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AtEDT1/HDG11 regulates stomatal density and water use efficiency via *ERECTA* and *E2Fa*

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Summary

- Improvement of crop drought resistance and water use efficiency (WUE) has been a major endeavor in agriculture. *Arabidopsis* ENHANCED DROUGHT TOLERANCE1/HOMEODOMAIN GLABROUS11 (AtEDT1/HDG11), a homeodomain-START transcription factor we previously identified from *enhanced drought tolerance1* mutant (*edt1*), has been demonstrated to significantly improve drought tolerance and WUE in multiple plant species when constitutively overexpressed.
- Here, we report the genetic evidence suggesting a genetic pathway, which consists of *EDT1/HDG11*, *ERECTA*, and *E2Fa* loci, and regulates WUE by modulating stomatal density. AtEDT1/HDG11 transcriptionally activates *ERECTA* by binding to HD *cis*-elements in the *ERECTA* promoter. ERECTA in turn depends on E2Fa to modulate the expression of cell cycle-related genes.
- This modulation affects the transition from mitosis to endocycle, leading to increased ploidy levels in leaf cells, and therefore increased cell size and decreased stomatal density.
- Our results suggest a possible EDT1/HDG11-ERECTA-E2Fa genetic pathway that reduces stomatal density by increasing cell size and provide a new avenue to improve WUE of crops.

Key words

EHANCED DROUGHT TOLERANCE1/HOMEODOMAIN GLABROUS11 (EDT1/HDG11), water use efficiency (WUE), stomatal density, ERECTA, transcription factor E2Fa

Introduction

Drought stress is one of the most severe environmental constraints that greatly restrict plant growth, distribution and crop production (Zhu, 2002; Kissoudis *et al.*, 2016). An effective strategy of drought resistance used by plants is reducing the transpirational water loss, which allows plants to maintain an adequate water status to sustain critical physiological and biochemical processes (Nilson & Assmann, 2007; Lawson & Blatt, 2014). However, a reduction in transpirational water loss often leads to a decline in biomass accumulation because of reduced carbon assimilation. Therefore, plant water use efficiency (WUE), which is defined as plant production per amount of water used, is critical for plant survival and crop yield (Yoo *et al.*, 2009). Stomata control gas and water exchanges between plants and environment. The regulation of stomatal density and stomatal movements influence WUE in plants (Lawson & Blatt, 2014).

Previously, we described a mutant of *edt1*, in which the homeodomain leucine zipper (HD-Zip) transcription factor *EDT1/HDG11* is constitutively activated by an activation tagging T-DNA insertion, leading to reduced stomatal density and water loss, improved WUE, but unaltered stomatal index (Yu *et al.*, 2008). When overexpressed, *AtEDT1/HGD11* improved WUE in *Arabidopsis*, tobacco (Yu *et al.*, 2008), and rice (Yu *et al.*, 2013). The *AtEDT1/HGD11*-conferred drought resistance and improved WUE have been successfully recapitulated by overexpressing *AtEDT1/HDG11* in cotton and poplar (Yu *et al.*, 2016), wheat (Li *et al.*, 2016), turf grass (Cao *et al.*, 2009), sweet potato (Ruan *et al.*, 2012), pepper (Zhu *et al.*, 2015), Chinese kale (Zhu *et al.*, 2016), *Salvia miltiorrhiza* (Liu *et al.*, 2017), and alfalfa (Zheng *et al.*, 2017), implicating that the underlying mechanisms might be well conserved in higher plants.

The enhanced WUE of *edt1* mutant is reminiscent of that found in *ERECTA*-overexpressing transgenic plants. *ERECTA*, the first reported WUE-related gene, was isolated by quantitative trait locus (QTL) analysis for carbon isotope discrimination (Masle *et al.*, 2005). Altering *ERECTA* gene expression displayed significant change of plant WUE (Masle *et al.*, 2005; Shen *et al.*, 2015). However, the causal molecular mechanism remains unclear. *ERECTA* encodes a leucine-rich repeat receptor-like kinase (LRR-RLK)

(Torii *et al.*, 1996) with many important functions in plant development (Shpak *et al.*, 2004; Pillitteri & Torii, 2012; Bemis *et al.*, 2013; Cui *et al.*, 2014; Jorda *et al.*, 2016; Tameshige *et al.*, 2016; Ikematsu *et al.*, 2017). ERECTA and its two highly homologous receptor-like kinases (RLKs), ER-LIKE1 (ERL1) and ER-LIKE2 (ERL2), synergistically inhibit stomatal differentiation and enforce proper stomatal spacing (Shpak *et al.*, 2005; Lau & Bergmann, 2012; Lee *et al.*, 2012b; Lee *et al.*, 2015; Meng *et al.*, 2015; Qi *et al.*, 2017).

It was reported that increased WUE was correlated with reduced stomatal density (Doheny-Adams *et al.*, 2012). Furthermore, stomatal density can be genetically manipulated to improve WUE (Franks *et al.*, 2015; Hepworth *et al.*, 2015). The changes in stomatal density of leaves can arise from variations in stomatal development as well as variations in epidermal cell size. Unlike stomatal development and patterning defective mutants, the *ERECTA* single-gene knockout mutant *er-105* exhibits increased stomatal density but no changes to stomatal index (Shpak *et al.*, 2005). When overexpressed, *ERECTA* reduced stomatal density without changing stomatal patterning (Masle *et al.*, 2005), resembling the phenotypes observed in the *edt1* mutant we previously reported (Yu *et al.*, 2008). The WUE in *er-105* and *edt1* is altered because of altered stomatal density, along with the change of epidermal cell size (Masle *et al.*, 2005; Yu *et al.*, 2008).

The final cell size within an organ is often, but not always, correlated with the ploidy level resulting from the nuclear endoreduplication (Breuer *et al.*, 2010). Transcription factor E2Fa is a key regulator in cell cycle-endocycle progression. Overexpression of *E2Fa* in *Arabidopsis* can stimulate cell proliferation via activating S-phase genes expression and inducing extra rounds of DNA replication (endoreduplication). Through binding or dissociating with retinoblastoma-related protein (RBR), E2Fa can control cell cycle and endocycle processes (Magyar *et al.*, 2012).

In this report, we continue to investigate the mechanisms of *AtEDT1/HDG11*-conferred drought resistance and WUE improvement. We provide evidence to suggest a genetic pathway composed of EDT1/HDG11, ERECTA, and E2Fa as key components in reducing

stomatal density and increasing WUE. *ERECTA* is a major target of EDT1/HDG11 and is transcriptionally upregulated by the EDT1/HDG11, which leads to a higher polyploidy level in leaf cells through an E2Fa-dependent pathway. The elevated ploidy levels increase leaf cell size and thus decrease stomatal density, which consequently improves plant WUE.

Materials &Methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia-0 ecotype (Col-0) was used as wild type. All mutants and transgenic plants used in the present work are in the Col-0 background. Some plant materials used in this study were previously described:*edt1* (Yu *et al.*, 2008), *er-105* (Shpak *et al.*, 2005), *35S::HA-HDG11* (Xu *et al.*, 2014), *e2fa* (Xiong *et al.*, 2013). CS89504 (*er-105*) and CS855653 (*e2fa*) mutant seeds were obtained from Arabidopsis Biological Resource Center.

CaMV 35S promoter-driven *gERECTA* overexpression lines were generated and selected on BASTA, the independent transgenic T2 lines were screened by qRT–PCR, the lines which showed high level of ERECTA transcript in young leaves were selected for further analysis and cross. Double mutants were generated by genetic crosses. Plants of a correct genotype were isolated from the F2 populations. The activated expression of *EDT1/HDG11* in *edt1*, and overexpression of *ERECTA* in *ER* lines, conferred resistance to Basta. Thus, progenies of each cross were first tested for Basta resistance, and subsequently the genotype of individual plants, homozygous lines were identified by PCR-based genotyping, the presence of the *er-105* mutation was determined by RT-PCR using the primer pairs: ERECTA-RT-F and ERECTA-RT-R (see Table S1). Similarly, *35S::ER e2fa* double mutant plants were genotyped with primers listed in Table S1.

Arabidopsis seeds were surface sterilized for 10 min in 10% bleach and washed at least five times with sterile water. Plant seeds were kept at 4 °C for 2 days in darkness before germination on horizontal agar plates containing solid Murashige and Skoog (MS) medium

with 1% (w/v) sucrose at 22 °C under 16-h light/8-h dark cycles. Adult plants were grown in peat under long-day conditions (16-h light / 8-h dark) at 22–24°C.

DNA constructs and plant transformation

To prepare the ERECTA overexpression construct, the primer set gERECTA-F and gERECTA-R (Table S1) and full-length genomic coding regions for ERECTA fragments were amplified and cloned into pDONR207, and subsequently shuttled it into the expression binary vector pCB2004 in which the cauliflower mosaic virus 35S promoter drives expression of the ERECTA gDNA (Lei, 2007). These constructs were then individually transformed into *Agrobacterium tumefaciens* strain (C58C1), and introduced into *Arabidopsis* plants by the floral dip method.

Cytological analyses

To visualize epidermal cell outlines, 7-day-oldseedlingswerestainedwith 0.2 mg/ml propidium iodide (PI) for 20 min and washed twice in water. Single optical sections of the adaxial side of PI-stained cotyledons from the wild type and the mutants were acquired under a fluorescence microscope with a 40×objective (ZEISS Axioskop2 plus): 543-nm of the laser was used for excitation, and emission was detected at 620 nm. For quantitative analysis of cell density and stomatal density, 30 leaves were sampled for each genotype grown in the same conditions.

Leaf transverse section image analysis was performed as described (Zhao *et al.*, 2010). Briefly, leave samples (5x5 mm^2) were fixed in the fixation buffer containing 50% (V/V) ethanol, 3.7% (V/V) formaldehyde and 5% (V/V) acetic acid for 24 hours before dehydrated with a graded series of ethanol (from 30%, 70%, to 100%). The samples were treated with xylene and embedded by paraffin through LEICA EG1150H. 10 μm sections were prepared by the LEICA RM2255 microtome. The embedding sections were placed on microslides, dried at 42℃, and treated with xylene to remove paraffin, then dehydrated with a graded series of ethanol until distilled water. The sections were stained with 0.1% Toluidine Blue in 0.1M phosphate buffer (pH=7.0), then observed under LEICA M165C microscope. Palisade

cell dimensions in the cross section images (length and width) were measured using Image J (https://imagej.nih.gov/ij/).

Physiological measurements

Photosynthesis (P) and transpiration (T) rates were measured on fully developed leaves of plants grown in a greenhouse, using a Li-6400XT portable gas-exchange system with 6400-15 Extended Reach 1 cm Chamber(LI-COR, Lincoln, NE, USA) and a custom-made light source as described (Yu *et al.*, 2008). All measurements were conducted between 9:00 a.m. and 11:00 a.m. Conditions in the Li-6400XT chamber were as follows: constant air flow rate, 500 μmol/s; CO_2 concentration, 400 μmol/mol;temperature,23± 2 °C; relative humidity (RH), 75-80%; and photosynthetic photon flux density, 1000 μmol (photon)/m^2 per second. Incoming air was aspirated through a custom-made humidifier consisting of a plastic bottle with wet paper towels. Gas exchange measurements were taken after steady state had been reached. WUE was defined as P/T ratio and derived from the measured P and T.

Flow cytometry analysis

For flow cytometry measurements, the fifth rosette leaf was collected and chopped with razor blades in nuclei extraction buffer and stained with propidiumiodide(PI) as described before (Jing *et al.*, 2009). The experiment was performed three times independently. A total of 10,000 nuclei were measured per analysis. Flow cytometry data were obtained using a FACS calibur flow cytometer (BD Biosciences).

qRT-PCR analysis

For E2Fa target genes expression analysis, RNA was prepared from whole seedlings (7 days after germination) using Trizol reagent (Invitrogen). RNA was reverse transcribed to cDNA using PrimeScript™ RT reagent Kit (Perfect RealTime) (TAKARA), according to the manufacturer's procedure. An aliquot of 1μL of the synthesized cDNA was used as template in a 10μL PCR reaction with gene specific primers (Table S1) and the SYBR® Premix Ex Taq™ II (TliRNaseH Plus) kit (TAKARA). *Ubiquitin5* (*UBQ5*, At3g62250) was used as the

internal control to normalize expression levels. Real time PCR is running on Stepone real-time PCR systems (Applied Biosystems). RNA was isolated using Trizol reagent (Invitrogen) from four-week-old rosette leaves of wild type and *edt1* for the transcript levels of *ERECTA*, *ERL1* and *ERL2*.

Yeast-one-hybrid and Yeast-two-hybrid analysis

Yeast strain YH187 and destination vectors (pHIS2 and pGADT7) were obtained from Jin-Song Zhang (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). For the Yeast-one-hybrid analysis, the prey vector harbouring*HDG11* (pAD-HDG11) and the bait vector pHIS2harbouringthe *ERECTA* promoter fragment *cis*-element 1 or *cis*-element 2 were used to transform yeast cells. The transformants were observed for their growth on SD/-Leu medium or SD/-Leu-His medium with3-amino triazole (3-AT) following the instruction of the BD Matchmaker Library Construction & Screening Kits (www.bdbiosciences.com). Transformation with empty vectors pGADT7 and pHIS2 were used as negative controls. The experiments were repeated three times with the same results. For the Yeast-two-hybrid analysis, the kinase domain of ERECTA was inserted into pDEST32vector to construct the bait plasmid, while the prey plasmid was constructed with pDEST22 vector and the full-length CDS of E2Fa. Positive clones were screened using SD/-Leu-Trp-His with 10mM 3-AT plates and X-gal assay. The primers could be found in Table S1.

ChIP-qPCR assay

The ChIP experiment was performed as described (Mukhopadhyay *et al.*, 2008) with minor modifications. One gram leaves of four-week-old wild type and the transgenic plants harboring *35Spro::HA-HDG11* (Xu *et al.*, 2014)were harvested and immersed in 1% formaldehyde under vacuum for 10 min. Glycine was added to a final concentration of 0.125 M, and incubation was continued for 5 min. After washing, the seedlings were ground into a fine powder with liquid nitrogen and resuspended in nuclei isolation buffer. The nucleus were then collected by centrifugation and resuspended with nuclei lysis buffer. The crosslinked DNA/protein complexes were fragmented by sonication with an Ultrasonic Process (Sonics),

resulting in fragments of ≤500 bp. After centrifugation (16,000 ***g***), the supernatant, 300μL was used as input, while the rest was divided into two samples, of which one sample was treated with an HA tag-specific monoclonal antibody (1:100 for ChIP assay, HA-Tag, 26D11, Mouse mAb, M20003, Abmart) and the other with no antibody. The samples were incubated overnight. Immunoprecipitates were collected with 100μL of supernatant precleared with 50μLof the protein A agarose/salmon sperm DNA (Millipore) and subsequently eluted from the beads. All centrifugation steps with bead-containing samples were done at 1000g.Proteins were de-cross-linked, and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were resuspended in 40μL of Tris-EDTA buffer (0.05 M Tris-HCl and 0.02 MEDTA, pH 6.5). Input and immunoprecipitated chromatin were used for ChIP-Quantitative Polymerase Chain Reaction (ChIP-qPCR) analysis using various *ERECTA* promoter-specific primers that were designed to amplify *ERECTA* promoter fragments (see Table S1). The ChIP-qPCR analysis was performed according to the procedure described previously (Mukhopadhyay *et al.*, 2008). The regions of ERECTA 3′ UTR that do not contain putative binding sites of HD class transcription factors (*cis* NC) was as negative control. The relative quantity value is presented as the DNA binding ratio (differential site occupancy).

Accession Numbers

EDT1/HDG11 (AT1G73360), *ERECTA* (AT2G26330), *E2Fa* (AT2G36010), *ERL1* (AT5G62230), *ERL2* (AT5G07180).

Results

EDT1/HDG11 depends on *ERECTA* in regulating stomatal density and WUE

To determine the genetic relationship between *ERECTA* and *EDT1/HDG11*, we introduced the activated *EDT1/HDG11* from the gain-of-function mutant *edt1* into *er-105* background by crossing and generated a double mutant *edt1 er-105*, in which *EDT1/HDG11* is overexpressed while *ERECTA* is completely knocked out. The double mutant *edt1 er-105*

displayed a similar morphological phenotype (Fig. 1a and b), photosynthesis and transpiration rates to *er-105* (Fig. 1c and d). In the double mutant *edt1 er-105*, leaf epidermal cell density and stomatal density were similar to that of *er-105*, significantly higher than that in *edt1* mutant as observed in both cotyledons (Fig. 1e and f) and the fifth rosette leaf of four-week-old plants (Fig. S1a and b). WUE of *edt1 er-105* double mutant was similar to that of *er-105*, but significantly decreased compared to that of *edt1* mutant (Fig. 1g), which was mainly contributed by increased transpiration rate (Fig. 1d). Stomatal density was negatively correlated with WUE (R^2 = 0.9623, Fig. 1h) and photosynthesis rate (R^2 = 0.9339, Fig. 1i), but positively correlated with transpiration rate (R^2 = 0.9595, Fig. 1j). These results suggest that *EDT1/HDG11* functions through *ERECTA* since a knockout of *ERECTA* is sufficient to block the function of *EDT1/HDG11* in regulating cell size, stomatal density and water use efficiency.

Cell density is negatively correlated with leaf cell size

We have previously showed that in *edt1*mutant, stomatal density change was caused by cell size enlargement and all leaf cells are proportionally enlarged (Yu *et al.*, 2008). To demonstrate whether this correlation between cell size and cell density also happens in the internal leaf cell, we measured more regularly shaped palisade cell dimension, as an indication or reflection of leaf cell size, among wild type, *edt1*, *er-105*, and *edt1 er-105*. The length and width of palisade cells in *edt1 er-105* double mutant was similar to the *er-105*, significantly reduced compared to that of *edt1* (Fig. S1c). A strong negative correlation was found between palisade cell length and epidermal cell density (R^2 =0.8689, Fig. S1d) as well as between palisade cell length and stomatal density (R^2 =0.9841, Fig. S1e), which validates cell density as an indication of cell size.

Cell density is negatively correlated with polyploidy level

Stomatal density is determined largely by changes in epidermal cell size (Carins Murphy *et al.*, 2016). To test whether the change of leaf cell size is accompanied by a change in leaf ploidy level via nuclear endoreduplication, we compared the DNA ploidy level of the fifth rosette leaf of four-week-old plants by flow cytometry. The fifth rosette leaf has the most

similar leaf size in different mutants (Fig. S2). The ploidy distributions revealed significant differences between *edt1, er-105* and *edt1er-105*. The ploidy of *edt1* cells ranged from 2C to 32C with a peak at 8C, whereas the ploidy of the cells in *er-105* and *edt1er-105* were from 2C to 32C with a peak at 4C (Fig. 2a). The average percentage of high polyploidy (8C or higher) cells was 45.95%, 57.82%, 34.54% and 35.04% for the wild type, *edt1*, *er-105* and *edt1 er-105*, respectively (Fig. 2b). The population of leaf cells with 8C and higher DNA ploidy level was significantly increased in *edt1* and reduced in *er-105* and *edt1 er-105* compared to that of the wild type. The cell density of the mutants is negatively correlated with their ploidy level ($R^2 = 0.8856$, Fig. 2c), suggesting that an activated expression of *EDT1/HDG1* in *edt1* stimulates endoreduplication. The leaf ploidy level distribution of *edt1 er-105* was similar to that of *er-105*, consistent with our hypothesis that the function of *EDT1/HGD11* is dependent on *ERECTA* in regulating cell and stomatal density. These results indicate that the enlarged leaf cell size of the *edt1* mutant is correlated with the increase of ploidy levels, and *ERECTA* is required in this regulation.

EDT1/HDG11 upregulates the expression of *ERECTA*

Quantitative real-time PCR (qRT-PCR) analysis showed a higher expression level of *ERECTA* in *edt1* compared with that of the wild type (Fig. 3a). This result suggests that *ERECTA* might be transcriptionally upregulated by EDT1/HDG11 in *edt1* mutant.

To investigate whether EDT1/HDG11 directly binds to the promoter of *ERECTA*, we analyzed the *ERECTA* promoter sequence and found two putative binding sites of HD class transcription factors, *cis*-element 1 (*cis 1*: AAATTAGT) and *cis*-element 2 (*cis 2*: TAATAATTA) (Fig. 3b). Yeast-one-hybrid assays showed that EDT1/HDG11was able to bind the *cis*-element 1 and *cis*-element 2 in yeast cells (Fig. 3c). We further performed a chromatin immunoprecipitation assay followed by qPCR (ChIP-qPCR) analysis using the *edt1*as described (Xu *et al.*, 2014). The results from ChIP-qPCR showed that the *cis*-element 1 and *cis*-element 2 were significantly enriched in the immunoprecipitate. For the negative control, we did not detect any significant enrichment of the control sequence *cis* NC within the *ERECTA* 3′ UTR (Fig. 3d). These results demonstrate that EDT1/HDG11 specifically binds to the promoter of ERECTA and potentially activates the transcription of *ERECTA*.

qRT-PCR analysis showed that the expression levels of *ER-LIKE1* (*ERL1*) and *ER-LIKE2* (*ERL2*), two closely related RLKs of *ERECTA*, were not significantly higher in *edt1* than that in the wild type (Fig. 3a), although promoter scanning results indicated that a *cis*-element 1 (*cis1*: AAATTAGT) is presented in *ERL2* promoter, and a variant *cis*-element 1 (*cis1m*: AAATTATT) in *ERL1* promoter (Fig. 3b). The ChIP-qPCR experiment results showed that these two regions were not enriched with anti-HA antibody (Fig. 3d), suggesting that the *cis* elements in the *ERL1* and *ERL2* promoter are not bound by EDT1/HDG11 *in planta*.

ERECTA is dependent on E2Fa in stomatal density modulation

Since cell size is positively correlated with ploidy level in the mutant *edt1* and *er-105*, to further investigate how the plasma membrane-localized ERECTA affects leaf ploidy level via nuclear endoreduplication, we performed a yeast-two-hybrid screening using the ERECTA kinase domain as a bait. A few nuclear factors involved in cell cycle regulation were identified (Fig. S3a). One of the ERECTA-interacting proteins is E2Fa, which was previously shown to regulate proliferation and endocycle in *Arabidopsis* (Magyar *et al.*, 2012). E2Fa was further analyzed to confirm its interaction with ERECTA by *in vitro* and *in vivo* interaction analyses (Fig. S3b-d), indicating that E2Fa might be a downstream target of this pathway.

To confirm the genetic relationship between ERECTA and E2Fa, we analyzed potential epistatic interaction by crossing the *ERECTA*-overexpressing line *35S:gERECTA-L1* (designated as 35S::*ER*) (Fig. 4a) with the *E2Fa* knockout mutant (*e2fa*). The transcript level of *ERECTA* in the double mutant*35S::ER e2fa* was comparable to that of the parental *ERECTA*-overexpressing line (Fig. 4a-b). The double mutant *35S::ERe2fa* showed similar plant growth morphology to *e2fa* but not to *35S::ER* (Fig. 4c and d). The leaf cell and stomatal density of *35S::ER e2f*a was similar to that of *e2fa*. The decreased leaf epidermal cell and stomatal density of *35S::ER* were completely repressed in *35S::ER e2fa* (Fig. 4e and f, Fig. S4a and b), indicating that ERECTA-modulated cell and stomatal density is E2Fa-dependent. The consistent results were obtained in the measurement of palisade cell dimension (Fig. S4c). The palisade cell length was negatively correlated with cell density (R^2

= 0.8688, Fig. S4d) as well as with stomatal density (R^2 = 0.9871, Fig. S4e). The ploidy levels of leaf cells were measured by flow cytometry. Average percentage of high polyploidy cells with 8C and higher DNA ploidy level was significantly higher in *35S::ER* leaf than that in the wild type (65.83% vs. 51.6%), while it was only 40.97% in *e2fa*, significantly lower than that in the wild type leaf. In contrast to 35S::*ER* plants, the high ploidy cell proportion of the double mutant *35S::ER e2fa* was reduced to 40.58%, which was similar to that of *e2fa* (Fig. 4g). A strong negative correlation was found between ploidy level and cell density (R^2 = 0.9314, Fig. 4h). These results show that cell size is positively correlated with cell ploidy level as previously shown for *edt1* and *er-105* mutants (Fig. 2), and E2Fa is required for endoreduplication in *35S::ER* plants. Moreover, WUE of *35S::ER e2fa* was similar to *e2fa* but significantly lower than that of *35S::ER* (Fig. 4i). Again, WUE was found negatively correlated with stomatal density (R^2 = 0.9882, Fig. 4j). Taken together, these results indicate that E2Fa is an important component downstream of ERECTA. ERECTA is dependent on E2Fa to control cell size, stomatal density, and WUE.

Expression levels of E2Fa-targeted genes are modulated by EDT1/HDG11 and ERECTA

To further confirm that ERECTA affects cell size through E2Fa, we measured the relative transcript levels of known E2Fa target genes that are required for G1/S transition and cell cycle progression by qRT-PCR in the wild type, *edt1* and *ERECTA* mutants. The target genes include *MINICHROMOSOME MAINTENANCE 5* (*MCM5*) and *CELL DIVISION CONTROL 6* (*CDC6*), which are essential for the initiation of the DNA replication (Xiong *et al.*, 2013); *CYCLIN-DEPENDENT KINASE B1;1* (*CDKB1;1*) and *CYCLIN A2;3* (*CYCA2;3*), which play a central role in the control of mitotic cell cycle and inhibit the endocycle; *CELL CYCLE SWITCH PROTEIN 52 A1* (*CCS52A1*) and *CELL CYCLE SWITCH PROTEIN 52 A2* (*CCS52A2*) that play important regulatory roles in the transition from mitosis to endocycle by stimulating the degradation of mitotic cyclins (Magyar *et al.*, 2012). Compared to wild type, the expression of *CDKB1;1* and *CYCA2;3*was repressed in*edt1* and *35S::ER*, but enhanced in *er-105*, *e2fa* and *ER e2fa*. On the contrary, the expression of *CCS52A1*, *CCS52A2*, *MCM5* and *CDC6* was increased in *edt1* and *35S::ER*, but decreased in *er-105*, *e2fa* and *35S::ER*

e2fa (Fig. 5). These results suggest that, through E2Fa transcription factor, ERECTA modifies the expression levels of cell cycle-related genes and regulates the transition from mitosis to endocycle, triggers cells to enter the endoreduplication cycle prematurely, and eventually results in larger-sized cells with higher polyploidy levels.

Discussion

Plant WUE is critical to plant survival and crop yield. In this study, we provided evidence to suggest an EDT1/HDG11-ERECTA-E2Fa genetic pathway that regulates plant WUE via leaf cell size and stomatal density. The homeodomain transcriptional factor EDT1/HDG11 upregulates the *ERECTA* expression, ERECTA promotes endoreduplication through E2Fa, leading to higher ploidy level and thus larger cells and reduced stomatal density, ultimately improving WUE. *AtEDT1/HDG11*-conferred drought resistance and improved WUE have been recapitulated by overexpressing *AtEDT1/HDG11*in many plant species including monocots and woody plant (Yu *et al.*, 2008; Cao *et al.*, 2009; Ruan *et al.*, 2012; Yu *et al.*, 2013; Zhu *et al.*, 2015; Li *et al.*, 2016; Yu *et al.*, 2016; Zhu *et al.*, 2016; Liu *et al.*, 2017), implicating a possible conserved mechanism in plants.

Arabidopsis ERECTA and its functional homologs, *ERL1*and *ERL2*, show synergistic interaction in promoting aboveground organ growth (van Zanten *et al.*, 2009). However, *ERECTA*, *ERL1*, and *ERL2* have subtle different roles in epidermal development (Shpak et al., 2005; Lee et al., 2012b; Pillitteri & Torii, 2012; Qi et al., 2017). Detailed analysis of higher order mutant combinations revealed that ERECTA primarily acts at the early steps of stomatal development to suppress entry asymmetric divisions (Pillitteri & Torii, 2012). Elevated expression of *ERECTA* leads to an increased epidermal cell size and reduced stomatal density in leaves, but the stomatal index is not significantly changed, consistent with the report that *er* single mutation displayed an increased stomatal density and smaller epidermal cells, without significant change in stomatal index (Masle *et al.*, 2005; Shen *et al.*, 2015). Our work revealed that the activated transcription of *ERECTA* by EDT1/HDG11 modulates stomatal density mostly through cell size rather than stomatal development, and also showed that the cell size is associated with the increase of ploidy levels (Figure 1 and

Figure 2). Considering that the phenotype of overexpressed *EDT1/HDG11* is largely blocked by knocking out *ERECTA* in the double mutant *edt1er-105*, we concluded that *ERECTA* is a major target of EDT1/HDG11 for augmenting leaf cell size and decreasing stomatal density, thereby improving plant WUE in *edt1*.

Previous studies have demonstrated key functions of *ERECTA* family genes in regulating the architecture of the whole plant and the development of stomata (van Zanten *et al.*, 2009; Pillitteri & Torii, 2012). ERECTA binds to the secreted Epidermal Pattern Factor peptide 2 (EPF2), while ERL1 binds to the EPF1 to regulate stomatal development (Lee et al., 2012a). In this study, a transcriptional regulation of *ERECTA* by transcription factor EDT1/HDG11 is shown to coordinate with other developmental processes to optimize stomatal density and water use efficiency. HDG11 was able to confer drought tolerance, how ERECTA coordinates developmental signals and drought stress response need to be further investigated. *ERECTA* family genes are known to regulate the architecture of the whole plant and the development of stomata mainly through the MAPK signaling cascade, influencing stomatal development and patterning by phosphorylating and regulating the stability of transcription factors SPCH, MUTE and FAMA (Lampard *et al.*, 2008; Lampard *et al.*, 2009; Meng *et al.*, 2012). These developmental regulations influence cell fate determination and have drastic effects on growth, sometimes even causing death or sterility of plants. However, enhanced ERECTA increases cell size and decreases stomatal density through the transcription factor E2Fa, influencing cell cycle and endocycle transition, which modifies the stomatal density without affecting the cell fate determination.

E2F transcription factors are required for progression in both mitotic and endocycle (De Veylder *et al.*, 2002). E2Fa is localized to both the cytoplasm and nucleus (Kosugi & Ohashi, 2002; Magyar *et al.*, 2012), while ERECTA is localized at the plasma membrane (Horst *et al.*, 2015). As a receptor-like protein kinase, ERECTA likely relays the signaling through phosphorylation of its targets. Although we genetically demonstrated that E2Fa is a downstream target of ERECTA (Fig. 4 and 5), how ERECTA biochemically transduces the signal to E2Fa awaits further investigation.

In conclusion, we have demonstrated that EDT1/HDG11 transcriptionally activates *ERECTA* expression. ERECTA influences E2Fa activity via a yet unknown mechanism, which regulates the expression of E2Fa target genes in the mitosis-to-endocycle transition and allows cells to decrease their division rate and promote endoreduplication. Consequently, these changes result in increased leaf cell size, reduced stomatal density, and improved water use efficiency.

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Authors' contribution

XYG and YW designed the research, performed the research, data analysis and interpretation. PXZ, PX, GHY, LYZ, and YX performed the research, data analysis and interpretation. XYG wrote the manuscript and CBX designed of the research, performed interpretation, edited the manuscript, and supervised the project. X-YG and YW authors contributed equally to this work

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Supporting information

Fig. S1. EDT1/HDG11 is dependent on ERECTA in regulating epidermal cell density and palisade cell size.

Fig. S2. Comparable leaf area of the fifth rosette leaf in different mutants.

Fig. S3. ERECTA interacts with E2Fa via its kinase domain (ERK).

Fig. S4. Epidermal cell density and stomatal density are negatively correlated with palisade cell size.

Table S1. Primers used in this study.

Figure 1. EDT1/HDG11 is dependent on ERECTA in regulating stomatal density and WUE.

(a) Morphological phenotype of *Arabidopsis thaliana* wild type (wt), *edt1*, *er-105* and *edt1 er-105* grown on soil under long-day conditions. Scale bar represents 2 cm.

(b) Leaf spread of four-week-old wild type, *edt1*, *er-105* and *edt1 er-105*. Scale bar represents 2 cm. The fifth rosette leaf was used for gas-exchange measurements and flow cytometry measurements, and highlighted in a yellow box.

(c) and (d) Comparisons of photosynthesis rate (c) and transpiration rate (d) among the wild type, *edt1*, *er-105*, and *edt1 er-105*. Data were analyzed for significant differences between the means based on Bonferroni's multiple comparison tests using one-way analysis of variance (ANOVA) ($P < 0.05$). Columns with different letters are significantly different from each other. Values are means ±SD (n = 10 plants per genotype).

(e) Confocal images of adaxial cotyledon epidermis from 7-day-old wild type, *edt1*, *er-105*, and *edt1 er-105*. Scale bar represents 100 μm.

(f) Quantitative analysis of adaxial cotyledon cell density and stomatal density in 7-day-old wild type, *edt1*, *er-105*, and *edt1 er-1*05. Values represent the average (± SD) (n = 30), letters indicate differences statistically significant among the genotypes for cell density and stomatal density, respectively ($p < 0.05$; two-way ANOVA with Bonferroni post-test).

(g) Comparison of WUE among the wild type, *edt1*, *er-105*, and *edt1 er-105*. WUE was measured as described in Methods. Values are mean ± SD (n = 10 plants per genotype).

Letters indicate statistical significance from one-way ANOVA followed by Bonferroni's multiple comparison test ($P < 0.05$).

(h-j) Correlation of stomatal density with WUE (h), photosynthesis rate (i) and transpiration rate (j). Simple linear regression analyses were made using the mean values of stomatal density in (f), photosynthesis rate in (c), transpiration rate in (d) and WUE in (g).

Figure 2. Leaf cell polyploidy level is reduced in *er* mutant.

(a) Ploidy histogram for nuclei isolated from *Arabidopsis thaliana* wild type (wt), *edt1*, *er-105*, and *edt1 er-105*. The nuclei isolated from the fifth rosette leaf of four-week-old plants were measured by flow cytometry. Data are from one out of three independent experiments with similar results.

(b) Ploidy level distribution of wild type (wt), *edt1*, *er-105* and *edt1 er-105*. The nuclei isolated from the fifth rosette leaf of four-week-old plants were measured by flow cytometry. 10,000 nuclei were counted for each sample. Data represent average ± SD (biological replicates of n=3; 6 leaves from at least 3 different plants per genotype). Significant differences are calculated for high ploidy level ($\geqslant$ 8C) by one-way ANOVA followed by Bonferroni's multiple comparison test ($P \leq 0.05$). Lower case letters indicate differences statistically significant among the genotypes.

(c) Correlation between ploidy level and cell density. A simple linear regression analysis was made using the mean values of cell density in Figure 1f and the average percentage of high polyploidy cells (8C and higher ploidy levels) in (b).

Figure 3. EDT1/HDG11 upregulates *ERECTA* expression by binding to its promoter region.

(a) Quantitative real-time PCR analysis of *ERECTA*, *ERL1* and *ERL2* expression between *Arabidopsis thaliana* wild type and *edt1*. All assays were carried out at least three times and levels of statistical significance were calculated using student's *t*-test (* $P \leq 0.05$).

(b) The schematic illustration of the locations of predicted HD-binding *cis*-elements (inverted triangles) in the *ERECTA*, *ERL1* and *ERL2* promoter, and the primers used for ChIP-qPCR (red short lines).

(c) EDT1/HDG11 protein binds to *ERECTA* promoter in yeast-one-hybrid assay. Yeast clones were grown on the synthetic dropout medium lacking Leu (left) or lacking Leu/His and containing 10 mM 3-aminotriazole (right). The experiments were repeated three times with the same results.

(d) ChIP-qPCR assays for EDT1/HDG11 binding to the promoter of *ERECTA*, *ERL1* and *ERL2 in vivo*. ChIP-qPCR analysis was performed as described in Methods using the *35Spro::HA-HDG11* transgenic plants and an HA tag-specific monoclonal antibody for immunoprecipitation. The *cis NC* in *ERECTA* 3′ UTR without HD binding sites was used as negative control. Values are mean± SD (*n*= 3 experiments, ***P ≤ 0.001, Student's *t*-test).

Figure 4. ERECTA and E2Fa share a common pathway to regulate cell size, stomatal density and WUE.

(a) Relative expression levels of *ERECTA* in rosette leaves of 4-week-old *Arabidopsis thaliana* wild type (wt) and *ER*-overexpressing lines (T2, lines *35S::gERECTA-L1-3*) detected by qRT-PCR. Values are means ± SD (n = 3).

(b) Relative expression levels of *ERECTA* in rosette leaves of 4-week-old wild type, *35S::gERECTA e2fa* double mutant (two independent lines, 3*5S::ER e2fa-L1* and *35S::ER e2fa-L2*), and *ER*-overexpressing line *35S::gERECTA-L1* (35S::*ER*). Values are means ± SD (n = 3).

(c) Morphological phenotype of wild type, *35S::ER*, *e2fa* and *35S::ER e2fa* grown on soil under long-day conditions. Scale bar represents 2 cm.

(d) Leaf spread of four-weeks-old wild type, *35S::ER*, *e2fa* and *35S::ER e2fa*. Scale bar represents 2 cm. The fifth rosette leaf was used for gas-exchange measurements and flow cytometry measurements, and highlighted in a yellow box.

(e) Confocal images of adaxial cotyledon epidermis from 7-day-old wild type, *35S::ER*, *e2fa* and *35S::ER e2fa*. Scale bar represents 100 μm.

(f) Quantitative analysis of adaxial cotyledon cell density and stomatal density in 7-day-old wild type, *35S::ER*, *e2fa* and *35S::ER e2fa*. Values are mean ± SD (n = 30), letters indicate differences statistically significant among the genotypes for cell density and stomatal density, respectively (p ≤ 0.05; two-way ANOVA with Bonferroni post-test).

(g) Enlarged cell size, equivalent to reduced cell and stomatal density, is correlated with the increased polyploidy level in leaf cells. Ploidy level distribution of the fifth rosette leaf in four-week-old wild type, *35S::ER*, *e2fa* and *35S::ER e2fa* was measured by flow cytometry. 10,000 nuclei were counted for each sample. Data represent average ± SD (biological replicates of n=3; 6 leaves from at least 3 different plants per genotype). Significant differences are calculated for high ploidy level (≥ 8C) by one-way ANOVA followed by Bonferroni's multiple comparison test ($P \leq 0.05$). Lower case letters indicate differences statistically significant among the genotypes for cell density and stomatal density, respectively.

(h) Correlation between ploidy level and cell density. A simple linear regression analysis was made using the mean values of cell density in (f) and the average percentage of high polyploidy cells (8C and higher ploidy levels) in (g).

(i) Comparisons of WUE between wild type, *35S::ER*, *e2fa* and*35S::ER e2fa*. WUE was measured as described in Methods. Values are mean ± SD (n = 10 plants per genotype). Letters indicate statistical significance from one-way ANOVA followed by Bonferroni's multiple comparison tests ($P \leq 0.05$).

(j) Correlation of stomatal density with WUE. A simple linear regression analysis was made using the mean values of stomatal density in (f) and WUE in (i).

Figure 5. Expression levels of E2Fa target genes are changed in *edt1* and *ERECTA* mutants.

The transcript levels of the indicated target genes of E2Fa were quantified in *edt1, er105, 35S::ER, 35S::ER e2fa,* and *e2fa* by qRT-PCR. Values were normalized to Arabidopsis *Ubiquitin5* expression level and represented as n-fold compared to the wild type (Col-0). All assays were carried out at least three times and levels of statistical significance were calculated using student's *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Values are mean ± SD.