

The *Arabidopsis* ethylene overproducer mutant *eto1-3* displays enhanced freezing tolerance

Rafael Catalá and Julio Salinas*

Departamento de Biología Medioambiental; Centro Investigaciones Biológicas (CIB-CSIC); Madrid, Spain.

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Low temperature is one of the most important environmental stresses constraining plant development and distribution. Plants have evolved complex adaptive mechanisms to face and survive freezing temperatures. Different signaling pathways regulating plant response to cold have been described, and some of them are mediated by hormones. Recently, we reported that ethylene (ET) acts as a positive regulator of plant freezing tolerance through the activation of cold-induced gene expression, including the CBF-regulon. Here, we present data demonstrating that the *Arabidopsis* ET overproducer mutant *eto1-3* has enhanced freezing tolerance. Moreover, we also show that this mutant exhibits increased accumulation of *CBF1*, *2* and *3* transcripts, which should account for its tolerant phenotype. All these results constitute new genetic evidence supporting an important role for ET in plant response to low temperature by mediating the CBF-dependent signaling pathway.

Ethylene (ET) has a cardinal role integrating developmental events with external stimuli, including abiotic stresses.¹ Data reported by several authors have provided evidence for a positive role of ET in plant defense mechanisms against low temperature. It is well documented that exposure to cold activates ET biosynthesis in different species such as *Arabidopsis* or winter rye.²⁻⁸ Moreover, blocking the perception or synthesis of ET severely decreases tomato and tobacco tolerance to cold, indicating that this hormone is necessary for a correct response to low temperature.⁹ Furthermore, exogenous application of ACC, the ET precursor, significantly enhances tolerance to temperatures under 0°C in *Arabidopsis*, tomato and tobacco.^{8,9} On the other hand, Yu and colleagues (2001) described that the activation of ET production elicited by the exposition to 4°C elevates the tolerance of winter rye to subzero temperatures through the accumulation of antifreeze proteins.

Recently, the analysis of ET-defective and -overproducer *Arabidopsis* mutants allowed us to demonstrate that ET has a key function as positive regulator of freezing tolerance and cold acclimation, an adaptive response by which plants increase their freezing tolerance after being exposed to temperatures between 10 and 4°C.⁸ We also showed that ET participates in the reprogramming of gene expression that undergoes when plants are exposed to low temperature, activating, among others, the CBF-mediated signaling pathway,⁸ one of the main pathways controlling the cold acclimation response.¹⁰ Interestingly, our results supported a prominent role in this pathway for the *Arabidopsis* Ψ 14-3-3 isoform RCI1A in controlling the stability of ACC synthase (ACS), the key enzymes in ET biosynthesis.⁸

To further characterize the role of ET in plant response to low temperature, we have analyzed the constitutive freezing tolerance of the *Arabidopsis* ethylene overproducer mutant *eto1-3*.¹¹ The *eto1-3* mutation produces a truncate ETO1 protein that is unable of targeting ACS5 for proteasomal degradation, which consequently provokes an increase of ET in mutant plants.¹² As a first step, we determined whether under our standard growing conditions *eto1-3* plants also displayed increased ET levels compared to the wild-type genotype. **Figure 1A** shows that, in fact, ET content in 2-week-old *eto1-3* plants grown on soil under long day conditions at 20°C is higher than in wild-type plants. Then, we assessed the freezing tolerance of 2-week-old wild-type and *eto1-3* plants grown under control conditions and subsequently exposed to different subzero temperatures during 6h. Tolerance was estimated as the percentage of survivals after one week of recovery at standard growing conditions. Results showed that *eto1-3* mutants are significantly more tolerant to freezing than wild-type plants (**Fig. 1B and D**). The determined LT₅₀ (temperature that causes 50% of lethality) values were -6,4°C and -5,5°C, respectively (**Fig. 1B and D**).

Genome wide transcriptomic analysis of *Arabidopsis ACS octuple* mutants, which have reduced ET content, unveiled that basal levels of this hormone are required for accurate constitutive expression of a number of cold-induced genes, including the CBF-target *KIN1*.¹³ Moreover, we have reported that ET positively regulates the constitutive expression of *CBF1* and *CBF2*, as well as that of several genes of the CBF-regulon.⁸ Since *eto1-3* plants have elevated levels of ET, we hypothesized that this mutant should have increased content of *CBF* transcripts. To test this possibility, the expression levels of *CBF1*, *2* and *3* were determined by

*Correspondence to: Julio Salinas; Email: salinas@cib.csic.es

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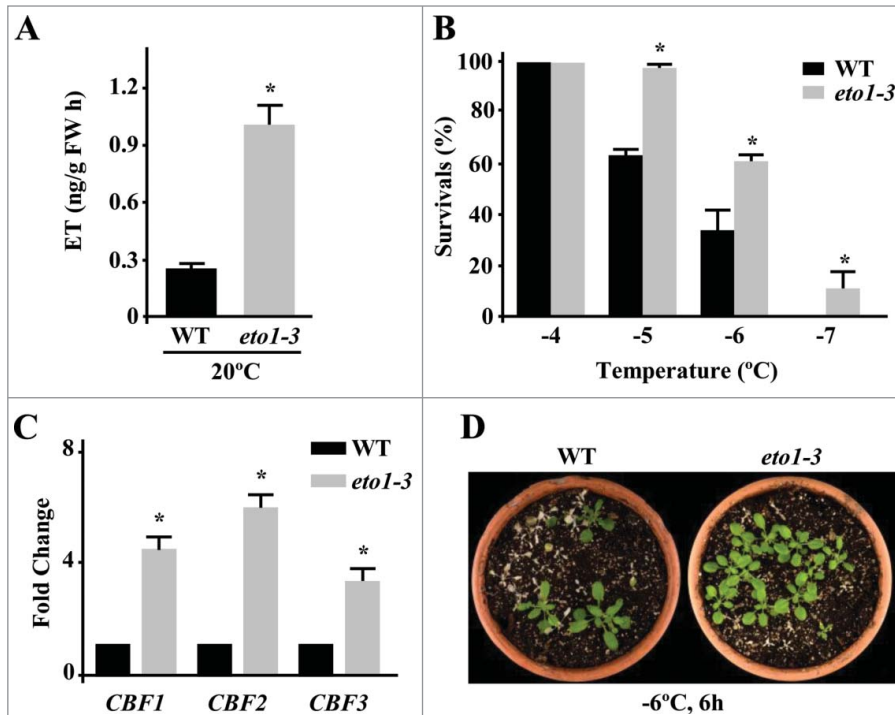


Figure 1. Arabidopsis *eto1-3* mutant plants display enhanced constitutive tolerance to freezing temperatures. (A) Levels of ET, as determined by gas chromatography, in 3-week-old Col-0 (WT) and *eto1-3* plants grown on soil under long day conditions at 20°C. Data are expressed as means of 3 independent experiments with 5 plants each. Bars indicate \pm SD. Asterisks indicate significant differences ($P < 0.05$) with WT plants. (B) Freezing tolerance of 2-week-old Col-0 (WT) and *eto1-3* plants grown on soil under long day conditions at 20°C and then exposed 6 hours to the indicated freezing temperatures. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7 days of recovery under control conditions. Data are expressed as means of 3 independent experiments with 50 plants each. Bars indicate \pm SD. Asterisks indicate significant differences ($P < 0.05$) with WT plants. (C) Expression levels of *CBF1*, *CBF2* and *CBF3* genes, as determined by qPCR, in 2-week-old Col-0 (WT) and *eto1-3* plants grown on soil under long day conditions at 20°C. Analyses were performed in triplicate with 3 independent RNA samples. Bars indicate \pm SD. Asterisks indicate significant differences ($P < 0.05$) with WT plants. (D) Freezing tolerance of representative WT and *eto1-3* plants 7 days after being exposed to -6°C for 6 hours (h).

qPCR in 2-week-old wild-type and *eto1-3* plants growing under standard conditions. Results revealed that, as hypothesized,

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transcripts corresponding to all *CBFs* accumulate significantly more in the mutant than in wild-type plants (Fig. 1C), which would account for its freezing tolerant phenotype. Consistent with these findings, we have described that an *RC1A* null mutant of *Arabidopsis* displaying enhanced ET levels exhibits increased constitutive expression of *CBF1* and *CBF2*, and augmented constitutive tolerance to subzero temperatures.⁸ In contrast with the results described here, Shi and colleagues (2012) reported that *eto1-3* plants display reduced tolerance to freezing temperatures. As already discussed in our previous work,⁸ these differences must be due, in all likelihood, to the differences existing between our growing conditions (soil in pots) and those used by Shi and colleagues (2012) (MS plates). In conclusion, the characterization of *eto1-3* freezing tolerance provides further genetic evidence hinting at the relevance of ET in the regulation of freezing tolerance through the control of the CBF gene expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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