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RESEARCH PAPER

The *Arabidopsis thaliana MYB60* promoter provides a tool for the spatio-temporal control of gene expression in stomatal guard cells

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Abstract

Plants have evolved different strategies to resist drought, of which the best understood is the abscisic acid (ABA)-induced closure of stomatal pores to reduce water loss by transpiration. The availability of useful promoters that allow for precise spatial and temporal control of gene expression in stomata is essential both for investigating stomatal regulation in model systems and for biotechnological applications in field crops. Previous work indicated that the regulatory region of the transcription factor AtMYB60 specifically drives gene expression in guard cells of *Arabidopsis*, although its activity is rapidly down-regulated by ABA. Here, the activity of the full-length and minimal *AtMYB60* promoters is reported in rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*), using a reporter gene approach. In rice, the activity of both promoters was completely abolished, whereas it was spatially restricted to guard cells in tobacco and tomato. To overcome the negative effect of ABA on the *AtMYB60* promoter, a chimeric inducible system was developed, which combined the cellular specificity of the *AtMYB60* minimal promoter with the positive responsiveness to dehydration and ABA of the *rd29A* promoter. Remarkably, the synthetic module specifically up-regulated gene expression in guard cells of *Arabidopsis*, tobacco, and tomato in response to dehydration or ABA. The comparative analysis of different native and synthetic regulatory modules derived from the *AtMYB60* promoter offers new insights into the functional conservation of the *cis*-mechanisms that mediate gene expression in guard cells in distantly related dicotyledonous species and provides novel tools for modulating stomatal activity in plants.

Key words: ABA, dehydration, guard cell-specific promoters, inducible promoters, stomata, synthetic regulatory modules.

Introduction

Drought represents a major threat to agriculture and food production. Even in the most productive cropping environment, short periods of water scarcity are responsible for considerable reductions in seed and biomass yields each year (Ciais *et al.*, 2005). Increasing temperature and changes in rainfall are expected to exacerbate the negative effects of water deficiency in agriculture (Lobell *et al.*, 2008). In this changing environment, yield stability will depend highly upon the ability to develop novel crop varieties with a more sustainable use of water and enhanced tolerance to water shortages.

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Plants have evolved different adaptive strategies to withstand drought, including the rapid closure of the stomatal pores distributed on the surface of leaves and stems. During drought, plants accumulate the stress hormone abscisic acid (ABA), which triggers in guard cells a signalling cascade that rapidly leads to stomatal closure to minimize water loss by transpiration (Kim *et al.*, 2010). Modelling studies predict that earlier and tighter stomatal closure would reduce desiccation and support yield stability under water stress (Sinclair and Muchov, 2001). Most importantly, data from multiple years of a field trial indicate that enhancement of ABA responses in guard cells can efficiently reduce water loss by transpiration and increase crop resilience to climate change (Wang *et al.*, 2005, 2009).

Genetic screens and gene profiling studies have greatly improved our understanding of the molecular networks that control guard cell activity in response to internal signals and environmental cues, and have identified several candidate genes for downstream biotechnological applications (Leonhardt *et al.*, 2004; Galbiati *et al.*, 2008; Yang *et al.*, 2008; Gardner *et al.*, 2009). Evidence indicates that stomatal closure can be effectively enhanced by disrupting negative regulators of ABA responses, or by overexpressing positive regulators of the ABA signalling pathway (Pei *et al.*, 1998; Gosti *et al.*, 1999; Klein *et al.*, 2003). Guard cell-related transcription factors have also proven to be valuable targets for modulating stomatal activity in plants (Cominelli *et al.*, 2010).

Most genes involved in the regulation of guard cell responses are also expressed in other tissues and control several yield-associated traits (Schroeder *et al.*, 2001). Consequently, genetic engineering strategies which incorporate the use of strong constitutive promoters [e.g. the *Cauliflower mosaic virus* (CaMV) 35S promoter] for conferring transgene expression will result in undesirable side effects on plant growth and productivity. Proper genetic manipulation of stomatal responses involves the use of effective expression systems, including guard cell-specific promoters, to confer precise spatial regulation of transgenes. Above all, regulatory modules which combine cellular specificity with responsiveness to environmental (e.g. dehydration) and/or internal (e.g. ABA) stimuli will prove invaluable in genetically engineering novel adaptive traits in crops.

In a previous work, the 1.3 kb genomic region upstream of the *Arabidopsis AtMYB60* gene (At1g08810) was identified as a guard cell-specific promoter (Cominelli *et al.*, 2005). More recently, it was shown that the activity of the *AtMYB60* promoter in stomata is rapidly down-regulated by exogenous application of ABA (Cominelli *et al.*, 2011). Serial deletion analysis of the 1.3 kb region allowed the discovery of the module responsible for the negative ABA-dependent regulation and identified a 246 bp sequence (from –262 bp to –16 bp upstream of the start codon) as the minimal regulatory module sufficient to confer guard cell-specific activity (Cominelli *et al.*, 2011).

In this work, the pattern of spatial and temporal activity of the *AtMYB60* promoter in rice, tobacco, and tomato is reported. Analysis of stable transgenic lines expressing the *β-glucuronidase* (*GUS*) reporter gene under the control of either the 1.3 kb or the 246 bp *AtMYB60* promoter revealed that these regulatory elements, although inactive in rice tissues, were specifically activated in guard cells of tobacco and tomato. Most importantly, a synthetic system which incorporates the guard cell-specific *AtMYB60* module and the ABA- and drought-inducible module from the stress-regulated *rd29A* promoter was developed. Tobacco and tomato transgenic lines expressing *GUS* under the control of the chimeric promoter revealed strong activation of reporter gene expression upon ABA application or dehydration treatment exclusively in guard cells. Taken together, these results highlight the usefulness of the *AtMYB60* promoter for designing modular expression systems suitable for the spatial and temporal control of gene expression in stomata, both for studying stomatal function in model systems and for engineering guard cell responses in crops.

Materials and methods

Plasmid construct

The previously described $AtMYB60_{pro}1.3$*:GUS* and $AtMYB60_{pro}246$*:GUS* constructs (Cominelli *et al.*, 2011) were used to generate stable transgenic tobacco and tomato lines. For rice transformation, the $AtMYB60_{pro}1.3$*:GUS* and $AtMYB60_{pro}246$*:GUS* cassettes were cloned into the *Hin*dIII–*Eco*RI sites in the pCAMBIA 1300 binary vector (CAMBIA, Canberra, Australia), carrying resistance to hygromycin. To generate the *rd29A-*$MYB60_{pro}246$ construct, the genomic region from –254 bp to –40 bp, located upstream of the *rd29A* gene, was amplified using the primers pDREABF1 (5′-*AAGCTT*ACATTTTAGGATGGAATAAATAT-3′) and pDREABR1 (5′-TCCCTTTATCTCTCTCAGT*AAGCTT*-3′), both containing a *Hin*dIII site (italics). The PCR fragment was inserted in the *Hin*dIII site upstream of the 246 bp promoter in the $AtMYB60_{pro}246$*:GUS* construct.

Plant material, plant transformation, and growth conditions

Arabidopsis transgenic lines (Col-0) were generated by *Agrobacterium*-mediated transformation as described (*A. tumefaciens* strain GV3101) (Clough and Bent, 1998). Transformed seeds were sterilized overnight in a sealed chamber using 100 ml of commercial bleach and 3 ml of 37% HCl and selected on Murashige and Skoog (MS) medium, 1% (w/v) sucrose, 0.8% (w/v), agar, and 50 μg ml^{-1} kanamycin. Plants were grown in a growth chamber under long-day conditions (16 h light; 8 h dark at 100 μmol m^{-2} s^{-1}) at 22 °C. Rice transgenics were produced in the *japonica* cv. Nipponbare, as described (Hiei *et al.*, 1994), using *A. tumefaciens* strain EHA105. Transgenic T_1 lines were selected by germinating seeds on MS medium, 1% (w/v) sucrose, 0.8% (w/v) agar and 50 μg ml^{-1} hygromycin. Resistant plants were transferred to pots containing a blend of loam sandy soil and peat (4:1, v/v) (VIGORPLANT, Fombio, Italy), fertilized with Guano (COMPO, Cesano Maderno, Italy) after repotting and before flowering, and grown in a greenhouse under a 12 h light/12 h dark cycle at 280 μmol m^{-2} s^{-1}, at 26 °C. Tobacco experiments were performed in *Nicotiana tabacum* cv Samsun. Transgenic lines were generated as described (Horsch *et al.*, 1985), using *A. tumefaciens* strain GV3101. T_1 seeds were sterilized with absolute ethanol for 2 min and 50% commercial bleach for 5 min, rinsed with sterile distilled water, and germinated on MS medium, 1.5% (w/v) sucrose, 0.7% plant agar, and 50 μg ml^{-1} kanamycin. Kanamycin-resistant plants were transferred to pots and grown as described for rice. Tomato transgenic lines were produced in both the Microtom and Moneymaker backgrounds as described (McCormick, 1991; Davuluri *et al.*, 2005) (*A. tumefaciens* strain AGL1). Rooted plants were transferred to pots and grown

in a greenhouse as described for rice and tobacco. T_1 kanamycin-resistant plants were selected as described for tobacco.

GUS assays

For detection of GUS activity, tissues were vacuum-infiltrated and incubated for 24–48 h at 37 °C, in 0.5 mg ml^{-1} X-glucuronic acid, 0.1% Triton X-100, and 0.5 mM ferrocyanidine in 100 mM phosphate buffer (pH 7). Tissues were cleared with a chloral hydrate:glycerol:water solution (8:1:2, v/v/v). Samples were examined using a Leica M205 FA stereomicroscope and Leica DM2500 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany).

ABA and dehydration experiments

ABA treatments were performed *in vivo*. Plants grown in soil were sprayed with 100 µM ABA (±-*cis*, *trans* ABA; SIGMA, Milano, Italy), dissolved in 100% ethanol, or with an equal amount of ethanol (mock solution). For dehydration experiments, leaves were detached from soil-grown plants and air dried for up to 6 h in a growth cabinet under continuous light, at 26 °C, with 30% relative humidity.

Quantification of mRNA expression

RNA isolation, reverse transcription, and quantitative PCRs (qPCRs) were performed as previously described (Galbiati *et al.*, 2011). *GUS* expression was analysed using primers qPCR_GUSF1 and qPCR_GUSR1, and normalized using the *AtACTIN2* gene (At3g18780) in *Arabidopsis* (Nishimura *et al.*, 2003), the *Elongation Factor 1α* gene (*NtEF1α*) in tobacco (Liu *et al.*, 2012), or the *LeEF1α* gene in tomato (Bartley *et al.*, 2003). In rice, the presence of the transgene and *GUS* expression were assessed by PCR and reverse transcription–PCR (RT–PCR), respectively, using the primers GUSRTF1 and GUSRTR1. The *OsActin* gene was used as a control (Zhao *et al.*, 2009). The sequences of all the primers used in expression studies are listed in Supplementary Table S1 available at *JXB* online.

Results

Activation pattern of the Arabidopsis AtMYB60 promoter in rice, tobacco, and tomato

To investigate the activity of the guard cell-specific *AtMYB60* promoter in cereals, stable transgenic lines of rice (spp. *japonica* cv. Nipponbare), expressing the reporter *GUS* under the control of the full-length (1.3 kb) or the minimal (246 bp) regulatory region of *AtMYB60*, were generated (*$AtMYB60_{pro}$1.3:GUS* and *$AtMYB60_{pro}$246:GUS* lines, respectively). Thirty hygromycin-resistant primary transformants (T_0) were recovered for each construct, and the presence of the transgene was investigated by PCR (Supplementary Fig. S1 at *JXB* online). Staining of developing T_0 *$AtMYB60_{pro}$1.3:GUS* or *$AtMYB60_{pro}$246:GUS* leaves did not reveal *GUS* expression in guard cells or in any other cell type (Fig. 1A, B). Consistently, RT–PCR analysis of the T_1 seedlings did not detect expression of the reporter gene (Supplementary Fig. S1). These findings suggest the absence of a MYB60-related regulatory network in the guard cell of rice, which might reflect the functional divergence in stomata between monocots and dicots (Serna, 2011).

Next, the conservation of the cellular specificity of the *AtMYB60* promoter was investigated in dicot systems, with the two *Solanaceae* crop species tobacco (cv. Samsun) and tomato (cv. Microtom and cv. Moneymaker). Histochemical analysis of 10–15 independent T_0 *$AtMYB60_{pro}$1.3:GUS* or *$AtMYB60_{pro}$246:GUS* lines for each genotype revealed specific GUS staining in guard cells distributed on developing leaves (Fig. 1C–H). In agreement with data from *Arabidopsis*, which demonstrate that the full-length 1.3 kb *AtMYB60* promoter possesses stronger activity in guard cells (Cominelli *et al.*, 2011), stomata from *$AtMYB60_{pro}$1.3:GUS* plants showed more intense GUS signals, compared with *$AtMYB60_{pro}$246:GUS* individuals, in both tobacco and tomato.

Independent T_2 *$AtMYB60_{pro}$1.3:GUS* and *$AtMYB60_{pro}$246:GUS* tobacco and tomato lines (n=10) were selected to investigate further the cell and tissue specificity of *GUS* expression during plant development. Ten-day-old

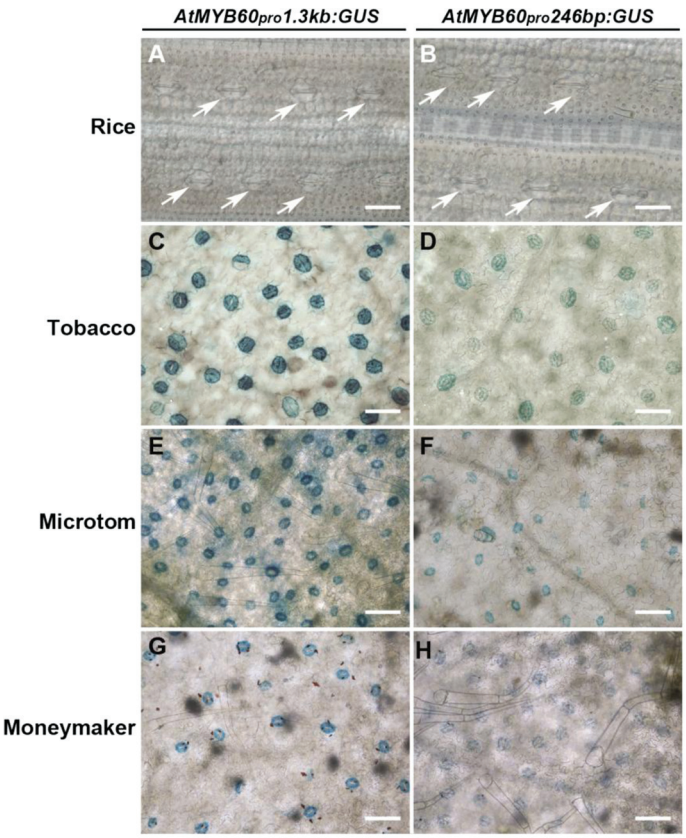

Fig. 1. Histochemical localization of GUS activity in transgenic rice, tobacco, and tomato T_0 plants. (A and B) Leaves from rice *AtMYB60pro1.3:GUS* and *AtMYB60pro246:GUS* plants, respectively. Arrows indicate stomata. (C and D) Leaves from tobacco *AtMYB60pro1.3:GUS* and *AtMYB60pro246:GUS* plants, respectively. (E and F) Leaves from Microtom *AtMYB60pro1.3:GUS* and *AtMYB60pro246:GUS* plants, respectively. (G and H) Leaves from Moneymaker *AtMYB60pro1.3:GUS* and *AtMYB60pro246:GUS* tomato plants, respectively. Leaves from the *AtMYB60pro1.3:GUS* lines were incubated in the staining solution for 24 h (A, C, E, G), whereas leaves from the *AtMYB60pro246:GUS* lines were incubated for 48 h (B, D, F, H). Bar=50 µm.

AtMYB60$_{pro}$*1.3:GUS* seedlings displayed expression of the reporter exclusively in guard cells, distributed on cotyledons, hypocotyls, and leaf primordia, in all the tobacco (Fig. 2A, B) and tomato (Fig. 2D, E) lines analysed. No GUS signals were detected in roots (insets in Fig. 2A, D). Similarly, *AtMYB60*$_{pro}$*246:GUS* tomato and tobacco seedlings showed *GUS* expression exclusively in guard cells, even though the intensity of the staining was reduced compared with seedlings harbouring the *AtMYB60*$_{pro}$*1.3:GUS* construct (data not shown). Analysis of developing and mature leaves confirmed the guard cell-specific expression of the reporter in both species (Fig. 2C, F). In flowers, consistent *GUS* expression in guard cells located on sepals was detected (Supplementary Fig. S2A, E at *JXB* online). Occasionally, diffuse and intense staining of anthers was observed in individual flowers from tomato, whereas weak localized signals were found in the inner part of tobacco anthers (Supplementary Fig. S2). These findings are in contrast to the lack of *GUS* expression in reproductive tissues reported for both the *AtMYB60*$_{pro}$*1.3:GUS* and *AtMYB60*$_{pro}$*246:GUS* constructs in *Arabidopsis* (Cominelli *et al.*, 2011). However, GUS activity was also observed in anthers of flowers from untransformed Moneymaker, Microtom, and tobacco plants (Supplementary Fig. S2). It is thus likely that *GUS* expression in male reproductive organs originated from an anther-specific endogenous *GUS* activity, which has been previously described for different members of the *Solanaceae* family, including tomato (Plegt and Bino, 1989).

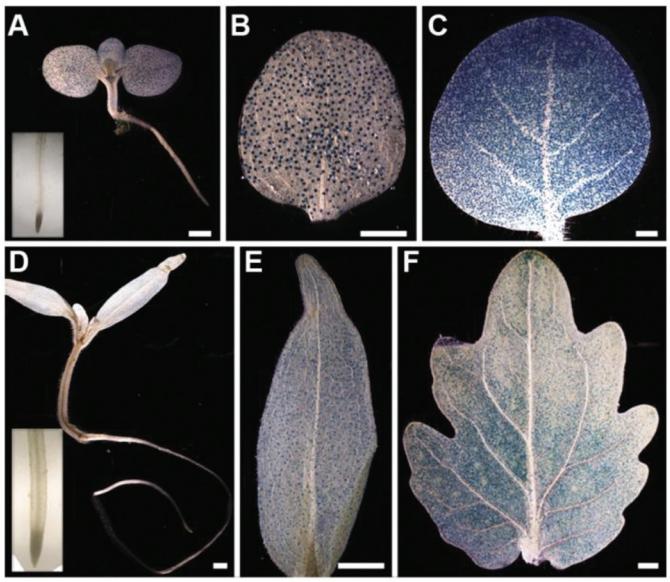

Fig. 2. Developmental *GUS* expression patterns in homozygous T_2 *AtMYB60pro1.3:GUS* tobacco and tomato lines. (A) A 15-day-old tobacco seedling. The inset represents a magnified view of the primary root, (B) Detail of a cotyledon. (C) Developing tobacco leaf. (D) A 15-day-old Microtom seedling. The inset represents a magnified view of the primary root. (E) Detail of a cotyledon. (F) Developing Microtom leaflet. All tissues were incubated in the staining solution for 24 h. Bar=1 mm.

The activity of the AtMYB60 promoter is negatively regulated by ABA and dehydration in tobacco and tomato guard cells.

In *Arabidopsis*, the activity of the full-length *AtMYB60* promoter is rapidly down-regulated following exogenous applications of ABA, whereas the 246 bp minimal promoter is not affected by the hormone (Cominelli *et al.*, 2011). ABA treatment of *AtMYB60*$_{pro}$*1.3:GUS* tobacco and Microtom plants caused a marked reduction in histochemical detection of GUS signals in guard cells compared with mock-treated plants (Fig. 3A). These results were substantiated by qPCR analyses of two randomly selected lines, which showed significant down-regulation of the level of *GUS* transcripts upon ABA application ($P < 0.001$ for all time points, paired Student's *t*-test) (Fig. 3B, D). A comparable down-regulation of GUS staining and *GUS* transcript abundance was also evident when leaves were subject to 6 h of dehydration (Fig. 3A, C, E). The same results were obtained when Moneymaker *AtMYB60*$_{pro}$*1.3:GUS* tomato plants were exposed to ABA or dehydration (data not shown).

ABA treatment of the *AtMYB60*$_{pro}$*246:GUS* lines yielded conflicting results. Nearly 80% of the Microtom (n=10), Moneymaker (n=10), or tobacco (n=15) lines analysed did not show obvious changes in the intensity of GUS staining in response to ABA (Fig. 4A). Surprisingly, the remaining 20% of the lines displayed marked down-regulation of GUS activity in guard cells, following exposure to ABA (Fig. 4A). These results were further confirmed by qPCR analysis of *GUS* expression in randomly selected lines (Fig. 4B, C). Nonetheless, dehydration treatments resulted in a drastic decrease of *GUS* expression in both ABA-insensitive and ABA-sensitive lines, suggesting a possible ABA-independent regulation of the 246 bp minimal promoter in response to stress (Fig. 4A, C, D).

Construction of an ABA- and dehydration-inducible guard cell-specific synthetic promoter.

The conserved cellular specificity of the *AtMYB60* promoter makes it a potentially valuable tool to manipulate guard cell activity in *Solanaceae* crops. However, an obvious pitfall for the general applicability of this tool resides in the strong ABA- and dehydration-induced down-regulation of its activity. In the attempt to reprogram the negative response of the *AtMYB60* promoter to ABA and dehydration, a novel chimeric promoter was constructed. Such a synthetic regulatory element combined the *AtMYB60* guard cell-specific module with the ABA- and stress-inducible *rd29A* promoter (At5g52310) (Yamaguchi-Shinozaki and Shinozaki, 1994). In more detail, the 246 bp *AtMYB60* minimal promoter was fused to the 214 bp region of the *rd29A* promoter (from –254 bp to –40 bp), which contains two dehydration-responsive elements (DREs; TACCGACAT), a DRE-core motif (GCCGAC), one activator sequence (*as1*; TGACGTCA), and one ABA-responsive element (ABRE; TACGTGTC) (Yamaguchi-Shinozaki and Shinozaki, 1994; Narusaka *et al.*, 2003). This regulatory region has been shown to activate gene

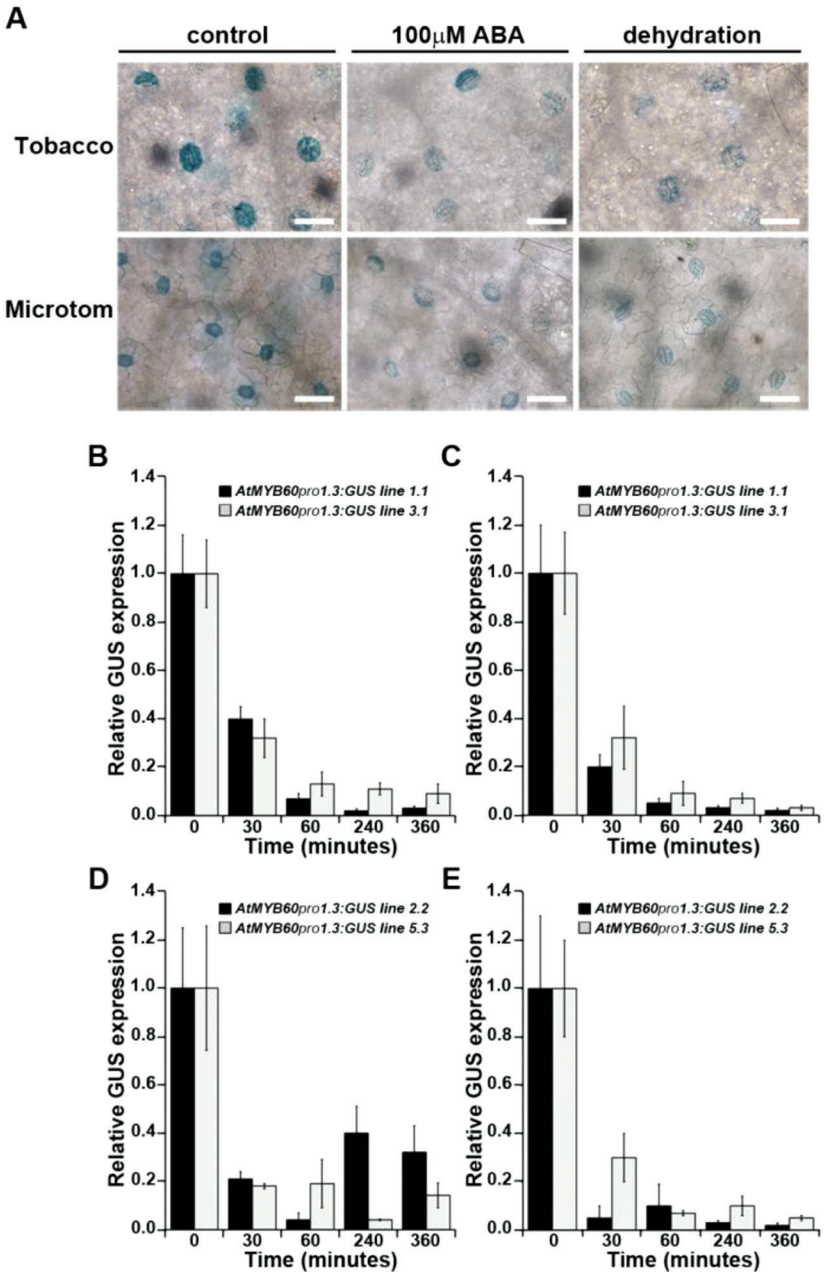

Fig. 3. ABA- and dehydration-induced down-regulation of *GUS* expression in the *AtMYB60pro1.3:GUS* lines. (A) Histochemical detection of GUS activity in guard cells from tobacco and Microtom *AtMYB60pro1.3:GUS* lines following 6 h of exposure to 100 μM ABA or dehydration. Control and treated tissues were incubated in the staining solution for 24 h. Bar=50 μm. (B–E) qPCR analysis of *GUS* expression in response to 100 μM ABA (B and D) or dehydration (C and E) in two independent tobacco (B and C) or Microtom lines (D and E). Total RNA samples were extracted at the indicated time points (minutes). Relative *GUS* transcript levels were determined using *GUS*-specific primers and normalized to the expression of the tobacco or tomato *EF1*α genes.

expression strongly in response to osmotic stress through both ABA-dependent and ABA-independent pathways in different plant species (Kasuga *et al.*, 1999, 2004; Wang *et al.*, 2005). The resulting *rd29A-MYB60*$_{pro}$*246* chimeric promoter was fused to the reporter *GUS* and transformed in *Arabidopsis* (Fig. 5A). Fifteen independent T_2 *rd29A-MYB60*$_{pro}$*246:GUS* lines were selected for analysis of *GUS* expression. A previously described *AtMYB60*$_{pro}$*246:GUS* line was used as a control for the experiments (Cominelli *et al.*, 2011). Under standard growth conditions, all the transgenic lines expressing *GUS* under the control of the chimeric promoter showed a weak stomatal GUS pattern, comparable in intensity and distribution to that of the control line (Fig. 5B). In agreement with a previous report (Cominelli *et al.*, 2011), the activity of the 246 bp control promoter was largely unaffected by ABA, albeit that it was down-regulated by dehydration. Conversely, both ABA and dehydration treatments triggered a strong increase of *GUS* expression in the *rd29A-MYB60*$_{pro}$*246:GUS* lines (Fig. 5B). Importantly, augmented GUS signals were only observed in guard cells and not in

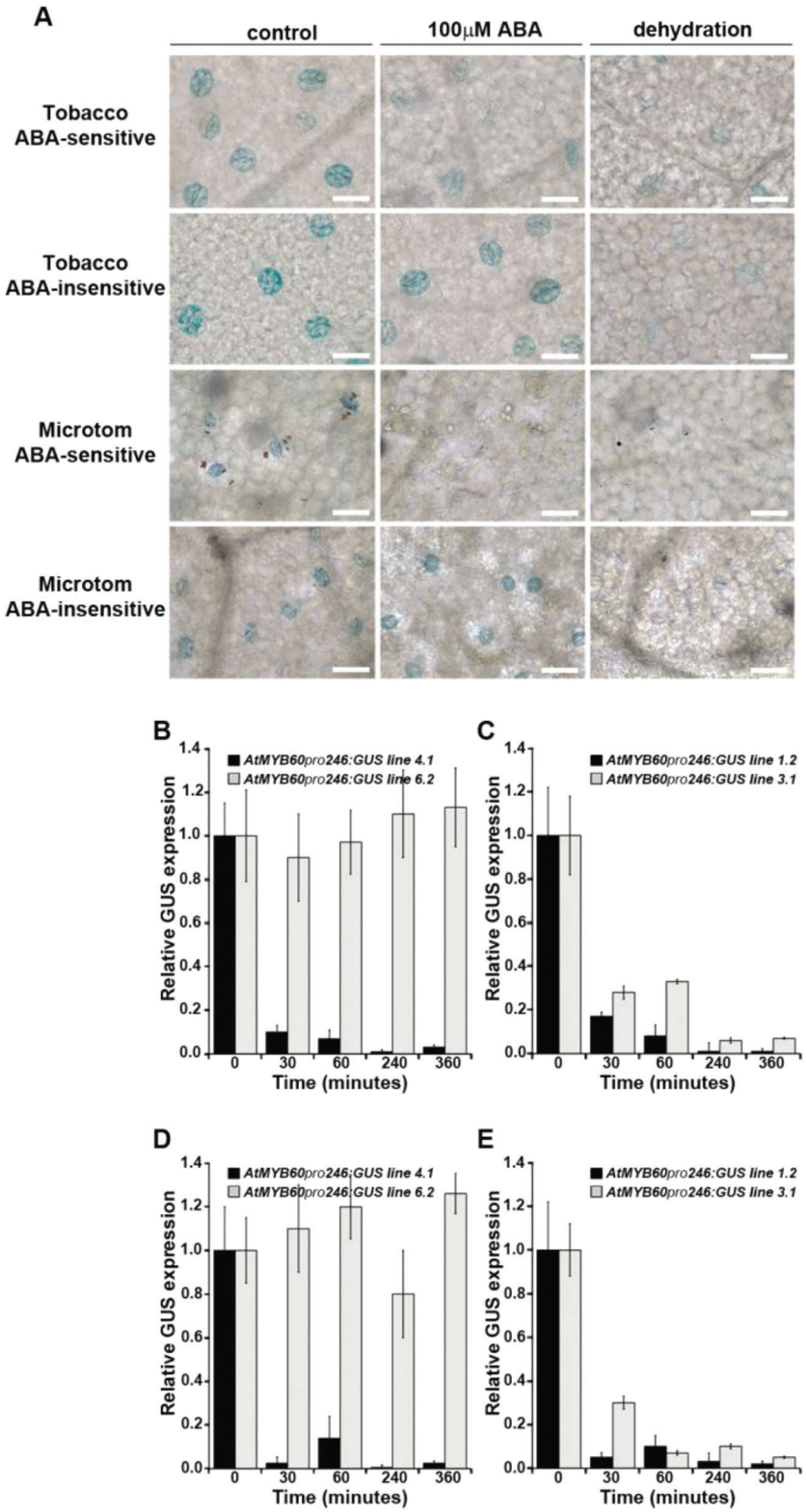

Fig. 4. Analysis of *GUS* expression in the *AtMYB60pro246:GUS* lines in response to ABA and dehydration treatments. (A) Histochemical detection of GUS activity in guard cells from tobacco and Microtom *AtMYB60pro246:GUS* lines following 6 h of exposure to 100 μM ABA or dehydration. Control and treated tissues were incubated in the staining solution for 48 h. Bar=50 μm. (B and C) qPCR analysis of *GUS* expression in response to 100 μM ABA (B) or dehydration (C) in ABA-sensitive (black bars) and ABA-insensitive (grey bars) tobacco lines. (D and E) qPCR analysis of *GUS* expression in response to 100 μM ABA (D) or dehydration (E) in ABA-sensitive (black bars) and ABA-insensitive (grey bars) Microtom lines. Total RNA samples were extracted at the indicated time points (minutes). Relative *GUS* transcript levels were determined using *GUS*-specific primers and normalized to the expression of the tobacco or tomato *EF1*α genes.

other cell types. qPCR analysis of three randomly selected lines confirmed the significant induction of *GUS* transcripts following ABA application or exposure to dehydration (up to 16-fold, $P < 0.001$, paired Student's *t*-test) (Fig. 5C, D).

Next, the *rd29A-MYB60$_{pro}$246:GUS* construct was introduced in tobacco and tomato to test whether the synthetic promoter retained its cellular specificity and its responsiveness in these two species. Histochemical analysis of 10 independent T_2 tobacco lines revealed expression of the reporter exclusively in stomatal guard cells, with an intensity of staining similar to the *AtMYB60$_{pro}$246:GUS* line, used as a control (Fig. 5E). Following application of ABA or exposure to dehydration, all the *rd29A-MYB60$_{pro}$246:GUS* lines showed significant up-regulation of *GUS* expression, as demonstrated by both the intensity of the staining (Fig. 5E) and the level of the *GUS* transcripts (Fig. 5F, G, up to 14-fold, $P < 0.001$, paired Student's *t*-test). Notably, after 6 h of exposure to dehydration, plants showed severe symptoms of wilting (Supplementary Fig. S3 at *JXB* online). Yet, the *rd29A-MYB60$_{pro}$246:GUS* lines still showed intense GUS staining in stomata distributed on the damaged tissue. Comparable results, in terms of both cellular specificity and ABA- and dehydration-induced up-regulation of *GUS* expression, were obtained in tomato plants transformed with the *rd29A-MYB60$_{pro}$246:GUS* construct (Supplementary Fig. S4).

As a whole, these findings validate the use of the *AtMYB60* minimal promoter to engineer synthetic regulatory modules to activate gene expression in guard cells in response to hormonal signals and environmental cues, in both model plant systems and crops.

Discussion

The availability of a wide repertoire of cell-specific and inducible promoters has become increasingly important for all levels of genetic engineering in plants, from primary research to development of commercial biotech crops. Previous studies indicated that the *AtMYB60* transcription factor is highly expressed in stomatal guard cells (Leonhardt *et al.*, 2004; Galbiati *et al.*, 2008; Bates *et al.*, 2012) and demonstrated that its promoter sequence specifically activates transgene expression in stomata of *Arabidopsis* (Cominelli *et al.*, 2005, 2011; Nagy *et al.*, 2009; Meyer *et al.*, 2010; Bauer *et al.*, 2013). In this work the cellular specificity of the *AtMYB60* promoter was investigated in rice, tobacco, and tomato. Analysis of several independent rice lines carrying the *GUS* gene under the control of either the minimal or the full-length *AtMYB60* promoter did not detect reporter activity in guard cells or in any other cell type (Fig. 1A, B). Stomata found in grasses and in dicots are highly divergent in terms of cell morphology and tissue patterning (Serna, 2011). The lack of activity of the *AtMYB60* promoter in rice probably reflects an evolution-driven difference in the transcriptional mechanisms that mediate gene expression in guard cells from monocots and dicots. Control of gene expression is largely determined by *cis*-regulatory modules localized in the promoter sequence of regulated genes and their cognate transcription factors. Clusters of DNA consensus sequences for DOF proteins ([A/T]AAAG), found upstream of the ATG codon of *AtMYB60*, have proved essential to activate gene expression in stomata (Cominelli *et al.*, 2011). Consistently, a DOF-type transcription factor (*Stomatal Carpenter 1*; *SCAP1*) has been shown to bind the *AtMYB60* promoter directly and to regulate *AtMYB60* expression in guard cells (Negi *et al.*, 2013). One likely possibility is that rice guard cells lack the *trans*-acting factors which bind to the [A/T]AAAG motifs in the *AtMYB60* promoter, suggesting that grasses employ *cis*-elements other than DOF motifs to regulate gene expression in stomata. Further evidence indicates the lack of a MYB60-related guard cell-specific regulatory network in rice. Two putative *AtMYB60* orthologues have been identified in the rice genome, namely *LOC_Os11g03440* and *LOC_Os12g03150* (Kawahara *et al.*, 2013). A comprehensive transcriptomic analysis of several cell types from rice revealed that both genes are widely expressed in leaves but their expression is strongly down-regulated in guard cells compared with other cell types, including blade mesophyll, bundle sheath, and vein (Jiao *et al.*, 2009).

In contrast to rice, in tobacco and tomato, activity of the *AtMYB60* promoter was regulated to the same developmental, spatial, and cell-specific stringency as in *Arabidopsis*. Transgenic lines harbouring the *AtMYB60$_{pro}$1.3:GUS* or the *AtMYB60$_{pro}$246:GUS* construct revealed *GUS* expression exclusively in guard cells throughout plant development (Fig. 2). Oh and colleagues reported that a 1.2 kb region of the *AtMYB60* promoter can drive reporter gene expression in roots of *Arabidopsis* (Oh *et al.*, 2011). Nevertheless, patchy patterns of GUS activity in root tissues were only detected in seedlings upon prolonged treatment (up to 24 h) with indole acetic acid (IAA), a conditon which might not reflect the physiological role of the *AtMYB60* promoter. Conversely, previous analysis of nearly 100 independent *Arabidopsis* lines carrying serial deletions of the *AtMYB60* promoter fused to the *GUS* gene did not reveal expression of the reporter in root tissues under standard growth conditions or following exposure to ABA (Cominelli *et al.*, 2011). Fully consistent with these results, no GUS staining was observed in roots from the tobacco and tomato lines described in this study (Fig. 2).

As previously observed in *Arabidopsis* (Cominelli *et al.*, 2011), the 246 bp *AtMYB60* regulatory region showed weaker activity in guard cells from tobacco and tomato, compared with the 1.3 kb full-length promoter (Fig. 1C–H). Different regions of the *AtMYB60* promoter can thus be exploited to produce cell-specific expression systems tailored to achieve various level of transgenes expression in stomata.

Results from tobacco and tomato point to the conservation of the *cis*- and possibly *trans*-mechanisms that modulate gene expression in the guard cell of *Arabidopsis* and *Solanaceae*. Interestingly, a preliminary survey of the closest homologue of *AtMYB60* found in the tomato genome (*Solyc10g081490*) revealed a high degree of similarity in the number, organization, and localization of DOF target sites with the promoter region of *AtMYB60* (Supplementary Fig. S5 at *JXB* online). As clusters of [A/T]AAAG motifs have also been identified in the promoter of the guard cell-specific potassium channel

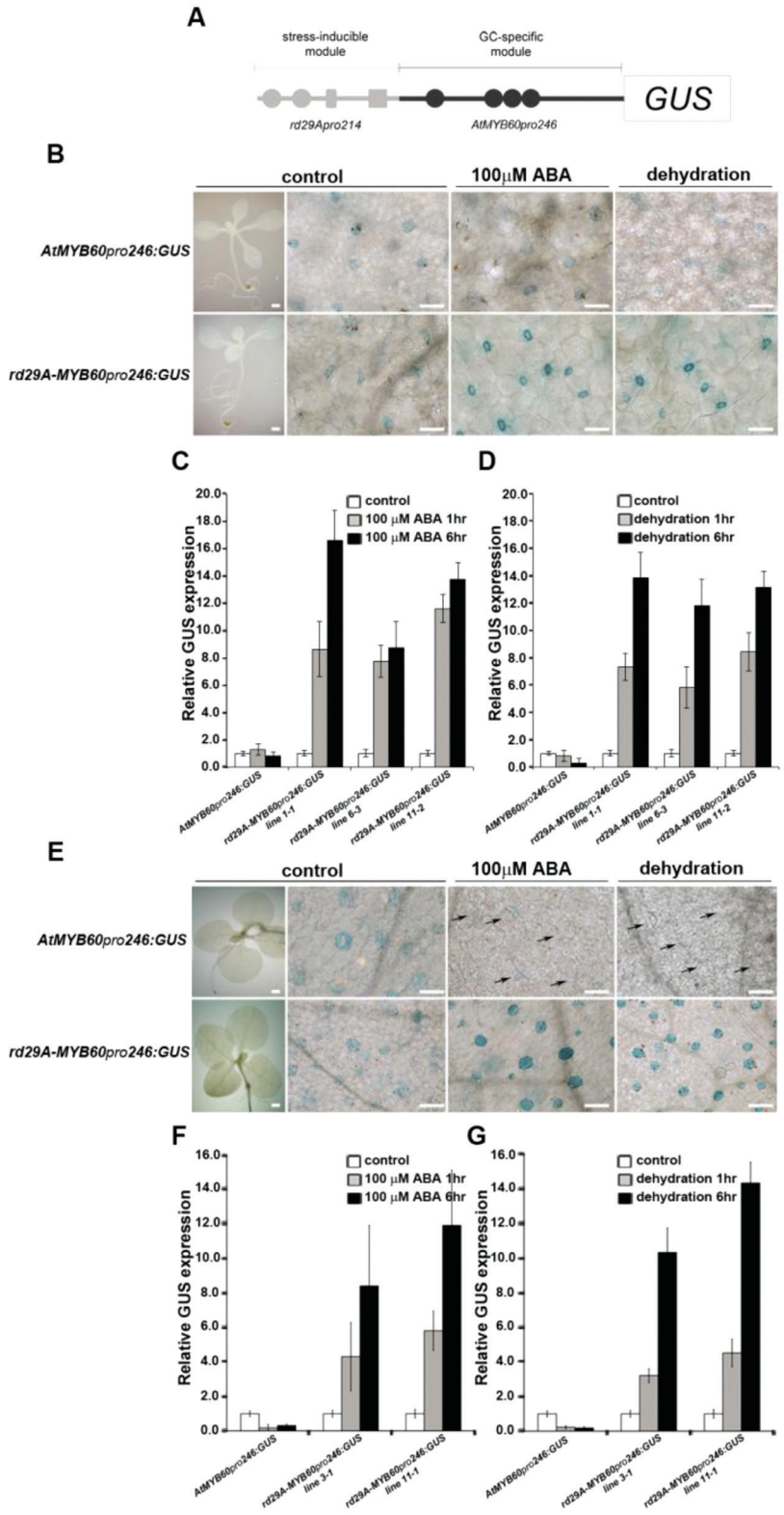

Fig. 5. Rewiring of the activity of the *AtMYB60* promoter in guard cells. (A) Schematic representation of the *rd29A-MYB60pro246:GUS* construct (not to scale). Grey circles represent DRE-core motifs (GCCGAC), the grey rectangle represents the *as1* motif (TGACGTCA), the grey square represents the ABRE motif (TACGTGTC), and black circles represent DOF motifs ([A/T]AAAG). (B) Histochemical localization of GUS activity in guard cells from a 15-day-old *AtMYB60pro246:GUS Arabidopsis* control plant (upper left panel) and from a *rd29A-MYB60pro246:GUS* plant (lower left panel), grown under standard conditions (bar=1 mm). Leaves from the *AtMYB60pro246:GUS* control line did not show evident changes in the intensity of the GUS staining following 6 h of exposure to 100 μM ABA, whereas they showed reduced staining after 6 h of dehydration treatment (upper panels). Conversely, ABA and dehydration tratments induced a drastic increase in GUS activity in the *rd29A-MYB60pro246:GUS* line (lower panels) (bar=50 μm). (C and D) qPCR analysis of *GUS*

KST1 from potato (Plesch *et al.*, 2001), as well as in the regulatory region of *VvMYB60*, the functional orthologue of *AtMYB60* from grape (Galbiati *et al.*, 2011), it is intriguing to speculate that the conservation of guard cell-specific *cis*-regulatory elements extends across a wide range of dicotyledonous plants.

Previous data demonstrated that the activity of the full-length *AtMYB60* promoter is negatively regulated by ABA in *Arabidopsis* (Cominelli *et al.*, 2011). In agreement with this observation, exogenous application of the hormone or exposure to severe dehydration resulted in the rapid down-regulation of *GUS* expression in tobacco and tomato *AtMYB60*$_{pro}$*1.3:GUS* lines (Fig. 3). The sequence comprised between –366 bp and –262 bp from the ATG start codon of *AtMYB60* has been identified as the region responsible for the negative effect of ABA on gene expression (Cominelli *et al.*, 2011). Consistent with this finding, *Arabidopsis* lines carrying the reporter gene under the control of the 246 bp minimal promoter (devoid of the –366/–262 bp region) did not show significant changes in *GUS* expression in response to ABA (Cominelli *et al.*, 2011). Even though the majority of the tobacco and tomato *AtMYB60*$_{pro}$*246:GUS* lines analysed in this study displayed insensitivity to ABA in terms of regulation of GUS activity, a few lines retained a negative response to the hormone (Fig. 4). This result could be the consequence of positional effects on the activity of the transgene, or could advocate the involvement of divergent *cis*-mechanisms that mediate ABA-induced gene repression in *Arabidopsis* and *Solanaceae*. Notably, both ABA-insensitive and ABA-sensitive lines revealed a drastic decrease of GUS activity in response to dehydration (Fig. 4A, C, D). This indicates that the *AtMYB60* minimal promoter probably encompasses *cis*-acting elements capable of down-regulating gene expression in response to stress in an ABA-independent manner and demonstrates the functional conservation of such elements in *Arabidopsis*, tobacco, and tomato.

The observed ABA- and dehydration-induced down-regulation of the *AtMYB60* promoter activity poses obvious limits to its applicability. Guard cell-specific regulatory modules suitable for the selective up-regulation of transgene expression upon stress imposition are highly desirable both for functional studies and for biotechnological applications in crops. Taking advantage of the well-characterized modular organization of the stress-activated *rd29A* promoter, a chimeric regulatory element (*rd29A-MYB60*$_{pro}$*246*) intended for rewiring the activity of the *AtMYB60* promoter was constructed. To this end, the 214 bp stress-responsive module from the *rd29A* promoter was conjugated to the 246 bp *AtMYB60* guard cell-specific element (Fig. 5A). Analysis of several independent transgenic lines demonstrated that, in contrast to the native 246 bp *AtMYB60* promoter, the chimeric *rd29A-MYB60*$_{pro}$*246* system was capable of boosting gene expression in response to ABA or dehydration not only in *Arabidopsis* (Fig. 5B–D), but also in tobacco (Fig. 5E–G) and tomato (Supplementary Fig. S3 at *JXB* online). This implies that the dehydration-induced down-regulation of *GUS* expression mediated by the *AtMYB60* minimal promoter is over-ruled by the stress-activated *rd29A* module. One likely possibility is that, under stress, repression of the 246 bp *AtMYB60* promoter is counteracted by the activity of the dehydration-induced CBF/DREB transcriptional activators, which directly bind the DRE motifs in the *rd29A* promoter (Shinozaki and Yamaguchi-Shinozaki, 2000).

Most importantly, the *rd29A-MYB60*$_{pro}$*246* regulatory module retained the tight cellular specificity of the *AtMYB60* minimal promoter, as ABA- and dehydration-induced up-regulation of reporter gene expression exclusively occurred in guard cells (Fig. 5B, E; Supplementary Fig. S3 at *JXB* online). Interestingly, the 214 bp region of the *rd29A* promoter, incorporated in the chimeric system, contains the root-specific activator sequence *as1* (Lam *et al.*, 1989). Lam and colleagues, demonstrated that the insertion of a single *as1* motif in a green tissue-specific promoter is sufficient to confer root expression (Lam *et al.*, 1989). Despite the presence of the *as1* motif, *GUS* expression in roots (or in any other tissue devoid of stomata) was not observed in any of the *rd29A-MYB60*$_{pro}$*246* lines analysed in this study. This suggests that the control exerted by the *rd29A-MYB60*$_{pro}$*246* synthetic promoter over gene expression is predominantly mediated by the cell-specific module and employs *trans*-regulatory mechanisms that are differentially expressed in guard cells. One possible scenario is that, in its default state, the *AtMYB60* promoter is inactivated by the binding of one or more transcriptional repressors. In the guard cell, the absence of such repressors (e.g. due to lack of expression or to selective protein degradation) allows for the binding of guard cell-specific *trans*-activators (e.g. SCAP1), to promote transcription.

A modular synthetic promoter for the spatio-temporal control of transgene expression in stomata has been reported by fusing a guard cell-specific element from the promoter of the potato phosphoenolpyruvate carboxylase (PEPC) gene with the ethanol-inducible gene switch *AlcR*/*alcA* (Xiong *et al.*, 2009). This system resulted in reliable activation of transgene expression upon ethanol application in *Arabidopsis*

expression in response to 100 μM ABA (C) or dehydration (D) in three independent *Arabidopsis rd29A-MYB60pro246:GUS* lines. (E) Histochemical localization of *GUS* expression in guard cells of 15-day-old tobacco plants from an ABA-sensitive *AtMYB60pro246:GUS* control line (upper left panel) and from a *rd29A-MYB60pro246:GUS* line (lower left panel), grown under standard conditions (bar=1 mm). After 6 h of exposure to 100 μM ABA or dehydration, *AtMYB60pro246:GUS* control plants showed a severe decrease in the intensity of the GUS staining (upper panels, arrows indicate stomatal guard cells). In contrast, ABA and dehydration treatments resulted in enhanced GUS activity in the *rd29A-MYB60pro246:GUS* lines (lower panels) (bar=50 μm). (F and G) qPCR analysis of *GUS* expression in response to 100 μM ABA (C) or dehydration (D) in two independent tobacco *rd29A-MYB60pro246:GUS* lines. qPCR experiments in *Arabidopsis* (C and D) and tobacco (F and G) included an *AtMYB60pro246:GUS* line as a control. Relative *GUS* transcript levels were determined using gene-specific primers and normalized to the expression of the *AtACTIN2* gene (At3g18780) in (C) and (D), or using the *NtEF1*α gene in (F) and (G).

stable transformants. Yet, expression of the transgene was not restricted to mature guard cells, as it was also observed in the guard cell lineage, including meristemoids and guard mother cells (Xiong *et al.*, 2009). Even though the PEPC–*AlcR*/*alcA* module represents a valuable tool to investigate stomatal development and activity in model systems, its exploitation in field crops is rather difficult as it relies on exogenous application of ethanol to activate the expression of downstream genes. Conversely, the *rd29A-MYB60*$_{pro}$*246* system described in this study provides a more suitable tool to engineer stomatal activity in crops. It only employs plant-specific *cis*-elements and it is directly activated in response to stress, allowing for the spatial and temporal regulation of gene expression in a more physiological context. Most importantly, its activity in crop species, including tobacco and tomato commercial varieties, has been directly validated. Several biotechnological applications that employ the use of this regulatory module can be envisaged. For instance, recent evidence indicates that the regulation of the guard cell-autonomous ABA synthetic pathway plays a major role in modulating stomatal activity in response to stress (Bauer *et al.*, 2013). In this respect, the *rd29A-MYB60*$_{pro}$*246* promoter represents a suitable tool to modulate the cell-specific and stress-regulated expression of key ABA biosynthetic genes (e.g. *ABA3*) in guard cells to tailor plant adaptation to the prevailing climatic conditions.

Taken together, results from this work corroborate the value of the *AtMYB60* promoter as a tool to design novel flexible expression systems suitable for modulating stomatal activity in dicotyledonous plant model systems and crops. In addition, they rationalize the combinatorial engineering of hormone- and stress-responsive *cis*-motifs upstream of cell-specific core promoters for the accurate control of gene expression.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. PCR and RT–PCR analysis of independent T_1 rice transgenic seedlings.

Figire S2. *GUS* expression pattern in flowers from tomato and tobacco *AtMYB60*$_{pro}$*1.3:GUS* lines.

Figure S3. Histochemical localization of *GUS* expression in tobacco *rd29A-MYB60*$_{pro}$*246:GUS* plants exposed to severe dehydration.

Figure S4. Rewiring of the activity of the *AtMYB60* promoter in tomato guard cells.

Figure S5. Occurrence of [A/T]AAAG motifs in the 1.3 kb regulatory region located upstream of the translational start codon of the *AtMYB60* and *Solyc10g081490* genes.

Table S1. Sequence of oligonucleotides used in this study for analysis of *GUS* expression.

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