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Ethylene response factors (ERFs) are plant transcriptional regulators mediating ethylene-dependent gene expression via binding to the GCC motif found in the promoter region of ethylene-regulated genes. We report here on the structural and functional characterization of the tomato *Sl-ERF2* gene that belongs to a distinct class of the large ERF gene family. Both spliced and unspliced versions of Sl-ERF2 transcripts were amplified from RNA samples and the search in the public tomato expressed sequence tag (EST) database confirmed the existence of the two transcript species in a number of cDNA libraries. The unspliced transcript contains two open reading frames yielding two hypothetical proteins, a small highly truncated version lacking the APETALA2 domain and a bigger protein lacking the N-terminal MCGGAAI$^{I}/_{L}$ consensus peptide specific to ERF members from subfamily IV. Nevertheless, functional Sl-ERF2 protein may only derive from spliced transcripts since, depending on the tissue, the level of the spliced transcript is much higher than that of the unspliced transcript. *Sl-ERF2* is expressed in all plant tissues tested, though its transcript accumulates preferentially in germinating seeds and ripening fruit. Overexpression of the *Sl-ERF2* gene in transgenic tomato lines results in premature seed germination and enhanced hook formation of dark-grown seedlings, which is indicative of increased ethylene sensitivity. The expression of the *mannanase2* gene is upregulated in *Sl-ERF2*-overexpressing seeds, suggesting that *Sl-ERF2* stimulates seed germination through the induction of the *mannanase2* gene. It is noteworthy that the exaggerated hook phenotype is abolished when ethylene perception is blocked, strongly suggesting that Sl-ERF2 requires other ethylene-dependent components to impact the hook formation process.

Keywords: ABA — ERF — Ethylene — Germination — MAN2 — Tomato.

Abbreviations: ABRE, ABA-responsive element; AP2, APETALA2; CaMV35S, cauliflower mosaic virus 35S; Ct, threshold cycle; ERE, ethylene-responsive element; ERF, ethylene response factor; EST, expressed sequence tag; 1-MCP, 1-methylcyclopropene; MAN2, mannanase 2; ORF, open reading frame; RT–PCR, reverse transcription–PCR; UTR, untranslated region.

Introduction

Ethylene is an important phytohormone involved in many plant developmental processes. Notably, this plant hormone is involved in germination, fruit ripening, abscission and senescence (Abeles et al. 1992). Ethylene response factors (ERFs) are known to act at the last step of the ethylene signaling pathway (Ohme-Takagi and Shinshi 1995). ERF-type transcription factors are specific to plants and belong to the large AP2/ERF family which accounts for >70 genes in *Arabidopsis thaliana* (Riechmann et al. 2000). Proteins encoded by this family have a highly conserved DNA-binding domain known as the AP2 domain made up of 58–59 amino acids involved in the high affinity binding to the target DNA sequences (Allen et al. 1998). The ERF proteins specifically bind the so-called GCC box with a strictly conserved GCCGCC core domain to modulate transcription of genes such as *PDF1.2* or *NtChitinase* harboring this type of *cis*-element on their promoter (Ohme-Takagi and Shinshi 1995, Gu et al. 2002). It is known that *ERF* genes are not only induced by ethylene but can also respond to jasmonate, ABA, NaCl (Finkelstein et al. 1998, Zhang et al. 2004), salicylic acid (Gu et al. 2000), wounding (Tournier et al. 2003) and biotic stress (Fujimoto et al. 2000, Onate-Sanchez and Singh 2002, Brown et al. 2003, Lorenzo et al. 2003).

It was reported recently that tomato ERFs belong to four distinct classes, and expression analyses revealed that representatives from each class display a differential pattern of expression in a tissue- and developmental-specific manner (Tournier et al. 2003). The tomato *Sl-ERF2* (AY192368) gene belongs to class IV characterized by the presence of a conserved short N-terminal domain (MCGGAAI$^{I}/_{L}$) of unknown function. Sl-ERF2 was capable of binding the GCC box found in the promoter of ethylene-responsive genes and shows a distinctive

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ripening- and wound-associated expression, yet its transcript accumulation was unaffected by ethylene treatment in tomato leaves (Tournier et al. 2003).

ERFs have been shown to be involved in normal and abnormal plant developmental processes such as plant defense (Zhou et al. 1997, Thara et al., 1999, Brown et al. 2003, Chakravarthy et al. 2003, Cheong et al. 2003), osmotic stress tolerance (Park et al. 2001, Zhang et al. 2004) and seed germination (Finkelstein et al. 1998; Song et al. 2005). Seed germination is one of the earliest and most important steps of the plant life cycle as it allows embryos to develop into seedlings. In many plant species, germination is preceded by dormancy which is known to be maintained by ABA (Hilhorst et al. 1995) while gibberellin is required for breaking dormancy and inducing germination (Karssen et al. 1989, Debeaujon and Koorneef 2000). Germination is characterized by radicle protrusion as a result of weakening of the endosperm region enclosing the radicle tip, termed the endosperm cap (Groot and Karsen 1987). The ABA-insensitive Arabidopsis mutant *abi4* affected in seed germination displays altered expression of seed-specific genes (Finkelstein et al. 1998) and the *abi4* mutation is caused by a single pair deletion within an *APETALA2* gene (Finkelstein et al. 1998). While it has been known for a long time that ethylene impacts seed germination, it was demonstrated only recently that ERFs are involved in ethylene-dependent regulation of seed germination (Song et al. 2005). It was reported that AtERF7 acts as a transcriptional repressor of the ABA response and that transgenic Arabidopsis lines expressing an RNAi construct targeted to down-regulate the AtERF7 gene are more sensitive to ABA and germinate later than the wild-type seeds.

While direct evidence for the involvement of ERFs in the seed germination process is still scarce, we report in the present study that overexpression of the *Sl-ERF2* gene in transgenic tomato lines results in premature seed germination and causes altered ethylene response as assessed by the triple response. Moreover, our data suggest that Sl-ERF2 may stimulate seed germination through the activation of the *Sl-Man2* gene encoding mannanase.

Results

Structure of the Sl-ERF2 gene

In order to gain more information on the structure of the tomato *Sl-ERF2* gene, a 2,517 bp genomic fragment was isolated and fully sequenced, allowing delineation of the promoter region (1,367 bp) and the transcribed region (1,150 bp). The isolated gene is composed of two exons and one intron and contains an open reading frame (ORF) of 783 bp. As shown in Fig. 1A, the first exon starts at nucleotide 151 and ends at nucleotide 292, and the second exon encompasses the region from nucleotide 368 to 1,150. Upstream of the first exon there is a 5′-untranslated region (5′UTR) of 150 bp and downstream of the second exon a 3′UTR of 141 bp. The *Sl-ERF2* gene contains a single, small intron of 75 bp (Fig. 1A). The closest Arabidopsis homolog of *Sl-ERF2* is *AtEBP* (AT3G16770.1) which also contains a single intron though of a larger size (237 bp). Sl-ERF2- and AtEBP-encoded proteins display 52% identity and 64% similarity at the amino acid level.

Features of the Sl-ERF2 promoter

The tomato *Sl-ERF2* genomic clone contains a 1,367 bp fragment upstream of the transcription site corresponding to the promoter region that is likely to harbor most regulatory elements necessary for driving the regulated transcription of the gene. In silico analysis of the promoter performed by PlantCare software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) identified three putative ABA-responsive elements (ABREs) containing the consensus sequence GTACGTGGCGC lying at positions −929, −1,183 and −1,194 (Fig. 1A). A putative regulatory element, known as an RY-element, found in the promoter region of seed-specific regulated genes, was also identified at position −605. Finally, at least five putative ethylene-responsive elements (EREs) were found at positions −747, −631, −431, −322 and −32 (Fig. 1A).

Tomato Sl-ERF2 gene undergoes alternative splicing

Two Sl-ERF2 transcripts were detected and the corresponding cDNAs cloned and sequenced. The presence of the intronic region in one of these mRNA species raises the possibility that Sl-ERF2 undergoes alternative splicing (Fig. 1B). The search in the tomato expressed sequence tag (EST) database (sgn.cornell.edu) identified a number of contigs (SGN-E375112, SGN-E378950, SGN-E377694, SGN-E258222, SGN-E233294 and SGN-E231194) that contain an intronic region, confirming the co-existence of spliced and unspliced versions of Sl-ERF2 transcripts and suggesting that alternative splicing may play a role in controlling the expression of the *Sl-ERF2* gene. Sequence analysis of the unspliced Sl-ERF2 transcript revealed two putative 'Stop codons', the first being located in the intron region at position 153 from the 'Start codon' and the second at position 858. Two proteins can therefore be derived from this transcript: (i) a low molecular weight predicted peptide (5.5 kDa) of 50 amino acids; and (ii) a higher molecular weight protein of 209 amino acids (24 kDa). Compared with the protein derived from the spliced version of the Sl-ERF2 transcript, the short putative protein corresponds to the N-terminal part lacking the AP2 domain, while the larger one corresponds to the C-terminal moiety containing the AP2 domain but lacking the

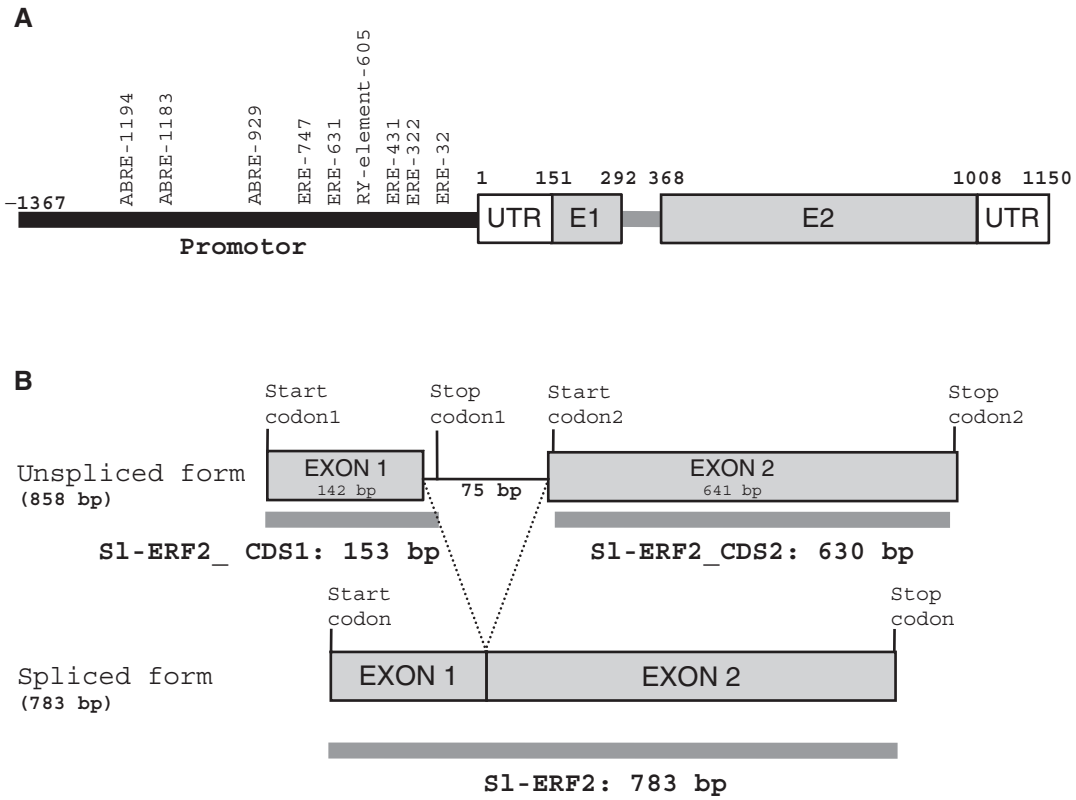

Fig. 1 Structure of the *Sl-ERF2* gene. (A) Genomic structure of the tomato *Sl-ERF2* (AAO34704) gene. The black line represents the promoter region, the gray line the intron, the gray boxes the exons, and white boxes the untranslated region. (B) Structure of Sl-ERF2 unspliced and spliced forms. Gray boxes represent the exons, the black line the intron, and the gray lines represent complete open reading frames.

N-terminal MCGGAAI$^{I}/_{L}$ consensus peptide specific to members of subfamily IV of the ERF gene family (Fig. 1B).

In order to assess the relative abundance of each version of the Sl-ERF2 transcripts, we performed a comparative analysis of the accumulation of the spliced and unspliced Sl-ERF2 transcripts. Specific primers allowing discrimination between the two mRNA species were used in a quantitative reverse transcription–PCR (RT–PCR) experiment with RNA samples extracted from different plant tissues. Table 1 shows that accumulation of the spliced transcript is 82–70,000 times higher than that of the unspliced version, suggesting that the spliced version accounts for most of the Sl-ERF2 transcripts in all tissues tested. Hence, because it is more likely that functional Sl-ERF2 protein only derives from the spliced transcript, we decided to target subsequent expression studies to this type of transcript.

Sl-ERF2 is mainly expressed in ripening fruit

To uncover the expression pattern of the *Sl-ERF2* gene at the transcriptional level, quantitative RT–PCR analyses were performed using different tomato plant tissues.

Table 1 Abundance of the unspliced and spliced forms of the Sl-ERF2 transcript in different tissues of tomato plants

	Spliced/unspliced	±SD
Red fruit	72,744	2,395
Flower	10,026	3,113
Root	5,451	6,22
Seed	82	13
Stem	10,822	1,523

Transcript accumulation was monitored using specific primers, allowing complete discrimination between the spliced and unspliced forms. The expression level of both forms was assessed by real-time PCR, and the data are expressed in fold differences in the abundance of the Sl-ERF2 spliced transcript relative to the spliced transcript.

The data presented in Fig. 2A reveal a ubiquitous expression of *Sl-ERF2* in various plant tissues even though transcripts appear to accumulate preferentially in germinating seeds and ripening fruit. Pre-germinating seeds display the highest level of transcript accumulation, whereas the lowest expression is found in roots where *Sl-ERF2* transcript accumulation is 10 times lower than that in fruit.

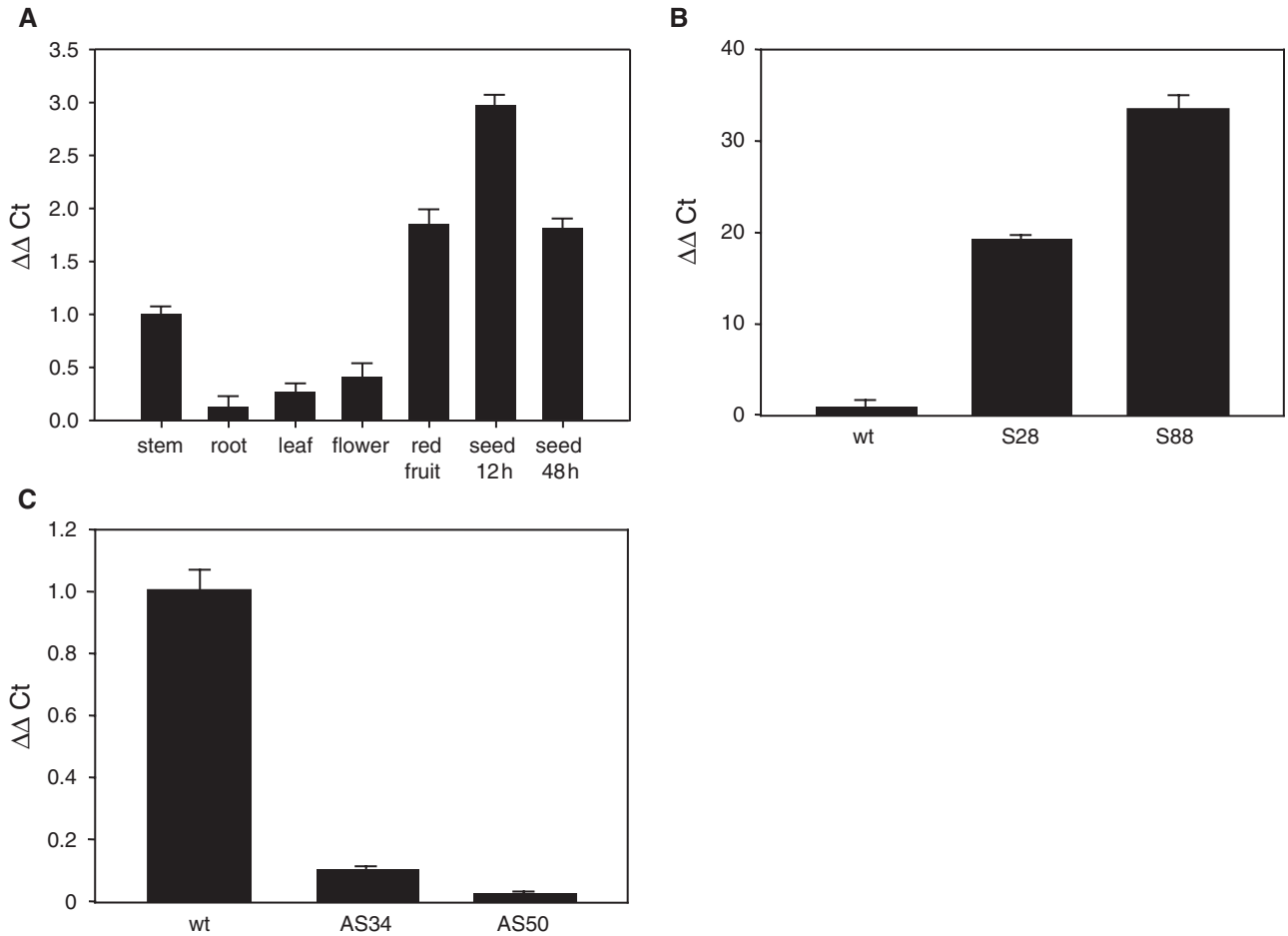

Fig. 2 Expression pattern of *Sl-ERF2*. (A) Tissue-specific expression of Sl-ERF2 in tomato. The levels of Sl-ERF2 transcripts were assessed by real-time quantitative PCR and the data are mean values of three independent experiments. Accumulation of spliced Sl-ERF2 transcript was monitored in stem, root, leaf, flower, red fruit (69 d post-anthesis) and seeds after 12 and 48 h of imbibition in water. ΔΔCt refers to the fold difference in Sl-ERF2 abundance relative to stem taken as a reference tissue. (B) Overexpression of the *Sl-ERF2* gene in transgenic tomato seeds. The levels of Sl-ERF2 transcripts in transgenic sense lines (S28 and S88) were assessed by real-time quantitative PCR, and ΔΔCt refers to the fold difference in Sl-ERF2 transcript accumulation relative to wild type (WT). The data are mean values corresponding to three independent experiments. (C) Down-regulation of the Sl-ERF2 transcript in transgenic tomato plants expressing an antisense construct of the *Sl-ERF2* gene. The level of Sl-ERF2 transcripts in transgenic antisense lines (AS34 and AS50) was assessed by real-time quantitative PCR, and ΔΔCt refers to the fold difference in Sl-ERF2 transcript accumulation relative to wild type (WT). The data are mean values corresponding to two independent experiments.

Overexpression of Sl-ERF2 results in enhanced ethylene sensitivity and premature seed germination

In an attempt to unveil the physiological significance of *Sl-ERF2* and to better explore its role in seed germination, we generated tomato lines under- and overexpressing this gene by stably transforming tomato plants with either sense or antisense constructs under the control of the constitutive 35S promoter. A number of homozygous transgenic lines corresponding to independent transformation events were obtained and analyzed. It is noteworthy that no visible phenotypes could be detected in any of the *Sl-ERF2*-suppressed lines (Fig. 2C), which may be due to functional redundancy among *ERF* genes. In contrast, *Sl-ERF2*-expressing lines showed visible phenotypes associated with seed germination and ethylene response. Two transgenic lines S28 and S88 showing 19 and 33 times higher accumulation of Sl-ERF2 transcript, respectively, were selected for subsequent studies (Fig. 2B).

Hook formation in the seedlings is one component of the typical triple response displayed by dark-grown seedlings in response to the plant hormone ethylene. Compared with the wild type, dark-grown overexpressing lines exhibited exaggerated apical hook formation in the absence of exogenous ethylene treatment (Fig. 3A). Table 2 shows that when grown in the dark, 1% of wild-type seedlings exhibit complete hook formation, whereas this

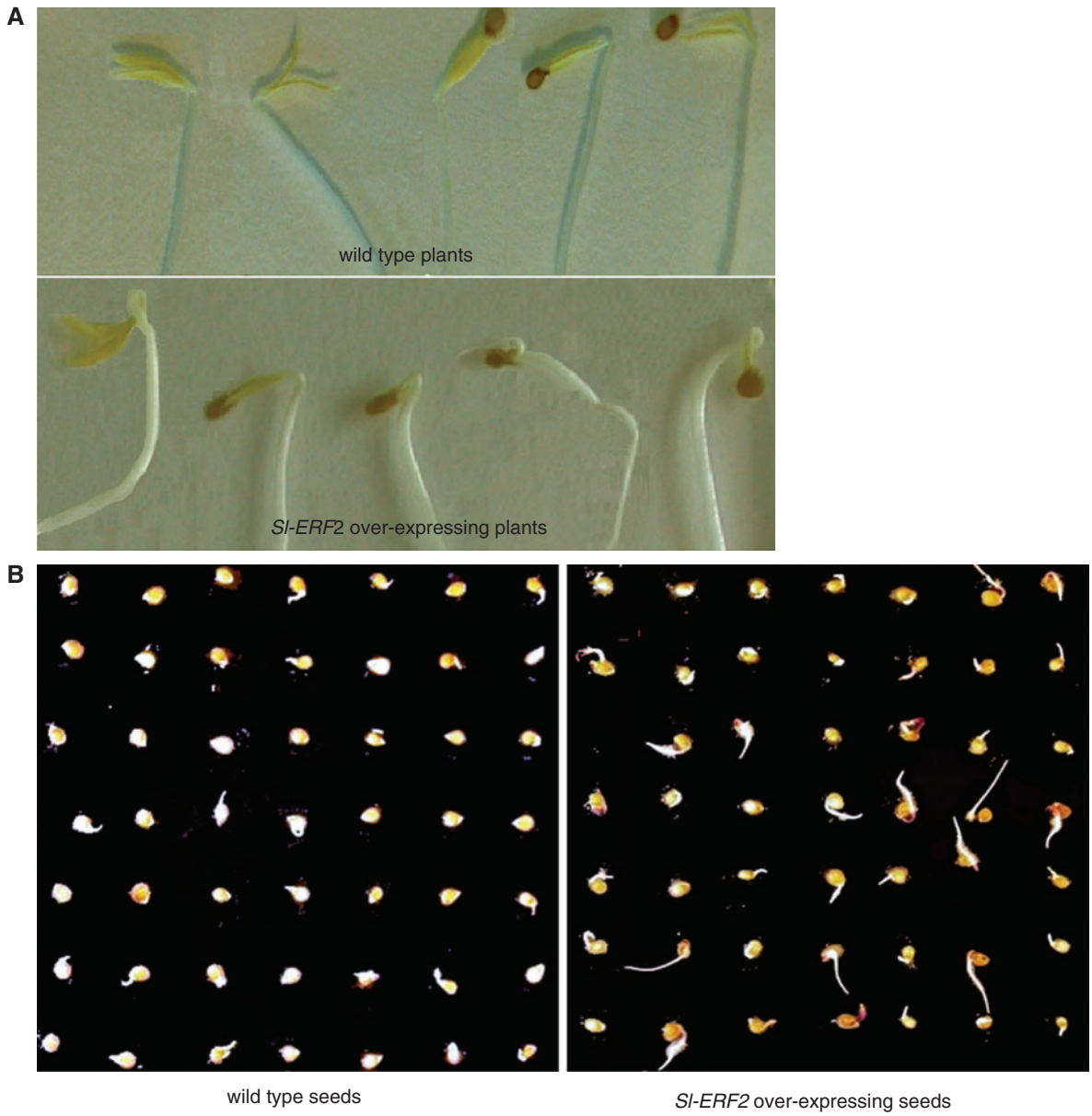

Fig. 3 Phenotypes of the transgenic *Sl-ERF2*-overexpressing lines. (A) Exaggerated hook curvature displayed by overexpressing lines compared with the wild type. Hook curvature was monitored on 4-day-old etiolated seedlings. (B) Early germination phenotype displayed by *Sl-ERF2*-overexpressing tomato lines. Seeds were imbibed on water-prepared 1% agar, and germination was scored at root protrusion.

proportion increased up to 16 and 18% for S28 and S88 overexpressing lines, respectively. Furthermore, treatment with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene perception, abolished formation of a complete hook in the sense lines (Table 2).

Sl-ERF2 is involved in seed germination

Considering the presence of the RY-element, a motif present in seed-specific promoters, and the ABREs in the *Sl-ERF2* promoter, we sought to assess the effect of the overexpression of the *Sl-ERF2* gene on seed germination. Figs. 3B and 4A show that both S28 and S88 lines displayed early germination compared with the wild type. Indeed, after 78 h imbibition in water, up to 72 and 66% of S28 and S88 overexpressing seeds germinated, respectively, whereas <25% of wild-type seeds initiated the germination process. Fig. 4A also indicates that both wild-type and transgenic seeds display full germination potential (100% germinated seeds) after 142 h. Because these data suggest that *Sl-ERF2*

might be involved in triggering the seed germination process, we tested whether *Sl-ERF2* overexpression is capable of overcoming the typical ABA inhibition of seed germination. We therefore assessed the effect of this hormone on the germination of transgenic seeds. Fig. 4B shows that in the presence of 3 μM ABA, 85% of wild-type seeds failed to germinate, while in the same condition, inhibition of seed germination was reduced to 45 and 68% for S88 and S28 transgenic seeds, respectively. However, higher ABA concentrations resulted in almost complete inhibition of germination of both wild-type and *Sl-ERF2*-expressing seeds. We addressed the impact of ABA treatment on the expression of *Sl-ERF2* in germinating seeds. Fig. 4C shows that the seed-associated expression of *Sl-ERF2* is reduced when seed germination is inhibited by exogenous ABA treatment. However, ABA inhibition of *Sl-ERF2* gene expression in wild-type germinating seeds occurs only after 48 h imbibition but not at earlier stages (Fig. 4C).

In order to gain better insight into the mechanism by which *Sl-ERF2* impacts seed germination, we assessed its transcript accumulation during this process. Taking into

Table 2 Apical hook formation in etiolated wild-type and *Sl-ERF2*-overexpressing tomato lines

	Air		1-MCP	
	%	±SD	%	±SD
Wild type	1	0.08	0	0
S88	16	2.9	1.5	0.21
S28	18	1.1	0	0

Complete hook formation (exceeding 270°) in wild-type and *Sl-ERF2*-overexpressing seedlings in the dark in air or in air + 1-MCP was scored. The data correspond to the mean value of three biological replicates corresponding to the seedlings.%, percentage of seedlings with complete hook formation. $P < 0.05$.

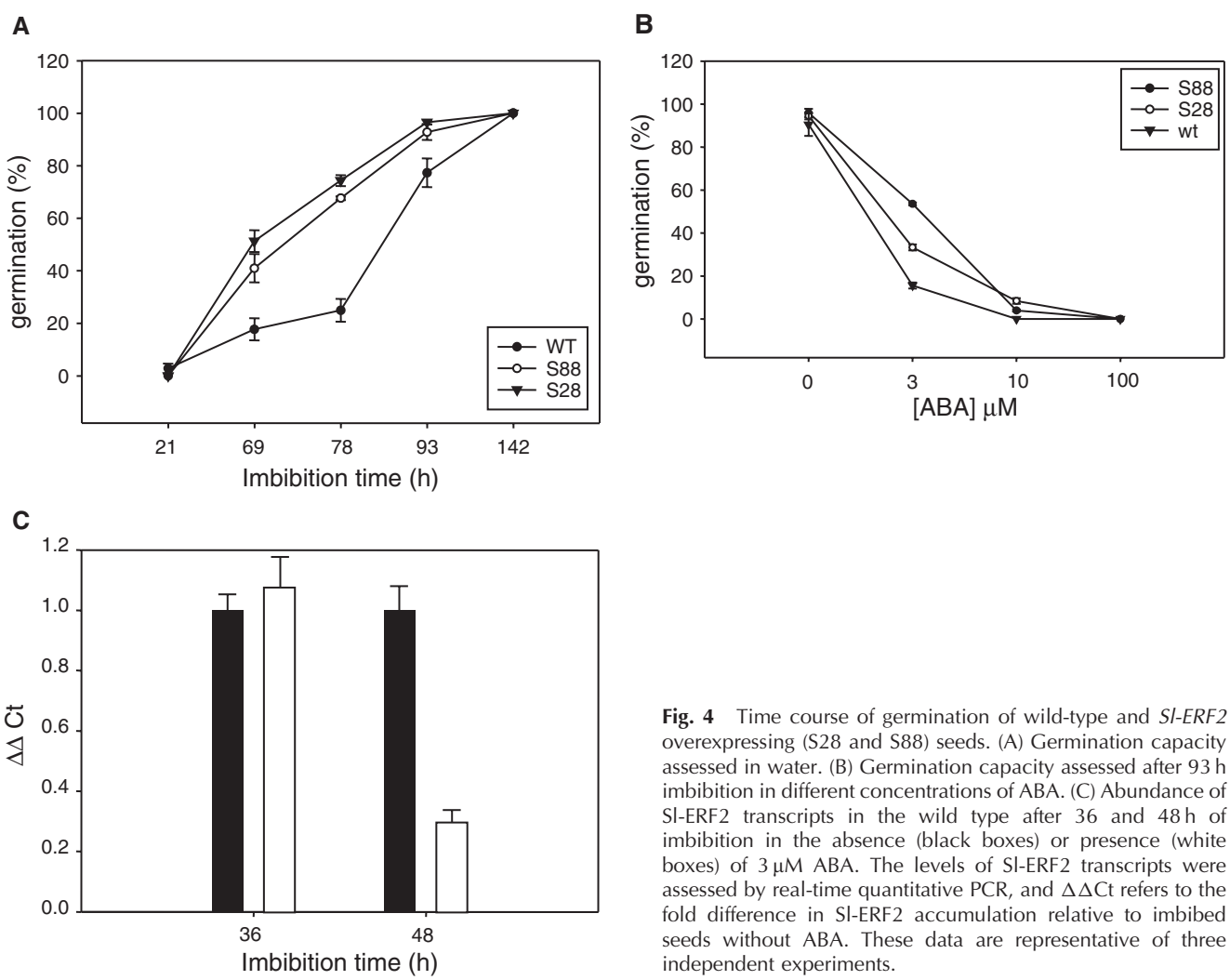

Fig. 4 Time course of germination of wild-type and *Sl-ERF2* overexpressing (S28 and S88) seeds. (A) Germination capacity assessed in water. (B) Germination capacity assessed after 93 h imbibition in different concentrations of ABA. (C) Abundance of Sl-ERF2 transcripts in the wild type after 36 and 48 h of imbibition in the absence (black boxes) or presence (white boxes) of 3 μM ABA. The levels of Sl-ERF2 transcripts were assessed by real-time quantitative PCR, and ΔΔCt refers to the fold difference in Sl-ERF2 accumulation relative to imbibed seeds without ABA. These data are representative of three independent experiments.

account that *Sl-ERF2* can give rise to two types of transcripts, specific primers were designed to target exclusively the spliced mRNA in all quantitative RT–PCR experiments. Fig. 5A shows that Sl-ERF2 transcript accumulation in germinating wild-type seeds decreases after 24 h imbibition and then increases at 48 h, coincident with the start of the germination process (see Fig. 4A).

The mannanase2 gene is up-regulated in the Sl-ERF2 overexpressing lines

Because the *mannanase2* gene (AF184238) is considered as a marker of seed germination (Nonogaki et al. 2000), we assessed the accumulation of the tomato mannanase2 transcript (Sl-MAN2) in germinating seeds. In wild-type seeds, the level of Sl-MAN2 transcript decreases slightly after 6 h of imbibition in water and then undergoes a dramatic increase, reaching 15 times its initial level after 48 h imbibition (Fig. 5B). In order to uncover whether the overexpression of *Sl-ERF2* impacts the accumulation of Sl-MAN2 transcripts during the germination process, we assessed the level of Sl-MAN2 transcripts in the transgenic lines. Quantitative RT–PCR data (Fig. 6) reveal that after 12 h imbibition, accumulation of *Sl-MAN2* transcripts is substantially higher in *Sl-ERF2*-expressing seeds than in the wild type. The level of *Sl-MAN2* transcripts in S28 and S88 is three and six times higher than in the wild type, respectively, clearly indicating that the *Sl-MAN2* gene is up-regulated in the overexpressing lines (Fig. 6).

To explore further whether the *Sl-MAN2* gene is under direct regulation by *Sl-ERF2* and in order to address whether seed germination is dependent on the expression of the *mannanase2* gene, we assessed its expression in wild-type and transgenic seeds upon ABA treatment. Fig. 7 indicates that accumulation of *Sl-MAN2* transcripts completely collapses in the presence of 3 μM ABA in both wild-type and *Sl-ERF2*-overexpressing lines. However, the ABA-induced inhibition of *mannanase2* expression is higher in wild type (39 times) than in transgenic S28 and S88 lines where it reaches 11 times and six times, respectively, (Fig. 7). As a result, the level of *Sl-MAN2* transcripts in transgenic seeds remains significantly higher than that in the wild type, which correlates with the higher germination capacity exhibited by S88 and S28 lines (see Fig. 4B).

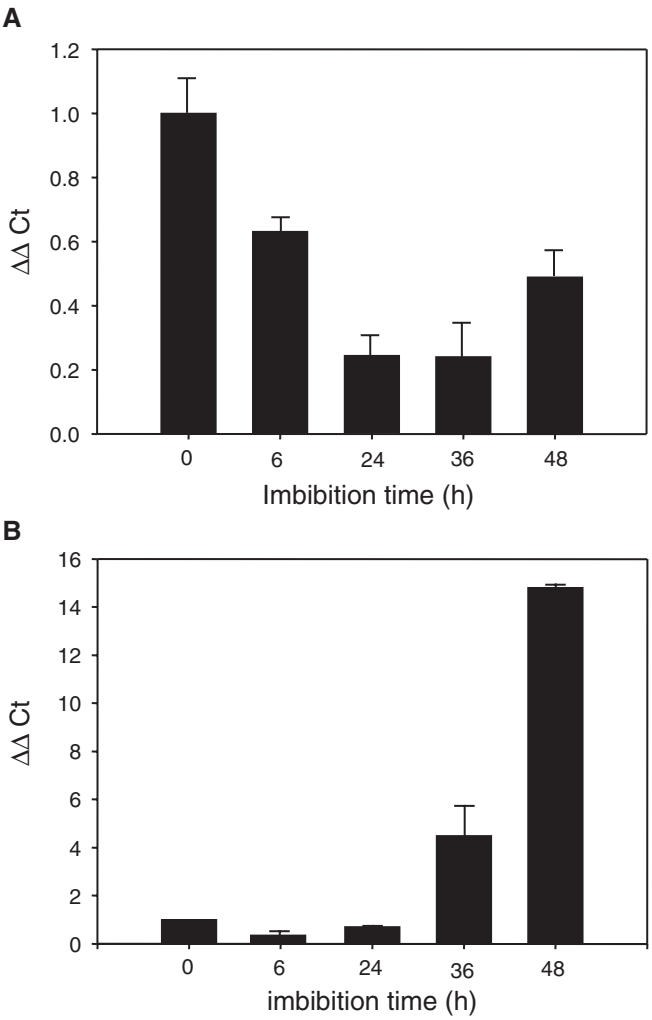

Fig. 5 Time course of accumulation of Sl-ERF2 and Sl-MAN2 transcripts during seed imbibition. Transcript accumulation of Sl-ERF2 (A) and *Sl-MAN2* (B) assessed by real-time quantitative PCR. The experiment was carried out in triplicate, and ΔΔCt refers to the fold difference in *Sl-ERF2* and *Sl-MAN2* expression relative to time 0 h.

Discussion

ERF proteins are defined as a large family of transcription factors involved in ethylene-mediated regulation of gene expression (Ohme-Takagi and Shinshi 1995). We have described previously four new members of the ERF gene family in tomato and showed that, among these, *Sl-ERF2* exhibits a ripening-associated pattern of expression (Tournier et al. 2003). We report here that *Sl-ERF2* is also involved in other ethylene-dependent developmental processes such as apical hook formation and seed germination. Comparative analyses show that the tomato *Sl-ERF2* gene shares a similar structure with *AtEBP*, its putative Arabidopsis ortholog. Both genes are composed of two exons and a single small intron. However, while the regulation through alternative splicing has not been described so far for any member of the ERF family, an important feature of the *Sl-ERF2* gene is the presence of two different transcripts corresponding to spliced and unspliced versions. The search in the tomato EST database (sgn.cornell.edu) confirmed the existence of the two mRNA species, opening up the possibilty that alternative splicing

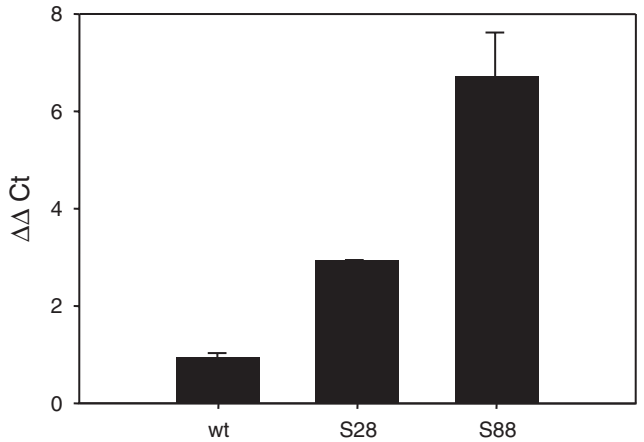

Fig. 6 Quantitative RT–PCR analysis of Sl-MAN2 transcript accumulation in *Sl-ERF2*-overexpressing tomato seeds. ΔΔCt refers to the fold difference in *Sl-MAN2* expression relative to the wild type.

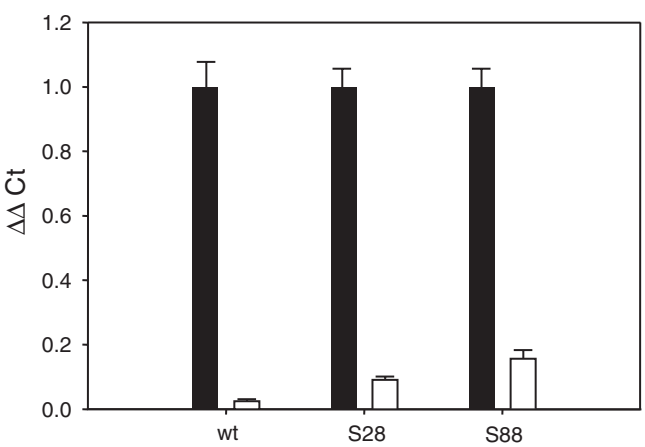

Fig. 7 ABA responsiveness of *Sl-MAN2* in imbibed seeds. RNAs were extracted from wild-type (WT) or *Sl-ERF2*-overexpressing (S28 and S88) seeds after 48 h of imbibition in either the absence (black boxes) or presence (white boxes) of 3 μM ABA. The levels of *Sl-MAN2* transcripts were assessed by real-time quantitative PCR in triplicate, and ΔΔCt refers to the fold difference in *Sl-MAN2* expression relative to imbibed seeds without ABA.

might play a role in the regulation of the *Sl-ERF2* gene in this species. Yet it appears that the unspliced Sl-ERF2 transcript is unlikely to give rise to functional protein since two truncated proteins can be derived from the two ORFs, one corresponding to the N-terminal part lacking the AP2 domain and the other corresponding to the C-terminal part which lacks the N-terminal MCGGAAI$^{I}/_{L}$ consensus peptide specific to ERF members of subfamily IV (Tournier et al. 2003). Moreover, in all tomato tissues considered, the abundance of the spliced Sl-ERF2 transcripts is several thousand times higher than that of the unspliced form and therefore accounts for most of *Sl-ERF2*-derived transcripts. Taking into account that the abundance of the unspliced transcript is several thousand fold lower than that of the spliced form, and that the putative proteins derived from the unspliced form are truncated proteins, it can be speculated that functional proteins may only derive from the spliced transcript.

The *Sl-ERF2* promoter harbors a number of putative *cis*-regulatory elements, among which are three ABREs, an RY-element found in seed-specific regulated genes (Fujiwara and Beachy 1994, Reidt et al. 2000) and five putative EREs. The presence of these regulatory elements suggests a role for *Sl-ERF2* in the associated developmental processes. The physiological significance of the *Sl-ERF2* gene was therefore addressed here by the analysis of up- or down-regulated transgenic tomato lines. However, none of the *Sl-ERF2*-suppressed lines showed any visible phenotype which may result from functional redundancy among members of the ERF gene family. In contrast, consistent with the presence of the RY and ERE *cis*-elements in the promoter region of the *Sl-ERF2* gene, overexpressing lines showed altered phenotypes associated with seed germination and ethylene response. The enhanced ethylene response in *Sl-ERF2*-expressing lines is revealed by exaggerated hook formation in the absence of ethylene treatment. While these data strongly suggest that *Sl-ERF2* is actively involved in hook formation, they also indicate that in the absence of ethylene perception, Sl-ERF2 alone is unable to induce hook formation. Therefore, Sl-ERF2 protein seems to require some other ethylene-dependent components to impact this developmental process.

Up-regulation of *Sl-ERF2* also results in premature seed germination concomitant with enhancement of *mannanase2* gene expression. A number of studies showed that mannanase activity correlates with the germination process (Dahal et al. 1997, Dutta et al. 1997). It was reported that the endosperm cell walls contain approximately 60% mannan (Groot et al. 1988, Dahal et al. 1997) probably in the form of galactomannan or galactoglucomannan polymers which constitute the major carbohydrate reserves of the endosperm and contribute to its rigidity. Endo-(1,4)-β-mannanase, which hydrolyzes internal bonds within mannan polymers, has been associated with the mechanism of seed germination in many plant species (Watkins et al. 1985, Dutta et al. 1994, Downie et al. 1997, Dutta et al. 1997, Sanchez and De Miguel 1997). Mannanase activity was found to be high in the endosperm tissue and, among the tomato mannanase genes expressed in this tissue, *Sl-Man2* was shown to be preferentially expressed in the endosperm cap of seeds prior to radicle emergence. In contrast, *Sl-Man1* is expressed at the post-germinative phases (Nonogaki et al. 2000). Therefore, the expression of the *Sl-Man2* gene can be considered as a good marker of seed germination and the Sl-Man2 protein as a

germination-specific protein. Mannanase gene expression seems to be required for seed germination, and in the *Sl-ERF2*-expressing lines the premature seed germination correlates with an enhanced expression of the *Sl-Man2* gene, suggesting that Sl-ERF2 impacts seed germination through the positive regulation of the *Sl-Man2* gene. While we confirm in this study that ABA plays a role in the inhibition of seed germination (Toorop et al. 2000), we show for the first time that ABA exerts a negative regulation on both *Sl-ERF2* and *Sl-Man2* genes. Our data indicate therefore that ABA and Sl-ERF2 have opposite effects on the expression of the mannanase gene and hence on seed germination. During the process of seed germination, Sl-ERF2 may allow functional integration of ethylene and ABA signals, leading to a fine coordination of this crucial developmental process. The role of ERF-like proteins in integrating ABA and ethylene responses has been recently demonstrated for AtERF7, an Arabidopsis ERF, expressed during drought stress responses of plants (Song et al. 2005).

Materials and Methods

Plant material

Tomato (*Solanum lycopersicum* cv MicroTom) plants were grown under standard greenhouse conditions. For growth in chamber rooms, the conditions are as follow: 14 h day/10 h night cycle, 25/20°C day/night temperature, 80% hygrometry, 250 μmol m^{-2} s^{-1} intense luminosity.

Plant transformation

A sense construct consisting of the full-length coding sequence of *Sl-ERF2* (from ATG to the Stop codon) under the transcriptional control of the cauliflower mosaic virus 35S (CaMV 35S) promoter and the nopaline synthase (NOS) terminator was introduced into tomato plants using the pGA643 binary vector. *Agrobacterium tumefaciens*-mediated transformation of tomato plants was carried out according to Jones et al. (2002), and transformed lines were selected as in Wang et al. (2005). All experiments were carried out using homzygous lines from F_3 or later generations.

Germination assay

After fruits were harvested, seeds were collected and stored at 20°C until use. For germination experiments, 100 tomato seeds were placed in Petri dishes on one layer of filter paper moistened with 10 ml of water, and incubated at 25°C in the dark. For ABA treatment, seeds were imbibed in the presence 3, 10 and 100 μM ABA.

Apical curvature test

Sterilized seeds were put on Murashige and Skoog agar medium plates and placed in the dark for 2 d at 4°C. Hook formation was assessed on 3-day-old dark-grown seedlings with or without MCP, and the apical curvature was estimated visually. Fifty seedlings were used for each experiment and three biological replicates were performed.

Isolation of the genomic clone

Genomic DNA was extracted from 1 g of ground tomato (*S. lycopersicum*) leaf tissue. The resulting powder was mixed with 5 ml of extraction buffer [2% (w/v) hexadecyl-trimethyl-ammonium bromide, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris–HCl, pH 8] and warmed at 65°C for 10 min. After a phenol/chloroform/isoamylalcohol and chloroform extraction, DNA was precipitated with 1 vol. of isopropanol for 20 min on ice. After centrifugation (5 min at 2,000×*g*), the pellet was re-suspended in 10 ml of washing buffer [76% (v/v) ethanol and 10 mM ammonium acetate]. After centrifugation (10 min at 2,000×*g*), the DNA was re-suspended in 200 μl of sterile water. An RNase treatment was done at 37°C for 10 min. A pair of primers was chosen based on the cDNA sequence, and PCRs were performed on the genomic DNA. The amplified fragments were cloned and fully sequenced. Comparative analysis between the genomic clone and cDNA sequences allowed the delimitation of introns and exons.

Isolation of the Sl-ERF2 promoter

The Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used to isolate the *Sl-ERF2* gene promoter region. Each tomato genomic DNA aliquot was digested with four 6 bp-recognizing and blunt end-forming restriction enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. Adaptor DNA which harbored two primer-binding sites for AP1 and AP2 primers provided by the Genome Walker Kit was linked to both ends of the restricted tomato DNA fragment at 16°C. AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and AP2 (5′-ACTATAGGGCACGCGTGGT-3′) primers were used for PCR amplification, and were paired with two *Sl-ERF2* gene-specific antisense primers. The tomato genomic DNA fragment with adaptors at both ends was used as a template for the amplification of the promoter region. The generated PCR product was cloned into pGEMT-easy vector (Promega) and fully sequenced. DNA sequences were analyzed with BLAST network services at the National Center for Biotechnology Information (Altschul et al. 1997), and by PlantCARE, (Lescot et al. 2002).

RNA extraction and quantitative PCR

RNA was extracted by the phenol–chloroform method according to Zegzouti et al. (1999). Extractions from seed tissue were performed at different times of imbibition: 0, 6, 24, 36 and 48 h before root protrusion. The same protocol was used for RNA extraction from stem, leaf, root, flower and fruit tissues. DNase-treated RNA (2 μg) was then reverse-transcribed in a total volume of 20 μl using the Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed using cDNAs corresponding to 2.5 ng of total RNA in a 10 μl reaction volume using the SYBR Green PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers for Sl-ERF2 and Sl-MAN2 transcripts. To assess the relative abundance of the Sl-ERF2 spliced and unspliced transcripts, we designed specific primers capable of discriminating between the two mRNA species. *Actin* was used as a reference gene with constitutive expression in various tissues. The following gene-specific primers were used: Sl-ERF2F spliced, GTTCCTCTCAACCCCAAACG; Sl-ERF2R spliced, TTCATCTGCTCACCACCTGTAGA; Sl-ERF2F_unspliced, TCGACCCTCTACAGGTACTAGTTAATCATATATA; Sl-ERF2R_unspliced, TTCACTCGCTCACCACCTGTTT;

Sl-MAN2F, GAATTGGGAAAAAATCCATCCA; Sl-MAN2R, TCATGGCATGAGACTGACTTGTAAT; Sl-Actin-51F, TGTC CCTATTTACGAGGGTTATGC; Sl-Actin-51R, AGTTAAATC ACGACCAGCAAGAT.

For *Sl-ERF2* and *Sl-MAN2*, the optimal primer concentration was 300 nM and for Sl-Actin the primers were used at 50 nM concentration. Real-time PCR conditions were as follow: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min, and finally one cycle at 95°C for 15 s and 60°C for 15 s. For all real-time PCR experiments, two biological replicates were made and each reaction was run in triplicate. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the Sl-Actin-51 (accession No. Q96483) as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value for *Sl-ERF2* and *Sl-MAN2* genes was normalized to the Ct value for Sl-Actin-51 and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

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