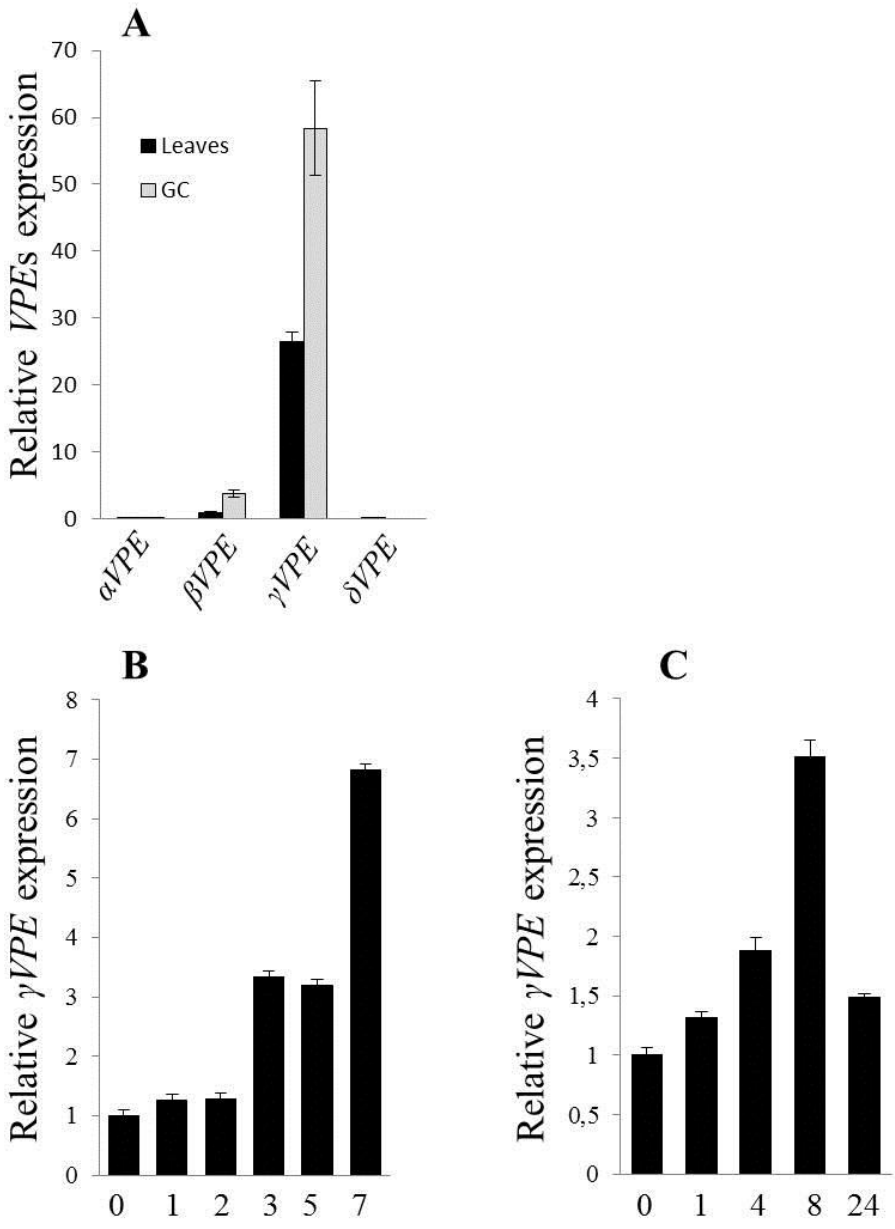

A
Relative VPEs expression
70
60
50
40
30
20
10
0
Leaves
GC
αVPE
βVPE
γVPE
δVPE
B
Relative γVPE expression
8
7
6
5
4
3
2
1
0
0 1 2 3 5 7
C
Relative γVPE expression
4
3,5
3
2,5
2
1,5
1
0,5
0
0 1 4 8 24

Figure 2

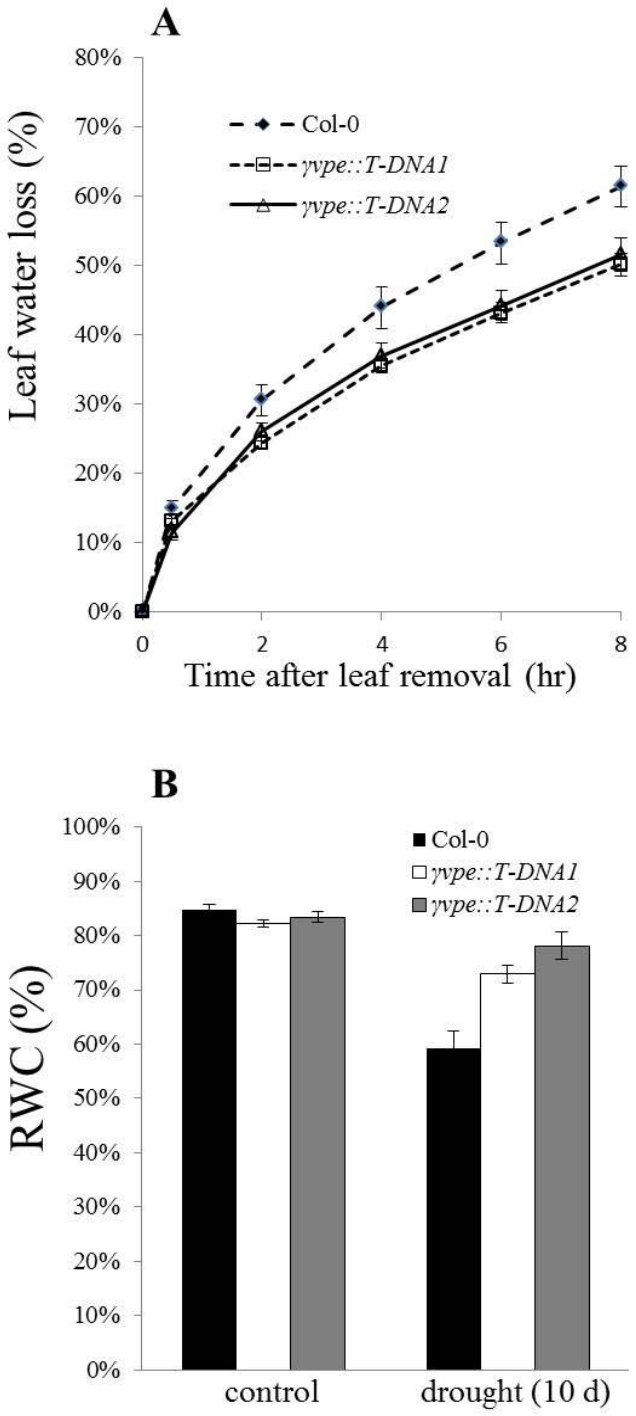

A
Leaf water loss (%)
80%
70%
60%
50%
40%
30%
20%
10%
0%
Col-0
γvpe::T-DNA1
γvpe::T-DNA2
0
2
4
6
8
Time after leaf removal (hr)
B
RWC (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Col-0
γvpe::T-DNA1
γvpe::T-DNA2
control
drought (10 d)

Figure 3

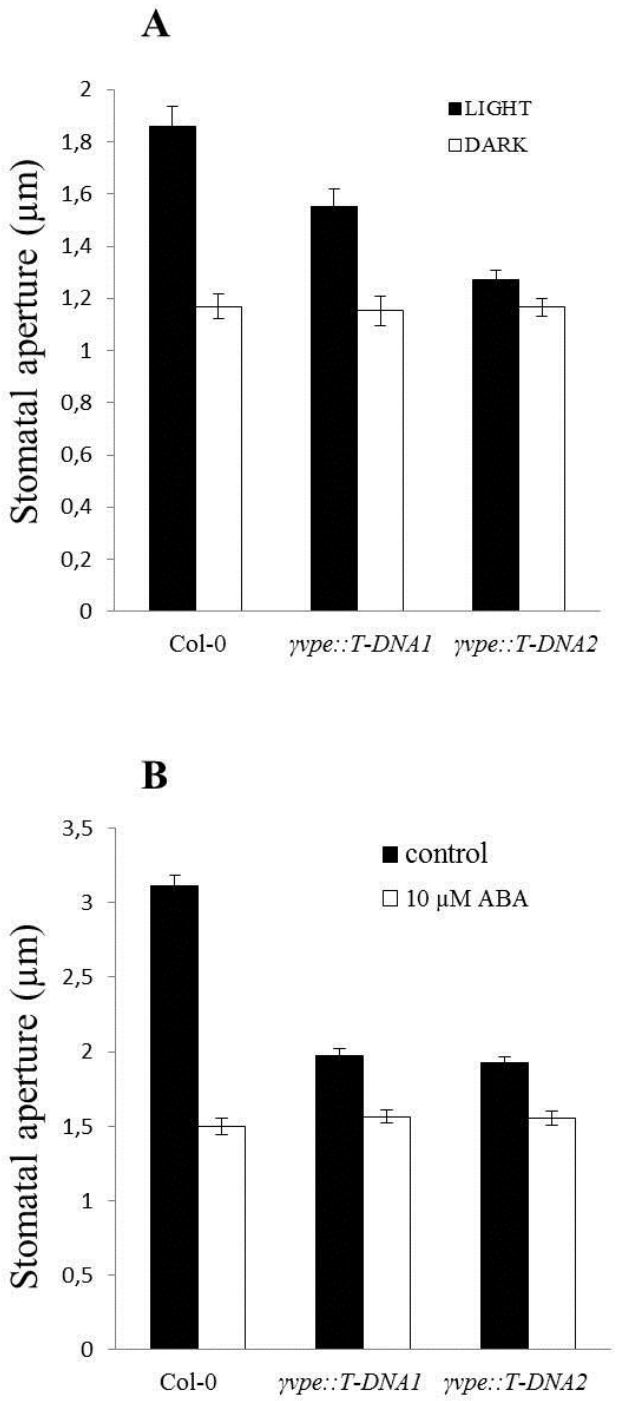
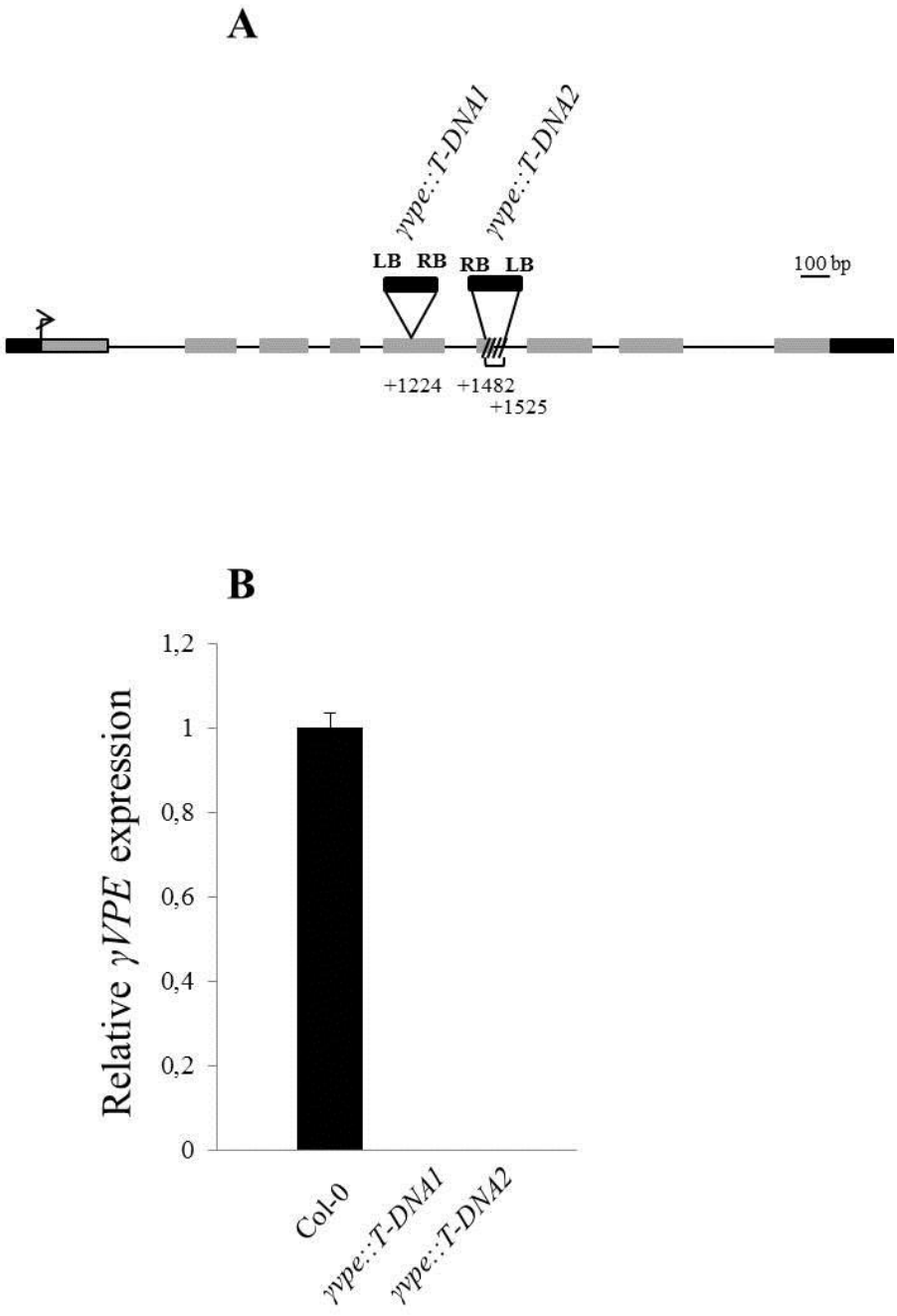

A
Stomatal aperture (µm)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
LIGHT
DARK
Col-0
γvpe::T-DNA1
γvpe::T-DNA2
B
Stomatal aperture (µm)
3,5
3
2,5
2
1,5
1
0,5
0
control
10 µM ABA
Col-0
γvpe::T-DNA1
γvpe::T-DNA2

Figure S1


A
γvpe::T-DNA1
γvpe::T-DNA2
LB RB RB LB
100 bp
+1224 +1482
+1525
B
Relative γVPE expression
1,2
1
0,8
0,6
0,4
0,2
0
Col-0
γvpe::T-DNA1
γvpe::T-DNA2