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RESEARCH PAPER

Ethylene receptor ETR2 controls trichome branching by regulating microtubule assembly in *Arabidopsis thaliana*

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Abstract

The single-celled trichome of *Arabidopsis thaliana* is a widely used model system for studying cell development. While the pathways that control the later stages of trichome development are well characterized, the early signalling events that co-ordinate these pathways are less well understood. Hormones such as gibberellic acid, salicylic acid, cytokinins, and ethylene are known to affect trichome initiation and development. To understand the role of the plant hormone ethylene in trichome development, an *Arabidopsis* loss-of-function ethylene receptor mutant, *etr2-3*, which has completely unbranched trichomes, is analysed in this study. It was hypothesized that ETR2 might affect the assembly of the microtubule cytoskeleton based on analysis of the cytoskeleton in developing trichomes, and exposures to paclitaxol and oryzalin, which respectively act either to stabilize or depolymerize the cytoskeleton. Through epistatic and gene expression analyses it is shown that ETR2 is positioned upstream of CHROMATIN ASSEMBLY FACTOR1 and TRYPTICHON and is independent of the GLABRA2 and GLABRA3 pathways. These results help extend understanding of the early events that control trichome development and identify a signalling pathway through which ethylene affects trichome branching.

Key words: Cytoskeleton, endoreduplication, epigenetic, hormone, signal transduction, tubulin.

Introduction

Trichome development in *Arabidopsis thaliana* has six distinct steps (Syzmanski *et al.*, 1998) that are controlled by over 30 genes, the majority of which signal to affect trichome branching (reviewed in Schellmann and Hülskamp, 2005). Endoreduplication during early trichome development requires the activity of the transcription factor GLABRA3 (GL3), which is thought to facilitate rapid growth of the cell (Hülskamp *et al.*, 1994). Once the trichome has attained a designated size, the microtubule cytoskeleton re-orients to cause two or more branching events controlled by several independent pathways. GL3 is thought to act upstream of *FURCA4* (*FRC4*) to regulate trichome branching positively (Luo and Oppenheimer, 1999), while in another pathway, ANGUSTIFOLIA (AN) is negatively regulated by the MYB transcription factor NOEK (NOK) to restrict branching (Folkers *et al.*, 1997; Jakoby *et al.*, 2008). TRYPTICHON (TRY) has been found, through epistatic analysis, to act upstream of *FRC4*, *ZWICHEL* (*ZWI*), and *STICHEL* (*STI*) to restrict branching and has also been proposed to affect GL3 negatively (Esch *et al.*, 2003). STI, ZWI, and AN are implicated in the assembly of microtubules with consequences for branching pattern and number (Oppenheimer *et al.*, 1997; Mathur and Chua, 2000; Folkers *et al.*, 2002). STI is considered to be one of the most important contributors to trichome branching, as mutations to this gene yield predominantly unbranched trichomes, while mutations to the other genes mentioned above yield predominantly two-branched trichomes (Hülskamp *et al.*, 1994). The epigenetic state of the cell also appears to play a role in the final shape of the trichome based on loss-of-function mutations to the trimeric protein CHROMATIN ASSEMBLY FACTOR1 (CAF1) (Exner *et al.*, 2006, 2008; Ono *et al.*, 2006). Mutations either to the FASCIATA1 (FAS1) or to the

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FASCIATA2 (FAS2) subunits of CAF1 show increased trichome branching and are thought to act through regulation of STI (Exner *et al.*, 2008).

Hormones can also affect trichome development. For example, the gibberellic acid (GA) mutant *spy-5* has increased trichome branching (Perazza *et al.*, 1998). Exogenous application of ethylene meanwhile, has been found to increase branch number in cucumber trichomes (Kazama *et al.*, 2004) and increased ethylene synthesis has been correlated with branch extension in cotton trichomes (Shi *et al.*, 2006; Qin *et al.*, 2007). Ethylene is a gaseous plant hormone that, in *Arabidopsis*, is sensed by five receptors (ERS1, ERS2, ETR1, ETR2, and EIN4) (Hua and Meyerowitz, 1998). These receptors, which localize to the endoplasmic reticulum (ER), form homo- and heterodimers with each other and associate with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), which represses activation of downstream pathways in the absence of ethylene (Grefen *et al.*, 2008). Upon binding of ethylene through a copper co-factor, repression of the ethylene response pathways is relieved through inactivation of CTR1 (Rodríguez *et al.*, 1999). It has also been shown that ethylene can affect downstream responses in a CTR1 independent pathway (Hass *et al.*, 2004). There are two classes of ethylene receptor mutants in *Arabidopsis*. Gain-Of-Function (GOF) mutations disrupt ethylene binding (Bleeker *et al.*, 1988; Hua *et al.*, 1995, 1998; Sakai *et al.*, 1998; Hall *et al.*, 1999), while Loss-Of-Function (LOF) mutations result in a protein that is unable to associate with CTR1 (Hua and Meyerowitz, 1998). LOF mutations cause truncations to the signalling portion of the ethylene receptor such that CTR1 is no longer activated (thus a *loss* of function). Loss of CTR1 activity alleviates the repression on the ethylene signalling pathway. Therefore LOF mutants have a constitutively *active* ethylene response pathway. GOF mutants, conversely, can never bind ethylene and thus CTR1 is constantly activated by the mutated ethylene receptor (thus a *gain* of function). These mutants, therefore, have a complete inhibition of the ethylene signalling pathway. Single loss-of-function mutants generally result in no obvious phenotype, although *etr1-7* has been shown to have increased sensitivity to ethylene (Cancel and Larsen, 2002). In the past, only triple LOF mutants, such as *etr1-7etr2-3ein4-4*, exhibit developmental characteristics consistent with a constitutive ethylene response (Hua and Meyerowitz, 1998). Study of both GOF and LOF ethylene receptor mutants help determine the impact of receptor function in ethylene responsive developmental pathways.

To better understand the role of ethylene in trichome morphogenesis, a screen of GOF and LOF ethylene receptor mutants for alterations in trichome branching was undertaken. Unlike all the other mutants, the *etr2-3* mutant has only unbranched trichomes, suggesting that this mutation impacts the early stages of trichome development. Here it is demonstrated that signalling through ETR2 participates in the control of microtubule dynamics, and that it is an upstream regulator of the TRY-mediated trichome branching pathway.

Materials and methods

Plant growth conditions

Arabidopsis thaliana seeds (source: *Arabidopsis* Biological Resource Center) were stratified for 4 d at 4 °C in the dark and grown in soil under a long photoperiod (16/8 h light/dark) at a light intensity of 180 $\mu E\ m^{-2}\ s^{-1}$ at the rosette level at 22 °C. For paclitaxol and oryzalin experiments, plants were grown on Murashige and Skoog basal salt mixture agar plates (Sigma, Oakville, ON, Canada) pH 5.7–5.8, 0.6% (w/v) agar for one week. Paclitaxol (MP Biomedicals, Solon, OH, USA) tests were performed according to Mathur and Chua (2000) (n=60 plants). Plant roots were soaked in an oryzalin (Riedel-de Haën, Pestanal) solution (0, 0.01, 0.1 or 1 μM) for 72 h and then root hairs were counted (n=900 root hairs per concentration). For epistatic analysis, all double mutants were followed to homozygous F_3 plants and were back-crossed to the wild type (Col-0) to ascertain the parental genotype.

Microscopy

The degree of trichome branching was analysed on leaf 5 of 30-d-old soil-grown *Arabidopsis* plants using a Carl Zeiss Stemi 2000-C dissecting microscope (Carl Zeiss, Germany). Representative images of leaves for the wild type (Col-0) and all single and double mutants were taken using a cryo-Scanning Electron Microscope as per Harrison *et al.* (2007). For analysis of trichome nuclear ploidy, trichomes were fixed and removed as per Zhang and Oppenheimer (2004) and stained with 4′6-diamino-2-phenylindole (DAPI) as per Folkers *et al.* (1997) and pictured using a Carl Zeiss Axioplan Fluorescent microscope (Carl Zeiss, Germany). Images were captured using an AxioCam HRc CCD camera and fluorescence levels of nuclei for the wild type and *etr2-3* were analysed using AlphaEase FC Imaging System software (Alpha Innotech; San Leandro, CA). Levels of endoreduplication were determined by comparing the fluorescence of stomatal guard cell nuclei, which are known to be 2C (Melaragno *et al.*, 1993), to the fluorescence of wild-type and *etr2-3* trichome nuclei. Levels of endoreduplication were normalized to the nearest multiple of 2C.

To determine the effect of the *etr2-3* mutation on the microtubule cytoskeleton, double mutants were constructed between *etr2-3* and *35S:MAP4-GFP* and the GFP-labelled cytoskeleton in the trichomes was viewed as per Mathur and Chua (2000).

Histochemical GUS assay

To analyse the cellular expression of *ETR2*, the entire 5′ upstream region of the gene was cloned using the primers: $ETR2_{pro}$ fw: 5′-GTCGACAGAAGAACGCATGAGAG-CC-3′; $ETR2_{pro}$ rv: 5′-CCATGGCACCACCATTGATA-GTATC-3′ into the pCAMBIA 1305.1 vector (Centre for the Application of Molecular Biology to International Agriculture, Canberra, Australia; http://www.cambia.org) and transformed into wild-type *Arabidopsis*, Columbia

ecotype, as per Clough and Bent (1998). Leaves from young and mature homozygous T_3 plants were GUS stained according to Regan *et al.* (1999) and photographed using either a Carl Zeiss Axioplan microscope or Carl Zeiss Stemi 2000-C dissecting microscope with an AxioCam HRc CCD camera.

Quantitative PCR

Quantitative PCR was used to determine the expression differences of several genes. Plants of the wild type (Col-0 and Enk-2), *etr2-1*, *etr2-3*, and *fas1-1* were grown as outlined above for 10 d at which point they were harvested and frozen in N_2. Total RNA was extracted using the Qiagen RNeasy Plant Kit (Mississauga, ON) according to the manufacturer's instructions and 1 μg of total RNA was used for the synthesis of cDNA using AMV reverse transcriptase (Promega; Madison, WI). At least three independent biological replicates were used for the wild type and each mutant with at least two technical replicates. Quantitative PCR was performed using the Cepheid OmniMix HS system following the manufacturer's instructions (Sunnyvale, CA) on the SmartCycler system (Cepheid; Sunnyvale, CA). Ubiquitin-10 was used as an internal control. Primers used for the analyses were as follows: *ANGUSTIFOLIA (AN)* fw: 5′-TCGCATACAGAAACA-AGGACAC-3′, *AN* rv: 5′-ACACGTCAAAACTATGG CTAGC-3′; *STICHEL (STI)* fw: 5′-GCTTTAGTAAAC-GAGCTAGTTGG-3′, *STI* rv: 5′-CTAGCTCGCTTAA-CAGTCTCTG-3′; *TRIPTYCHON (TRY)* fw: 5′-TCG-CCCTCCAT GACTCTGAAGAAG-3′, *TRY* rv: 5′-CTC-TTCCTGCTATCAAATCCCACC-3′; *ZWICHEL (ZWI)* fw: 5′-CCACAGTGTCTGATGCTGTTGAGGAG-3′; *ZWI* rv: 5′-CTGGAGGAGATCTCCAATATACTTGT-TATC-3′; *FASCIATA1 (FAS1)* fw: 5′-TTCTGAATCT-GTCTTTGGTGCTGGGAGACG-3′; *FAS1* rv: 5′-CCAT-GAATGATCGAATATCCACCTCACTCAGT-3′; *UBIQ-UITIN-10 (UBQ10)* fw: 5′-GTCCTCAGGCTCCGTGG-TG-3′; *UBQ10* rv: 5′-GCCATCCTCCAACTGCTTTC-3′.

Results

Loss-of-function ETR2 mutants display altered trichome branching

To determine if any of the *Arabidopsis* ethylene receptor GOF or LOF mutants had altered trichome development, as compared to the wild type (Columbia), mutant lines of all five *Arabidopsis* receptors were screened. Only LOF mutations to the ETR2 receptor caused trichome branching abnormalities among the ethylene receptor mutants tested (*etr1-1*, *etr1-5*, *etr1-6*, *etr1-7*, *ers1-1*, *ers1-3*, *ers2-1*, *ers2-3*, *etr2-1*, *etr2-2*, *etr2-3*, *ein4-1*, *ein4-4*, *ein4-7*). Wild-type rosette leaves in *Arabidopsis* have 1% two-branched trichomes, 97% three-branched trichomes, and 2% four-branched trichomes (as measured on leaf 5; n=798 trichomes). The LOF mutants of *ETR2* differed from the wild type (Fig. 1). While the GOF mutant, *etr2-1* (Fig. 1B), had trichomes similar to the wild type (Fig. 1A), with 2% two-branched trichomes, 93% three-branched trichomes, and 5% four-branched trichomes (n=950 trichomes), *etr2-2* (a LOF mutant; Fig. 1C) lacked four-branched trichomes and instead had 17% two-branched trichomes, and 83% three-branched trichomes (n=1349 trichomes). The most altered phenotype was observed in the LOF mutant *etr2-3* (Fig. 1D), which had 100% unbranched trichomes (n=1253

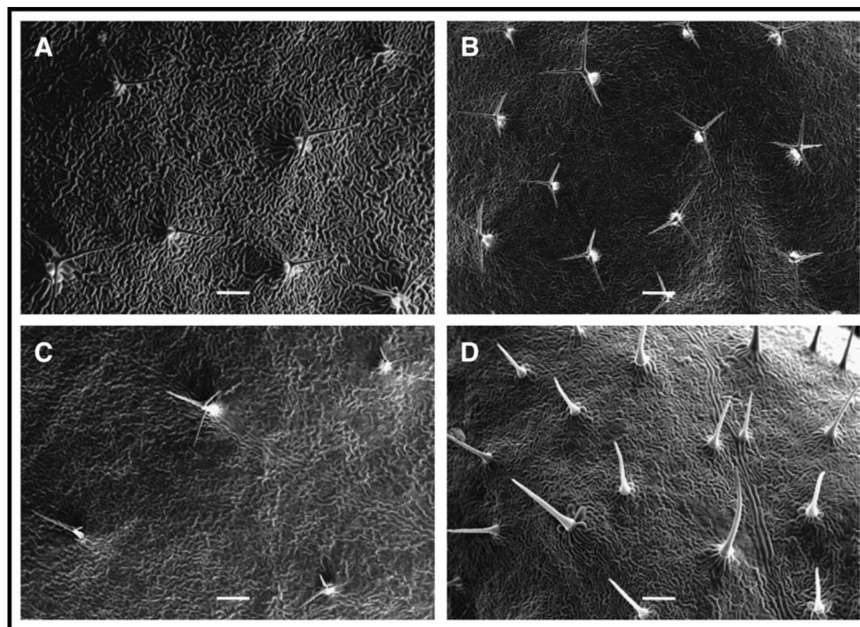

Fig. 1. Representative trichome branching in wild type and three *etr2* mutants. Trichomes of the wild type are predominantly three-branched (A) as are trichomes of *etr2-1* (B). The *etr2-2* mutant has a higher number of two-branched trichomes (C) while *etr2-3* has only unbranched trichomes (D). Scale bars=200 μm.

trichomes). This was true on rosette leaves, flowering stalks, and on sepals, as compared to the wild type which has both two-branched and unbranched trichomes on the flowering stalks and sepals. Representative images of the wild type, *etr2-1*, *etr2-2*, and *etr2-3* are shown in Fig. 1.

It has previously been shown that *ETR2* is expressed in mature leaves (Sakai *et al.*, 1998; Grefen *et al.*, 2008). Transgenic *Arabidopsis* expressing an $ETR2_{pro}$*:GUS* construct were created to determine if there is a cellular specific expression related to trichomes. It was found in all 13 lines recovered that *ETR2* is expressed in juvenile leaves, and that GUS expression was observed in young trichomes in half of these lines (Fig. 2A). During leaf expansion, *ETR2* expression became restricted to trichomes (Fig. 2B), while in fully expanded leaves no GUS staining was found in either the leaf epidermal cells or trichomes as has also been shown by Jakoby *et al.* (2008). These data demonstrate that *ETR2* is expressed in trichomes early in development during which the initiation of trichome branching occurs.

Microtubule cytoskeleton organization is altered in etr2-3

To date, mutations that cause a reduction in trichome branching either affect the level of endoreduplication within the cell or the organization of the microtubule cytoskeleton (Mathur and Chua, 2000). Trichomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) to determine the DNA content of *etr2-3* trichomes as compared to the wild type. Epidermal pavement cells generally have between 2C and 16C of DNA, while trichomes have between 4C to 64C, with the majority containing an average of 32C (Melaragno *et al.*, 1993). It was found that average DNA content between *etr2-3* and wild-type trichomes was not significantly different (Fig. 3A; P <0.05). This indicates that *ETR2* affects trichome branching in an endoreduplication-independent manner.

Assembly and structural arrangement of microtubules are maintained in part by MICROTUBULE-ASSOCIATED PROTEINs (MAPs) (Igarashi *et al.*, 2000; Smertenko *et al.*, 2000). This association has been exploited through the use of a GFP-labelled MAP4 protein to image the microtubule cytoskeleton in a number of living plant cell types (Olson *et al.*, 1994; Marc *et al.*, 1998). To image the microtubule cytoskeleton in developing trichomes, *etr2-3* was crossed with transgenic *Arabidopsis* expressing *35S:MICROTUBULE-ASSOCIATED PROTEIN4-GFP (35S:MAP4-GFP)*. Overexpression of the MAP4 has also been shown to cause stabilization of microtubule assembly (Marc *et al.*, 1998). In *35S:MAP4-GFP* lines this can lead to artefacts such as bulging hypocotyl cells, thickened trichomes or induced trichome branching. Despite this drawback, with careful screening these lines have been widely used throughout the literature (Olson *et al.*, 1995; Marc *et al.*, 1998; Kragler *et al.*, 2003; Shevchenko *et al.*, 2008). The *etr2-3 35S:MAP4-GFP* double mutants were therefore carefully screened for lines which did not display these artefacts. It was found that in the early stages of wild-type trichome outgrowth, the cortical microtubule cytoskeleton appears to criss-cross the stalk of the trichome. A similar pattern was observed in very young trichomes of *etr2-3*. Following trichome branching, the microtubule cytoskeleton re-aligns into a longitudinal orientation (Fig. 3B, inset). It is at this stage that differences between wild-type and *etr2-3* trichomes were observed. While some of the *etr2-3* trichomes showed normal longitudinal microtubules in nearly mature trichomes, a subset of trichomes exhibited a random and disorganized microtubule network that showed evidence of depolymerization (Fig. 3C, inset). Trichomes of *etr2-3* also lacked the dense knots of microtubules typically present in branching trichomes. There was no visible GFP signal in fully mature *etr2-3* trichomes. Thus, microtubule assembly in *etr2-3* trichomes nearing maturity do not consistently show the same organization as in the wild type.

In previously characterized mutants that show altered microtubule assembly, it has been shown that pharmacological stabilization of microtubules can induce branching in trichomes (Mathur and Chua, 2000). When *etr2-3* was treated with high concentrations of paclitaxol, a known stabilizer of microtubule assembly (Schiff *et al.*, 1979; De Brabander *et al.*, 1981; Verde *et al.*, 1991; Hyman and Karsenti, 1998), approximately 50% of the trichomes developed branch initials or unbranched stalks with incipient branch bulges (Fig. 3D). The cytoskeleton of 2 *etr2-3, 35S:MAP4-GFP* double mutant lines that were identified as having a highly stabilized microtubule cytoskeleton (evidenced by bulging hypocotyl cells, intense GFP signal, as

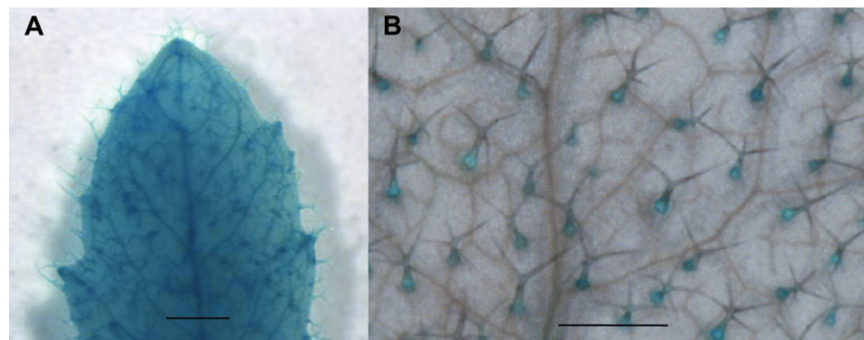

Fig. 2. *ETR2* is expressed in young leaves and mature trichomes. (A) $ETR2_{pro}$*:GUS* is expressed in very young leaves and trichomes (Scale bar=1 mm). (B) $ETR2_{pro}$*:GUS* is restricted to fully branched trichomes only (Scale bar=1 mm).

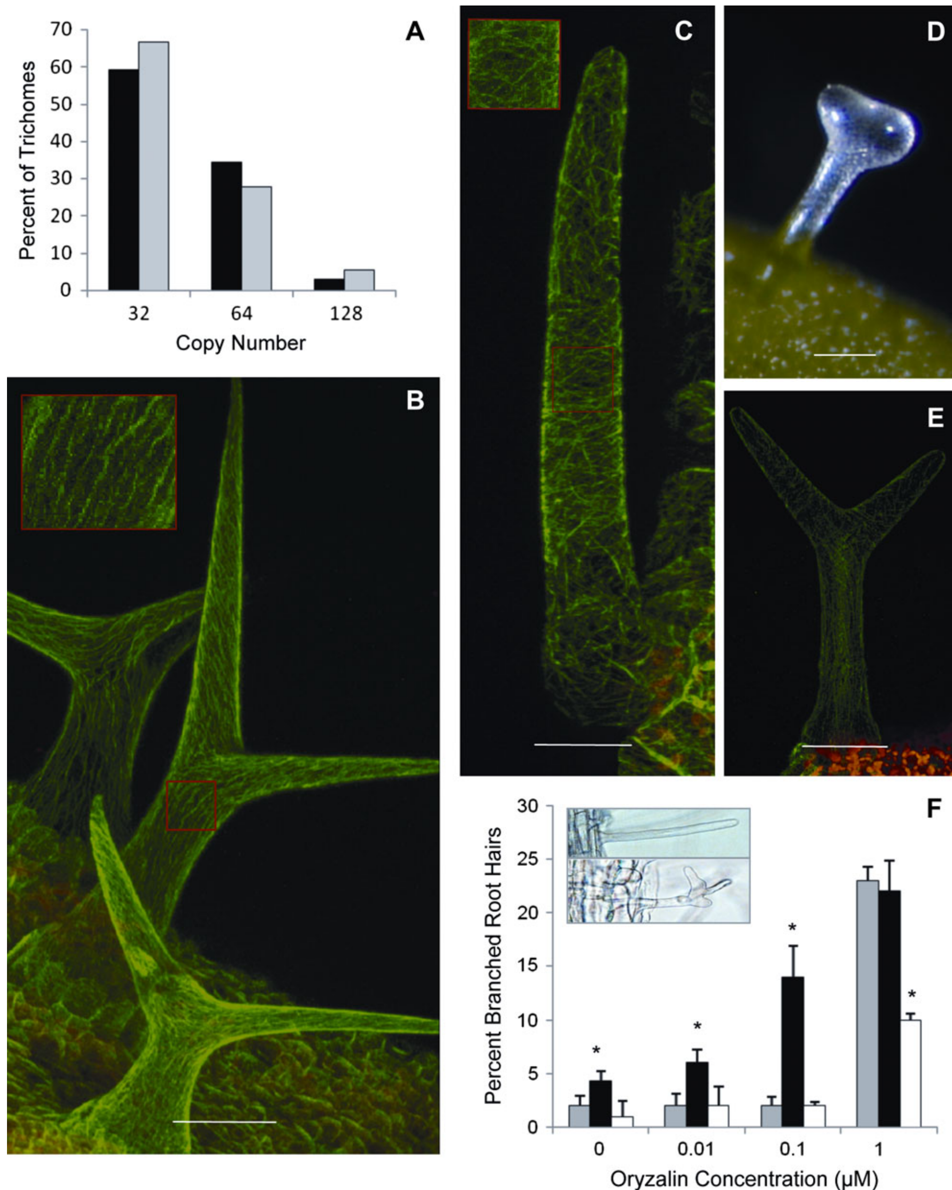

Fig. 3. ETR2 affects microtubule assembly. (A) Both wild-type (black bar) and *etr2-3* (grey bar) nuclei show the same level of endoreduplication (P <0.05, t test). (B) Wild-type trichomes expressing *35S:MAP4-GFP* show a longitudinal fine mesh of microtubules (scale bar=50 μm). Inset is a magnification of longitudinally oriented microtubules in the stalk of the trichome. (C) Some *etr2-3×35S:MAP4-GFP* trichomes nearing maturity show disorganized microtubule cytoskeleton and early signs of depolymerization at the base of the trichome (scale bar=30 μm). Inset is a magnification of disorganized microtubule assembly discussed in the text. (D) Branching is induced in *etr2-3* when treated with 20 μM paclitaxol for 2 h and then grown on MS medium until new leaves are produced (scale bar=100 μm). (E) *etr2-3×35S:MAP4-GFP* lines which express the highest level of MAP4-GFP show trichome branching and normal microtubule assembly (scale bar=70 μm). (F) Oryzalin causes depolymerization of microtubules and induces unbranched root hairs to become branched (inset). Bar chart of dose-response of the wild type (grey bars), *etr2-3* (black bars), and *etr2-1* (white bars) to different oryzalin concentrations. Asterisk indicates statistically significant difference from the wild type (P <0.05, t test).

well as a mixture of unbranched and branched trichomes) were also analysed. The two-branched trichomes in these lines had blunt-ended branch tips, a phenotype that has been associated with stabilized microtubules in *sti* (Mathur and Chua, 2000) and the microtubule cytoskeleton appeared normal (Fig. 3E). Therefore stabilization of microtubule assembly by either overexpression of MAP4 or application of paclitaxol can partially rescue the *etr2-3* mutant phenotype.

If the *etr2-3* mutant is affected in the assembly of the microtubule cytoskeleton, it should be possible to disrupt cytoskeletal assembly more easily in the *etr2-3* mutant compared to the wild type. This was studied by treating *etr2-1*, *etr2-3*, and the wild type with increasing concentrations of oryzalin. Oryzalin has been shown to depolymerize microtubules, leading to root hair branching, an increase in the girth of trichomes, and alteration of the shape of the root tip (Baskin *et al.*, 1994). While analysis of

root hair branching is not directly associated with trichome branching, branching of the root hairs can be accurately quantified and used to support our findings that cytoskeletal assembly is impaired in these mutants. In MS media, *etr2-3* had a higher number of branched root hairs than the wild type (Fig. 3F). Three days after the addition of 0.1 μM oryzalin to the media, the number of branched root hairs in *etr2-3* increased significantly as compared to the wild type or *etr2-1* (P <0.05). The wild type required a concentration of 1.0 μM oryzalin before a significant increase in root hair branching was detected. By contrast, the GOF mutant *etr2-1*, had a significantly lower number of branched root hairs at 1.0 μM oryzalin compared to the wild type (P <0.05). Therefore, LOF and GOF mutations to *ETR2* have opposite effects on the sensitivity to oryzalin.

Epistatic crosses and expression analysis place ETR2 in the trichome development pathway

The *etr2-3* mutant was crossed with several trichome branching mutants to gain insight into the position of ETR2 in the known trichome morphogenesis signalling pathway. Representative pictures of the original mutants (Fig. 4B, D, F, H, J) and the resulting double mutants (Fig. 4C, E, G, I, K, L) are shown and percentages of branch types are shown in Table 1. When crossed with *glabra3* (*gl3*), which has two-branched trichomes (Fig. 4B), the double mutant had puddle-like, unbranched trichomes that grew parallel to the leaf surface (Fig. 4C). When crossed with *spy-5* (Fig. 4D), a mutant in the gibberellin pathway with increased trichome branching, the resulting double mutant had a mixture of two- and three-branched trichomes (Fig. 4E). Crossing of *etr2-3* with *glabra2 (gl2)* (Fig. 4F) resulted in leaves with a mixture of two branched and unbranched trichomes (Fig. 4G). The *glabra2* and *etr2-3 glabra2* double mutants have approximately the same proportion of unbranched and two branched trichomes (Table 1). There were, however, distinct differences in the morphology of the trichomes between *gl2* and the double mutant. Trichomes of the double mutant were fully extended and sharp tipped. Conversely, trichomes on the first leaves of *gl2* either did not fully extend (for unbranched trichomes) or showed a high proportion of branched trichomes with very short branches. When *etr2-3* was crossed to *fasciata1 (fas1)*, a loss-of-function mutation to *CAF1* that has trichomes with increased branching (Fig. 4H), the resulting double mutant had 100% unbranched trichomes (Fig. 4I). Based on the epistatic relationship between *FAS1* and *ETR2*, *etr2-3* was crossed to *try*, a mutant with clumped trichomes that exhibits supernumerary branches. The *try etr2-3* double mutant had unbranched trichomes that, in a few cases appeared clumped (Fig. 4K). A cross was also performed between *fas1-1* and *try* and the double mutant had the trichome branching and clustering phenotype of *try* (Fig. 4L). Taken together, these results suggest that ETR2 signals in a pathway that is parallel to GL2, GL3, and gibberellic acid, but that ETR2 operates in the same pathway as CAF1 and TRY.

To characterize further how ETR2 affects the expression of genes involved in the regulation of trichome branching, the expression levels of five key trichome branching regulators were analysed (Fig. 4M). Expression was performed in *etr2-1*, *etr2-3*, and *fas1-1* and compared to their respective wild type (Col-0 for the first two mutants and Enk-2 for *fas1-1*). Because epistatic analysis suggested that ETR2 signals in the same pathway as CAF1, the expression level of *FAS1* in the *etr2-3* and *etr2-1* mutants was also tested. It was found that expression levels of *FAS1* in *etr2-3* were significantly increased, while they were not significantly affected in *etr2-1* (P <0.05). This would support the epistatic results which suggest that ETR2 acts upstream of CAF1. It was found that *TRY* expression was significantly increased in *etr2-3*, while it was significantly repressed in *etr2-1* (Fig. 4M). In the *fas1-1* mutant *TRY* expression is also significantly repressed. As TRY is a known regulator of *STI* and *ZWI*, the expression patterns of these two genes were considered. It was determined that *STI* was significantly down-regulated in *etr2-3* and increased in *etr2-1* but was unaffected in *fas1-1*. There was no significant difference in expression of *ZWI* in any of the mutants tested. As a control, the level of expression of *AN* was tested as it is a gene known to be in a separate trichome branching pathway from TRY, and presumably unaffected by ETR2. The expression levels in both ethylene mutants was not significantly different from the wild type (Fig. 4M). These data confirm that ETR2 is in the same signalling pathway as CAF1 and that downstream components of this regulatory network are TRY and STI.

Discussion

Re-orientation of cellular growth to produce branching in *Arabidopsis* trichomes is a highly orchestrated process that requires input from several independent pathways (Hülskamp *et al.*, 1994, 1999; Folkers *et al.*, 1997; Krishnakumar and Oppenheimer, 1999; Luo and Oppenheimer, 1999). There is some evidence that the plant hormone ethylene is implicated in regulating the development of trichomes in cucumber (Kazama *et al.*, 2004) and cotton (Shi *et al.*, 2006; Qin *et al.*, 2007). This study has demonstrated that LOF mutations to the *Arabidopsis* ethylene receptor *ETR2* yield leaf trichomes with fewer branches. This reduction in branching is most likely due to the altered stability of microtubule assembly, while the mutation does not affect the level of endoreduplication. Based on the epistatic and gene expression analyses presented here, a model whereby ETR2 affects *CAF1*, in the TRY branching pathway has been proposed (Fig. 5).

Multiple ethylene receptors have been conserved throughout the plant kingdom. While these receptors show a high degree of homology in the ethylene binding domain, they show sequence variability in the C-terminal signalling domain. Recent work has shown that this variability may allow for differential transduction of the ethylene signal (Cancel and Larsen, 2002; Grefen *et al.*, 2008; Gao *et al.*,

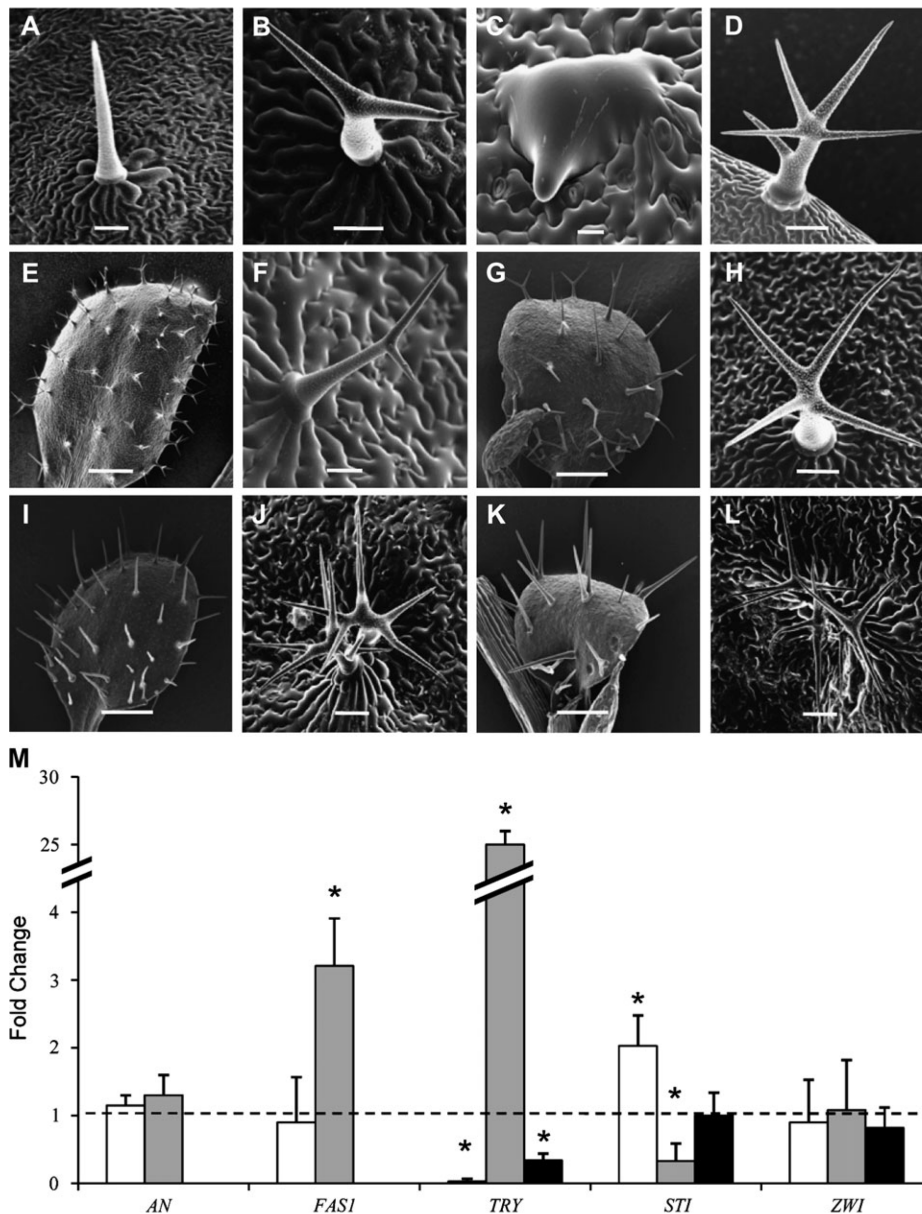

Fig. 4. Epistatic analysis places ETR2 in the TRY and CAF1 controlled pathway. Representative cryo-SEM images of (A) *etr2-3* (scale bar=100 μm), (B) *gl3* (scale bar=100 μm) (C) *etr2-3 gl3* (scale bar=10 μm), (D) *spy-5* (scale bar=100 μm), (E) *etr2-3 spy-5* (scale bar=1 mm), (F) *gl2* (scale bar=100 μm), (G) *etr2-3 gl2* (scale bar=1 mm), (H) *fas1* (scale bar=100 μm), (I) *etr2-3 fas1* (scale bar=100 μm). (J) *try* (scale bar=100 μm), (K) *try etr2-3* (scale bar=100 μm), (L) *try fas1-1* (scale bar=100 μm), (M) quantitative PCR analysis of *ANGUSTIFOLIA*, *TRYPTICHON*, and *STICHEL* expression in *etr2-1* (white bars), *etr2-3* (light grey bars), and *fas1-1* (black bars) (n=3–5) as compared to wild-type levels (dashed line). asterisk indicates significant differences from Col-0 (for *etr2-1* and *etr2-3*) and Enk-2 (for *fas1-1*) (P <0.05).

2008). The data presented here has shown that truncations to the signalling domain of the *Arabidopsis* ETR2 receptor affect trichome development and that the extent of these truncations is correlated with the severity of the trichome branching phenotype. In the *etr2-3* mutant, only 45% of the protein is made (Hua and Meyerowitz, 1998) and only unbranched trichomes are observed on the leaf surface. While in the *etr2-2* mutant, 73% of the protein is produced and this correlates with the appearance of a significantly higher number of two-branched trichomes, compared to the wild type. A similar dependence of phenotype on protein length has been previously described in LOF mutations to ETR1 with regard to plant growth when exposed to ethylene (Cancel and Larsen, 2002). This trichome phenotype is unique to the ETR2 receptor, as loss of part, or all, of the signalling domains in any of the other four *Arabidopsis* ethylene receptors did not affect trichome morphology.

Previous research suggests that two major pathways which contribute to alterations in trichome branch number is a result of different levels of endoreduplication in the cell or due to altered microtubule assembly (Hülskamp *et al.*, 1994,

Table 1. Trichome branching phenotypes of single and double mutants in the trichome branching pathway

Trichomes on leaf 2 or 3 of five plants were counted, and the relative percentages of each type of trichome recorded.

	Unbranched	2 Branches	3 Branches	4 Branches	5 Branches
Wild type		1	97	2	
etr2-3	100				
gl2	59	41			
etr2-3 gl2	57	41	2		
gl3	78	22			
etr2-3 gl3	97	3			
spy5			37	47	16
etr2-3 spy5		6	81	13	
fas1-1			52	27	21
etr2-3 fas1-1	100				
try[a]			34	54	12
etr2-3 try	100				

[a] Values from Perazza *et al.* (1999).

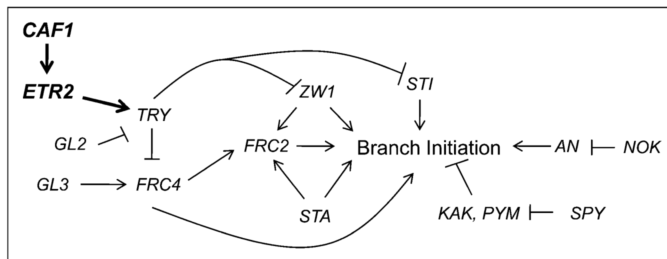

Fig. 5. Placement of ETR2 and CAF1 within the trichome branching pathway. Known network of trichome branching with *ETR2* and *CAF1* (in bold) as negative regulators of trichome branching through *TRY* (adapted from Luo and Oppenheimer (1999) with kind permission from The Company of Biologists).

1999; Oppenheimer *et al.*, 1997; Folkers *et al.*, 2002; Downes *et al.*, 2003; Castelano *et al.*, 2004; El Refy *et al.*, 2004; Desvoyes *et al.*, 2006; Ramirez-Parra *et al.*, 2007; Larson-Rabin *et al.*, 2009). Using analysis of ploidy level in both wild-type and *etr2-3* trichomes, it was demonstrated that average endoreduplication levels were not significantly different between the two plant lines. This led to the hypothesis that mutations to ETR2 were affecting the microtubule cytoskeleton. There is a growing body of evidence that ties ethylene to the control of microtubule orientation. Applications of 1-aminocyclopropane-1-carboxylic acid (ACC) to lettuce roots have been shown to induce randomization of the microtubule array and aid the induction of root hairs (Takahashi *et al.*, 2003). ACC and ethylene are also known to alter the re-orientation of microtubules into a longitudinal orientation (Apelbaum and Burg, 1971; Roberts *et al.*, 1984; Yuan *et al.*, 1994; Le *et al.*, 2004). Analysis of the microtubule cytoskeleton in developing *etr2-3* trichomes indicated that there was a higher degree of microtubule disorganization, compared to the wild type, and a complete lack of microtubule knots in the cytoskeleton that are normally associated with branch initiation. By stabilization of microtubule assembly through either pharmacological means (paclitaxol administration) or through transgenic strategies (overexpression of MAP4) induction of either branch initials or full trichome branching was achieved. Conversely, destabilization of the cytoskeleton was more easily accomplished at lower concentrations of oryzalin in *etr2-3*, compared to the wild type. These data would indicate that branch formation in *etr2-3* is impeded by the lack of proper microtubule organization, and that the LOF mutant still retains the ability to branch if microtubule assembly is artificially stabilized.

Two other proteins, STI and ZWI, have been proposed to have a similar stabilizing effect on microtubule assembly as their loss can also be partially replaced by paclitaxol (Ilgenfritz *et al.*, 2003). Based on epigenetic and gene expression analysis in the *etr2-3* mutant, a model is proposed concerning ETR2's role in trichome branching (Fig. 5). As *etr2-3* is a LOF mutant, ethylene responses controlled by ETR2 are constitutively active in this background. Therefore, in the model proposed, as *CAF1* expression was found to be increased in *etr2-3*, it can be hypothesized that ethylene bound to ETR2 would likewise cause an increase in *CAF1* expression. Through epigenetic silencing, CAF1 would then initiate a signal cascade that culminates in the repression of STI through increased activity of TRY. Exner and colleagues (2008) have implicated CAF1 in the control of STI and ZWI activity, although it was not known through what intermediate. Ethylene has previously been proposed to affect transcriptional control through the recruitment of histone deacetylases which would alter chromatin structure and block *cis*-elements (Pazin and Kadonaga, 1997; Fujimoto *et al.*, 2000; Zhou *et al.*, 2005). The demonstration that ethylene controls the epigenetic state of a cell through transcriptional control of CAF1 opens a new avenue through which ethylene signal transduction can control a suite of genes through the regulation of one intermediate. Interestingly, it was found that there were changes to *STI* expression in *etr2-3* and *etr2-1* but not in *fas1-1* (the CAF1 mutant), even though epigenetic analysis places ETR2 upstream of CAF1 (Fig. 5). This may indicate that CAF1 is not the only regulator through which ETR2 signals affecting STI. The results of this study, together with those of Exner and colleagues (2008), also indicate that CAF1 and ETR2 affect trichome branching independent of the GL2 and GL3 endoreduplication pathways (Fig. 5). This is striking as TRY, also implicated in the same pathway, is known to affect the level of endoreduplication in cells. However, as TRY can affect multiple cell developmental pathways independently (Hülskamp *et al.*, 1994; Schnittger *et al.*, 1999; Schellmann *et al.*, 2002), this may be a separate mechanism whereby TRY affects trichome branching independent of its role in the cell cycle.

It has been shown in this study that ETR2 is an important regulator of trichome development through the modulation of *CAF1*, *TRY*, and *STI* expression. The data from imaging of the microtubule cytoskeleton and through

drug treatment also suggest that signalling through ETR2 is responsible for maintaining microtubule stability. As mutations to the other ethylene receptors do not affect trichome morphology in a similar manner, this suggests that the ETR2 receptor has a unique role in mediating ethylene's involvement in the establishment of cell shape. This study is an important step forward in the understanding of how ethylene affects cellular development.

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