Genes Genom (2014) 36:427–441
DOI 10.1007/s13258-014-0177-x

RESEARCH ARTICLE

The cloning and characterization of a poplar stomatal density gene

Shaneka S. Lawson · Paula M. Pijut · Charles H. Michler

Received: 4 December 2013 / Accepted: 20 January 2014 / Published online: 8 February 2014

Abstract EPIDERMAL PATTERNING FACTOR1 (EPF1) is a well characterized negative regulator of cell division in *Arabidopsis thaliana* (*At*EPF1) where the primary region of localization is the leaf. However, little data have been reported on the role of EPF1 in other plant species. In this study, the *EPF1* gene from *Arabidopsis* and the newly identified poplar ortholog from *Populus trichocarpa* (*Pta*EPF1) were overexpressed in a hybrid poplar genotype. We attempted to identify the physiological role of *Pta*EPF1. Gene overexpression experiments were performed to determine if and how stomatal density (SD) numbers were affected. The poplar 717-1B4 (*P. tremula* × *P. alba*) genotype was used in the study. Results presented here suggest that overexpression of *Pta*EPF1 and *At*EPF1 in poplar led to significantly altered SD and also affected transgenic water stress tolerance. Overexpression of *At*EPF1 in 717-1B4 led to the most dramatic decrease in SD while overexpression of *Pta*EPF1 in 717-1B4 significantly increased SD in several transgenic lines, an indication that EPF1 may have additional functions in poplar. Also, abnormalities in leaf morphology were discovered that indicated overexpression of *At*EPF1 or *Pta*EPF1 in poplar triggered aberrant phenotypes not seen in other published *Arabidopsis* studies, an indication of additional pathway involvement.

Electronic supplementary material The online version of this article (doi:10.1007/s13258-014-0177-x) contains supplementary material, which is available to authorized users.

S. S. Lawson (✉) · P. M. Pijut · C. H. Michler
USDA Forest Service, Northern Research Station, Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC), Purdue University, 715 West State Street, PFEN126A, West Lafayette, IN 47907, USA
e-mail: shaneka.lawson@gmail.com; sslawson@fs.fed.us

Keywords Stomatal density · Tree physiology · Poplar · Transgenics

Introduction

The *Arabidopsis* genome was the first plant genome to be sequenced (Arabidopsis Genome Initiative AGI 2000). Fully annotated, the release of the *Arabidopsis* sequence provided a foundation for the analysis of a number of plant-specific signaling and cellular developmental pathways. With the completion of the poplar genome sequencing project (Tuskan et al. 2006), use of precise gene integration and altering techniques for the generation of transgenic poplar trees were soon utilized based on the ability to manipulate particular genes within the genome. Poplar research programs began because of the ease in overexpression or gene manipulation due to the already available poplar transformation protocol (Parsons et al. 1986). With a small genome, roughly 480 Mbp coding for 45,000 genes across 19 chromosomes, poplar is one of the most popular choices for tree research (Tuskan et al. 2006).

The majority of poplar species and many hybrids between poplar species are highly sensitive to water stress. In plants, transpiration and water stress tolerance are regulated by a number of genes and pathways working together. A mutation in a gene that regulates transpiration may lead to the inability of the plant to regulate its water intake to compensate for excessive water loss. The process of transpiration and plant water stasis have been the most common targets used for modification of WUE in agricultural crops, and now hardwood tree species (Schroeder et al. 2001; Shinozaki et al. 2003; Condon et al. 2004; Zhang et al. 2004; Nilson and Assmann 2007; Vinocur and Altman 2005). By using a unique approach to improving water-use efficiency (WUE)

such as decreasing stomatal density (SD), it may be possible to reduce total water consumption and allow surplus water to be used in other capacities.

The generation of individual stoma in plants is the result of a series of asymmetric and symmetric cell divisions controlled by a number of genes. There exists a trio of stomatal precursor cells: meristemoids (M), meristemoid mother cells (MMC), and guard mother cells (GMC). When a cell first enters the stomatal lineage, a MMC divides asymmetrically to generate an M that is usually triangular in shape. At this point the cell can adopt one of two fates: (1) additional asymmetric divisions or (2) proceed directly to form a GMC. Once formed, the GMC divides symmetrically to produce two immature guard cells that will proceed to form a single stoma (Bergmann and Sack 2007). The *EPIDERMAL PATTERNING FACTOR1* (*EPF1*) gene functions to control meristemoid initiation, additional regulatory steps involved in guard cell formation (von Groll et al. 2002), and is expressed in M and GMCs. EPF1 is a secretory peptide (Hara et al. 2007) that serves to maintain appropriate stomatal distribution by enforcing the one-cell spacing rule. This rule proposed that two stomata are not permitted to form adjacent to one another, and must be separated by at least a single pavement cell. Mutations in EPF1 lead to aberrant stomatal spacing where clusters, pairs, or groups of stomata form in close proximity to one another without the required one-cell separation. Overexpression of *EPF1* leads to decreased stomatal numbers indicating its role as a negative regulator of stomatal development (Hara et al. 2007).

In the present study overexpression of *Arabidopsis thaliana EPF1* (*At*EPF1) and *Pta*EPF1 in poplar was evaluated. It was hypothesized that overexpression of both the *At*EPF1 and *Pta*EPF1 genes would result in decreased stomatal numbers, modified WUE, carbon and nitrogen content, and in some cases, altered stomatal morphology. The results of this study serve to increase the knowledge available about methods available to alter tree morphology using gene sequences from other species.

Materials and methods

Gene selection and polymerase chain reaction (PCR) verification

The EPF1 candidate gene was selected based on availability of mutant seeds, gene size, and quality of previously published results for *Arabidopsis*. BLASTn and BLASTp alignments indicated the sequence of a putative ortholog available in poplar. Total RNA from *A. thaliana* leaves and poplar (717-1B4) leaves and stems was isolated with TRIZOL reagent (Invitrogen) and the resultant concentrations determined using a Nanodrop 8000 (Thermo Scientific). One microgram total RNA from each sample was reverse-transcribed using iScript cDNA Synthesis kit (Bio-Rad), and 1 µl of cDNA was used as a template for PCR. Eight different primer pairs were prepared on the basis of genomic sequences provided by TAIR, GenBank (NCBI), Phytozome, and the poplar Joint Genome Initiative (JGI) (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). The *Arabidopsis* version of the *EPF1* gene was isolated using primers F1 (5′-GCTCTAGAATGAAGTCTCTTCTTCTCC TTG -3′) and R1 (5′-TCAAGGGACAGGGTAGGACTTAT TGTTG-3′) while the putative poplar version (later described as *Pta*EPF1) was isolated using the primers F2 (5′-GCTCTAGAATGGCAAACCAACTTCCCCAG-3′) and R2 (5′-CCCGAGCTCTTAAAACTTTTTCACCTGAAC TAGTGC-3′). Confirmation of genetic amplification led to the generation of cDNA with *At*EPF1 amplified by F3 (5′-AT GAAGTCTCTTCTTCTCCTTGC-3′) and R3 (5′-TCAAGG GACAGGGTAGGAC-3′) and *Pta*EPF1 by F4 (5′-ATGAA GATTTTTGTTGCAACATTAGTC-3′) and R4 (5′-GGAT AAGACTTGTTATGACACATGC-3′). The kanamycin gene was amplified using F7 (5′-ATGACTGGGCACAACA GACA-3′) and R7 (5′-AATATCACGGGTAGCCAACG-3′) and the 35S promoter F8 (5′-TTTTCAATTTCAGAAA GAATGCTAA-3′) and R8 (5′-CGTGTTCTCTCCAAATG AAATG-3′). PCR products were electrophoresed on a 0.8 % agarose gel.

Gene sequences

All gene sequences were initially isolated from *Arabidopsis*, 717-1B4 genomic DNA. The *EPF1* gene sequences used in this project were amplified using gene-specific primers designed against the entire gene coding sequence obtained from exhaustive screening of *Arabidopsis* (TAIR) http://arabidopsis.org/, *Populus trichocarpa* JGI (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html), and Phytozome (http://www.phytozome.net) databases. The EPF1 sequences were amplified from *Arabidopsis*, 717-1B4 cDNA. Online sequence data from this work can be accessed on the *Arabidopsis* TAIR or GenBank/EMBL (http://www.ncbi.nlm.nih.gov/genbank/, http://www.ebi.ac.uk/embl/) databases under the following accession numbers: pBI121, Af485783; *At*EPF1, At2g20875; *Pta*EPF1, XM_002329376 (XP_002329412, eugene3.01200060 Identity = (54/61) 88 %, Positives = (59/61) 96 %) and neomycin phosphotransferase II (nptII), protein ID AAL92039.1.

Vector construction

Constructs were developed using the pBI121 vector system. Removal of the β-glucuronidase (GUS) gene allowed a cassette containing the *EPF1* gene to be inserted into the

vector. The assembled constructs contained the *At*EPF1 gene, nptII, and the putative *P. trichocarpa EPF1* (*Pta*EPF1) gene under the control of the Cauliflower mosaic virus (CaMV) 35S promoter to generate increased expression levels for each gene within the transgenic plants.

Generation of transgenic plants

Arabidopsis (Columbia, Col-0) transformation was performed via the floral dip method using the *Agrobacterium* strain EHA105 and binary vectors (Clough and Bent 1998). This protocol had been used successfully to achieve transformation efficiencies up to 3 %. Greenhouse-grown starting material (3–5 cm shoot tips) was disinfested by submerging with shaking (200 rpm) in 1 % Tween 20 for 5 min, 70 % ethanol for 1 min, and 20 % commercial bleach (5.25 % sodium hypochlorite) for 15 min (Kang et al. 2009). Tissues were then rinsed five times in sterile water.

Explants were propagated at 4 week intervals and cultured in Magenta GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml medium composed of 1 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 0.5 mM 1,2,3,5/4,6-hexahydroxycyclohexane (myo-inositol), 1 mM L-glutamine, 20 g L^{-1} sucrose, 4.3 g L^{-1} Murashige and Skoog Basal salts (MS; M499, PhytoTechnology Laboratories; Shawnee Mission, KS) (Murashige and Skoog 1962), liquid MS organic vitamins, and 7.6 g L^{-1} Difco-Bacto agar.

Poplar in vitro shoot cultures were propagated every 4 week, and after the generation of ~100 shoot cultures per genotype, *Agrobacterium*-mediated transformation of in vitro leaf explants were conducted using binary vectors and protocols adapted from Noë1 et al. (2002), Ma et al. (2004), and Cseke et al. (2007). Poplar transformations were based primarily on the protocol of Ma et al. (2004), however specific changes were introduced. The *Agrobacterium* strain used here was EHA105 (Cseke et al. 2007) rather than C58. The binary vector backbone used was pBI121 rather than pART27. Kanamycin concentrations used for selection processes were 50 mg L^{-1} rather than 25 mg L^{-1} (Cseke et al. 2007). Use of timentin was restricted to 200 mg L^{-1} as opposed to 400 mg L^{-1} during the pre-selection process.

Pre-culture on callus induction medium was done for 12 days rather than 14 days based on data from Nöel et al. (2002). All other aspects of the Ma et al. (2004) transformation protocol were followed however SOC media was used instead of LB media to improve transformation efficiencies. To control *Agrobacterium* contamination 1.6 mM Timentin (300 mg L^{-1}) was added to callus and shoot induction media. *Arabidopsis* plants and poplar shoot cultures were grown under cool-white light (275 µmol m^{-2} s^{-1}) at 22–24 °C (16 day: 8 night) on shelves in a growth room.

Transformations were verified via PCR with the use of primers specific for each individual transgene. Sequencing of several PCR products confirmed that the PCR analysis was amplifying the gene of interest. This project produced ≥100 transgenic lines with ten ramets per line for use in molecular characterization. After greenhouse and growth chamber studies were started, additional ramets were propagated so that aseptic materials were available should more in-depth study be required.

Plant growth conditions

Transgenic plants were grown in the greenhouse with a 16-h photoperiod at an average daily temperature between 22 and 23 °C. Light levels were 200–350 µmol m^{-2} s^{-1} based on natural light. Nighttime temperatures were fairly steady at 18.3 °C. Measurements were recorded using Priva greenhouse control software (Priva, Netherlands, www.priva.nl).

All plants were grown in standard round 20 cm pots for the duration of the study. The soil mix used was a 4:1 mixture of Sun Gro Redi Earth plug and seedling mix (Sun Gro Horticulture). Plants were watered every 3 days by placing individual pots onto watering trays (28 cm × 43 cm × 5 cm) that were constantly refilled over the course of 1 h. The pots were then removed and allowed to drain when replaced on the greenhouse bench.

All plants were fertilized upon initial placement in the greenhouse and once every 3 week afterwards with acidified water supplemented with a combination of two water-soluble fertilizers (3:1 mixture of 21 N–2.2 P–16.6 K and 15 N–2.2 P–12.5 K, respectively; The Scotts Co., Marysville, OH) to provide the following (in mg L^{-1}): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 Mn and Zn, 0.24 Cu and B, and 0.1 Mo. This nitrate form was 76 % of nitrogen provided. Irrigation water was supplemented with 93 % sulfuric acid (Brenntag, Reading PA) at 0.08 ml L^{-1} to reduce alkalinity to 100 mg L^{-1} and pH to a range of 5.8–6.2. (http://www.hort.purdue.edu/hort/facilities/greenhouse/soilFert.shtml). The plants were placed in watering trays filled with liquid fertilizer for approximately 1 h before the trays were emptied and the plants were placed back on the greenhouse benches. Because stomatal densities can be influenced by a number of external factors including humidity, all transgenic and control plants were grown under the same conditions and humidity levels.

Complementation

Arabidopsis *At*EPF1 mutant seeds obtained from The *Arabidopsis* Information Resource (TAIR) were planted along with Col-0 wild-type seeds in 10 cm square pots. Col-0 seeds were transformed with a pBI121 binary vector

that had the GUS gene removed. Col-0 seeds transformed to carry the *At*EPF1 overexpression vector, and Col-0 seeds transformed to carry the *Pta*EPF1 overexpression vector were also produced. Complementations of the *At*EPF1 gene were done by overexpressing the *At*EPF1 gene in the *Arabidopsis epf1* mutant background. The putative homolog for *Pta*EPF1 was transformed into *Arabidopsis* Col-0 plants and the five transgenic lines generated all displayed SD phenotypes similar to those of Col-0 plants overexpressing *At*EPF1. Arabidopsis *epf1* mutants were transformed with the *At*EPF1 binary vector to determine if the *At*EPF1 SD phenotype could be complemented. Five wild-type (Col-0) plants were grown in identical conditions as the control (plants overexpressing the empty vector) to determine if there was compromised growth or phenotypic abnormalities as a result of the process of transforming the empty vector into the *Arabidopsis* Col-0 line. All plants were grown under long-day (16/8 h) conditions.

Safranin-O staining

Phyllotaxic leaf one from greenhouse grown poplar and rosette leaves from *Arabidopsis* plants were bleached in a 9:1 solution of ethanol to acetic acid with orbital shaking at 100 rpm for 2 h (up to overnight). Longer incubation times resulted in extremely fragile leaves that broke easily. Leaves were removed from the ethanol:acetic acid solution and blotted dry before being added to a 1 % Safranin-O solution and left to shake at 200 rpm overnight. Leaves were then removed from the Safranin-O solution, blotted, and soaked in distilled water for 10 min before being placed in a fresh 50 ml tube of distilled water and left to shake overnight at 100 rpm. The overnight shaking step could be reduced to 1 h if the water was drained and replaced after 30 min.

Stomatal calculations

Cell counts were taken from 1 mm^2 areas. SD was determined to be the number of stomata within a 1 mm^2 area and was used for all species in comparative analysis. Stomatal index (SI) was calculated to be the SD × 100 % divided by [SD + pavement cells (per 1 mm^2)].

Microscopy

Differential interference contrast (DIC) images were captured with a Zeiss LSM710 microscope (Carl Zeiss, Inc.,). Images of three sections of each leaf were taken and leaves from three phyllotaxic levels (leaf one, five, and ten) of poplar plants while all *Arabidopsis* leaves were taken from the uppermost leaf of the rosette. Determinations of average stomatal number were made by examining five plants and leaves from each genotype according to phyllotaxy. Photographic analysis using a scoring method that visually ascertained trichome density in comparison with WT plants were generated and reported.

Cryogenic Scanning Electron Microscopy (Cryo-SEM) tissue samples were cut from freshly harvested poplar transgenic leaves. Sections of tissue that averaged 1.0 cm × 0.4 cm were all cut from Region 2 (see Supplemental Fig. 1S) and were gently, without touching more than the edges, affixed to the slide using Tissue Tek (Sakura Finetek) to prevent further movement. Samples were frozen in slush liquid nitrogen to −160 °C and then transferred to a GATAN CT2500 (Abingdon, Great Britain) pre-chamber, which was cooled to −160 °C. The samples were sublimed for 3 min, then sputter coated with platinum before being transferred to the FEI NOVA nanoSEM field emission SEM microscope (http://www.fei.com) cryostage. Images were recorded using the Through-the-Lens (TLD) or Everhart–Thornley (ET) detector operating at 5 kV accelerating voltage and ~4.8–5.0 mm working distance. Cryo-SEM does not require a critical point dehydration step common to non-cryogenic SEM (González-Méijome et al. 2006; Cochard et al. 2007). Images in this work were acquired from plants grown side by side under the same environmental growth conditions.

Isotope analysis

Measurements of percent carbon (%C) and percent nitrogen (%N) were obtained from 2 mg of ground, desiccated leaf tissue using a continuous flow isotope ratioing mass spectrometer (C-FIRMS). Individual baseline standards were analyzed four times each to assure the accuracy of the machine. A single analysis of each leaf sample was conducted for each of the transgenic lines. Carbon and nitrogen percentages were calculated using acetanilide, the industry standard, as a control. Reported carbon and nitrogen isotope values were calculated with a two-point normalization using acetanilide and keratin. National Institutes of Standards and Technology (NIST) peach protocols were used as a check of the normalization. Carbon isotope discrimination data obtained were used to represent time-integrated WUE_{ti} ($\delta^{13}C$) rather than instantaneous WUE (WUE_i). Nitrogen and carbon isotope data are reported as values where $\delta^{15}N$ [(^{15}N:$^{14}N_{leaf}$) relative to (^{15}N:$^{14}N_{atm}$)] is represented by $\delta^{15}N$ (‰) and $\delta^{13}C$ [(^{13}C:$^{12}C_{leaf}$) relative to (^{13}C:$^{12}CO_{2atm}$)] is represented by $\delta^{15}C$ (‰). Isotope analyses were performed at the Idaho State Stable Isotopes Laboratory in Moscow, Idaho. (http://www.cnrhome.uidaho.edu/isil).

Water withholding

Three 4-month-old representative plants from each transgenic line and three of each of the wild-type controls were examined for water stress tolerance in the greenhouse. All plants were age-matched, had stem heights that ranged between 20 and 23 cm, and were arranged in a random complete block design. Stomatal conductance (g_s), transpiration (E), and CO_2 assimilation (A) were measured with a steady-state gas-exchange system (for photosynthesis–transpiration) that incorporates an infrared gas analyzer (LI-6400XT, Li-Cor, Lincoln, Nebraska, USA) beginning at 11 A.M. for each day of the sampling period. Transgenic and control plants were grown in the Whistler Greenhouse (Purdue University) under normal growth conditions (as described in plant growth conditions) for 3 week before starting water withdrawal. Leaf A, E, and g_s were determined in response to changes in the intercellular CO_2 concentration (C_{in}).

The artificial drought was implemented 24 h after all transgenics had been watered. LI6400XT readings were taken in triplicate from each plant and were indicated as day 0. Water was then withheld for five consecutive days. At day 5 and day 10 after water withdrawal Li-Cor readings were again recorded. After 14 days, the plants were watered and allowed to recover for 24 h before Li-Cor readings of stomatal conductance (g_s), transpiration (E), and CO_2 assimilation (A) were taken. This study was repeated in triplicate. Data were analyzed using SAS (SAS Institute Inc. 2008) to determine the response to water stress tolerance for each individual plant. WUE data collected here was referred to as instantaneous (WUE_i).

Total leaf area (TLA)

Leaves were removed from three individual transgenic plants from each line and photographed for analysis at the beginning of the water withdrawal study. Values obtained from each plant were added together and averaged according to transgenic line. Data readings were presented for each transgenic line as the standard error of the mean (±SEM) where $n = 3$. TLA was determined using the ImageJ Software Package (http://rsb.info.nih.gov/ij/). TLA was not measured at the end of the experiment as many plant leaves were brittle, fractured when handled, or had fallen from the plant during the water withdrawal period.

Leaf size in transgenic and control plants were determined using ImageJ software. Leaves removed from plants for analysis of TLA were also analyzed for variations in size and a total of 25 plants from each category (717-1B4-*At*EPF1, 717-1B4-*Pta*EPF1) were measured.

Instantaneous water-use efficiency (WUE_i)

Three 4-month-old representative plants from each transgenic line and three of each of the wild-type controls arranged in a random complete block design were examined for WUE in the greenhouse. All plants were age-matched and had stem heights that ranged between 20 and 23 cm. Transpiration (E) and CO_2 assimilation (A) were measured with a steady-state gas-exchange system (for photosynthesis–transpiration) that incorporates an infrared gas analyzer (LI-6400XT, Li-Cor, Lincoln, Nebraska, USA) beginning at 11 A.M. All plant material was grown in the Whistler Greenhouse (Purdue University) under normal growth conditions (as described in plant growth conditions) for 3 week before readings were taken. LI6400XT readings were taken in triplicate from each plant and leaf A and E were determined in response to changes in the intercellular CO_2 concentration (C_{in}). Data were analyzed using SAS (SAS Institute Inc. 2008).

Statistical analysis

Calculation of standard deviation and use of balanced analysis of variance was performed on SD data and results of $p < 0.05$ were deemed significant. All analyses were performed using SAS statistical software (SAS Institute Inc. 2008).

Results

Sequence analysis of the *EPF1* gene

Ten EPF1 homologs have been found in *Arabidopsis*, but not all function in stomatal formation and development (Hara et al. 2009). Phylogenetic analysis of the protein sequences from four homologs (EPF1, EPF2, epidermal patterning factor-like6 (EPFL6 aka CHALLAH), and EPFL9 (aka STOMAGEN) indicated particular regions of homology (Shimada et al. 2011). While no conserved domains exist in these sequences, inspection of the most homologous sequences across species indicated similar regions of overlap when compared to a putative poplar EPF1 homolog (*Pta*EPF1). Analysis of the 15 genes with the greatest homology to *At*EPF1 showed genes from *Vitis vinifera*, *P. trichocarpa*, *Ricinus communis*, and *Oryza sativa* species to be the most prevalent (Fig. 1a). These genes also contained the six conserved cysteine residues described by Shimada et al. (2011) to represent disulfide bridges in EPFL9 and one additional pair whose role has yet to be determined (Fig. 1b).

Discovered during a BLAST search (NCBI, Phytozome) of the poplar genome in 2008, POPTR_0019s13390

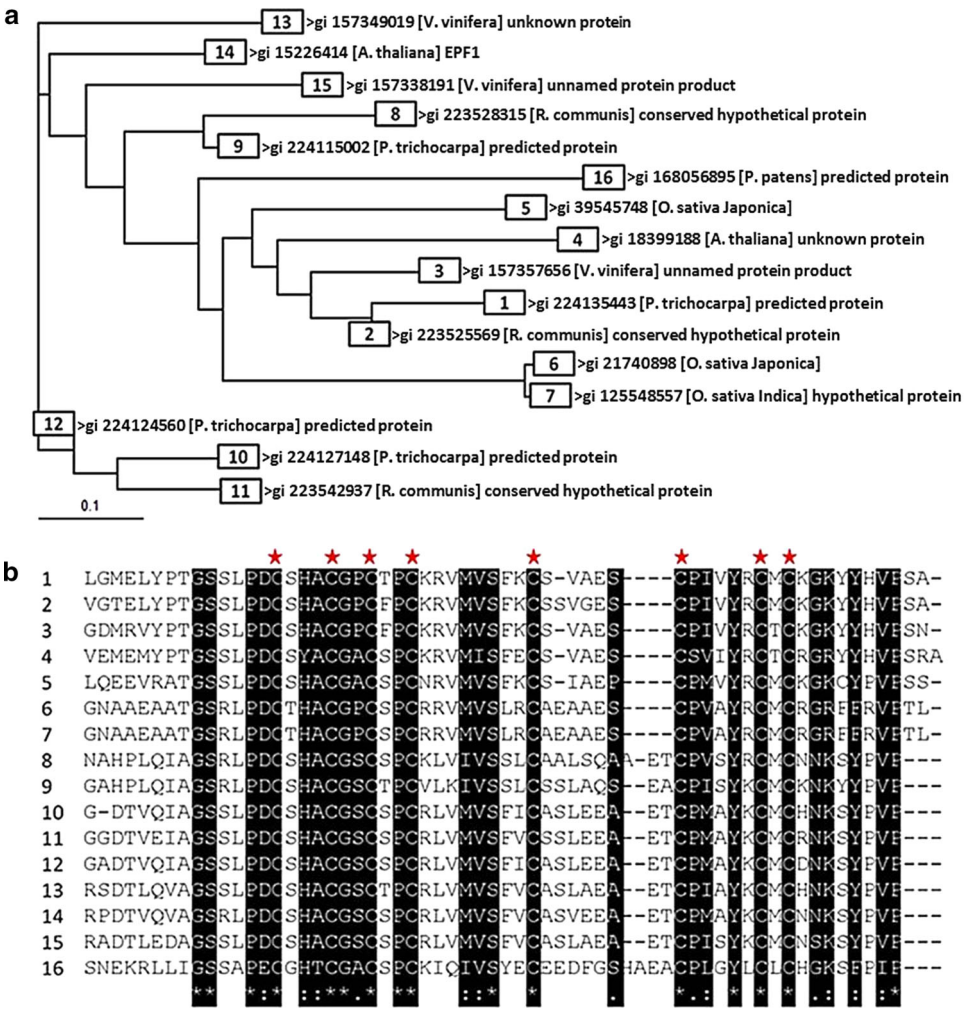

Fig. 1 Comparison of *At*EPF1 to all species in the NCBI database. **a** Hierarchical phylogenetic tree of the 15 proteins most homologous to *At*EPF1 (*Line 14*). *Scale bar* represents mean number of mutational changes per residue and indicates divergence of these genes from *At*EPF1. **b** Amino acid sequences for proteins described above. *Stars* are used to indicate the eight cysteines proposed to form disulfide bridges between EPF1 superfamily members

(labeled: >gi_224127148 in Fig. 1a) was isolated as a potential ortholog of the *Arabidopsis EPF1* gene based on nucleic acid and protein sequence information. Now officially annotated as a co-ortholog of At2g20875 (*At*EPF1) (see Supplemental Fig. 2S), *Pta*EPF1 and *At*EPF1 were cloned into a pBI121 overexpression vector and used to transform the poplar INRA 717-1B4 (*P. tremula* × *P. alba*) genotype. The putative full-length EPF1 coding sequence was isolated from poplar by comparative analysis followed by PCR amplification and sequencing. The EPF1 gene sequence from *A. thaliana* (*At*EPF1), which had no conserved domains, shared several short regions of homology with poplar genes when analyzed using the CLUSTALW alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (see Supplemental Fig. 3S). These regions of similarity were conserved across several species (Fig. 1a, b), and may be important in discovering additional EPF1 homologs in other species. The regions that were not included in these alignments were of low complexity, or contained extensive sequence repeats in both species as was determined by the PredictProtein program (http://www.predictprotein.org). Other means of determining similarities between the sequences proved futile. Sequence alignments of the four poplar sequences most homologous to *Pta*EPF1 revealed several highly conserved regions of similarity (Fig. 2a, b). This comparison highlighted the eight cysteine residues mentioned previously and provided the supportive background data needed to postulate that these disulfide bridges may be important in identifying additional family members based on protein sequence analysis.

Arabidopsis thaliana EPF1 (*AtEPF1*) expression levels

Expressed in several *Arabidopsis* developmental structures, *At*EPF1 expression levels were highest in the *Arabidopsis* rosette and first true leaf and also in newly developing poplar leaf primordial tissue (nearest the shoot apical meristem), as well as to a lesser extent in the stem tissue nearest the apical meristem (see Supplemental Fig. 4S).

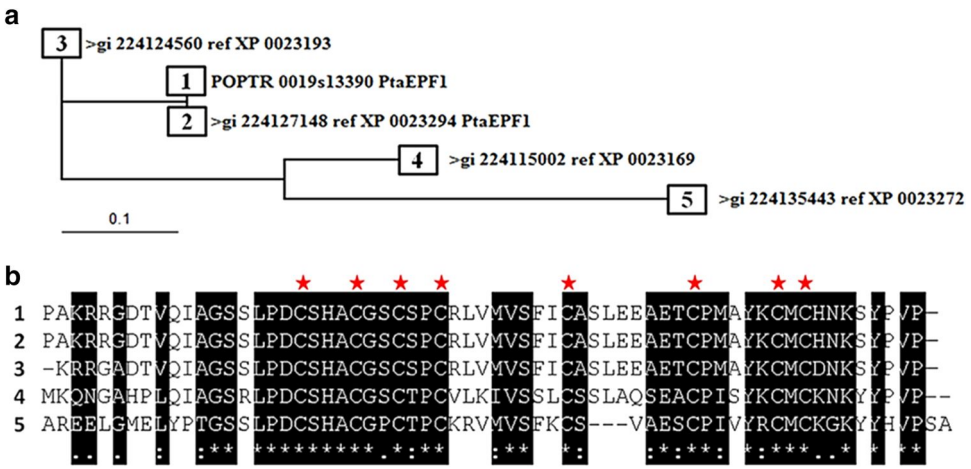

Fig. 2 Comparison of POPTR 0019s13390 with closest poplar genome matches. **a** The initial gene POPTR 0019s13390 (*Sequence number 1*) was compared to the newly annotated genome of *P. trichocarpa* and was confirmed to be *Pta*EPF1. The eight cysteines proposed to form disulfide bridges (*stars*) between EPF1 superfamily members were also present in these matches and **b** phylogenetic analysis showed the divergence of these poplar genes from POPTR 0019s1339

Considerable expression was seen in both leaf and stem tissue of poplar plants. Semi-quantitative RT-PCR analysis indicated no EPF1 expression in either poplar or *Arabidopsis* root tissue (data not shown). Analysis of EPF1 cellular expression levels indicated that RNA gene expression was found in Ms, GMCs, and immature guard cells (Hara et al. 2007). The presence of EPF1 was suggested to be involved in controlling identity of nearby cells, preventing additional guard cell formation (Hara et al. 2009).

Leaf sampling and phyllotaxy

Stomatal densities vary with respect to sampling section, location, and leaf age. Plant leaves develop quickly and physiological traits could be drastically different over a short period of time. Examination of immature leaf primordia nearest the apical meristem at the plant shoot apex showed that stomatal densities were higher than in older (fully developed) leaves for the aspen (717-1B4) genotype studied. Examinations of other poplar genotypes indicated that SD naturally varied dramatically between genotypes.

The greatest variabilities in SD were seen between newly developing leaves (primordia) and leaf one rather than older leaves (see Supplemental Fig. 5S). This observation emphasized the importance of consistency in the leaf selection process. Analysis of the 717-1B4 transgenics indicated an increase in SD by roughly 10 % for the immature leaf primordia when compared to the first mature leaf. Density also differed depending on leaf sampling location. Therefore all counts recorded in this study were taken from a consistent region (Region 2) on each leaf (see "Materials and methods" section).

Phenotype complementation analysis

Complementation analysis of the *Arabidopsis epf1* mutant was performed in order to determine functionality of the constructs designed in this study (Fig. 3a, b). The results were similar to the previous study by Hara et al. (2007), however rather than a two to threefold increase in density observed in the *epf1* mutants when compared to controls, less than one-and-one-half-fold difference was seen in the complementation experiment conducted. Overexpression of the *At*EPF1 construct resulted in a four-fold decrease in SD while Hara et al. (2007) reported a two- to three-fold decrease with their *At*EPF1 construct (Fig. 3c). Overexpression of *Pta*EPF1 consistently decreased stomatal densities and complementation studies showed overexpression of both *At*EPF1 and *Pta*EPF1 in *Arabidopsis* resulted in decreased SD. This observation was the first step in the determination of the function of the *Pta*EPF1 gene isolated in this study. No other data have been published to assign or postulate about the function of the *Pta*EPF1 gene.

Stomatal observations from transgenic poplar

Overexpression of the *Arabidopsis* EPF1 gene in 717-1B4 resulted in many lines with significantly lower stomatal densities. Examination of the 15 lines that displayed the greatest difference when compared to wild-type (WT) 717-1B4 plants indicated that stomatal densities of these transgenics were dramatically lower than those overexpressing *Pta*EPF1 (Table 1). Interestingly, two lines designated 7-*Pta*EPF1-88 (7P-88) and 7P-94 had increased stomatal densities when compared to WT. The 7P-109 line also exhibited an increase in SD on the adaxial side from

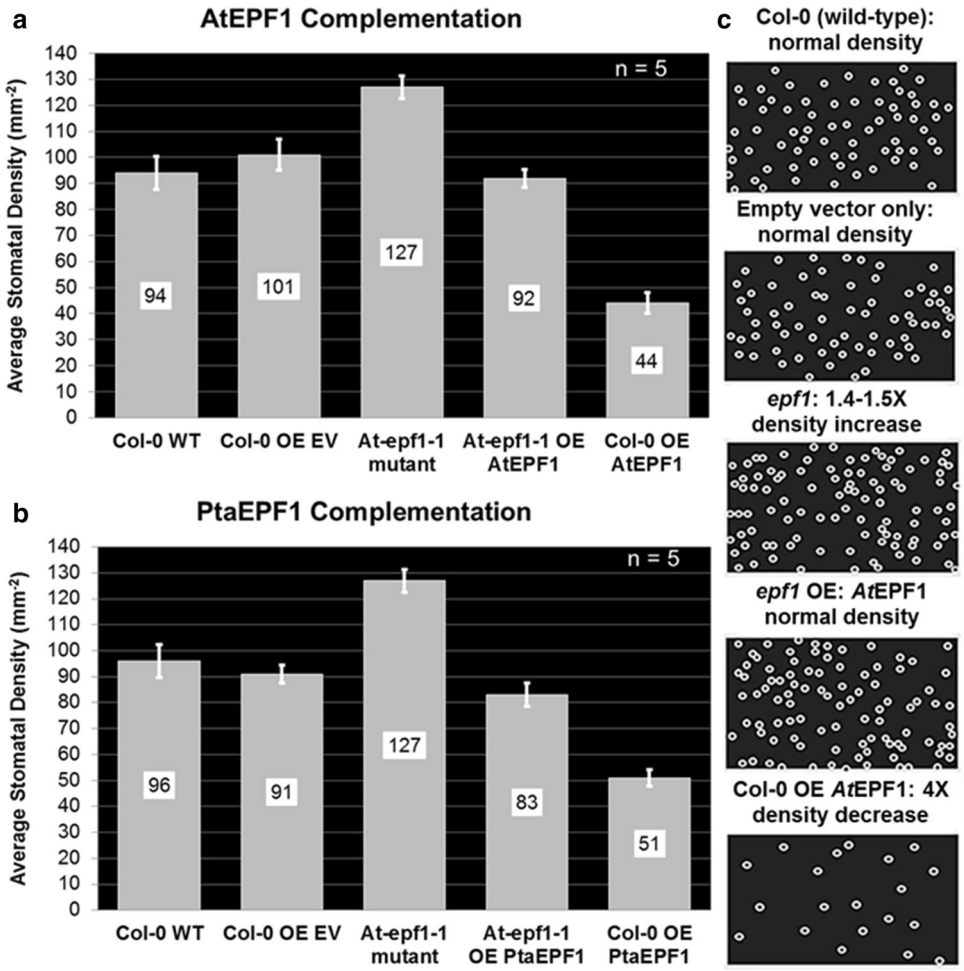

Fig. 3 Complementation analysis of *At*EPF1 and the overexpression of *Pta*EPF1 in *Arabidopsis epf1* mutants. **a** Complementation analysis of the *epf1* mutant phenotype with *At*EPF1. **b** The overexpression of *Pta*EPF1 in wild-type *Arabidopsis* plants. **c** Visual representation of the complementation analysis of *At*EPF1. Each individual *panel* was used to represent the results of an individual experiment. *Circles* were used to represent the individual stomata observed in each *panel* and were not drawn to *scale*. Each *circle* was an accurate depiction of the location where individual stomata were found on the leaf. The numerical data were presented in *panel A*. Each *panel* was a representation of a 1 mm^2 section of the *Arabidopsis* leaf. *Error bars* (±SEM)

zero to an average of 3.3 ± 1.1 stomata mm^{-2}. This line was the only 717-1B4 line to demonstrate a differential change in SD on both leaf surfaces. Calculations of SI in 717-1B4 transgenics showed that plants from line 7P-110 were the only ones that had a SI greater than control plants, and were the only line not statistically different from controls when compared with overexpression lines (Table 1).

Stomatal density and leaf size analysis

Decreased SD was not followed by a decrease in TLA in most 717-1B4-*At*EPF1 transgenic lines (Table 1), and was an indication that the plant was still able to obtain a sufficient amount of carbon to maintain growth under the study conditions. The 717-1B4-*Pta*EPF1 lines demonstrated a strong positive correlation with TLA that was not observed in any of the other transgenics (Fig. 4a). The TLA of a number of the transgenic plants was higher than the controls. In addition, the average leaf sizes for the majority of transgenics were significantly smaller than the WT leaves (Fig. 4b).

Stomatal size analysis

Examination of 100 717-1B4-*At*EPF1, 717-1B4-*Pta*EPF1, and 717-1B4 control plant stomata indicated significant differences in sizes and apertures of transgenic stomata when compared to WT leaves collected at the same time (Fig. 5). Extensive measurements of stomatal aperture for all transgenic lines were not performed because of the high degree of stoma size variability within a single leaf. Initial attempts to draw conclusions using aperture were inconclusive as a result of abnormal cell sizes and shapes among the numerous transgenic lines when a small number of stomata were examined, as the introduction of these variables represented an increased risk of error. Inspection of guard cell morphology indicated that, in general, stoma length and stomatal apertures of transgenic poplar lines were often smaller than WT, a trend that was followed in all of the lines examined. A shorter stoma was thought to be an indication of the overall variability in stomatal sizes often found in trees. The stomatal size variations seen in transgenics were also seen in control plants although to a lesser extent.

Table 1 Summary of transgenic 717-1B4 poplar leaf characteristics

Genotype	Line	Leaf stomatal density (mm^{-2})		Ratio (AD/AB)	% of WT		SI (%)		TD (versus WT)	Total leaf area (cm^2)	TLA (versus WT)	% of WT (TLA)
		AB	AD		AB	AD	AB	AD				
717-1B4												
35S::*At*EPF1												
	1	$113.9 \pm 8.5^{c,d}$	n/a	n/a	31.4	n/a	14.6	n/a	↓↓	$292.3 \pm 4.9^{c,d,e}$	↑	107.5
	6	120.7 ± 9.1^{c}	n/a	n/a	33.2	n/a	19.0	n/a	↓	237.8 ± 3.2^{h}	↓	87.4
	9	152.4 ± 4.5^{b}	n/a	n/a	42.0	n/a	17.3	n/a	↓↓↓	$251.2 \pm 8.8^{f,g,h}$	↓	92.3
	12	57.2 ± 9.2^{f}	n/a	n/a	15.7	n/a	11.0	n/a	↓	$261.1 \pm 6.7^{f,g}$	↓	96.0
	26	63.5 ± 10.2^{f}	n/a	n/a	17.5	n/a	12.2	n/a	↓	331.1 ± 11.7^{b}	↑	121.7
	42	$77.2 \pm 2.7^{e,f}$	n/a	n/a	21.3	n/a	14.0	n/a	↓	$186.5 \pm 7.4^{i,j}$	↓	68.6
	43	88.4 ± 4.3^{e}	n/a	n/a	24.3	n/a	16.1	n/a	↓	$293.9 \pm 12.0^{c,d,e}$	↑	146.0
	46	$95.3 \pm 5.8^{d,e}$	n/a	n/a	26.2	n/a	18.1	n/a	↓	$303.3 \pm 9.5^{b,c}$	↑	108.1
	50	$76.0 \pm 12.6^{e,f}$	n/a	n/a	20.9	n/a	15.0	n/a	↓	$299.6 \pm 5.3^{c,d}$	↑	111.1
	55	$114.3 \pm 8.5^{c,d}$	n/a	n/a	31.5	n/a	17.5	n/a	↓↓	205.9 ± 9.2^{i}	↓	24.3
	58	89.6 ± 4.2^{e}	n/a	n/a	24.7	n/a	14.7	n/a	↓↓↓	$187.9 \pm 7.5^{i,j}$	↓	30.9
	81	$76.6 \pm 2.6^{e,f}$	n/a	n/a	21.1	n/a	15.6	n/a	↓	$239.9 \pm 5.5^{g,h}$	↓	11.8
	83	$73.4 \pm 3.5^{e,f}$	n/a	n/a	20.2	n/a	14.1	n/a	↓	$285.7 \pm 2.8^{d,e}$	↑	105.0
	89	88.9 ± 4.2^{e}	n/a	n/a	24.5	n/a	14.6	n/a	↓	173.4 ± 5.8^{j}	↓	63.8
	101	$95.3 \pm 5.8^{d,e}$	n/a	n/a	26.2	n/a	15.8	n/a	↓	375.8 ± 5.5^{a}	↑	138.2
	717-1B4	363.2 ± 14.3^{a}	n/a	n/a	–	–	26.9	n/a	–	$272.0 \pm 3.4^{e,f}$	–	–
35S::*Pta*EPF1												
	88	374.7 ± 6.9^{b}	n/a	n/a	103.2	n/a	13.1	n/a	↓↓	298.7 ± 4.9^{b}	↑	109.8
	94	457.2 ± 7.2^{a}	n/a	n/a	125.9	n/a	14.7	n/a	↓↓	322.3 ± 3.2^{a}	↑	118.5
	109	317.5 ± 12.5^{c}	3.3 ± 1.1	0.010	87.4	n/a	12.2	n/a	↓	282.6 ± 6.8^{c}	↑	103.9
	110	302.7 ± 14.3^{c}	n/a	n/a	83.3	n/a	28.7	n/a	↓↓	$260.9 \pm 8.3^{d,e}$	↓	95.9
	111	258.2 ± 12.2^{d}	n/a	n/a	76.7	n/a	25.7	n/a	↓↓	253.0 ± 6.5^{e}	↓	93.0
	717-1B4	363.2 ± 14.3^{b}	n/a	n/a	–	–	26.9	n/a	–	$272.0 \pm 3.4^{c,d}$	–	–

The table includes statistical significance for stomatal density and the leaf area data generated by overexpression of 35S::*AtEPF1* and 35S::*PtaEPF1* in the 717-1B4 genotype of poplar as well as stomatal ratios and index values for each line. Differences in trichome density and TLA when compared to wild-type plants are also displayed. Means with same letter were not significantly different at the $p < 0.05$ level of probability

AB average abaxial stomata; *AD* average adaxial stomata; *Decreased* (↓); *Increased* (↑); *SI* stomatal index (stomata per 100 epidermal cells; see "Materials and methods" section); *TD* trichome density; *TLA* total leaf area; *WT* wild-type

Inspection of water stress data from individual lines

A water withholding experiment was used to seek out differences between control plants and transgenics with respect to physiological characteristics. The experiment lasted 15 days and 14 of those days the plants were without water. Transgenic lines demonstrated similar patterns of *A*, g_s, and *E* at the start of the water withdrawal period, but diverged on day 10 with respect to *A* where both 717-1B4-*At*EPF1 and 717-1B4-*Pta*EPF1 lines exhibited a more gradual decrease in *A* than controls (Fig. 6). Both sets of transgenics showed increased g_s on day 5 with respect to day 0. On day 10 of the study all 717-1B4-*At*EPF1 transgenics and controls showed decreased g_s rates followed by increased g_s on day 15 with the exception of 717-*Pta*EPF1 lines which all continued to decline. An increase in *E* was seen on day 5 for both transgenic groups. A slower adjustment of *E* in several 717-1B4-*Pta*EPF1 lines was seen at day 10. On day 15, control plants and nearly all 717-1B4-*At*EPF1 lines showed elevated rates of *E* not seen in 717-1B4-*Pta*EPF1 lines. Upon completion of the water withholding experiment the majority of 717-1B4-*At*EPF1 transgenic lines had recovered while 717-1B4-*Pta*EPF1 lines lagged behind or died off. The majority of 717-1B4-*At*EPF1 lines renewed *A* at rates higher than controls. These lines also recovered g_s to pre-study levels. When compared to controls, *E* was slightly increased as well.

Time-integrated water-use efficiency (WUE_{ti})

The WUE results reported in this section refer to time-integrated WUE (WUE_{ti}, $\delta^{13}C$) rather than instantaneous WUE (WUE_i). Comparison of carbon content among the transgenic lines indicated that the majority of the poplar transgenics, possessed a higher percentage of total carbon (21 of 25) and lower $\delta^{13}C$ (used synonomously with WUE_{ti}) values (17 of 25). Only eight lines of 717-1B4-*At*EPF1 transgenics demonstrated greater WUE_{ti} values

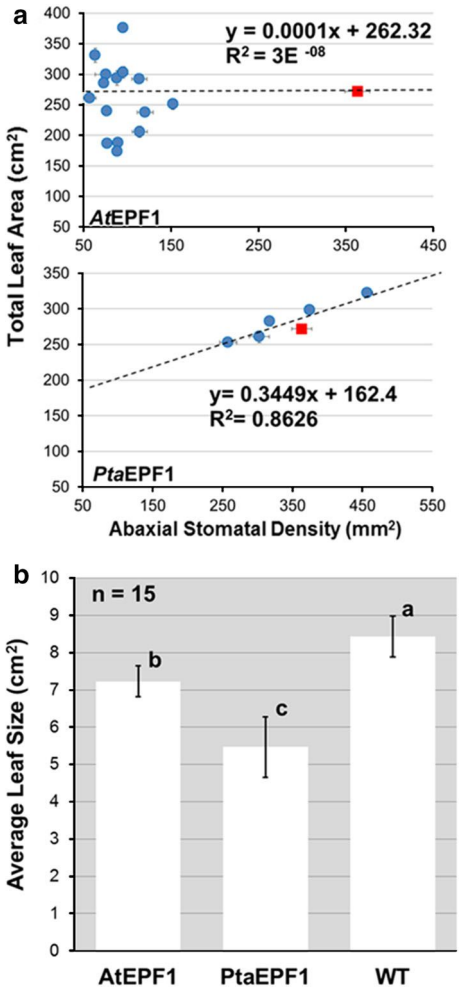

Fig. 4 Average leaf sizes of control and transgenic plants. Measurements of leaf sizes were conducted to determine if the leaves of transgenics were smaller than wild-type. Means with the *same letter* were not significantly different at $p < 0.05$. *Error bars* (±SEM)

while none of the 717-1B4-*Pta*EPF1 lines were shown to possess a greater WUE_{ti}. Comparison of nitrogen content among transgenic lines indicated that the majority, 18 of 25, possessed lower leaf nitrogen content than controls (Table 2).

Of the 20 transgenic lines overexpressing *At*EPF1, fewer than half showed greater percentages of total nitrogen than WT (Table 2). Examination of natural nitrogen abundance ($\delta^{15}N$) indicated that all 20 lines had lower values than controls. Total carbon analysis indicated that 18 lines had greater amounts of carbon and only two lines showed less carbon than controls. Only 5 transgenic lines were generated when the poplar EPF1 gene (*Pta*EPF1) was overexpressed in 717-1B4 plants. Three of these lines had greater total carbon contents while a single line had 10 % greater nitrogen content. None of these transgenics had greater WUE_{ti} than the control plants.

Discussion

The EPF superfamily in *Arabidopsis* contains ten members. However, in poplar a lack of detailed genome annotation information prevents conclusive identification of other potential group members. The possibility exists that several family members may be involved in plant abiotic stress responses such as temperature and humidity, but do not play a direct role in stomatal development as was reported for *At*EPFL6 (Rychel et al. 2010). The putative *Pta*EPF1 gene used in this study is >80 % homologous to the *At*EPF1 gene and exhibited a motif similar to other *Arabidopsis* EPF superfamily members. The shared motif region (LPDCSHACGSC(S/T)PC) is also found in several other poplar genes with unknown functions.

Extensive measurements of transgenic leaf SD showed nearly all lines were statistically different from WT. Plants with the most significant decrease in SD also exhibited significantly decreased TLA, however it cannot be stated conclusively that this was a result of altered biomass partitioning as root biomass data was not observed. Consistent decreases in aboveground biomass with overexpression of either *At*EPF1 or *Pta*EPF1 in poplar were not found. This may indicate that genes controlling biomass partitioning may also work to decrease carbon allocation to leaves and increase deposition in roots. According to Bascó et al. (2008), decreased SD did not automatically decrease photosynthesis, although more greenhouse and field studies will be needed to prove this conclusively. Aganchich et al. (2009) noted that decreased carbon content in olive trees (*Olea europaea* L.) was a result of increased production of detoxification enzymes (cytochrome P450s, metabolic sinks for environmental contaminants) in an attempt to minimize damage to photosynthetic apparatus during water stress conditions. Those trees were also shown to have increased proline levels for added protection.

All of the lines in this study had decreased trichome densities when compared to the control hence it is suspected that additional pathways were activated by the overexpression of both forms of the EPF1 gene. Interestingly, the overexpression of *Pta*EPF1 did not result in the dramatically decreased SD numbers observed when *At*EPF1 was overexpressed. It is believed that the identified *Pta*EPF1 works in tandem with another gene or has a slightly different function in poplar when compared to *Arabidopsis*. Additional studies addressing leaf physiology and its relation to growth were needed as Monclus et al. (2009) stated that there was no clear evidence WUE was related to growth performance or whether or not some leaf traits could be used as predictors for productivity in *Populus deltoides* × *P. nigra* hybrids. Eight transgenic lines in this study demonstrated improved WUE and five of those

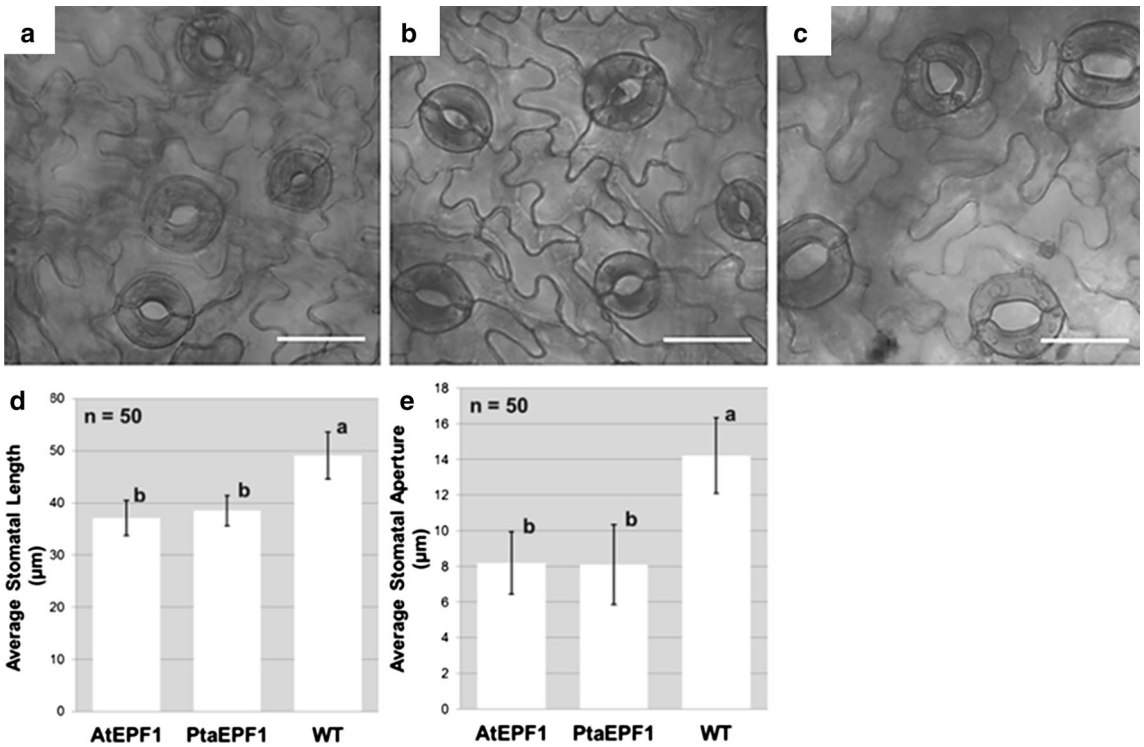

Fig. 5 Stomatal sizes in transgenics were smaller than wild-type. **a** 717-1B4 *At*EPF1 and **b** 717-1B4 *Pta*EPF1 overexpressing transgenic lines were used to illustrate the difference in sizes between transgenics and the **c** Wild-type 717-1B4 plants. **d** Data analysis of guard cell length and **e** aperture. Means with the *same letter* were not significantly different at $p < 0.05$. *Bar* = 50 µm. *Error bars* (±SEM)

lines had a greater TLA, an indication that decreased SD played a role in improving biomass and WUE.

Studies of tropical tree species in the Amazonian region indicated that decreased stomatal densities were not necessarily connected to altered conductance rates (Ainsworth and Rogers 2007; Bonal et al. 2011). Bonal et al. (2011) determined that decreased $\delta^{13}C$ in several Amazonian tree species resulted from increased *A* however examinations of SD alone were not conclusively linked to a corresponding change in carbon assimilation in this study. A potential explanation for this observation was that the young trees in this experiment were grown in a less dense, open-canopy environment with little competition for light. Measurements of $\delta^{15}N$ were used to determine the amount of nitrogen taken up by natural sources (soil) and to ascertain whether or not nitrogen limitation was a factor in this experiment. Lower $\delta^{15}N$ values would be indicative of a higher accumulation of nitrogen and a more efficient system of nitrogen fixation than would be found in natural sources. With respect to $\delta^{15}N$, only one line failed to have a lower value than control plants. Alternatively, changes in leaf nitrogen content could be indicative of a more rapid system of utilization in transgenics. Nitrogen content of an average poplar leaf in the field ranges between 2.0 and 2.5 % (Isaakidis et al. 2004) and increased nitrogen content may indicate increased photosynthetic processes however, this would likely only occur when *A* rates were highest (Meziane and Shipley 2001). Nitrogen content in this study fluctuated and no patterns were observed. Yadollahi et al. (2011) indicated that leaf nitrogen content, TLA, and SD were not useful in screening water stress tolerance in almond however many poplar species have shown different responses when exposed to water-limited conditions (Monclus et al. 2009). It is important to emphasize that all data regarding carbon and nitrogen percentages were the result of a single analysis of each transgenic line using highly specialized equipment. The time and prohibitive costs associated with replication in triplicate of an isotope study were the only deterrents to generation of those additional datasets.

Morphological abnormalities such as guard cell pairing or ablation, decreased trichome density, irregular stoma formation, and arrested stomata were also seen in some of the regenerated transgenic lines (see Supplemental Fig. 6S). These observations led to the supposition, as was true for *Arabidopsis*, that additional genes were involved in the poplar stomatal regulatory pathway. Manipulation of *EPF1* with the use of a constitutively active promoter (CaMV 35S) may have caused expression of another gene in the regulatory pathway to be altered. Minimal data exists

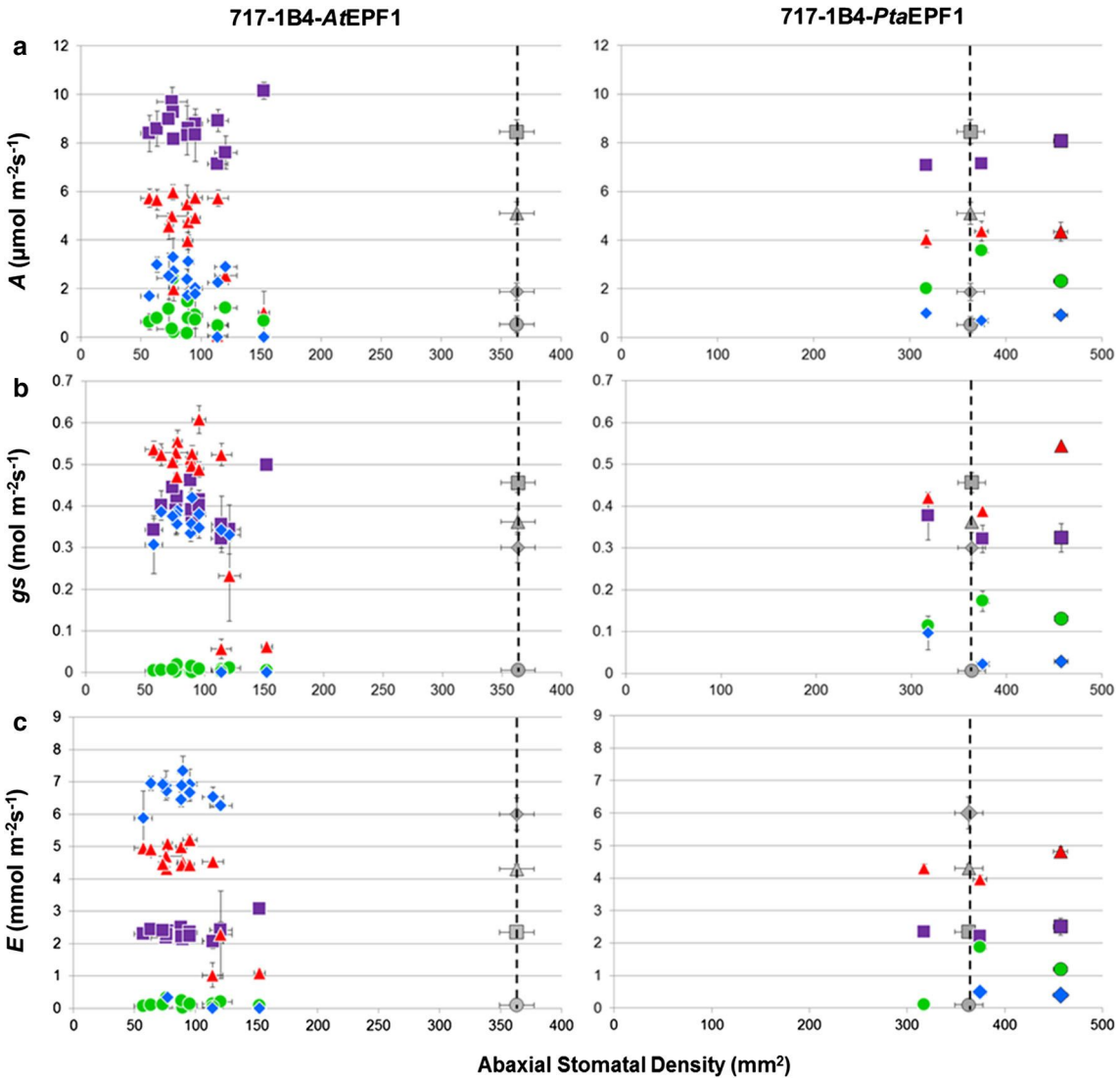

Fig. 6 Abaxial stomatal density and **a** CO_2 assimilation (*A*), **b** stomatal conductance (g_s), and **c** transpiration (*E*) in 717-1B4 lines. Analysis of stomatal density and physiological responses over the course of the water withholding experiment. The 717-1B4 control plants were indicated by a *dotted black line*. Absence of a *marker* indicated the plant did not survive the artificial drought conditions. Readings after a normal watering regime are considered day 0 (*square*), after 5 days without water (day 5; *triangle*), after 10 days without water (day 10, *circle*), and after 14 days without water and 1 day after rewatering (day 15, *diamond*). *Error bars* (±SEM)

in poplar concerning the stomatal regulation as opposed to the plethora of information available for *Arabidopsis*. It was hypothesized that the observed aberrant morphological phenomenon resulted from mixed signaling events, however additional explanations may could involve bacterial or viral infections such as those described in *Vicia faba* (Willmer et al. 1996), which prevented stomatal closure. Another explanation may be that abnormally large, misshapen stomata were an artifact from tissue culture. In potato (*Solanum tuberosum*) abnormally large stomata were observed that remained locked in the open position unresponsive to both exogenous application of ABA and water stress conditions (Willmer et al. 1996). Rose plants that displayed a similar "locked open" phenotype have also been reported (Sallanon et al. 1993). Previously published results with other transgenics such as (*Nicotiana tabacum* cv. SR1) indicated that drought tolerant transgenics exposed to water stress conditions were significantly greener and exhibited less wilting (Rivero et al. 2007), an observation also made in this study. These data indicated that mechanisms involved in poplar stomatal development may follow a scheme similar to that of *Arabidopsis* with respect to SD and size. Additional abnormal morphologies seen in poplar may stem from activation of gene functions not described in *Arabidopsis*. Other *EPF1*-like genes have recently been identified in poplar and may work in tandem

Table 2 Research findings using carbon and nitrogen isotope analysis using leaf tissue

Genotype	Line	Nitrogen isotope		Carbon isotope		% of WT		WUE_{ti} (vs WT)
		%N	∂^{15}N (‰)	%C	∂^{13}C (‰)	%N	%C	
717-1B4								
35S::*At*EPF1								
	1	3.51	−8.37	47.74	−18.40	68.6	104.1	↑
	6	3.88	−7.02	48.11	−21.66	75.8	105.0	↓
	9	4.51	−7.57	48.45	−20.64	88.1	105.6	↓
	12	4.48	−7.41	48.46	−21.12	87.5	105.7	↓
	26	5.0	−6.72	49.62	−19.39	97.7	108.2	↑
	42	5.8	−4.5	47.56	−18.36	113.3	103.8	↑
	43	3.26	−9.39	48.25	−19.45	63.7	105.3	↑
	46	5.25	−5.25	48.22	−19.61	102.5	105.2	↑
	50	5.3	−4.58	48.26	−22.07	103.5	105.3	↓↓
	51	5.12	−0.95	45.5	−30.77	100.0	99.3	↓↓↓
	55	5.21	−5.53	47.56	−18.36	101.8	103.7	↑
	58	4.22	−4.26	47.75	−22.77	82.4	104.2	↓↓
	81	3.98	−5.35	47.71	−22.64	77.7	104.1	↓↓
	83	2.63	−5.33	47.2	−21.50	51.4	103.0	↓
	87	2.17	−3.93	47.53	−20.38	42.4	103.7	↓
	89	5.1	−6.78	47.73	−20.87	99.6	104.1	↓
	93	5.77	−3.56	43.06	−20.52	112.7	93.9	↓
	94	5.63	−3.26	46.9	−18.42	110.0	102.3	↑
	98	3.59	−2.02	48.07	−21.00	70.1	104.9	↓
	101	3.15	−8.19	47.29	−18.24	61.5	103.2	↑
	717-1B4	5.12	−0.67	45.84	−19.74	–	–	–
35S::*Pta*EPF1								
	83	5.63	−3.39	45.41	−22.65	110.0	99.1	↓↓
	88	2.49	−1.09	47.03	−22.51	48.6	102.6	↓↓
	109	3.76	0.16	44.78	−22.47	73.4	97.7	↓↓
	110	3.07	−3.11	48.34	−20.80	60.0	105.5	↓
	111	2.42	−3.45	47.78	−19.96	47.3	104.2	↓
	717-1B4	5.12	−0.67	45.84	−19.74	–	–	–

The table includes carbon and nitrogen percentages as well as isotope data for comparison to wild-type. Water-use efficiency based on carbon isotope analysis is also included

$\partial^{13}C$ carbon isotope; *Decreased* (↓); *Increased* (↑); $\partial^{15}N$ nitrogen isotope; *%C*, percent carbon; *%N* percent nitrogen; *WT* wild-type; WUE_{ti} time-integrated water-use efficiency

with the *Pta*EPF1 gene described in this study to perform a host of additional duties in stomatal development (Phytozome 2011). The lack of additional studies in poplar that use SD transgenics for WUE_{ti} experimentation limit the ability to compare results found here to other publications.

The goal of this research was to determine if gene information from *Arabidopsis* could be used in other species that had not been fully annotated to improve the quality of research until sequencing and subsequent annotation of the genome were completed. While it was not the intention or aim of this work to identify all aspects of EPF1 structure and function, this work has provided evidence that findings in one model species could have potential for use in another system. If additional overexpression studies were conducted, it is plausible that a further increase in *At*EPF1 expression levels may lead to a decrease in stomatal number that adversely affected growth. The data obtained in this study confirmed that the overexpression of the poplar and *Arabidopsis* homologs of the *EPF1* gene led to an alteration of SD in the 717-1B4 model tree genotype. Future experiments with these transgenics would include additional parameters (measurements of ABA content, additional isotope analysis, electrolyte leakage, leaf temperature, and proline content) to provide a more complete picture of decreased SD responses in poplar. Follow-up studies using Quantitative Trait Loci (QTL) analysis may

identify particular poplar genome regions or additional poplar genes involved with or upregulated by overexpression of *Pta*EPF1. It is possible that the *Pta*EPF1 gene works in tandem with another gene to control stomatal density or that it functions along a pathway similar but not identical to that found in *Arabidopsis*. The *Pta*EPF1 gene may also serve multiple roles in poplar, such as an involvement in ABA-regulation, stomatal closure events, or its incorporation and overexpression may affect susceptibility of the transgenics regenerated in this study to biotic factors (insect pests, pathogens, etc.). This research has provided information regarding the function of a previously uncharacterized gene within the poplar genome. This approach could also be used to identify a number of as yet uncharacterized genes within the poplar genome listed as unknown proteins with unknown functions.

Acknowledgments This study was financed by fellowships from the Fred M. van Eck Foundation and the Alliance for Graduate Education and Professoriate (AGEP) at Purdue University. The authors thank Drs. Martin-Michel Gauthier and John Gordon for their comments and critiques of earlier versions of this manuscript. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Conflict of interest The authors declare no conflict of interest regarding this article.

References

Aganchich B, Wahbi S, Loreto F, Centritto M (2009) Partial root zone drying: regulation of photosynthetic limitations and antioxidant enzymatic activities in young olive (*Olea europaea*) saplings. Tree Physiol 29:685–696

Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30:258–270

Bacsó R, Janda T, Galiba G, Papp A (2008) Restricted transpiration may not result in improved drought tolerance in a competitive environment for water. Plant Sci 174:200–204

Bergmann DC, Sack FD (2007) Stomatal development. Annu Rev Plant Biol 58:163–181

Bonal D, Ponton S, Le Thiec D, Richard B, Ningre N, Hérault B, Ogée J, Gonzalex S, Pignal M, Sabatier D et al (2011) Leaf functional response to increasing atmospheric CO_2 concentrations over the last century in two northern Amazonian tree species: a historical $d^{13}C$ and $d^{18}O$ approach using herbarium samples. Plant Cell Environ 34:1332–1344

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Cochard H, Casella E, Mencuccini M (2007) Xylem vulnerability to cavitation varies among poplar and willow clones and correlates with yield. Tree Physiol 27:1761–1767

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2004) Breeding for high water-use efficiency. J Exp Bot 55:2447–2460

Cseke LJ, Cseke SB, Podila GK (2007) High efficiency poplar transformation. Plant Cell Rep 26:1529–1538

González-Méijome JM, López-Alemany A, Almeida JB, Parafita MA, Refojo MF (2006) Microscopic observations of superficial ultrastructure of unworn siloxane-hydrogel contact lenses by cryo-scanning electron microscopy. J Biomed Mater Res B 76:419–423

Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T (2007) The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Gene Dev 21:1720–1725

Hara K, Yokoo T, Kajita R, Onishi T, Yahata S, Peterson KM, Torii KU, Kakimoto T (2009) Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERING FACTOR2 in *Arabidopsis* leaves. Plant Cell Physiol 50:1019–1031

Isaakidis A, Sotiropoulos T, Almaliotis D, Therios I, Stylianidis D (2004) Response to severe water stress of the almond *Prunus amygdalus*, 'Ferragnès' grafted on eight rootstocks. New Zeal J Crop Hort 32:355–362

Kang B-G, Osburn L, Kopsell D, Tuskan GA, Cheng Z-M (2009) Micropropagation of *Populus trichocarpa* 'Nisqually-1′: the genotype deriving the *Populus* reference genome. Plant Cell Tiss Org 99:251–257

Ma C, Strauss SH, Meilan R (2004) *Agrobacterium*-mediated transformation of the genome-sequenced poplar clone, Nisqually-1 (*Populus trichocarpa*). Plant Mol Biol Rep 22:1–9

Meziane M, Shipley B (2001) Direct and indirect relationships between specific leaf area, leaf nitrogen and leaf gas exchange effects of irradiance and nutrient supply. Ann Bot-London 88:915–927

Monclus R, Villar M, Barbaroux C, Bastien C, Fichot R, Delmotte FM, Delay D, Petit JM, Bréchet C, Dreyer E et al (2009) Productivity, water-use efficiency and tolerance to moderate water deficit correlate in 33 poplar genotypes from a *Populus deltoides* × *Populus trichocarpa* F1 progeny. Tree Physiol 29:1329–1339

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–497

Nilson SE, Assmann SM (2007) The control of transpiration. Insights from Arabidopsis. Plant Physiol 143:19–27

Noël N, Leplé J-C, Pilate G (2002) Optimization of in vitro micropropagation and regeneration for *Populus* x *interamericana* and *Populus* × *euramericana* hybrids (*P. deltoides*, *P. trichocarpa*, and *P. nigra*). Plant Cell Rep 20:1150–1155

Parsons TJ, Sinkar VP, Stettler RF, Nester EW, Gordon MP (1986) Transformation of poplar by *Agrobacterium tumefaciens*. Nat Biotechnol 4:533–536

Phytozome (2011) http://www.phytozome.net. Accessed 17 Nov 2013

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci USA 104:19631–19636

Rychel AL, Peterson KM, Torii KU (2010) Plant twitter: ligands under 140 amino acids enforcing stomatal patterning. J Plant Res 123(3):275–280

Sallanon H, Tort M, Coudret A (1993) The ultrastructure of micropropagated and greenhouse rose plant stomata. Plant Cell Tiss Org 32(2):227–233

SAS Institute Inc. (2008) http://www.sas.com/. Accessed 17 Nov 2013

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol 52:627–658

Shimada T, Sugano SS, Hara-Nishimura I (2011) Positive and negative peptide signals control stomatal density. Cell Mol Life Sci 68(12):2081–2088

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6(5):410–417

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A et al (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. and Gray). Science 313:1596–1604

Vinocur A, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotech 16(2):123–132

von Groll U, Berger D, Altmann T (2002) The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during *Arabidopsis* stomatal development. Plant Cell 14(7):1527–1539

Willmer C, Fricker M (1996) Stomata, 2nd edn. Chapman & Hall, London, pp 25–50

Yadollahi A, Arzania K, Ebadi A, Wirthensohn M, Karimia S (2011) The response of different almond genotypes to moderate and severe water stress in order to screen for drought tolerance. Sci Hortic-Amsterdam 129(3):403–413

Zhang C, Guo J, Xie H, Zhang W (2004) Comparison in leaf cell water relations between four species seedlings under drought. Nanjing Shida Xuebao (Ziran Kexue Ban) 27(4):72–75