Over-expression of a chimeric gene of the transcriptional co-activator MBF1 fused to the EAR repressor motif causes developmental alteration in *Arabidopsis* and tomato

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Abstract

Transcriptional co-activators of the Multiprotein Bridging Factor1 (MBF1) type belong to a multigenic family that encode key components of the machinery controlling gene expression by communicating between transcription factors and the basal transcription machinery. Knocking-down the expression of one member of the family has proved difficult probably due to functional redundancy. We show here that a fusion of *SlER24*, an *MBF1* type gene of tomato, to the Ethylene-responsive element-binding associated Amphiphilic Repression (EAR) motif is capable of slowing down significantly the expression of the GFP protein driven by a synthetic ethylene-responsive GCC-rich promoter in a single cell transient expression system. A fusion of *AtMBF1c* of *Arabidopsis* to *EAR*, driven by the 35S promoter, caused a reduction of the percentage of seed germination and dwarfism of the plant. Similar fusion with the *SlER24* of tomato in the MicroTom cultivar induced a delay of seed germination and no obvious effect on plant growth. Besides giving information on the role of the *MBF1* genes in plant development, this study demonstrates that the EAR strategy is efficient not only for regular transcription factors as demonstrated so far, but also in the case of co-activators known to not bind directly to DNA.

Keywords: Tomato; *Arabidopsis*; Multiprotein Bridging Factor1; EAR repressor motif; Seed germination; Plant dwarfism

1. Introduction

Transcriptional regulatory proteins play a central role in the expression of genome information during complex biological processes by integrating environmental and cellular signals. Among these proteins, transcriptional co-activators are key components of eukaryotic gene expression by communicating between transcription factors and/or other regulatory elements and the basal transcription machinery [1,2]. The highly conserved Multiprotein Bridging Factors 1 (MBF1) were first identified as co-activators in the silkworm *Bombyx mori* [2] and were shown to contribute with other proteins to the building of TAF complexes (TATA box protein Associated Factors) that are essential for transcriptional initiation. MBF1s mediate this transcriptional activation by bridging the general factor TBP (TATA box Binding Protein) and specific transcription factors bound to their target promoters [2–7].

The first plant *MBF1-like* gene, named *SlER24*, was identified on the basis of its ethylene responsiveness in tomato fruit and shown to be induced during fruit ripening [8]. Three stress-regulated *MBF1* genes were identified in *Arabidopsis* and the encoded proteins were shown to be able to bridge, *in vitro*, TBP and to functionally complement the MBF1 deficiency in yeast [9]. Moreover, it was reported that *ER24-like* genes in plants were induced by drought, heat shock, osmotic stress, pathogen attack, oxidative stress, wounding and in response to ethylene and salicylic acid (SA) treatments [10–14]. Direct evidence of the involvement of MBF1 in plant responses to environmental stresses was obtained by enhancing tolerance to heat and osmotic stresses in transgenic *Arabidopsis*

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lines expressing the *AtMBF1c* gene and more recently *AtMBF1a* [11,14]. These data indicate that *MBF1-like* genes can be associated with a variety of developmental processes in plants such as environmental stress tolerance and suggest that they may represent primary targets of physiological signals.

In tomato, *Sl*MBF1 is encoded by a multigene family of four members (*SlMBF1a-c* and *SlER24*) in which *Sl*ER24 is the most divergent one. Tomato *MBF1-like* genes as well as *SlER24*, encode functional transcriptional co-activators as demonstrated by their capacity to complement yeast *mbf1* mutant and expression studies revealed a distinct pattern of expression for *SlER24* compared to other *MBF1-like* genes, suggesting a specific role for *Sl*ER24 in ethylene and abiotic stress responses and in fruit ripening (unpublished data). Attempts to knock-out the function of *MBF1* genes in tomato have been so far unsuccessful, probably due to the functional redundancy of this type of transcriptional co-activator.

In plants, chimeric repressors in which transcription factor is fused to a repressor domain have been used successfully for targeted dominant repression of the expression of genes of interest [15–17]. So far, the repressive activity has not been demonstrated for transcriptional co-activators. In order to test whether a dominant repressor domain could overcome the gene redundancy of the *MBF1* family, the Ethylene-responsive element-binding factor associated Amphiphilic Repression (EAR) motif was fused to *SlER24* and its *Arabidopsis* ortholog *AtMBF1c* under the control of cauliflower mosaic virus 35S promoter. In this paper, we present the resultant loss-of-function phenotypes induced by the repressor domain including alteration of seed germination and plant dwarfism.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato (*Solanum lycopersicum*, cv MicroTom) and *Arabidopsis thaliana* (Wassilewskija ecotype) plants were grown in a culture chamber under the following conditions: 14 h day/10 h night cycle, 25/28 °C day/night temperature for tomato and 20/25 °C day/night temperature for *Arabidopsis*, 80% humidity, 250 mmol m^{-2} s^{-1} light intensity.

2.2. Plasmid construct

The coding sequences of *SlER24* and *AtMBF1c* without stop codon were PCR-amplified (ISIS polymerase, Qbiogene, Illkirch, France) from respectively tomato cDNA, *Arabidopsis* cDNA. Amplifications were performed with forward (5′ATGCCGAGCGACCAACAGGGGG3') and reverse (5′TGACTTGTGAATTTTACCTCTAAG3′) primers for *SlER24*, forward (5′ATGCCGAGCAGATACCCAGGAGC3′) and reverse (5′TTTCCCAATTTTACCCCTAAGTTTAAC3′) primers for *AtMBF1c*. PCR fragments were ligated into the p35SSRDXG vector described by Mitsuda et al. [18] digested with SmaI, between the cauliflower mosaic virus 35S promoter and the EAR-motif repression domain SRDX [15]. For plant stable transformation, SRDX-fusions in entry clones 35S::*SlER24SRDX* and 35S::*AtMBF1cSRDX* were transferred to the pBCKH plant expression vector [18] using the Gateway system (Invitrogen, Carlsbad, CA). For protoplast transformation, *SlER24* complete ORF, *SlER24SRDX* fusion and *SRDX* sequence alone were PCR amplified from respectively, cDNA, 35S::*SlER24SRDX* vector and p35SSRDXG vector. Then, PCR products were cloned in SmaI digested pGreenII expression vector [19] between the 35S promoter and the Nos terminator to form effector constructs. Vectors, pBCKH and p35SSRDXG were kindly provided by Masaru Ohme-Takagi, Gene Function Research Center, AIST, Tsukuba, Japan.

2.3. Expression analyses by semi-quantitative and quantitative RT-PCR

Total RNAs were extracted by the cetyltrimethylammonium bromide method [8]. After quantification, 10 μg of total RNA were treated with DNAse I (Promega, Madison, Wi, USA) and cleaned up with phenol–chloroform extraction. The reverse transcription reaction was carried out with the Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA) using 2 μg of total RNA.

Semi-quantitative RT-PCR was performed to detect the expression of chimeric transgene mRNAs. Polymerase chain reactions were carried out in a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using ∼100 ng of cDNA, 5 pmoles of each oligonucleotide primer, 200 μM of each dNTP, one unit of Taq Polymerase and 1X Taq polymerase buffer in a 20 μl volume. The PCR program initially started with a 95 °C denaturation for 5 min, followed by 28–38 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. The PCR samples were submitted to electrophoresis on 2.5% agarose gels in TBE buffer (89 mM Tris-base, pH 7.6, 89 mM boric acid, 2 mM EDTA). Gels were stained with ethidium bromide (10 μg mL^{-1}) and photographed on top of a 280 nm UV light box. Primers used were the following:

AtMBF1cF 5′TGTTCCTTTCTCTCAATTCATCG3′
SlER24F 5′CGTTGGCAGTTAATGTAAGAAAGCTAG3′
SRDX_R 5′CTTAAGCGAAACCCAAACGGAGTTC3′

Real-time quantitative PCR was performed using cDNAs corresponding to 2.5 ng of total RNA in a 10 μl reaction volume using SYBR GREEN PCR Master Mix (PE-Applied Biosystems) on an ABI PRISM 7900HT sequence-detection system. *SlActin*-51 (GenBank accession number Q96483) was used as a reference gene with constitutive expression in various tissues. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers:

AtMBF1aF 5′ACTGATGTAGCAAGTAACAAGAATC3′
AtMBF1aR 5′CAACTATGTGATGAAAAGACC3′
AtMBF1bF 5′AAGTGTAGAACAAAGCTCTTAAAG3′
AtMBF1bR 5′ATAATGACAAAAGGTTCCAAACAGC3′
AtMBF1cF 5′TGTTCCTTTCTCTCAATTCATCG3′
AtMBF1cR 5′CATTTATCAAACAAAACAACAAGAC3′
Atβ-tub4F 5′GAGGGAGCCATTGACAACATCTT3′
Atβ-tub4R 5′GCGAACAGTTCACAGCTATGTTCA3′

SlMBF1aF 5′CTTTAATCATTGGCTATGTTTTTGCT3′
SlMBF1aR 5′CAGAAGAAACACTAATTCAACAGAGAA3′
SlMBF1bF 5′GAACGTCTACATCGTTTGGGTTCT3′
SlMBF1bR 5′CGTCCCGAATCCAGACACA3′
SlMBF1cF 5′CAACTTTTTCTGTTAGCCCTCTTTCTAT3′
SlMBF1cR 5′ATGTAGCCAAGAAATCCAGAACCA3′
SlER24F 5′CGTTGGCAGTTAATGTAAGAAAGCTAG3′
SlER24R 5′TCCACCGGCAATTTCTCAA3′
SlActin-51F 5′TGTCCCTATTTACGAGGGTTATGC3′
SlActin-51R 5′CAGTTAAATCACGACCAGCAAGAT3′

RT-PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and one cycle 95 °C for 15 s and 60 °C for 15 s. Samples were run in triplicate in 384-well plates. For each sample, a threshold cycle (Ct) value was calculated from the amplification curves by selecting the optimal Rn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. To determine relative fold differences for each sample in each experiment, the Ct value for *MBF1* gene transcripts was normalized to the Ct value for *SlActin* or *Atβ-tubulin* and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

2.4. "Single cell system" for transient co-transformation

Protoplasts used for transfection were isolated from BY2 tobacco cells. BY2 cells were cultured in a modified Murashige–Skoog medium (Duchefa, Roubaix, France). Other additives were as described by Nagata et al. [20], except the further inclusion of 100 mg L^{-1} myo-inositol. Cell cultures (50 mL in 250 mL Erlenmeyer flasks) were kept in the dark at 25 °C under agitation (100 rpm). The cells (2 g) were digested in 20 mL solution containing 1.0% (w/v) cellulase 345 (Cayla, Toulouse, France), 0.2% (w/v) pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 0.6 M mannitol and 25 mM Tris MES [2-(4-morpholino)-ethane sulfonic acid] pH 5.5, at 37 °C for 1 h. Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis [21]. Two constructs containing synthetic promoter fused to GFP coding sequence were used as expression reporter vector. The first one was an ethylene-inducible synthetic promoter named GCC promoter consisting of a 35S minimal promoter (−46) associated to four GCC-box repeats fused to GFP coding sequence [22]. The second one was the DR5 reporter construct consisting of a 35S minimal promoter (−46) associated to a synthetic auxin response element (DR5) made of nine tandem copies of the consensus AuxRE motif fused to GFP coding sequence [23]. The DR5-GFP construct was a gift of Klaus Palme, Institut für Biologie II-Botanik, Universität Friburg, Germany. Aliquots of 0.5×10^6 protoplasts were transformed with 10 μg of reporter construct in combination with 10 μg of a pGreen II-based effector construct. For each transformation three technical repeats were performed. After 17 h of incubation, GFP expression was analyzed by flow cytometry (FCM) that allows quantification of thousands of events to obtain rapidly significant values. FCM analyses were performed using FACS Calibur instrument (Becton-Dickinson, San Jose, CA) equipped with a 200-μm nozzle and a water-cooled Enterprise coherent argon-ion laser (15 mW output) tuned to 488 nm. The sheet-fluid used was PBS buffer. GFP fluorescence was detected with an FITC 530/30-nm band-pass filter. For each sample, 100–1000 protoplasts were gated on forward light scatter, and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells above the background threshold (set arbitrarily based on a zero DNA transformed control, so that all control cells fall below this threshold). Data were analyzed using CellQuest software (Becton-Dickinson). Each data point is the mean value of three biological experiment repeats.

2.5. Plant transformation

Tomato plants harbouring the *35S::SlER24SRDX* insert were generated by *Agrobacterium tumefaciens*-mediated transformation according to Jones et al. [24]. Transformed lines were selected on hygromycin (25 mg L^{-1}) and analyzed by PCR using cDNA as template to check transgene expression. The protocol for *in planta* transformation of *Arabidopsis* was as described by Clough and Bent [25] and used with modifications. *A. tumefaciens* strain C58 carrying *35S::AtMBF1cSRDX* binary construct were grown to stationary phase in LB medium at 28 °C, 250 rpm. Cells were harvested by centrifugation for 20 min, at room temperature, at 5500 *g* and then re-suspended to final OD_{600} of two in inoculation medium containing 5% (w/v) sucrose and 0.05% (v/v) silwet L-77 (OSI Specialties, Inc., Danbury, CT, USA). Four-week-old plants were inverted into this suspension in order to submerge all floral buds and were then removed after 30 s of gentle agitation. Plants were left in a low-light location overnight and returned to the greenhouse the next day. Plants were grown for further 5–6 weeks until siliques were dry. The selection of putative transformants was done on a 25 mg L^{-1} hygromycin-containing agar medium. Heterozygous lines, four of *Arabidopsis* (C2, C4, C12 and C17) and two of tomato (with two plants for each: 15-1 and 2; 82-1 and 2) have been retained for molecular and phenotypic analysis.

2.6. Germination assay

Seeds were collected from wild-type and heterozygous transgenic tomato fruit at the orange stage, treated with 0.05% HCl, washed abundantly with water and dried quickly on paper in ventilated area. In wild-type *Arabidopsis* and heterozygous transgenic plants grown together, seeds allowed to ripen for 2 months and then collected and stored at room temperature. *Arabidopsis* seeds were stratified at 4 °C for 5 days prior to being placed in germination condition at 23 °C. Tomato and *Arabidopsis* seeds were first surface-sterilized in 50% bleach solution for 10 min, rinsed seven to nine times in sterile distilled water and germinated on filter paper with gibberellic acid (GA_3) 150 μM or water as control solution.

2.7. Histochemical GUS analysis

The *SlER24* promoter sequence (GenBank accession number EU240881) was ligated in pLP100 binary expression

vector harboring GUS coding sequence as reporter gene. This construct was used to transform tomato MicroTom plant as described above. Seeds and seedling of transgenic lines bearing the *pSlER24*::*GUS* fusion construct, were incubated at 37 °C overnight with GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton, and 0,5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) to reveal GUS activity. Following GUS staining, samples were washed several times to extract chlorophyll using graded ethanol series and stored at 4 °C.

2.8. Confocal microscopy

The epidermal structures of *Arabidopsis* rosette leaves and inflorescence stem tissues were revealed using Congo red and observed by confocal microscopy [26]. *Arabidopsis* tissues from wild-type and *MBF1cSRDX* harvested from 5-week-old plants were first treated with ethanol step gradient (from 50% to 100% ethanol) for chlorophyll removal followed by a re-hydration step gradient (50% to 100% water). Samples were incubated in a 0.2% aqueous solution of Congo red (Merck, Darmstadt, Germany) overnight and rinsed in water. Congo red is a fluorescent dye that binds in a highly ordered fashion to cellulose fibrils. Confocal images of *MBF1cSRDX* and wild-type plant tissues were acquired with a confocal laser scanning system (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) using a 40× water immersion objective (numerical aperture 0.75). The samples were observed with the 543 nm ray line of a helium neon laser for excitation and emitted light were collected in the 560–800 nm spectral range. Cell length and surface were calculated using Image-pro Plus software (Media Cybernetics, MD). Up to 100 epidermal stem cells and leaf cells were used respectively for cell length and cell surface measurement.

3. Results

3.1. The EAR motif fused to ER24 is capable of repressing the activity of a GCC box-rich synthetic promoter in a "single cell system"

In order to evaluate the ability of the EAR domain to act as a repressor when fused to the transcriptional co-activator MBF1, we have first tested this chimeric construct using a transient expression driven by a synthetic promoter in a "single cell system" using protoplasts. The GCC box is a well known ethylene inducible *cis*-acting element that binds specifically transcription factors of the Ethylene Response Factor (ERF) family [22]. Tobacco BY2 protoplasts were co-transfected with the GCC-rich promoter fused to GFP as reporter construct and *35S::SRDX*, or *35S::SlER24* or *35S::SlER24SRDX* as effector constructs or an empty vector as control. Fig. 1A shows that, on the basis of 100% fluorescence for the empty vector, over-expression of EAR motif alone had no effect on expression of the *GFP* reporter gene, while over-expression of *SlER24* alone stimulated expression to 140%. This result shows that *Sl*ER24 is able to act as a transcriptional activator on a GCC-containing promoter. The presence of the EAR motif in the ER24 construct caused a 2-fold repression of the GFP reporter gene as compared to ER24 alone or 32% repression compared to an empty vector. A reporter construct consisting of an auxin-inducible synthetic promoter (DR5) fused to GFP was also used to evaluate the specificity of SlER24SRDX repression activity. Over-expression of *SlER24* alone or fused to EAR motif did not affected DR5 promoter activity (Fig. 1B). This result shows that EAR motif associated to a *MBF1* gene is able to repress transcription specifically on ethylene-responsive reporter gene *in vivo*.

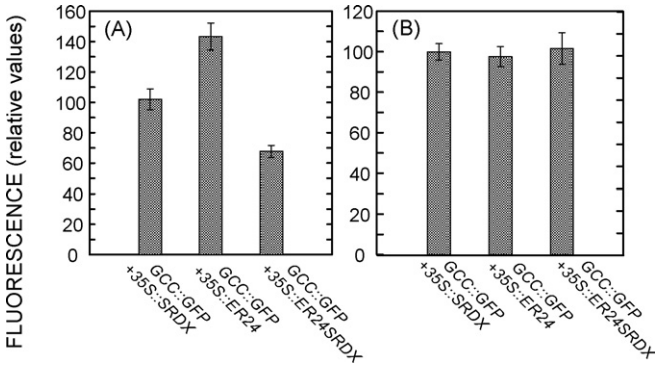

Fig. 1. Effect of *SlER24* or *SlER24SRDX* transient expression on GCC-rich synthetic promoter activity (A) and DR5 auxin-responsive synthetic promoter (B) evaluated in a "single cell system". Tobacco BY2 protoplast were co-transfected with one of the three effectors constructs (*35S::SRDX*, *35S::SlER24* or *35S::SlER24SRDX*) and an ethylene inducible synthetic GCC-rich promoter or a DR5 auxin-responsive promoter associated to the *GFP* reporter gene. Promoter activity was determined by quantification of fluorescence intensities by flow cytometry. Relative values on the *Y*-axis are expressed as percent of fluorescence obtained after co-transfection with the *GCC::GFP* or *DR5::GFP* constructs and an empty vector as effector. Values represent the mean ± S.E. of three independent biological measurements.

3.2. Expression of the transgene and members of the MBF1 family

Four independent transgenic lines of *Arabidopsis* and tomato plants were generated *via Agrobacterium* transformation. All of them express the SRDX-chimeric transgene at a high level (Fig. 2A and B) without affecting the expression of both endogenous *SlER24* in tomato and *MBF1c* in *Arabidopsis* that were targeted for EAR repression as well as of other members of the *MB1* gene family (Fig. 2C and D).

3.3. The presence of an EAR repressor motif in a MBF transgene affects the germination process

In considering that *SlER24* was an ethylene-responsive gene and that ethylene is known to stimulate germination, we sought to assess the effect of expressing *SlER24* and *AtMBF1c* fused to the EAR motif on seed germination in *Arabidopsis* and tomato. Fig. 3 shows substantial differences in germination between wild-type and two independent transformed lines. But global observation of transgenic seeds morphology, *i.e.*: size, shape and testa color, did not show any difference compare

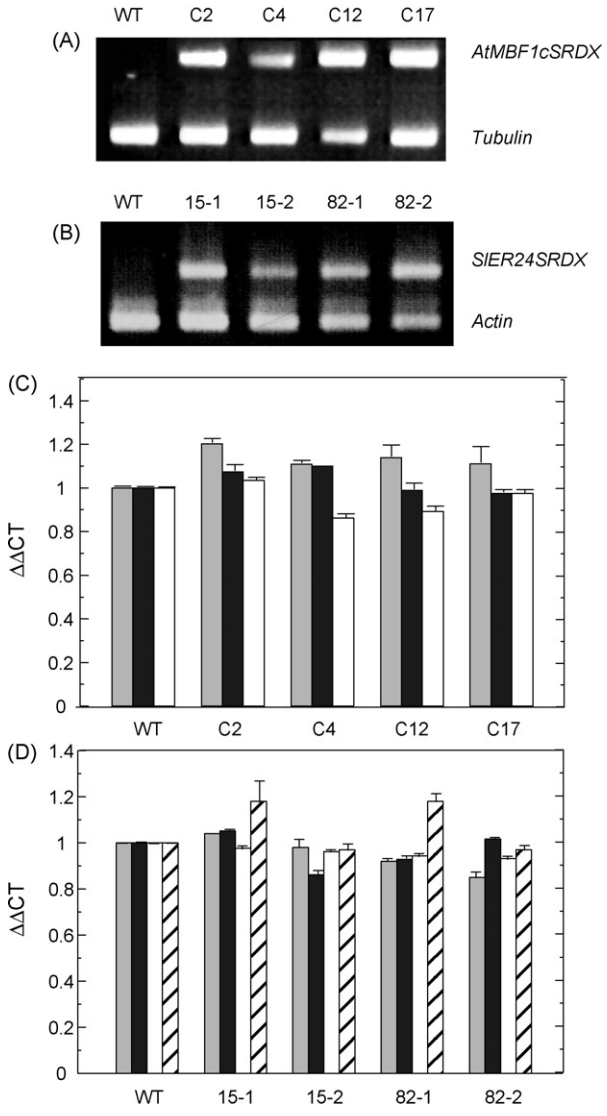

Fig. 2. Expression of the *SRDX* transgenes and genes of the MBF1 family in four transgenic lines and wild-type of *Arabidopsis* and tomato. Expression of *AtMBF1cSRDX* in Arabidopsis (A) and *SlER24SRDX* in tomato (B) by semi-quantitative RT-PCR. Expression levels of Arabidopsis *MBF1a* (grey bar), *MBF1b* (black bar) and *MBF1c* (open bar) (C) and tomato *MBF1a* (grey bar), *MBF1b* (black bar), *MBF1c* (open bar) and *SlER24* (dashed bar), (D) transcripts assessed by real-time quantitative PCR. In C and D, ΔΔCt refers to the fold difference in each transcript level relative to the wild-type taken as a reference sample and the data represent the mean values ± S.E. of two independent replicates. C2, C4, C12 and C17 correspond to *AtMBF1cSRDX* transgenic lines of *Arabidopsis*. 15-1, 15-2, 82-1 and 82-2 correspond to *SlER24SRDX* transgenic lines of tomato.

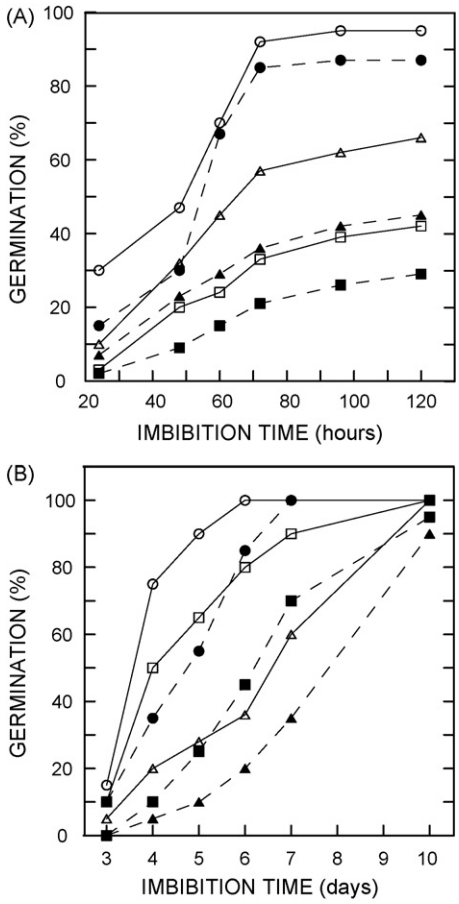

Fig. 3. Time course of germination of wild-type and two transgenic lines of *Arabidopsis*, C2 and C4 (A) and MicroTom tomato seeds, 15-1 and 82-1 (B). Seeds of two independent heterozygous transgenic lines (triangle and square symbols) and of wild-type (round symbols) were incubated at 22 °C in absence (dark symbols) or in presence (empty symbols) of 150 μM GA_3. The number of seeds showing visible radicle protrusion was counted (over 100 per experiment). Values correspond to the mean of two independent biological measurements.

to wild-type. In *Arabidopsis*, the presence of the *AtMBF1c* gene fused to the repressor caused considerable reduction of germination (Fig. 3A) with only 30% and 45% of the seeds capable to germinate after 120 h for the two transgenic lines as compared to almost 90% for wild-type seeds at 72 h. The presence of GA_3 increased the rate of germination and the final number of seeds capable to germinate rose to over 40% and 65% after 120 h for the two transgenic lines and over 95% for wild-type seeds (Fig. 3A). In tomato, unlike *Arabidopsis*, almost 100% of the seeds expressing *SlER24* fused to the repressor were able to germinate, but the rate of germination was considerably lowered as compared to wild-type (Fig. 3B). The presence of GA_3 increased the rate of germination for all seeds (Fig. 3B).

3.4. ER24 promoter expression pattern is consistent with a role of ER24 in the germination process

The expression pattern conferred to GUS by the *SlER24* promoter was performed by histochemical analysis of *pER24::GUS* tomato seeds during germination and radicle elongation (Fig. 4). GUS activity could not be detected before 72 h after soaking (Fig. 4A and B). Activity was first detected in the radicle and the micropylar endosperm, just before the radicle protrusion (Fig. 4C). Thereafter, GUS activity was high during the first steps of elongation of the radicle (Fig. 4D–F). At later stages, GUS activity was persisting in the tip of the root and was high in the hypocotyl, particularly at the neck between root and hypocotyl and at the base of cotyledons (Fig. 4G and H). In separate experiments carried out with 2-week-old plants, GUS activity became undetectable even in the root tip.

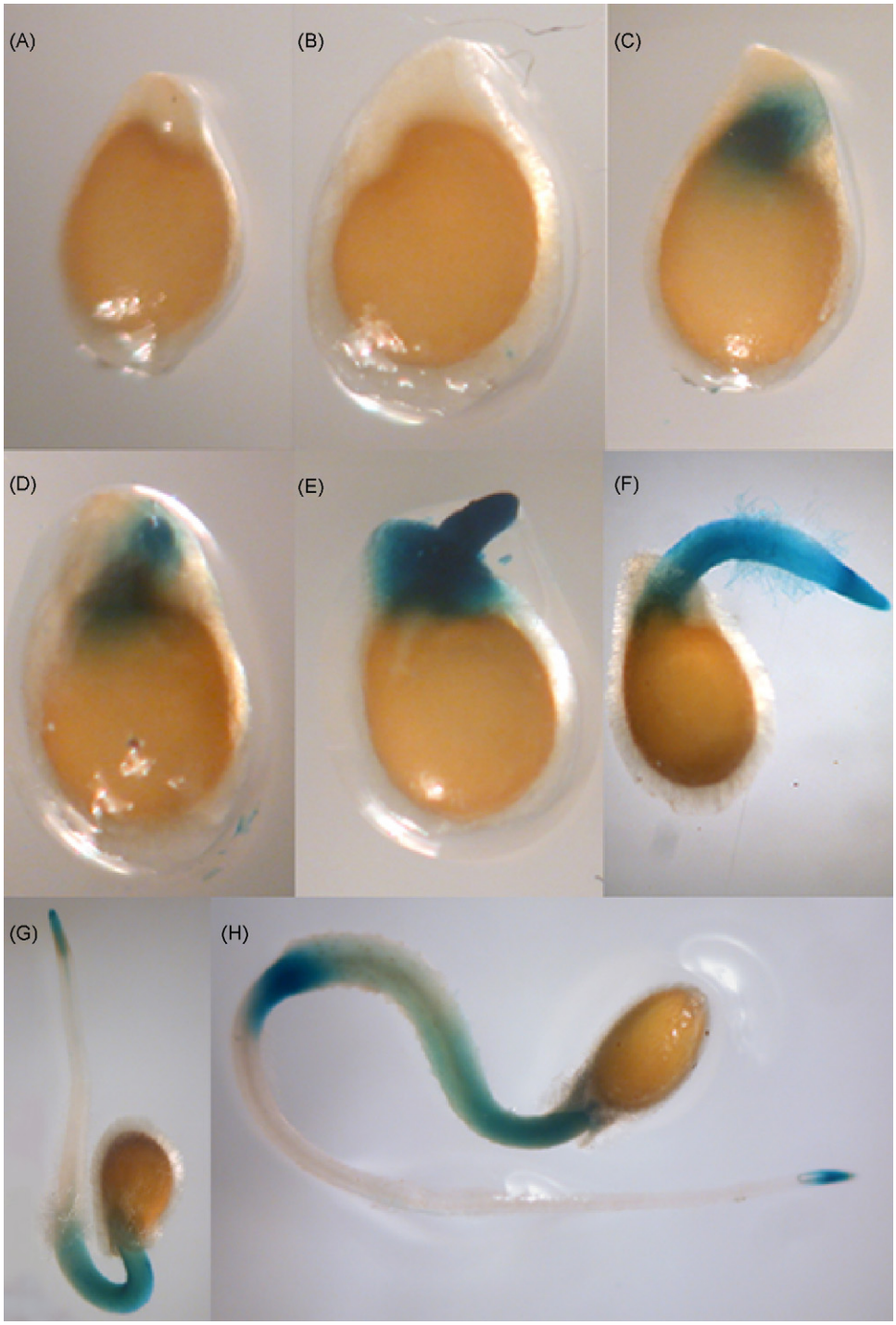

Fig. 4. Detection of GUS activity in transgenic *pSlER24::GUS* tomato seeds during germination and early developmental stages. Histochemical staining was monitored during the germination process at 24 h (A), 48 h (B) and 72 h (C) after imbibition, at radicle protrusion (D–F) and during seedling development (G–H).

3.5. The presence of an SRDX repressor motif in the Arabidopsis MBF1c transgene results in plant dwarfism

Four heterozygous independent lines of *Arabidopsis* plants harboring the *AtMBF1cSRDX* transgene have been generated. They clearly exhibit different levels of dwarfism (Fig. 5). Observations of the elongation of the first flower stalk indicated that the time of bolting remained unaffected but the stem elongated more slowly, resulting in at least a 2-fold reduction in height (Fig. 5A) compared to the wild-type. Severe reductions in the length of petioles and leaf blades (Fig. 5B) and decrease in length and seed number (Fig. 5C–E) were also obvious in *AtMBF1cSRDX* plants (Fig. 5C). Alterations of plant development were associated with morphological modifications at the cell surface of stem and leaves (Fig. 6). Concerning the stems, the *AtMBF1cSRDX* plants exhibited strong reduction of cell length with a number of cells per stem width that was reduced to eigth as compared to 12 in the wild-type (Fig. 6A

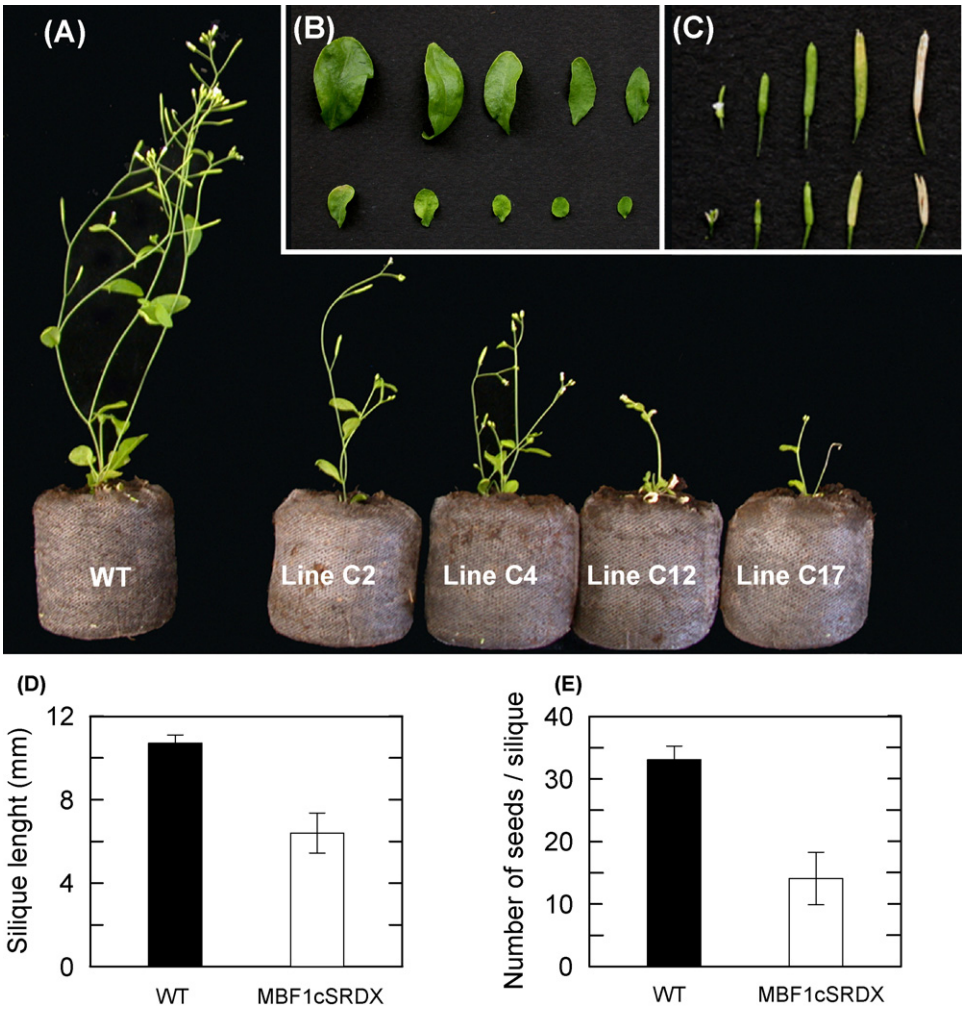

Fig. 5. Phenotypic comparison between *AtMBF1cSRDX* heterozygous transgenic lines and the wild-type grown under long-day conditions. (A) Five-week-old plants of wild-type (WT) and four *AtMBF1cSRDX* independent heterozygous lines (C2, C4, C12 and C17). (B) Inflorescence leaves from 8-week-old WT (top) and *AtMBF1cSRDX* (bottom) plants (C2). (C) Siliques from 8-week-old WT (top) and *AtMBF1cSRDX* (bottom) plants (C2). (D) Silique lengths and (E) number of seeds per silique of 8-week-old WT (dark bar) and *AtMBF1cSRDX* (empty bar) plants (C2). Each value represents the mean ± S.E. for around 30 siliques.

and B) and a shift of the distribution of the cell length classes towards the smaller size range (Fig. 6E). The leaves of plants harbouring the *AtMBF1cSRDX* transgene showed a significant reduction of the cell area with a higher number of cells per leaf (Fig. 6C and D). Accordingly, the cell surface of the *AtMBF1cSRDX* plants is predominately spread over the small classes (Fig. 6F). Surprisingly, the two MicroTom tomato lines in which *SlER24* was repressed and for which germination was affected, did not exhibit significant alteration of plant growth and no visible alteration of the timing of color changes during fruit ripening (data not shown).

4. Discussion

The use of an EAR-motif chimeric repressor, denominated CRES-T system [15], has been successful for analyzing the functions of transcription factors in cases where single gene knock out or antisense lines failed to display any obvious abnormal phenotypes. The major advantage of this system is to overcome functional redundancy of transcription factors belonging to multigene families [16,17]. Nevertheless, this strategy has not been used yet for transcriptional co-activators. Since no visible phenotype could be observed by using VIGS or antisense strategy, we decided to use the CRES-T system for disturbing the function of members of the MBF1 co-activators gene family. Single cell system consisting of transient protoplast transformation is a rapid and reliable method to evaluate the capability of different types of effectors to regulate the transcriptional activity of a target promoter fused to a reporter gene [27]. Since ER24 was identified as an ethylene-responsive gene [8], a synthetic promoter containing four GCC boxes was used in the present work for evaluating the repressor activity of *Sl*ER24SRDX. Data from Fig. 1 show that when *Sl*ER24 was fused to the EAR motif, the expression of the GCC containing promoter was significantly reduced. This suggests that, within the MBF1 transcriptional complex, ERFs are engaged in the binding to GCC boxes. This hypothesis is supported by the recent observation that ERFs were capable to interact with MBF1 co-activators in tobacco [28]. The ER24-EAR fusion protein is unable to recognise DR5, an auxin-

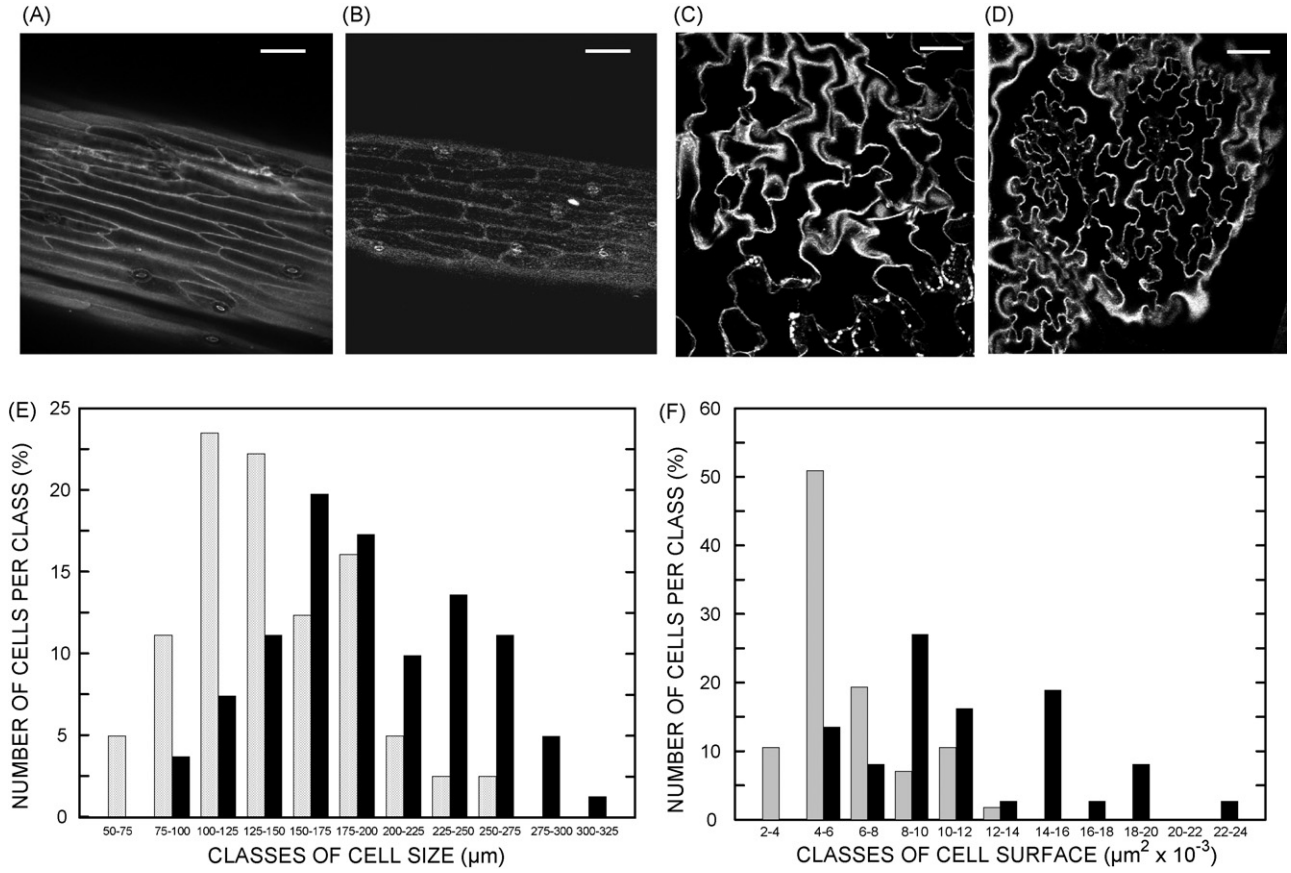

Fig. 6. Confocal microscopy analysis of 8-week-old *Arabidopsis* stem and leaves after Congo red staining. View of WT (A) and *AtMBF1cSRDX* (B) stem epidermis surface. View of WT (C) and *AtMBF1cSRDX* (D) leaf epidermis surface. Horizontal white bars of A, B, C and D correspond to 50 µm. Distribution of cell size of stems (E) and cell surface of leaves (F) in a population of 100 stem or leaf cells of WT (dark bars) or *AtMBF1cSRDX* (grey bars).

responsive synthetic promoter, again supporting the specificity of ER24 binding to the GCC box. Taken together, these data are ruling out a non-specific effect of the fusion protein. Our data also show that over-expression of *SlER24* alone stimulated transcriptional activity of the reporter gene as compared to empty vector, indicating that the *Sl*ER24 protein was present at limiting levels in the protoplasts.

Having demonstrated the capability of the EAR motif to inhibit the activity of MBF1 co-activators, we sought to evaluate the effects of the EAR motif on the development of whole plants of *Arabidopsis* and tomato after stable transformation. Our experiments demonstrate that both *Arabidopsis* and tomato transgenic lines show developmental alterations. One of the most obvious phenotype was related to a reduction of the number of seeds capable to germinate in *Arabidopsis* and to an important delay in the germination rate in tomato (Fig. 3). The role of *Sl*ER24 in the germination process is further substantiated by the pattern of GUS activity driven by *SlER24* promoter that shows strong expression at the early stages of root emergence.

Seed germination is a complex physiological process promoted by water imbibition after the eventual release of dormancy mechanism by appropriate triggers. Seed germination is controlled by the antagonistic action of gibberellic acid (GA) and abscissic acid (ABA) [29]. However, ethylene is also known for playing a role in seed germination where it counteracts ABA effects [30] and induces endosperm weakening by regulating the activity of cell wall-modifying proteins such as endo-β-mannanase [31] and β-Glucanase I [32]. Indeed, the ethylene insensitive *Arabidopsis* mutant *ein2* and *etr1* have enhanced dormancy [33] and ethylene is able to fully rescue the germination defect of the GA mutant of *Arabidopsis ga-1* [34]. The capability of GA treatments to overcome the effect of the *SRDX-SlER24* or *SRDX-AtMBF1c* transgene over-expression suggests the possible involvement of *SlER24* and *AtMBF1c* in the ethylene transduction pathway. This hypothesis is further supported by the fact that up-regulation of the ethylene response factor *Sl*ERF2, which interacts with MBF1, stimulates seed germination through the induction of the *mannanase2* gene [35]. Altogether these data suggest that MBF1 could be a component of the ethylene signal transduction pathways by regulating gene expression in seeds and thus mediating germination. However, MBF1 is probably not participating in all aspects of ethylene action. Over-expression of *SRDX-SlER24* or *SRDX-AtMBF1c* had no effect on ethylene triple response of etiolated seedlings. In addition, MBF1 may also be involved in the response to other hormones that are known to participate in GA/ABA cross-talk for the control of dormancy release and germination [36]. Elements putatively involved in the response to several hormones

(ethylene, GA, salicylic acid and methyl jasmonate) are present in the promoter sequence of *SlER24* (data not shown).

In *Arabidopsis*, contrary to tomato, *SRDX* repression of MBF1c gene caused a decrease in the percentage of seeds capable to germinate and GA could not fully restore the germination capacities to the level of the wild-type. Such a difference could be related to the growth reduction of *Arabidopsis* plants that could affect the viability of a certain percentage of seeds, while in tomato plant growth was not affected.

The EAR driven dominant repression causes a reduction in size of *Arabidopsis* plants by affecting cell size and cell number. Cell size reduction is particularly apparent in leaf epidermis where most of the cells have significant cell surface reduction comparatively to the wild-type. Cell division and elongation are under the control of hormones. Because ethylene is acting as a growth inhibitor in dicotyledonous plants, it is difficult to assess a role for MBF1 co-activators in ethylene action. If such was the case, MBF1-repressed plants would exhibit growth stimulation. More probably, repression of MBF1 prevents some of the hormones involved in cell division and elongation from being active. Candidate hormones are numerous (gibberellins, cytokinins, auxins, brassinosteroids) and it is difficult to suspect those whose action would be affected. However, we have observed very little or no effect of EAR repression of *SlER24* on the growth of the MicroTom genotype tomatoes. This genotype is affected in the synthesis of brassinosteroids which could explain the dwarfism of the plant [37]. These elements put together would suggest that EAR repression of MBF1 has no significant effect on MicroTom plant growth due to the absence of brassinosteroid biosynthesis, contrary to *Arabidopsis*. In this respect, it would be useful to perform transformation of regular genotypes of tomato. More experiments are anyway necessary to determine whether EAR repression of MBF1 function results in an alteration of the synthesis brassinosteroids or other hormones. As already mentioned above, analysis of the promoter sequence of *SlER24* revealed the presence of predicted *cis*-elements putatively involved in the response to ethylene, gibberellins, salicylic acid and methyl jasmonate.

The expression of the transgene in all four independent transgenic lines of *Arabidopsis* and tomato did not affect the expression of the corresponding endogenous gene and other members of the MBF1 family, indicating that the phenotypes observed are directly linked to the inhibition of the functionality of *SlER24* or *AtMB1c* by the EAR motif. However, although our experiments have been targeted to repress the function of *SlER24* in tomato and *AtMBF1c* in *Arabidopsis*, it may happen that other members of the *MBF1* family be functionally affected maybe through competitive occupation of their transcription activation site as hypothesized by Takase et al. [17].

Nevertheless, our data show that the dominant repressor domain EAR can be used successfully with transcriptional co-activators that do not interact directly with DNA such as MBF1 in *Arabidopsis* and tomato in the presence of the corresponding endogenous factors and of functionally redundant transcriptional co-activators.

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