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The Arabidopsis EIN2 restricts organ growth by retarding cell expansion

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The growth of plant organ to its characteristic size is a fundamental developmental process, but the mechanism is still poorly understood. Plant hormones play a great role in organ size control by modulating cell division and/or cell expansion. ETHYLENE INSENSITVE 2 (EIN2) was first identified by a genetic screen for ethylene insensitivity and is regarded as a central component of ethylene signaling, but its role in cell growth has not been reported. Here we demonstrate that changed expression of EIN2 led to abnormity of cell expansion by morphological and cytological analyses of *EIN2* loss-of-function mutants and the overexpressing transgenic plant. Our findings suggest that EIN2 controls final organ size by restricting cell expansion.

Introduction

The plant organs develop from meristems, the reservoirs of pluripotent cells of the plant, and then further grow to their specific size by coordinating cell proliferation and cell expansion.[1] The relative constancy of organ size within a given species, but its tremendous variance among species, suggests that the final size of an organ is monitored by intrinsically developmental programs.[2] Given their sessile and post-embryonic organogenesis lifestyle, the growth of plants are greatly influenced by the environmental signals, such as plant hormones, light, temperature and nutrients.[3,4]

Genetic studies have identified a number of factors influencing either cell proliferation or cell expansion. In plant, alteration of cell number within an organ usually results from a change in the duration of cell proliferation.[5] This process is modulated by positive factors, including AINTEGUMENTA (ANT), GROWTH-REGULATING FACTORS (AtGRFs), GRF-INTERACTING FACTORS (AtGIFs), ARGOS, ORGAN SIZE RELATED 1 (OSR1), JAGGED and KLUH.[6-13] Overexpression of these genes results in enlarged organs with increased cells due to extended duration of cell proliferation. In contrast, several factors restricting organ size by limiting the period of cell proliferation have been characterized, such as CINCINNATA (CIN), BIG BROTHER (BB), AUXIN RESPONSE FACTOR 2 (ARF2), DA1, DA2 and MEDIATOR COMPLEX SUBUNIT 25 (MED25).[14-19] The loss-of-function mutants of these genes exhibit enlarged organs. However, a few of factors influencing plant organ size by regulating cell expansion have been identified. For example, AtEXP10, ANGUSTIFOLIA (AN), ROTUNDIFOLIA 3 (ROT3), ARGOS-LIKE (ARL) and KUODA1 (KUA1) enhance organ growth by promoting cell expansion.[20-25] Whereas, BIGPETALp (BPEp) and its interacting protein AUXIN RESPONSE FACTOR8 (ARF8), control petal size by limiting cell expansion.[26,27]

The gas hormone ethylene plays numerous roles in the development and environmental responses of the plant, including seed germination, seedling growth and senescence, fruit ripening, stress and pathogen responses.[11] ETHYLENE INSENSITVE 2 (EIN2), acting downstream of CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), is the central regulator of ethylene signaling pathway.[28,29] The EIN2 protein consists of a hydrophobic N-terminus with the 12-transmembrane domains and a hydrophilic C-terminal domain that contains a conserved nuclear localization sequence.[29] EIN2 is localized at the ER membrane and physically interacts with the kinase domain of the ethylene receptors in the absence of ethylene, and is cleaved in ethylene-induced manner with the C-terminal fragment translocated into nucleus to stabilize the short-lived protein ETHYLENE INSENSITVE 3 (EIN3).[30,31]

Previous works about EIN2 primarily focused on its pivotal roles in ethylene signal transduction and functions in abiotic and biotic stress. Its effects on organ size regulation and cell growth control were always neglected. In order to explore these roles of EIN2, we analyzed the loss-of-function mutants of *EIN2* and its overexpressing transgenic plant, finding that mutants of *ein2-1* and *ein2-5* exhibit enlarged organs due to expanded cells, whereas the *EIN2* overexpressing transgenic plants which show constitutive ethylene response produce small organs with smaller cells. The marker genes related to cell expansion process display relevant change within the mutants and transgenic plants. These results suggest that EIN2 plays a role in restricting cell expansion and keeping plant final organ size in check.

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Materials and Methods

Plant materials and growth conditions

Wild-type Arabidopsis ecotype Columbia Col-0 (WT), *ein2-1* and *ein2-5* mutant were used in this study. Sterilized seeds were plated on 1/2 MS medium containing 1% sucrose and 0.6% agar, and then vernalized at 4°C in darkness for 2 d For seed germination, the plate was then transferred to a culture room at 22 ± 1°C with illumination of 80–90 μmol m^{-2} s^{-1} with a 16-h light /8-h dark photoperiod. The 7-day old seedlings after germination were planted in soil for further growth.[32]

Morphological and cytological analyses

The fully expanded leaves were used to determine the size of leaf and palisade cells. They were excised and photographed, and then cleared with chloral hydrate as previously described.[32] The palisade cells at the central position of leaf were visualized under a microscope and photographed. Areas of leaves and cells were measured with IMAGE J software (http://rsbweb.nih.gov/ij/), and the total number of palisade cells per leaf was estimated by the total leaf area multiplied by the average cell number per area.

Plant transformation

The 3885-bp *EIN2* coding sequence was amplified by reverse transcription polymerase chain reaction (RT-PCR) and cloned into pVIP96 for generation of the *35S-EIN2* construct. The *35S-EIN2* transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation.[34] Twenty independently transgenic lines harboring a single T-DNA insertion were used for morphological analyses and three of T3 generation plants were used for gene expression analysis.

Gene expression analysis

Total RNA was isolated with a guanidine thiocyanate extraction buffer, and the reverse-transcribed PCR (RT-PCR) was performed to monitor the expression of cell expansion related marker genes as described previously.[33] Real-time quantitative RT-PCR (qRT-PCR) was carried out using a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) with the SYBR® Premix Ex™ Taq II kit (Takara, Dalian, China). The normalization and relative values of expression level for each gene were calculated from three biological replicates, as previously described.[35]

Flow cytometric assay

The fully expanded (25 d after initiation) fifth leaves of WT, *ein2-1*, *ein2-5* and *35S-EIN2* were chopped with a razor, suspended in cold nuclear isolation buffer and flow cytometric analysis was carried out as described with a FACS Caliber flow cytometer (BD Biosciences, http://www.bdbiosciences.com/).[34]

Results

The enlarged organs of *ein2-1* and *ein2-5* and small organs of *35S-EIN2* transgenic plant

In order to investigate the roles of EIN2 in plant organ size control, the well-known *EIN2* loss-of-function mutant *ein2-1* and *ein2-5* were measured for the morphological analyses. The areas of fully expanded cotyledons of *ein2-1* and *ein2-5* increased by 47% and 55% respectively, when compared with those of wild-type (WT) plant (**Fig. 1A**). Detailed characterization of the fifth rosette leaf showed that the average leaf blade areas of *ein2-1* and *ein2-5* increased by 56% and 48% when compared with those in the WT, respectively. The other aerial organs, including stems, flowers and siliques, were also enlarged to some extent, resulting in bigger plants compared with WT plants (**Fig. 1**; **Table 1**). These observations demonstrate that the mutation in *EIN2* results in the excessive growth of aerial organs.

To further determine the organ size regulating role of EIN2, we generated *35S-EIN2* transgenic plants. All of 20 independently transgenic lines overexpressing *EIN2* displayed obvious smaller organs. The areas of fully expanded cotyledons and the fifth rosette leaves of *35S-EIN2* transgenic plants decreased by 39% and 62% respectively, when compared with those of WT plants (**Fig. 2**). These results, together with the above morphological analyses of *ein2* mutants, state clearly that EIN2 impedes organ growth during plant development.

EIN2 controls organ size by limiting cell expansion

To assess the contributions of cell division and cell expansion to the phenotypes of *EIN2* loss-of-function mutants and overexpressing transgenic plants, their palisade cells of the fully

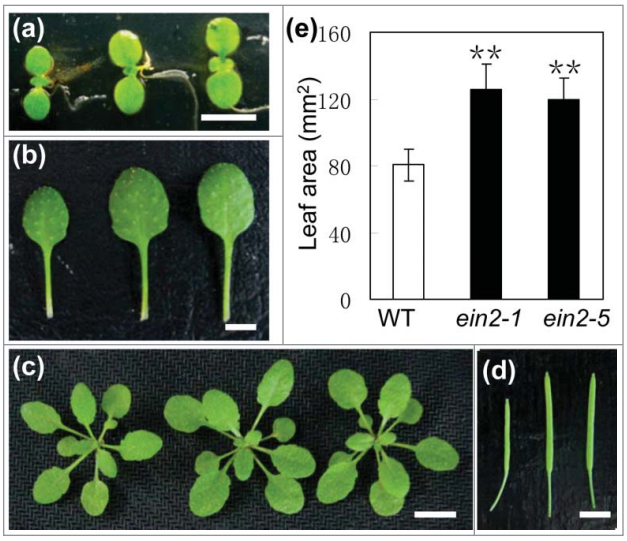

Figure 1. Mutation in *EIN2* led to enlarged organs. (**A**) Cotyledons, (**B**) fully expanded fifth leaves, (**C**) whole plants and (**D**) siliques of Wild-type (WT) (left), *ein2-1*(middle) and *ein2-5* (right). Bar, 5 mm in (**A, B, D**) and 1 cm in (**C**). (**E**) The areas of fully expanded fifth leaf blades in WT, *ein2-1* and *ein2-5* plants. Mean ± SD; Student's t-test: **, $P < 0.01$.

Table 1 Phenotype of *ein2-1* and *ein2-5* Plants

Variable	WT	*ein2-1*	*ein2-5*
Cotyledon area (mm^2)	2.16 ± 0.31	3.17 ± 0.34**	3.35 ± 0.42**
Petal area (mm^2)	1.35 ± 0.16	1.87 ± 0.19*	1.74 ± 0.15*
Flowering time (days)	25.1 ± 1.15	32.3 ± 1.32	31.5 ± 1.31
Silique length (mm)	10.6 ± 0.92	13.3 ± 1.57*	12.8 ± 1.44*
Plant height (cm)	27.6 ± 1.92	36.1 ± 3.04*	34.9 ± 3.32*

Mean ± SD; Student's t-test: **, $P < 0.01$; *, $P < 0.05$.

expanded fifth leaves were visualized under a microscope. As shown in **Fig. 3**, the average size of palisade cells in *ein2-1* and *ein2-5* increased by 52% and 45% respectively when compared with that of WT plants, while the average size of palisade cells in *35S-EIN2* transgenic plants decreased to 41% of that of WT plants. Furthermore, the estimated palisade cell number per leaf of all these lines remains comparable, demonstrating that EIN2 control organ size by limiting cell expansion and not by manipulating cell proliferation.

Cell expansion restricted by EIN2 is related to expansins but not to endoreduplication

The final size of a plant cell is often associated with nuclear polyploidy, which results from nuclear endoreduplication when a cell undergoes differentiation and cell expansion.[37] Analysis of the DNA content of fully expanded fifth leaves by flow cytometry with nuclei indicated that no enhanced endoreduplication in the fifth rosette leaves of *ein2-1* and *ein2-5* or reduced endoreduplication in *35S-EIN2* transgenic plant were observed when compared to WT plants (**Fig. 4**). These findings demonstrated that the cell size increase or decrease observed in leaves of the *ein2-1* and *ein2-5* or *35S-EIN2* transgenic plants was not resulting from the changed endoreduplication.

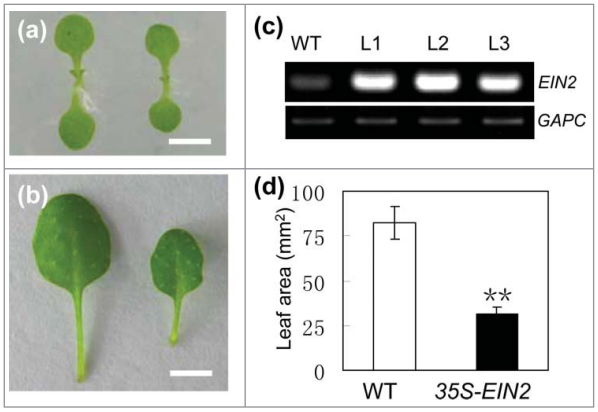

Figure 2. Morphology of *35S-EIN2* transgenic plants. (**A**) Cotyledons and (**B**) fully expanded fifth leaves of Wild-type (WT) (left), and *35S-EIN2* (right) plants. Bar = 5 mm. (**C**) Expression analyses of *EIN2* in three independent lines (L1 to L3) of *35S-EIN2* transgenic plants. The transcript abundance of *GAPC* was used as an internal control in the RT-PCR. (**D**) The areas of fully expanded fifth leaf blades in WT and *35S-EIN2* plants. Mean ± SD; Student's t-test: **, $P < 0.01$.

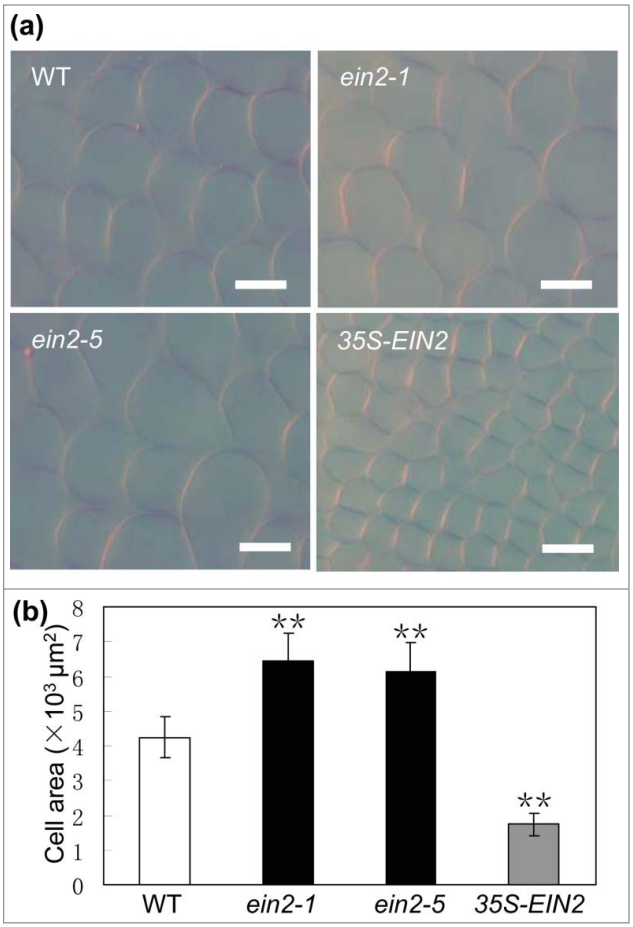

Figure 3. Cytological characterization of *ein2-1*, *ein2-5* and *35S-EIN2* transgenic plants. (**A**) Palisade cells of the fully expanded fifth leaf in WT, *ein2-1*, *ein2-5* and *35S-EIN2* transgenic plants. Bars = 100 μm. (**B**) Estimated palisade cell area in WT, *ein2-1*, *ein2-5* and *35S-EIN2* transgenic plants. Mean ± SD; Student's t-test: **, $P < 0.01$.

Expansins play essential roles in cell enlargement as key regulators of cell wall extension.[37,38] In the Arabidopsis genome, there are 26 α-expansin genes and at least five β-expansin genes,

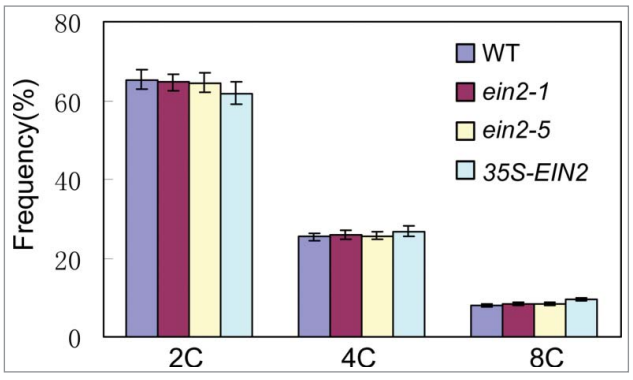

Figure 4. Ploidy levels of WT, *ein2-1*, *ein2-5* and *35S-EIN2* transgenic plants. Percentage of cells with different classes of nuclear ploidy. Mean ± SD.

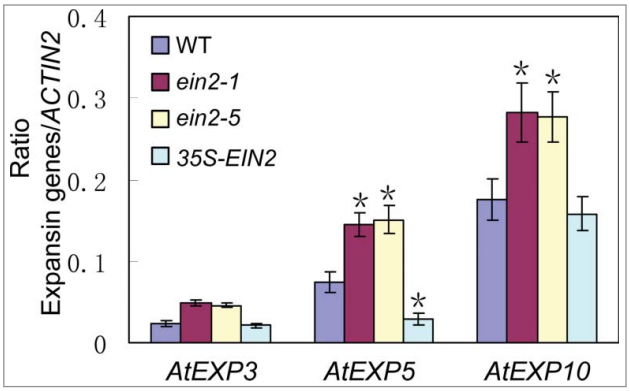

Figure 5. The expressions of expansin genes in WT, *ein2-1*, *ein2-5* and *35S-EIN2* transgenic plants. Expression levels of *AtEXP3*, *AtEXP5*, *AtEXP10* are higher in *ein2-1* and *ein2-5* leaves than in wild-type, and decreased expression levels of *AtEXP5* in *35S-EIN2* transgenic plants. Mean ± SD. Student's t-test: *, $P < 0.05$.

which might act redundantly to regulate plant growth and development.[37,38] In order to monitor the cell expansion processes within the *EIN2* loss-of function mutants and *35S-EIN2* transgenic plants, we examined the expression levels of the expansin genes. *AtEXP3, AtEXP5* and *AtEXP10* showed a higher expression level in the *ein2-1* and *ein2-5*, while the expression of *AtEXP5* decreased in *35S-EIN2* transgenic plants compared to WT plants (**Fig. 5**), and other expansin genes didn't show any significant expressive changes (data not shown), suggesting that repressing the expression of particular expansin genes accounts for cell expansion restricting by EIN2.

Discussion

To date, many genes participating in plant organ size control have been identified, and they are referred to as intrinsic yield genes (IYGs) due to their potential to boost the biomass and yield of agronomic plants.[34,39] However, relatively few of these genes coalesce into pathways, indicating that the mechanisms of plant organ size control are more complicated than those in animals.[5] In this study, we characterized EIN2 as a player in plant organ size control, with loss and gain of function producing opposite effects on organ size. Failure to maintain correct cell proliferation and/or expansion often, but not always, results in an alteration of plant organ size, because of compensation.[40,41] The enlarged cells in *EIN2* loss-of-function mutants and the smaller cells in *35S-EIN2* transgenic plants suggests that EIN2 restricts organ growth by limiting cell expansion, and the unchanged cell numbers in the organs of different lines illustrates that compensation does not take place. Our report discovered a novel role of EIN2 in regulating organ growth by affecting cell expansion in a general manner.

Within an organ, the final size of cells is often, but not always, associated with the ploidy level owning to the nuclear endoreduplication, which occurs when cells undergo differentiation and expansion.[2] A matter of size: developmental control of organ size in plants. Curr Opin Plant Biol 4(6):533–539. Although the mechanism is still obscure, the ploidy level has been regarded as an important factor in determining cell size.[36,42] Alteration of cell size in the plants with abnormal expression of *EIN2* provides another example that cell size is not correlated to the ploidy level. Expansion of plant cells necessarily requires a loosening of the cell wall and concomitant water uptake to a vacuole of high osmolarity to maintain turgor, and the involvement of expansins make this process more convenient.[43] The increased expression levels of *AtEXP3, AtEXP5* and *AtEXP10* in the *ein2-1* and *ein2-5* mutants and decreased expression levels of *AtEXP5* in *35S-EIN2* transgenic plants indicate that EIN2 might act on some unknown factors to reduce expansins expression level to restrict cell expansion.

Plant hormones, such as auxin, cytokinin, ethylene and abscisic acid, intensely influence organ growth and have been demonstrated to play a significant role in cell proliferation and/or cell expansion regulation.[41] However, how these hormonal signals are integrated into the program of plant organ size control remains largely unclear. the loss-of-function mutants of *EIN2* are not only insensitive to ethylene, but also have been found in screens for the mutants resistant to auxin transport inhibitors, cytokinins, or abscisic acid,[28] giving a hint that EIN2 might integrate these hormones signaling to fine-tune plant cell growth considering its role in cell expansion regulation presented by our results. Future work on investigation of molecular mechanism of EIN2 from the point view of cell growth regulation will bring about more interesting discoveries.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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