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RESEARCH PAPER

SlERF36, an EAR-motif-containing ERF gene from tomato, alters stomatal density and modulates photosynthesis and growth

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Abstract

The AP2 domain class of transcription factors is a large family of genes with various roles in plant development and adaptation but with very little functional information in plants other than *Arabidopsis*. Here, the characterization of an EAR motif-containing transcription factor, *SlERF36*, from tomato that affects stomatal density, conductance, and photosynthesis is described. Heterologous expression of *SlERF36* under the CaMV35S promoter in tobacco leads to a 25–35% reduction in stomatal density but without any effect on stomatal size or sensitivity. Reduction in stomatal density leads to a marked reduction in stomatal conductance (42–56%) as well as transpiration and is associated with reduced CO_2 assimilation rates, reduction in growth, early flowering, and senescence. A prominent adaptive response of *SlERF36* overexpressors is development of constitutively high non-photochemical quenching (NPQ) that might function as a protective measure to prevent damage from high excitation pressure. The high NPQ leads to markedly reduced light utilization and low electron transport rates even at low light intensities. Taken together, these data suggest that *SlERF36* exerts a negative control over stomatal density and modulates photosynthesis and plant development through its direct or indirect effects.

Key words: ERF, flowering, non-photochemical quenching, photosynthesis, repressor, senescence, stomata, stomatal conductance, tobacco, tomato.

Introduction

Plant development is a tightly regulated process that is controlled by both internal and external cues. One of the major factors that govern growth and survival is the ability to respond and adapt to the changing environment during different times of the day and in different seasons of the year. This requires responding rapidly to both biotic and abiotic factors, through changes in gene expression and activating responses that bring about physiological changes. At the genetic level, plants have evolved several gene families that control their development and govern their interactions with the environment (Reichmann and Ratcliffe, 2000). One of these is the plant-specific AP2/ERF domain family of transcription factors that is characterized by the presence of a conserved 58–59 amino acid AP2/ERF domain with different DNA-binding specificities (Sharma *et al.* 2010). This domain, singly or in combination with other motifs, can activate or repress gene expression in response to developmental or environmental signals, such as heat, drought, cold, and flooding, or in

response to pathogenic attack, often by mediating hormonal responses (Mizoi *et al.*, 2012; Gutterson and Reuber, 2004). The AP2/ERF domain family is one of the largest transcription factor families with 122 members in *Arabidopsis*, 139 in rice, and 146 in tomato (Nakano *et al.*, 2006; Pirrello *et al.*, 2012). Although several members of the family in different plants have been characterized, the function of a large number of AP2/ERF members is still unknown especially outside *Arabidopsis*. Thus, the functional analysis of AP2/ERF family genes, particularly in target crops like cereals, legumes, and fruits remains a major challenge.

A subclass of the AP2 domain family is the class II type family that functions as dominant repressors of gene expression (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). These family members possess a C terminal motif L/FDLNL/FxP designated as the ERF-associated Amphiphilic Repression (EAR) motif that has been shown to be involved in repression not only in association with the AP2 domain but also in other transcription factors, such as C2H2 zinc finger proteins and AUX/IAA proteins, that lack the AP2 domain (Kagale *et al.*, 2010; Kagale and Rozwadowski, 2011). In combination with the AP2 domain, there are eight members each of the EAR motif containing genes in *Arabidopsis* and rice (Nakano *et al.*, 2006) and at least seven in tomato (Sharma *et al.*, 2010). Functional analysis of a few of these genes reveals diverse roles such as in wounding and ethylene response (e.g. *AtERF4*, Yang *et al.*, 2005), ABA and salt stress signalling (e.g. *AtERF7*, Song *et al.*, 2005; *SlERF3*, Pan *et al.*, 2010), ABA-ethylene crosstalk (e.g. *AtERF11*, Li *et al.*, 2011), cold and drought stresses (e.g. *RAP2.1*, Dong and Liu, 2010), and herbivory (*OsERF3*, Lu *et al.*, 2011) Apart from *Arabidopsis*, the function of this family of genes remains poorly understood. This paper shows that ectopic expression of *SlERF36*, an EAR motif containing ERF gene, negatively regulates stomatal density and adversely affects several photosynthetic parameters in tobacco, leading to early flowering and senescence.

Materials and methods

Plant material and treatments

All studies were carried out on *Solanum lycopersicum* var. Ailsa Craig. Plants were grown in soil in glasshouse at 25–27 °C with a 16/8 light/dark photoperiod at a light intensity of about 400 µmol photons m^{-2} s^{-1}.

For studies on expression in different tissues such as root, stem, leaves, and flowers, these were collected separately from 3-month-old plants, frozen in liquid nitrogen and kept at –70 °C until use for RNA isolation.

RNA isolation, cDNA preparation, and real-time PCR

RNA was isolated from different tissues as described by Asif *et al.* (2000). RNA was reverse transcribed using REVERTAID MMLV reverse transcriptase (Fermentas) with the 3′-AP primer from Invitrogen (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′). Two degenerate primers ERF-F1 (5′-GTGGGGDAAATKSGYN KCKGARAT-3′) and ERF-R1 (5′-CTWAAAR SYGCBDYRTCGT ARGC-3′ that were designed based on an alignment of the AP2/ERF domain sequences of various ERF genes were used to amplify a fragment of 115 nt. This fragment showed sequence identity with a tomato BAC clone that was similar to the *NtERF5*. Based on this sequence, forward and reverse primers SlERF36-OF (5′-ATGGATCC TATGAGAAGAGGCAGAGC-3′) and SlERF36-OR (5′-GAAACC AGGATCCTTCAAAGACATAG-3′) were used to amplify the complete open reading frame of 666 nucleotides that encoded a protein of 221 amino acids (SGN-U313854). The fragment was cloned in the T/A cloning vector pTZ57R (Fermentas).

For real-time (RT) PCR analysis, primers SlERF36-FSQ (5′-ACTCATCGTCTTCTGTTGTTGA-3′) and SlERF36-RSQ (5′-ATTAGCATAAACATCATCCATCG-3′) were designed and used in combination with the internal control actin, amplified using the primers ActF1 (5′-ATGACATGGAGAAGATCTGGCATCA-3′) and ActR1 (5′-AGCCTGGATGGCAACATACATAGC-3′). The reactions were run using the Power SyBr Green PCR master mix on a ABI Prism 7000 real-time PCR machine (Applied Biosystems, USA). RNA was isolated from pooled tissue samples collected from several plants at specific stages mentioned above. Reactions were run in triplicates with cycling conditions as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Values were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. For calculating relative change in expression, the control sample data was averaged and considered as 1 and averaged values of all other samples plotted against the control data.

Development of transgenic plants

The complete *SlERF36* open reading frame was cloned in pBI121 at the *Bam*HI site. Transgenic tobacco plants, (*Nicotiana tabacum* var. Petit Havana) were generated by *Agrobacterium*-mediated transformation of leaf disc explants as described by Horsch *et al.* (1988). Independent transformants, selected on kanamycin on agar, were transferred to soil for hardening and plants grown in a glasshouse maintained at 25 °C. Plants in the T1 generation were selected for phenotypic analysis while homozygous progeny of three independent lines, viz. SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4, in the T2 generation were chosen for comparative physiological analysis.

Physiological studies

For each experiment, homozygous progeny of three independent transgenic lines (five or six plants per line) were grown under natural light in pots as described. Leaf gas exchange and fluorescence were simultaneously measured with a GFS-3000 Portable Gas Exchange Fluorescence System attached with a fluorescence module (PAM-fluorometer 3055-FL, Walz, Germany) on a leaf (generally the fourth leaf from bottom in a 30-day-old plant or fifth leaf from bottom in a 45-day-old plant) enclosed in a temperature-, light-, and humidity-controlled leaf chamber 3010-S (Walz). All comparative measurements were made on plants of the same age (30–45-day-old plants) between 08.00 and 11.00. The leaves were dark adapted at vapour pressure deficit (VPD) ranging between 0.5 and1 kPa, leaf temperature of 25 °C, and CO_2 concentration of 400 µmol mol^{-1}, and Fv/Fm measurements were made using a light pulse of 2000 µmol photons m^{-2} s^{-1}. The leaves were then exposed gradually to increasing photosynthetic photon flux density (PPFD) to 400 µmol photons m^{-2} s^{-1} and measurements of steady-state photosynthesis rate (A), stomatal conductance (g_s), transpiration (E), internal CO_2 concentration (C_i), and quantum yield of PSII in the light (ϕ) were made. Intrinsic water use efficiency was calculated as the A/E ratio.

Light response curves for photosynthesis were constructed using various PPFDs (ranging from 0 to 1200 µmol photons m^{-2} s^{-1}) incident on the uppermost, fully expanded dark-adapted leaves (generally the fourth and fifth leaves from bottom) as described in Escalona *et al.* (1999). A/C_i curves were performed over a range of CO_2 concentrations between 40 and 2500 µmol mol^{-1} on 3–5 plants per transgenic line as described in Escalona *et al.* (1999). VPD response curves for gas exchange parameters A, g_s, and E were constructed by exposing the leaves to a range of VPD levels (0.5–3.0 kPa) at 400 µmol

photons m^{-2} s^{-1} PPFD, leaf temperature of 25 °C and CO_2 concentration 400 µmol mol^{-1}. A, g_s, and E were recorded simultaneously after steady-state conditions (generally after 10–20 min) had been re-established at each VPD level. Light response curves for fluorescence (PSII) and P-700 (PSI) were measured with DUAL-PAM-100 (Walz) as described by Schreiber and Klughammer (2008). Maximal fluorescence and maximal P700 changes were obtained from dark adapted leaves (as described above) and then leaves were exposed to high light (i.e. 1200–1500 µmol photons m^{-2} s^{-1}) for 30 min to obtain a steady state before commencing measurement of several fluorescence parameters every 5 min at each PPFD (ranging from 11–2000 µmol photons m^{-2} s^{-1}). The quantum yield of PSI – Y(I) – is defined by the proportion of overall P700 that is reduced in a given state and not limited by the acceptor side. It is calculated from the complementary PSI quantum yields of non-photochemical energy dissipation due to donor-side limitation and acceptor-side limitation– Y(ND) and Y(NA) (Schreiber and Klughammer, 2008). The quantum yields of PSII, non-photochemical quenching, and non-light-induced non-photochemical fluorescence quenching – Y(II), Y(NPQ), and Y(NO) – were calculated from the measurement of chlorophyll fluorescence, as described by Kramer *et al.* (2004).

The electron transport rates ETRI and ETRII was calculated as ETR(I or II) = Y(I or II) × PPFD × 0.5 × abs, where Y is the apparent quantum yield, 0.5 is the proportion of absorbed light reaching PSI or PSII, and absI is absorbed irradiance, taken as 0.84 of incident irradiance. NPQ was calculated as (Fm – Fm′)/ Fm′. Chlorophyll content was measured by isolating chlorophyll from leaf discs and calculated according to Arnon (1949).

Estimation of stomatal density

Leaf epidermis (about 1 cm^2) from the abaxial surface of fully expanded leaves (fourth leaf from bottom of 45-day-old plants) was peeled off with a pair of forceps and placed immediately in water and later mounted in 10% glycerol and observed under a light microscope (Eclipse TE300 inverted microscope, Nikon). Stomata were counted in an area of 0.25 mm^2 in three different regions from three independent leaves of the same position from three independent lines.

Statistical analysis

The significance of correlations was tested by using linear regression, with $P < 0.05$ considered statistically significant. Means were compared by using one-way analysis of variance and post-hoc means comparison (Scheffé Test). All data analysis and plotting were performed with SigmaPlot version 8 0.

Results

The gene identified in these studies encoded a protein of 221 amino acids (Fig. 1) and was designated in a recent study of the tomato ERF family as *SlERF36* by Sharma *et al.* (2010) and as SlERF.F.1 by Pirrello *et al.* (2012). The gene encoded an ERF-type protein based on the presence of alanine at position 14 and aspartate at position 19 of the AP2/ERF domain (Sakuma *et al.*, 2002). In addition, there was a stretch of seven amino acids (FDLNFPP) at the C-terminal end that has been designated as an EAR motif (ERF-associated amphiphilic repression motif) and shown to be involved in repression of gene expression (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). As per the classification given by Fujimoto *et al.* (2000), *SlERF36* belongs to group II of ERF transcription factors, members of which have been shown to act as repressors of transcription, while as per the classification of Nakano *et al.* (2006) it belongs to family VIII B1 (Sharma *et al.*, 2010). BLAST analysis showed 73% amino acid identity with *NtERF5* of tobacco while the closest *Arabidopsis* homologues were *AtERF3* (58% amino acid identity) and *AtERF7* (50% amino acid identity).

Transcript accumulation during plant growth

As a first step towards the characterization of the function of *S1ERF36*, its expression pattern in different tissues was investigated using RT-PCR. Highest expression of *S1ERF36* was observed in leaves and roots whereas its expression was low in stem and barely detectable in flowers (Supplementary Fig. S1, available at *JXB* online). Transcript accumulation was low and did not vary much during fruit development and ripening (data not shown and Pirrello *et al.*, 2012).

SlERF36 overexpressors show early flowering and early senescence

Because of the large number of ERFs in plants and the possibility of redundancy, a functional analysis of *SlERF36* was carried out through ectopic expression of the gene under the CaMV35S promoter in transgenic tobacco. Of the 12 transformants obtained, three designated as SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 were selected for detailed study. RT-PCR with primers specific to tomato *SlERF36* showed the presence of the transcript in all three transgenic lines (Supplementary Fig. S2). Plants in T1 (second) generation were grown on soil in a glasshouse and monitored for various visible growth parameters such as height, time to flowering, leaf shape and size, and leaf senescence throughout the growth period (Fig. 2A). In general, transgenic plants were slow growing and appeared shorter in height with leaves having a lighter shade of green (Supplementary Fig. S3) as compared to control untransformed plants, but there was no change in leaf shape and size between the control and transgenic plants.

MRRGRATPAAAAAAVKPDGSGGLKEI**RFRGVRKRPWGRFAAEIRDPWKKTRVWLGTFDSAED**

AAKAYDAAARTLRGPKAKTNFPLPMYSQHHQFNRSLNPNDRLVDPRLYSQEAPIICQRPTSS

SMSSTVESFSGPRPPRQQTAVLPSRKHPRSPPVEPDDCRSDCDSSSSVVEDGDCEGGNDNIV

SSSLRNPLP***FDLNFPP***PMDDVYANSNDLYCTALCL

Fig. 1. Amino acid sequence of the SlERF36 polypeptide. The AP2 domain is shown in bold and underlined. The EAR motif at the C-terminal end has been boxed.

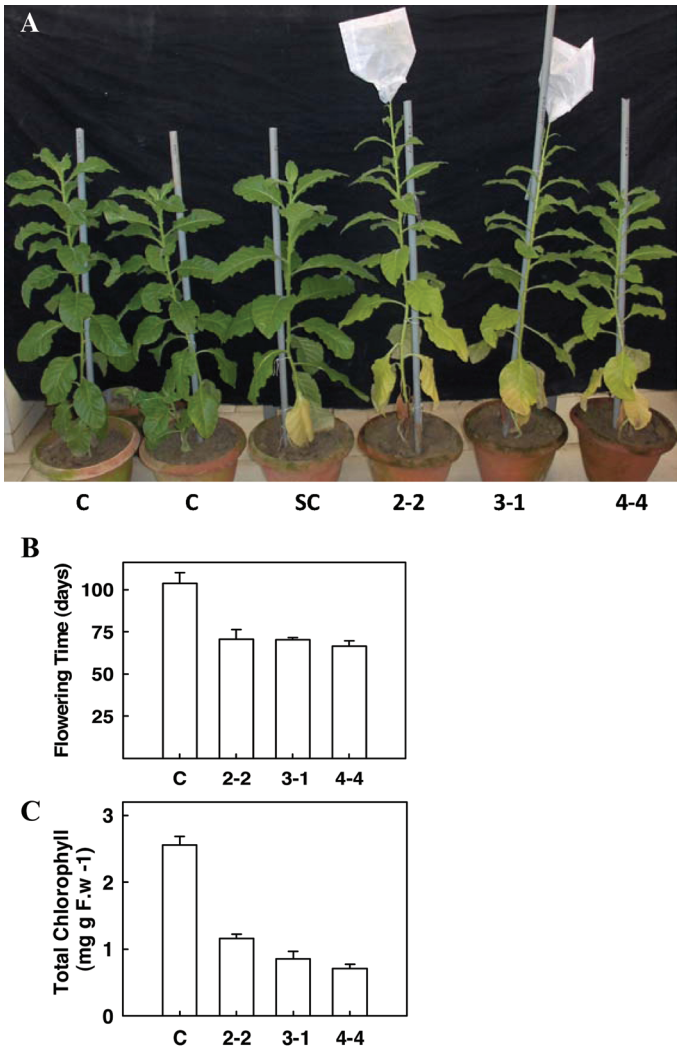

Fig. 2. (A) The early flowering and early senescence phenotype of transgenic lines expressing *SlERF36* under the CaMV35S promoter. Progeny of three independent transgenic lines SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 in the T1 (second) generation at about 90 days are shown. Plants were grown in the glasshouse at 25–27 °C at 16/8 light-dark cycles. C = Control (*Nicotiana tabacum* var Petit Havana), SC = segregating control. SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 are progeny of independent transgenic lines expressing *SlERF36*. (B) Graphical analysis of flowering time of control and transgenic tobacco plants expressing *SlERF36*. Values represent the average ± SD of 3–5 plants of each independent transformant. (C) Graphical analysis of total chlorophyll (6th leaf from bottom) of control and transgenic tobacco expressing plants *SlERF36*. Values represent the average ± SD of 5 leaves of each independent transformant (this figure is available in colour at *JXB* online).

Transgenic plants also showed early flowering with a difference of about one month between control and transgenic lines (Fig. 2B). While control plants began to flower at 103.25 ± 6.9 days, inflorescence was initiated in transgenic plants in 70.75 ± 5.61 (SlERF36-2-2), 70.25 ± 1.25 (SlERF36-3-1), and 66.5 ± 3.10 (SlERF36-4-4) days. Senescence of individual leaves in transgenic plants occurred earlier with early yellowing and death of the lower leaves as compared to corresponding control leaves (Fig. 2A). Chlorophyll levels of lower leaves (sixth leaf from bottom) were higher in controls (2.55 ± 0.12 mg (g FW)$^{-1}$) as against transgenic leaves where they were 0.71 ± 0.06 (SlERF36-4-4), 0.85 ± 0.10 (SlERF36-3-1), and 1.15 ± 0.06 (SlERF36-2-2) mg (g FW)$^{-1}$ at the 3-month stage (Fig. 2C).

SlERF36 overexpressors show reduced photosynthesis, conductance, and transpiration

In order to understand the basis of altered growth and other phenotypes in *SlERF36* overexpressors, gas exchange and fluorescence parameters were studied in 1-month-old homozygous plants grown under natural light. Interestingly, even at this stage, when visible differences between the control and transgenic plants were not too apparent, marked differences in photosynthetic parameters were observed (Table 1). Control plants showed a photosynthetic rate (A) of 4.97 ± 0.93 µmol CO_2 m^{-2} s^{-1}. In contrast, progeny of transgenic lines SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 had considerably reduced photosynthetic rates of 2.85 ± 0.28, 1.79 ± 0.19, and 2.34 ± 0.54 µmol CO_2 m^{-2} s^{-1}, which represented a decrease of 43–64% compared to the control. Stomatal conductance (g_s) also showed a decrease of 42–56% in the transgenic lines, with 84.2 ± 5.67 mmol water m^{-2} s^{-1} in control lines as against 46.76 ± 4.52, 37.45 ± 4.2, and 49.04 ± 9.53 mmol water m^{-2} s^{-1} for the progeny of SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4. Transpiration rates were reduced by 34–49% in progeny of the transgenic lines as against control. Water use efficiency (A/E) in the transgenic lines was also reduced to about 70–90% of the control.

In order to obtain an insight into the reasons for the reduced photosynthesis and transpiration, fluorescence kinetics and total yield were checked using the GFS system. No change was observed in the Fv/Fm ratios between the control and transgenic lines with Fv/Fm ratios of around 0.83–0.84 in all lines, suggesting that the function of reaction centres was not altered in transgenic plants relative to the control. However, the apparent quantum yield and electron transport rate of PSII was reduced in transgenic lines (Table 1). Interestingly, NPQ was substantially higher in progeny of all transgenic lines as compared to control plants.

SlERF36 overexpressors show defects in light utilization and CO_2 assimilation capacities

In order to test whether the ability of the transgenic plants to utilize light was selectively disturbed, measurements of PPFD response curves were carried in the transgenic plants. The photosynthetic rates at various light intensities (at 400 µmol CO_2 mol^{-1}) in transgenic lines showed considerable decrease from those of control plants right from 50 µmol m^{-2} s^{-1} onwards (Fig. 3A). As light intensity increased, there was an increase in photosynthetic rates in both the control and the transgenic lines, although rates of transgenic lines continued to remain much lower than those of control plants. Interestingly, the

Table 1. *Analysis of various gas exchange parameters in 1-month-old control and transgenic SlERF36 expressing tobacco plants grown under natural light*

Fluorescence data was obtained simultaneously with gas exchange using the GFS-3000 system attached with fluorescence measurement head (3055-FL) which serves as light source for gas exchange and fluorescence measurements as well as quantum yield measurements. Electron transport rate and NPQ were calculated from Fm and apparent quantum yield (Y) measurements made on dark and light adapted leaves, respectively, as ETR = Y × PPFD × 0.5 × 0.84, where Y = apparent quantum yield, and NPQ = (Fm – Fm′)/Fm′. Values represent the average of six homozygous progeny plants of each independent line (2-2, 3-1, and 4-4). Parameters in bold are significantly different in at least two transgenic lines ($P < 0.001$). *$P < 0.05$, **$P < 0.01$**, ***$P < 0.001$.

Parameter	Control	SlERF36-2-2	SlERF36-3-1	SlERF36-4-4
Photosynthetic efficiency, A (μmol CO_2 m^{-2} s^{-1})	**4.97 ± 0.93**	**2.85 ± 0.28*****	**1.79 ± 0.19*****	**2.34 ± 0.54*****
Stomatal conductance, g_s (mmol H_2O m^{-2} s^{-1})	**84.20 ± 5.67**	**46.76 ± 4.52*****	**37.45 ± 4.2*****	**49.04 ± 9.53*****
Transpiration rate, E (mmol H_2O m^{-2} s^{-1})	**0.93 ± 0.18**	**0.59 ± 0.05*****	**0.48 ± 0.04*****	**0.62 ± 0.11*****
Water use efficiency (A/E)	5.34 ± 0.29	4.80 ± 0.51*	**3.73 ± 0.50*****	**3.78 ± 0.26*****
Apparent quantum yield	0.60 ± 0.01	0.50 ± 0.04	0.46 ± 0.04*	0.48 ± 0.06*
Electron transport rate, ETR (μmol m^{-2} s^{-1})	96.35 ± 3.47	84.78 ± 7.76	76.96 ± 7.25*	81.39 ± 11.33*
Non-photochemical quenching, NPQ	0.69 ± 0.16	0.95 ± 0.22	**1.22 ± 0.32*****	**1.34 ± 0.15*****
Maximum quantum yield (Fv/Fm)	0.835 ± 0.001	0.82 ± 0.01	0.84 ± 0.004	0.84 ± 0.05

decrease in photosynthetic rates was more prominent at lower light intensities than at higher light intensities in transgenic lines compared to control plants. This indicated that the ability to utilize light optimally was markedly affected in the transgenic lines. There was, however, no change in the point at which light saturation occurred. In control as well as transgenic lines, saturation of photosynthesis occurred at a PPFD of about 450 μmol m^{-2} s^{-1}. The photosynthesis rates were also calculated as a function of changing g_s obtained from light response curves. As shown in Supplementary Fig. S4, there was a linear relationship between A and g_s, although the rates of control plants were higher than in transgenic plants.

Measurements of A/C_i response curves were also carried out in control and transgenic plants at 400 μmol light to test whether the ability to utilize CO_2 was affected (Fig. 3B). Surprisingly, although increasing C_i in transgenic lines led to increased photosynthetic rates, these were considerably lower than those in control plants at any given point by 30–50%. The initial slope of A/C_i curve was similar in control and transgenic plants but the rates in transgenic plants were reduced at higher C_i (>200 μmol mol^{-1}).

The sensitivity of stomata was also tested by measuring stomatal conductance at different vapour pressure deficits. As shown in Fig. 3C, an increase in VPD led to an increase in stomatal conductance up to 1.2 kPa in both control and transgenic lines followed by a decrease thereafter, indicating that the stomata in these lines retained their sensitivity to changes in VPD. The absolute values of g_s were expectedly much lower in transgenic lines as compared to control.

SlERF36 overexpressors have reduced electron transport rate and high NPQ

In order to obtain a more detailed insight into the processes affecting light and CO_2 utilization in the transgenic lines, the limitation parameters of photosystems I and II were measured. Both ETR II (Fig. 4A) and ETR I (Fig. 4E) were considerably reduced in transgenic lines, showing a decrease of 30–50% over those in control lines. A further analysis of the decrease in the ETRs was performed by measuring three complementary quantum yields of energy conversion, namely Y(II), Y(NPQ), and Y(NO) for PSII and Y(I), Y(ND), and Y(NA) for PSI. As shown in Fig. 4B, Y(II) in all transgenic plants showed a considerable decrease particularly at light intensities less than 200 μmol m^{-2} s^{-1}. This decrease in Y(II) appeared to be primarily due to an increase in NPQ (Fig. 4C) and not Y(NO) since latter remained almost constant in control and transgenic plants (Fig. 4D). Like Y(II), Y(I) was also decreased in transgenic plants (Fig. 4F). This decrease seemed to be due to marked donor side limitation of PSI (due to lower PSII ETR) as reflected by the elevated Y(ND), which was 30% higher in transgenic lines than in control leaves (Fig. 4G) while the corresponding Y(NA) was similar in both the control and the transgenics (Fig. 4H).

SlERF36 expression leads to reduced stomatal density in transgenic lines.

Since several photosynthetic parameters including stomatal conductance and light and CO_2 utilization were grossly affected in transgenic lines, this study investigated whether there was a change in the stomatal density between the control and the transgenic lines by measuring the stomata in several leaves of the control and transgenic lines (three leaves/line at three different locations in all three transgenic lines). A considerable decrease in the stomatal density was observed in all the transgenic lines (Fig. 5A). While control leaves had about 120 ± 6.5 stomata mm^{-2}, the transgenic lines SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 showed 83 ± 6, 77 ± 6.8 and 89 ± 9.5 stomata mm^{-2} (Fig. 5B). This represented a decrease of 25–35% in the total abaxial stomatal density as compared to control plants. There was, however, no change in the stomatal size from that in the control plants.

Discussion

The growth of plants and their adaptation to the environment is fine-tuned by the controlled expression of

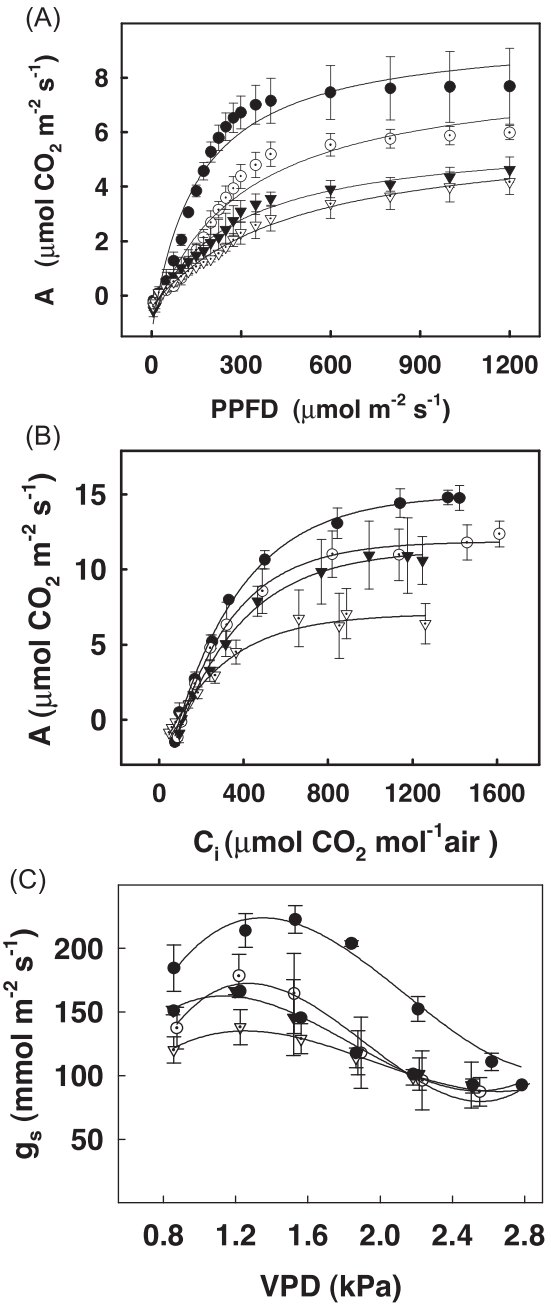

Fig. 3. Response of net photosynthesis, A, to PPFD (3A) and to sub-stomatal CO_2 concentrations C_i, (3B) and that of stomatal conductance g_s to VPD (3C) in leaves of control (●), SlERF36–2-2 (○), SlERF36–3-1 (▼) and SlERF36–4-4 (▽) lines of tobacco. Measurements were carried out as described in material and methods. Values are average ± SEs of three to five replicates. Curves were adjusted to a hyperbolic function $y=yo + a^*x/(b + x)$ for 3A and 3B and to a non-linear regression for 3C using the equation $g_s=aVPD^3+ bVPD^2+cVPD+ d$ (r^2 values ranging from 0.8–0.98).

various transcriptional regulators that include transcription factors, co-activators, repressors, and other accessory proteins. SlERF36/SlERF.F.1 is a C-terminal EAR-motif-containing AP2/ERF domain repressor protein that seems to control plant development and photosynthesis either by negatively regulating stomatal density and affecting stomatal conductance or through an independent effect on density.

One of the major effects of *SlERF36* expression in tobacco is a reduction of 25–35% in stomatal density (but without a change in size or sensitivity) in progeny of several independent transgenic lines. This is accompanied with a drastic effect on specific photosynthesis parameters that include a 42–56% decrease in stomatal conductance and transpiration, a lower rate of photosynthesis, decreased water use efficiency, lower rates of electron transport, and decreased quantum yield, leading finally to the onset of early flowering and senescence. While the decrease in density and conductance/transpiration are related and could in principle affect ETR and photosynthesis, they could also in turn be affected by the decrease in photosynthesis or be entirely independent effects from a direct or indirect action of SlERF36. Stomatal density is under the control of endogenous developmental cues but is also affected by changes in light intensity, CO_2 concentration, humidity, and water availability. Plants respond to changing CO_2 levels with a change in stomatal aperture as a short-term adaptation and with a change in both stomatal density and size over a longer timescale (Woodward, 1987; Woodward and Kelly, 1995). This adaptation mainly consists of a reduction in stomatal density in response to higher CO_2 levels (Woodward *et al.*, 2002; Hetherington and Woodward, 2003). Fossil data have also revealed that lower atmospheric CO_2 was associated with an increase in stomatal density and conductance and a reduction in size while higher atmospheric CO_2 was associated with decrease in stomata (Royer, 2001; Franks and Beerling, 2009). In fact, CO_2 doubling leads to an average decrease of almost 22% in stomatal density in 48 *Arabidopsis* accessions (Woodward *et al.*, 2002) and up to 29% in 110 other species (Woodward and Kelly, 1995). Stomatal conductance is also known to follow an inverse relationship with ambient CO_2 in angiospermic plants, decreasing at high CO_2 and increasing at low CO_2 levels (Brodribb *et al.*, 2009). This decrease in stomatal density and conductance most likely allows plants to fine tune their responses to the environment and control CO_2 assimilation and water utilization better (Franks and Beerling, 2009; Brodribb *et al.*, 2009). Given the approximately 22–30% decrease in stomata in response to CO_2 concentrations as high as twice the normal levels, it may be expected that an almost 25–35% decrease in stomatal density (and a 42–56% decrease in conductance) in transgenic *SlERF36* plants but without a corresponding increase in CO_2 concentration could severely restrict the uptake/availability of CO_2 to the plant and have a cascading effect on the other photosynthetic parameters.

One of the parameters that undergoes a drastic reduction along with the reduced stomatal density is the stomatal conductance which in turn could affect CO_2 uptake. This in turn severely limits the photosynthetic rate as evident from the 30–50% decrease in CO_2 assimilation in transgenic plants. Such an effect of reduced stomatal density and conductance on CO_2 uptake, photosynthesis, and productivity has also been reported in several previous studies in various plants (Freeland, 1948; Heichel, 1971; Fu *et al.*, 2010). Conversely, stomatal density is markedly affected by the environment and hence an effect of reduced photosynthesis on stomatal

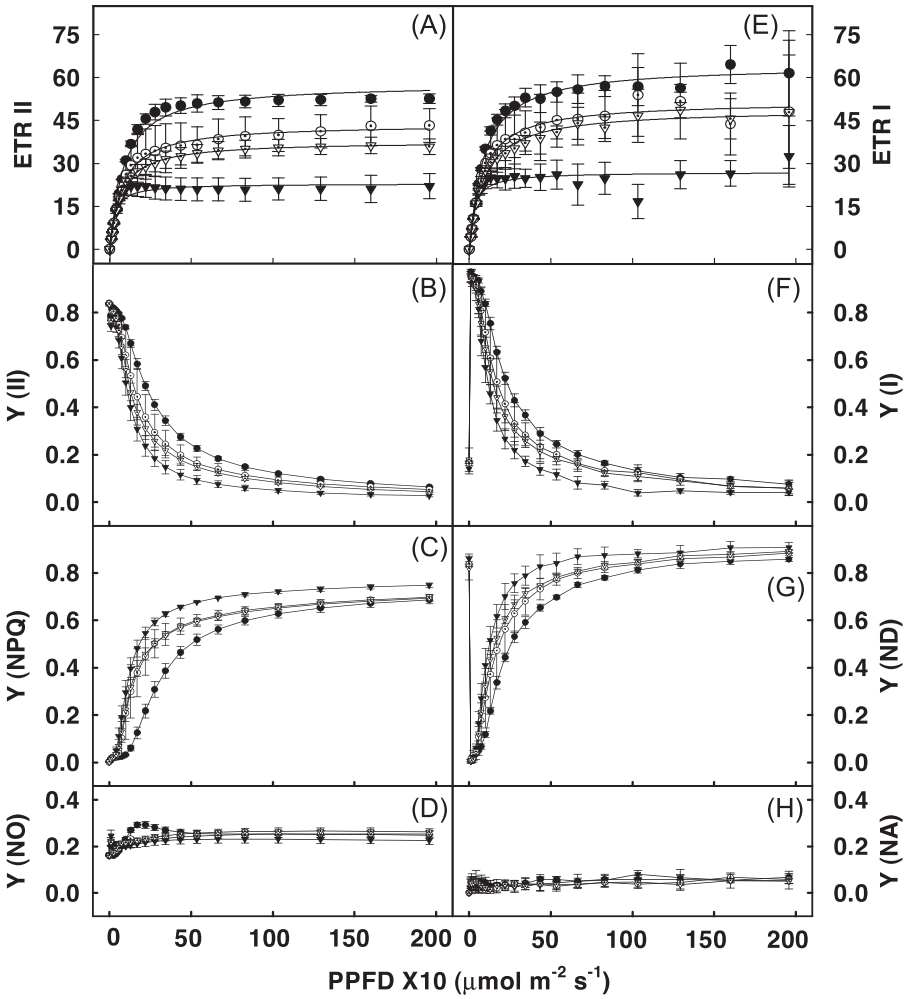

Fig. 4. Light response curve for electron transport rate of PSII (ETRII) and PSI (ETRI) and their respective quantum yields for leaves of control (●), SlERF36–2-2 (○), SlERF36–3-1 (▼) and SlERF36–4-4 (▽) lines of tobacco. (A) electron transport rate for PSII, ETRII; (B) photochemical quantum yield for PSII, Y(II); (C) quantum yield of light-induced non-photochemical fluorescence quenching for PSII, Y(NPQ); (D) quantum yield of non-light-induced non-photochemical fluorescence quenching for PSII, Y(NO); (E) electron transport rate for PSI, ETRI; (F) photochemical quantum yield for PSI, Y(I); (G) Quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation, Y(ND); (H) Quantum yield of non-photochemical energy dissipation in PSI due to acceptor side limitation Y(NA). Measurements were carried out as described in material and methods at light intensities ranging up to 2000. Values are average ± SEs of three to five replicates. Curves were adjusted to a hyperbolic function $y=yo + a*x/(b + x)$ for A and E.

density reduction cannot be ruled out. However, studies by Miyazawa *et al.* (2006) on Populus show that stomatal density of upper leaves was affected by conductance of lower leaves but was independent of photosynthesis. Similarly, Beerling and Woodward (1995) observed that the effect of increased CO_2 on stomatal density reduction was also seen in variegated leaves indicating that it was an effect independent of photosynthesis.

Interestingly, while a reduction in stomatal density and conductance could restrict CO_2 uptake and assimilation, increasing internal CO_2 does not restore the photosynthetic rates of the affected transgenic plants to those of the control. They continue to remain lower than control at any given C_i concentration (Fig. 3B). This shows that CO_2 is not the only limiting factor that is responsible for the photosynthetic defects in the transgenic plants. Rather, the plants appear to have undergone a reconditioning to an environment of reduced internal CO_2 that prevents them from making optimum utilization of light. This is evident from the fact that electron transport rates as well as the quantum yields of PSII and PSI are lower in transformants. The defect actually lies on the PSII side, leading to a reduction in the flow of electrons from PSII to PSI. The low PSII ETRs seem to result from considerably high levels of NPQ (Fig. 4C) as a protective mechanism against an environment of high excitation pressure due to low stomatal conductance and a limitation in CO_2 availability.

The increase in levels of NPQ is a response to avoid damage to the photosynthetic apparatus from high excitation pressure usually in response to high light conditions (Demmig-Adams and Adams, 1996; Oquist and Huner, 2003). But they may also result from a reduction in conductance beyond a certain

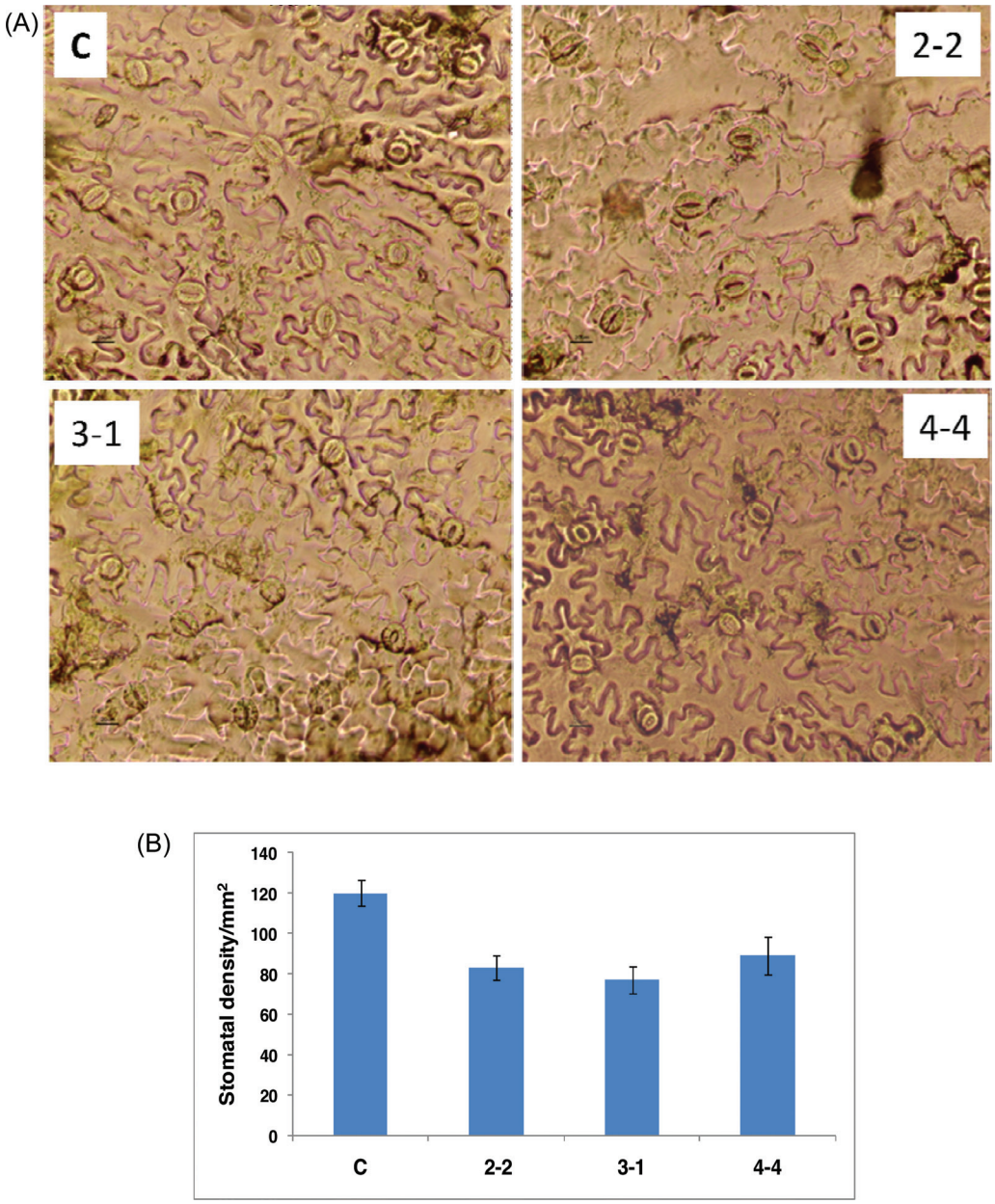

Fig. 5. (A) Leaf abaxial surface showing stomatal density in control (C) and transgenic *SlERF36* expressing tobacco plants of three independent lines, SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4. Stomatal density from leaf epidermal peels was estimated in the leaf sections at three different regions of three different leaves (4th leaf from bottom) under a light microscope (Nikon Eclipse TE300 Inverted microscope). The small black bar at the base of each picture on the left hand side represents a length of 20 µm. (B) Graphical estimation of the stomatal density of the lower leaf epidermis of control (C) and transgenic *SlERF36* expressing tobacco leaves of lines SlERF36-2-2, SlERF36-3-1, and SlERF36-4-4 shown in figure 5A. Values represent an average stomatal density ± SD in an area of 1 mm^2 of three independent leaves (from the same position) (this figure is available in colour at *JXB* online).

critical value, as shown previously in *Phaseolus vulgaris* by Omasa and Takayama (2003). By simultaneous measurement of NPQ and stomatal conductance after treating leaves with ABA, they observed that at conductance values greater than 80 mmol H_2O m^{-2} s^{-1}, the NPQ remained between 0.16 to 0.69. However, a reduction in stomatal conductance to less than 60 mmol m^{-2} s^{-1} resulted in drastic increase in NPQ with values reaching beyond 1.5. This is very similar to values observed in the current study wherein the *SlERF36*-expressing transgenic lines had stomatal conductances of lower than 50 mmol H_2O m^{-2} s^{-1} and high NPQ values ranging between 1.34 and 0.95 as against a conductance of 84 mmol H_2O m^{-2} s^{-1} and NPQ of only 0.69 in control plants. That this adaptation helps in protection of the reaction centres of the photosynthetic machinery in transgenic *SlERF36* plants is obvious from the fact that the openness of the reaction centres (as seen from an unchanged Fv/Fm ratio) is not affected in transgenic lines. What is surprising, however, is that, unlike most plants where high NPQ is a

response to high light intensity, the transgenic *SlERF36* plants seem to have constitutively high NPQ even at light intensities as low as 50 μE (where NPQ is almost 4-times higher in transgenic plants) (Fig. 4C). A consequence of the high NPQ is that only a fraction of the available light (even at low light intensities) is used for photosynthesis in the transgenic lines. This difference in light utilization between the control and the transgenic lines in addition to the reduced conductance and possibly reduced CO_2 availability leads to markedly reduced photosynthetic rates and lower quantum yield. The ensuing stress probably leads to early flowering and senescence.

These observations on the adverse effects of a 25–35% reduction in stomatal density on stomatal conductance, photosynthesis, and water use efficiency in tobacco are in contrast to several reports in *Arabidopsis* where a similar reduction in stomatal density in mutants like *gtl1* (Yoo *et al.*, 2010), *edt1* (Yu *et al.*, 2008), and *gpa1* (Nilson and Assman, 2010) actually leads to higher water use efficiency. However, in these mutants, although transpiration was considerably reduced, stomatal conductance was still quite high, as a result of which CO_2 assimilation rates only underwent a negligible change. These changes in conductances and CO_2 assimilation rates between *Arabidopsis* and tobacco for the same reduction in stomata may reflect inherent adaptive response strategies between *Arabidopsis* and tobacco. Understanding the effects of changes in stomatal density and conductance in the same genetic background and under the same growth conditions have so far been difficult and were only recently made through comparison of different stomatal density mutants in *Arabidopsis* (Doheny-Adams *et al.*, 2012). The SlERF36-expressing lines provide interesting study material for such a study in a different plant, tobacco.

How might SlERF36 exert its effects? SlERF36 could either have a direct effect on stomatal density that affects other components or a direct effect on photosynthesis that affects density or multiple independent effects. SlERF36 contains a C-terminal EAR motif and has recently been shown to actively repress the ethylene responsive GCC box *in vitro* (Pirrello *et al.*, 2012). Other studies have also reported the EAR motif to act as an active repressor. The mechanism of repression is believed to be through the interaction with histone deacetylases that mediate silencing of the target genes (Song *et al.*, 2005; Kagale and Rozwadowski, 2011). SlERF36 could target some component of the RuBP regeneration pathway leading to a cascading effect on ETRs and NPQ and possibly density and conductance. Conversely, SlERF36 could target a component of the stomatal development pathway. This pathway incidentally consists of several negative regulators such as TOO MANY MOUTHS, ERECTA, ERL1, ERL2, epidermal growth factors such as EPF1, EPF2, CHALLAH, and other negative regulators such as SDD1 and the MAPK kinase kinase, YODA (Shimada *et al.*, 2011). These control stomatal density through control of asymmetric cell division of the meristemoid mother cells such that at least one intervening cell separates adjacent stomata. Given the greater spacing in adjacent stomata amongst plants expressing *SlERF36*, SlERF36 might exert a control over the levels of some regulator that is possibly involved in the decision about spacing. The fact that SlERF36 suppresses the GCC box (Pirrello *et al.*, 2012) also raises the intriguing possibility of the involvement of ethylene in stomatal development and/or photosynthesis. Identifying the precise targets of SlERF36, the role of the EAR motif in their regulation and the direct or indirect action of SlERF36 targets on photosynthesis, NPQ, stomatal density and conductance would thus be the subject of more detailed studies.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Transcript accumulation of *SlERF36* in different tissues.

Supplementary Fig. S2. Detection of *SlERF36* transcripts in transgenic plants by reverse-transcription PCR.

Supplementary Fig. S3. Leaf colour of transgenic *SlERF36*-expressing plants.

Supplementary Fig. S4. Response of photosynthesis to changing stomatal conductance at different light intensities.

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