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# Ethylene signaling modulates tomato pollen tube growth, through modifications of cell wall remodeling and calcium gradient

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## RUNNING TITLE

Ethylene and pollen tube growth

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## SUMMARY

Ethylene modulates plant developmental processes including flower development. Previous studies have suggested ethylene participation in pollen tube (PT) elongation, and both ethylene production and perception seem critical at fertilization time. The full gene set regulated by ethylene during PT growth is unknown. To study this, we used various ETHylene Receptors (ETRs) tomato mutants: *etr3-ko*, a loss-of-function (LOF) mutant; and *NR* (*Never Ripe*), a gain of function (GOF) mutant. The *etr3-ko* PTs grew faster than its wild type, WT. Oppositely, *NR* PT elongation was slower than its wild type, and PTs displayed larger diameters. *ETR* mutations created feedbacks on ethylene production. Furthermore, the ethylene treatment of germinating pollen grains increased PT length in *etr-ko* mutants and WT, but not in *NR*. Treatments with the

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ethylene perception inhibitor, 1-MCP, decreased PT length in *etr-ko* mutants and wild types, but had no effect on *NR*. This confirmed that ethylene regulates PT growth. The comparison of PT transcriptomes in LOF and GOF mutants, *etr3-ko* and *NR*, both mutated on the *ETR3* gene, revealed that ethylene perception has major impacts on cell wall- and calcium-related genes as confirmed by microscopic observations showing a modified distribution of the methylesterified homogalacturonan pectic motif and of calcium load. Our results establish links between PT growth, ethylene, calcium and cell wall metabolisms, and also constitute a transcriptomic resource.

## INTRODUCTION

The human population growth and increasing food demand make food security a priority for the future (Gustavsson et al., 2011). Therefore, research on plant reproduction is critical for increasing crop productivity and fruit/seed yields. Sexual plant reproduction starts with pollen grain landing onto the stigma where it hydrates and starts to germinate; then, the pollen tube (PT), carrying the two sperm cells, grows rapidly through the style and the ovary (Figure S1), to reach the ovule where the fertilization takes place (An et al., 2020). PT elongation is essential for fertilization (Michard et al., 2017). Many studies suggested that ethylene participates in this process (An et al., 2020). For example, a few hours after the penetration of PTs in the tomato stigma, a burst of ethylene was detected in the pistil (Llop-Tous et al., 2000). Ethylene signaling starts with the binding of ethylene to specific receptors (Chang, 2016; Binder, 2020). In tomato, there are seven Ethylene Receptors (ETRs), *S/ETR1-7*, which are located in the endoplasmic reticulum, and their amount varies over plant development (Chen et al., 2020a). ETRs are negative regulators of ethylene responses. In absence of ethylene, they maintain the downstream signaling partners in a “OFF” status, but in presence of ethylene their phosphorylation level changes and this turns “ON” the signaling partners (Bisson & Groth, 2015; Chang, 2016; Binder, 2020).

Some studies have highlighted the critical role of ethylene perception in PT elongation. For example, in *Petunia inflata*, ethylene perception was shown to be correlated with PT elongation (Holden et al., 2003). Indeed, the treatment with 1-MethylCycloPropene (1-MCP), an ethylene perception inhibitor, led to a strong inhibition of PT growth during the first six hours after pollination. Besides, Jia et al. (2018) have observed that ethylene promotes PT growth by interacting with cyclic Guanosine MonoPhosphate (cGMP) in *Arabidopsis thaliana*, thus leading to

an increased number of actin filaments in PTs, which are important for their polarized growth. Moreover, in an ethylene-insensitive mutant (*etr1-1*), PT growth was reduced (Jia et al., 2018).

During PT growth, a number of cell wall components like pectins, cellulose, hemicelluloses and callose are required to build up the cell wall architecture (Mollet et al., 2013). Pectins constitute a major component of the PT apical region, and the pectin methylesterase (PME) activity is critical for PT growth (Bosch et al., 2005). During fruit ripening, ethylene was shown to modulate the expression of genes encoding cell-wall modifying enzymes such as *PMEs* and polygalacturonases (*PGs*) (Brummel, 2006). *PGs* are also known to be critical for PT growth (Dearnaley and Daggard, 2001). But a clear link between ethylene and cell wall remodelling during PT growth is yet to be demonstrated.

PT elongation, morphology, and orientation have been shown to be highly dependent on ions choreography. Namely, the combination of potassium, chloride, calcium and proton gradients play crucial roles in the development of pollen grains (Michard et al., 2017). Tip-focused  $\text{Ca}^{2+}$  gradients generated by the interplay of  $\text{Ca}^{2+}$  pumps and channels are observed during PT elongation and are correlated with pulses in growth rate (Zheng et al., 2019). The link between  $\text{Ca}^{2+}$  and ethylene has been reported, in other organs than flowers. It was found that  $\text{Ca}^{2+}$  is required for ethylene biosynthesis (Jung., 2000; Petruzzelli et al., 2003; Li., 2018). Moreover, it was shown that ethylene could enhance the endogenous  $\text{Ca}^{2+}$  concentration in tobacco cell suspension cultures by activating a plasma membrane  $\text{Ca}^{2+}$ -permeable channel (Zhao et al., 2007). A recent study has shown that exogenous application of  $\text{Ca}^{2+}$  to root tissues was able to down-regulate the expression of *ETR* genes, and up-regulate gene expression of downstream elements of the ethylene signaling pathway (Yu et al., 2019).

In the present study, we aimed at investigating the role of *ETRs* during PT growth in tomato. After identifying the main *ETRs* expressed during PT growth, we studied the effects of mutations affecting *ETRs* with opposite effects, a loss-of-function (LOF) mutation in the *etr3* mutant, and a gain-of-function (GOF) mutant carrying a constitutive functional *ETR* (*NR*, for *NEVER RIPE*). A RNA-seq comparative study between *etr3-ko*, *NR* and their respective wild-types, led us to focus on  $\text{Ca}^{2+}$  signaling- and cell wall-related genes, and on pectin remodeling to understand the observed phenotypes. In this study, we show links between metabolisms related to ethylene, calcium and cell wall during PT growth.

## RESULTS

### **Expression of *ETRs* in germinating pollen grains**

In WT PTs, we observed that *ETR3*, *ETR4*, and *ETR5* were the *ETR* genes with the highest expression levels 4 h after pollen imbibition, without significant differences between them (Figure 1A). *ETR1*, 2 and 7 were expressed at significantly lower levels, and *ETR6* transcripts were below the detection threshold. A preliminary experiment showed that 4 h after imbibition was the optimum time at which we were able to collect a sufficient amount of raw material and extract total RNA of good quality, with PTs being still in a linear phase of growth (Figure S2). In the following parts, we will focus on *etr3*, since we have LOF and GOF mutants for this gene, in the search for changes in gene expression between the two mutant types, to reveal the metabolisms driving PT growth under ETR control. Moreover, ETR3 has been shown to be involved in PT growth (Jegadeesan et al., 2018).

### **ETRs modulate pollen tube growth, but not ethylene production by pollen tubes**

To test whether ETRs modulate PT growth, we used two *ETR3* mutants: the *etr3-ko* line, a LOF mutant, and the *NR* line, a GOF mutant of *etr3*, and their corresponding wild types, *i.e.* WT and WTB, respectively (see Experimental Procedures for details). After 4 h of imbibition, PT growth was increased in *etr3-ko*, whereas PT growth was reduced in *NR* (Figure 1B). We observed a weak ethylene production by PTs, but without any significant difference between the tomato lines (Figure 1C). A similar stimulation of PT growth was also observed in another LOF affecting *ETR4*, *etr4-ko* (Figure S2A detailing growth kinetics), and in addition, we observed that the PTs of the *etr3-ko/etr4-ko* double mutant were growing faster than those of WT, but not significantly faster than those of *etr3-ko* or *etr4-ko* single mutants (Figure S2B).

### **Ethylene stimulates pollen tube growth, whereas 1-MCP inhibits it**

We decided to check the impact of adding an ETR effector, the ethylene itself, or adding an ETR inhibitor, 1-methylcyclopropene (1-MCP) in the headspace above germinating pollen grains. In *etr3-ko*, and in both WT lines, the ethylene treatment stimulates PT elongation (Figure 1B). This was not the case in *NR*, which is ethylene-insensitive. On the contrary, the addition of 1-MCP limited PT growth in *etr3-ko* (Figure 1B), but no significant change was observed in both WT and *NR* lines. After showing that functional ETRs limit PT growth, an effect reversed by adding ethylene, we performed an RNA-seq experiment to look at changes in gene expression possibly driven by ETR signaling and impacting PT growth.

### **Transcriptome profiling of *etr3-ko* and *NR* pollen tubes**

We compared the PT mRNAs of two *etr3* mutant lines, *etr3-ko* (*ETR3* LOF) and *NR* (*ETR3* GOF), and their respective WT. Total RNA was extracted from PTs collected 4 h after pollen grain imbibition. The overall quality of sequencing data was very good with a mean read quality of 35 (Illumina 1.9 encoding) (Figure S3A). A cleaning step was performed as 40.8% of the reads still had sequencing adaptors. After this step, more than 94% of the reads were mapped without ambiguity and less than 4 % were mapped at more than one location (Figure S3B). This showed a very good specificity of sequences confirmed by RNA-seq quantification. As shown in Figure S3C, more than 90% of sequenced reads were assigned to genes after the mapping process, making the analysis even more robust.

A principal component analysis (PCA), carried out on normalized RNA-seq counts, showed that the four biological replicates were well-clustered in the four conditions WT, WTB, *etr3-ko*, and *NR* (Figure 2A). This showed that all samples were of good quality, and that the biological variability was smaller than differences between the four conditions of interest. Moreover, the PCA revealed that the horizontal axis (first axis of the PCA), carrying 42% of the variability, discriminated the *NR* mutant from the three other conditions, which meant that *NR* was the condition creating the biggest differences. On the second axis carrying 26% of the variability, this PCA also underlined that both WT lines were close to each other. It could also be seen that *etr3-ko* was closer to WTs than *NR*.

There were 2452 Differentially Expressed Genes (DEGs) in *NR* vs WTB and 551 DEGs in *etr3-ko* vs WT (Figure 2B): 1131 were up-regulated genes in *NR* vs WTB, and 253 in *etr3-ko* vs WT, whereas the numbers of down-regulated genes were 1321 and 298, respectively. To validate the quality of the RNA-seq data by RT-qPCR analyses, we chose 15 genes, encoding proteins possibly impacting PT growth, and ranging from low to high expression levels in the RNA-seq experiment (Table S2). The chosen genes were associated to functions about ethylene perception or synthesis, or related to cell wall metabolism or calcium signaling. The data were generated with the same RNA samples as for the RNA-seq analysis. The values obtained from the four biological replicates were averaged, thus the correlation calculation between RNA-seq and RT-qPCR values was performed over 60 data points. The results showed a Pearson correlation coefficient of 0.864, a *P* value of  $6.7 \cdot 10^{-19}$  and a power of 1 (Table S2). These were good indicators of transcriptomic data quality. Because previous studies showed that tomato *ETRs* expression is altered in *ETR* mutants (Tieman et al., 2000, Chen et al., 2019), we checked if this was the case for the seven *ETRs* in PTs. The expression level of three *ETRs* was altered in the mutants: *ETR4* and *ETR5* were up-regulated in *NR*, whereas *ETR3* was down-regulated in

*etr3-ko* (Figure 2C). These results were confirmed by RT-qPCR (Table S2). The expression of the other genes, tested in the qPCR check, did not reveal any trend or will be described below.

The full list of DEGs, genes with a  $P < 0.05$ , is available in Table S3. In the following part of the study, we focused on cell wall modification and calcium signaling based on the observation of DEG families (Figure 2D). This revealed that cell wall-related genes were up-regulated in *etr3-ko* vs WT and down-regulated in *NR* vs WT, whereas calcium signaling-related genes were strongly down-regulated in *NR* vs WT. The key genes attracting our attention, with opposite differences between GOF and LOF mutants were: pectin methylesterases (PMEs) involved in cell wall remodeling, and calcium-dependent protein kinases (CPKs), calcium-pumps (ACAs), and calcium channels (CNGCs) for calcium signaling. They will be detailed below.

### **Transcriptomic analysis of the cell wall-related genes in *etr3-ko* and *NR* pollen tubes**

In both comparisons (*NR* vs WT and *etr3-ko* vs WT), around 10% of genes showing modified transcript levels encode proteins involved in cell wall protein or polysaccharide biosynthesis, in signaling at the plasma membrane interface or in cell wall metabolism, e.g. cell wall polysaccharides modifications (Table S4). Among these genes, there were 192 DEGs in *NR* vs WT, and 59 in *etr3-ko* vs WT. The encoded cell wall-related proteins belong to five main functional classes: proteins acting on cell wall polysaccharides (29% in *NR* and in *etr3-ko*), proteases (11 vs 16%), proteins possibly related to lipid metabolism (9 vs 11%), proteins with interaction domains with cell wall polysaccharides or proteins (5 vs 10%) and proteins of yet unknown function (33% vs 20%). Only a few genes encoding oxido-reductases, mostly multicopper oxidases showed modified transcripts levels (Sedbrook et al., 2002). Among the proteins of yet unknown function, six DEGs in *NR* encoded proteins with six conserved cysteines exhibiting the same spacing as thionins (Silverstein et al., 2007).

A main feature regarding the cell wall-related genes was that 17% and 28% of them encoded proteins possibly involved in the pectin metabolism in *NR* vs WT and in *etr3-ko* vs WT, respectively (Figure 3 and Table S4). These proteins belong to several families: polygalacturonases (PGs), of the glycosyl hydrolase family 28 (GH28), pectin methylesterases (PMEs), of the carbohydrate esterase family 8 (CE8), pectate lyases (PLs) of the PL1 family, PME inhibitors (PMEIs), trichome birefringence-like (TBLs) protein family and Pro-rich, Arabinogalactan conserved Cysteines (PAC) proteins (Hocq et al., 2017, Stranne et al., 2018, Nguyen-Kim et al., 2020). The number of DEGs encoding PGs and PMEs was higher in *NR* vs WT than in *etr3-ko* vs WT. In *NR*, four *PG* genes had increased transcript levels, whereas four

had decreased levels. Regarding the *PME* genes, three genes among the most highly expressed had slightly increased transcript levels, whereas seven *PME* genes were down-regulated (Table S4). In *etr3-ko*, four *PME* genes had higher transcript levels than in WT, among which two of the most highly expressed (Table S4). The *PL* genes showed the same kind of regulation in *NR* and *etr3*, with decreased transcript levels for four genes in *NR* and two in *etr3-ko*, and increased transcript levels for one gene in each case. In *etr3-ko*, two of the most highly expressed genes, *Solyc01g068120* and *Solyc06g084620*, encode PMEs and showed increased transcript levels compared to WT. The transcript level of two *PMEI* genes was decreased in *NR* whereas the situation was more contrasted in *etr3-ko* with one gene having more transcripts and another one less. *TBL* genes also exhibited a complex situation with a reduced transcript levels for one gene in *NR* and an increased transcript levels for two genes in *NR* or of one gene in *etr3-ko*. This could be due to the fact that the encoded enzymes could have different types of substrates, either hemicelluloses or pectins. Finally, the transcript levels of two genes encoding PAC proteins showed opposite variations in *NR*, whereas one of them had less transcripts in *etr3-ko*.

Altogether, a significant proportion of the cell wall-related DEGs encoded proteins (i) exhibiting enzymatic activities (PG, PME, PL, PAE, and possibly TBL) directed against pectin polymers such as homogalacturonans (HGs) or rhamnogalacturonan I (RG-I) (Hocq et al., 2017, Stranne et al., 2018), (ii) regulating the enzymatic activity of PME (PMEI) (Giovane et al., 2004) or (iii) able to interact with RG-I (Hijazi et al., 2014).

Moreover, we observed that exogenous ethylene application stimulated the expression of three genes encoding PMEs (*Solyc01g057220*, *Solyc05g052120* and *Solyc12g099410*) previously identified in the RNA-seq data set, and the application of 1-MCP slightly lowered their expression (Figure S4A). This further indicates that the expression of these genes is modulated by ethylene signaling.

### **Transcriptomic analyses of calcium signaling-related genes in *NR* and *etr3-ko* pollen tubes**

The expression of 47  $\text{Ca}^{2+}$  signaling-related genes was significantly altered in *NR* and *etr3-ko* (Figure 4). There were 34 DEGs (26 down- and 8 up-regulated) in *NR*, and 13 DEGs (6 down- and 7 up-regulated) in *etr3-ko* PTs. According to homologies with TAIR 11 annotation (Table S5), they belong to classes of  $\text{Ca}^{2+}$  pumps,  $\text{Ca}^{2+}$  channels, and  $\text{Ca}^{2+}$  sensors such as calmodulin-binding proteins (CaMBPs), calmodulin-like proteins (CMLs) or  $\text{Ca}^{2+}$  effectors including  $\text{Ca}^{2+}$ -dependent protein kinases (CPKs) and CBL-interacting protein kinases (CIPKs). The DEGs

coding for some proteins of similar function, were up-regulated in *NR* and down-regulated in *etr3-ko*, or inversely. This could be related to inverse PT growth response in these mutants. Among these, *CPKs* were down-regulated in *NR* and up-regulated in *etr3-ko*. In contrast, *CIPKs* were down-regulated in *NR*, but not significantly affected in *etr3-ko*. Globally in *NR*, there were more down-regulated *CML* and *CaM* genes than in *etr3-ko*. Some of these down-regulated genes exhibited a high number of RNA copies, like *Solyc06g068960*, *Solyc05g050750* and *Solyc02g067220*, when looking at the 'base mean' (Table S5), thus even DEGs with a small log2 fold change may have an impact on cell functions.

Additional results showed that exogenous ethylene application stimulated the expression of three  $Ca^{2+}$  signaling-related genes previously identified in the RNA-seq data set (*Solyc01g010020*, and *Solyc02g063340*, encoding calmodulins, and *Solyc03g123890* encoding a  $Ca^{2+}$ -transporting ATPase 1), and that the application of 1-MCP inhibited their expression (Figure S4B). This further shows that expression of these calcium signaling-related genes is modulated by ethylene signaling.

#### **Immuno-localization of homogalacturonans in *etr3-ko* and *NR* pollen tubes**

To check possible modifications of HGs in *etr3-ko* and *NR* PTs, we assessed the distribution of weakly and highly methylesterified HGs using LM19 and LM20, respectively. Labeling with LM20 revealed that the highly methylesterified HGs was, as expected, mainly located at the tip of the WT, WTB and *etr3-ko* PTs (Figure 5A). However, an unusual ring-like labeling pattern was observed along the *NR* PT cell wall (Figure 5A). Fluorescence intensity was measured in PT tips, using the same delimited area. The LM20 fluorescence intensity in *etr3-ko* PT apex slightly decreased compared to the WT, without being significant, while in *NR* PT tip, the intensity was significantly higher than in WTB and other lines (Figure 5B). Finally, the diameters of PTs grown in liquid and solid growth medium (GM) were measured in the tip region (Figure 5 C-D). No difference was observed in solid GM (Figure 5D), whereas in liquid GM, the diameters of *etr3-ko* PTs were smaller than in WT, while *NR* PT diameters were larger than in WTB (Figure 5 C-D).

The immuno-localization with LM19 of epitopes associated with weakly methylesterified HGs gave no significant difference (Figure S5A and B): 50% and 57% of the PT tips of WT and WTB were labeled, respectively. In *NR*, 39% of the PT tips were labeled, vs 67% in *etr3-ko*.

More LM19 labeling means an increase in cell wall stiffness (Parre and Geitmann, 2005), but no conclusion can be taken here. In addition to immuno-localization, the total PME activity was assayed on whole protein extracts of PTs, but no significant difference was observed, neither

looking at activity per fresh mass (Figure S5C), nor looking at specific activity per  $\mu\text{g}$  proteins (Figure S5D).

### **Calcium load in pollen tube tips is modulated by ETRs**

To verify the ethylene signaling ability to change  $\text{Ca}^{2+}$  ion dynamics in PT apex, PTs were loaded with fluo-4/AM to highlight  $\text{Ca}^{2+}$  gradient from the tip to the base (Figure 6). Calcium loading was observed in WT, WTB, *NR* and *etr3-ko* PT tips (Figure 6: panels A-D) and the zone over which fluorescence intensity was quantified is delimited by a yellow dashed line (see Methods for timing details). The fluorescence intensity in the apex region was higher in *etr3-ko* compared with WT, while it was lower in *NR* compared with WTB (Figure 6E), indicating that ethylene signaling modulates the  $\text{Ca}^{2+}$  accumulation in the PT apex. This corroborates RNA-seq results, as ethylene signaling modulates the expression of several genes encoding pumps and channels involved in  $\text{Ca}^{2+}$  homeostasis, or sensors (CaMs, CMLs) and effectors (CDPKs, CIPKs) involved in  $\text{Ca}^{2+}$  decoding. In addition to microscopic imaging, we observed the effects of lanthanum, an inhibitor of calcium transport (Qu et al., 2016) on the PT growth of the different lines. Figure S6 shows that the application of ethylene increased  $\text{Ca}^{2+}$  accumulation in the WT PTs and that application of 1-MCP decreased it. We also observed that the growth of all PTs was stopped after 2 h of lanthanum ( $\text{La}^{3+}$ ) treatment, regardless of the tomato line and that the  $\text{Ca}^{2+}$  accumulation in PT decreased within 30 min following the  $\text{La}^{3+}$  treatment.

## **DISCUSSION**

### **Ethylene signaling modulates pollen tube growth**

A few previous studies have suggested a role for ethylene in PT growth (Holden et al., 2003; Jia et al., 2018), but the expression of *ETRs* in PTs has been rarely analysed. We observed that three genes, *ETR3*, *ETR4* and *ETR5*, are expressed in tomato growing PTs, confirming recent observations showing expression of the same three *ETRs* in tomato flower tissues (Jegadeesan et al., 2018).

Using various *etr* mutants, we observed that LOF *etr3-ko* and *etr4-ko* led to longer PTs, and GOF *NR*, led to shorter PTs. This latter result confirms recent observations of shorter PTs in the *Arabidopsis etr1-1* GOF mutant (Jia et al., 2018). The ethylene being produced without significant difference between all lines, this suggests that PT growth depends on signaling arising from ETRs. Then, PT growth enhancement observed in WT and WTB after adding ethylene suggests that ethylene releases the blocking effect imposed by ETRs, while the dominant *NR* mutation imposes a strong inhibition that ethylene cannot release at the chosen dose. Additionally, our

results showing a significant increase in PT length upon ethylene treatment of the *etr3-ko* line, suggest that, as expected, knocking-out one *ETR* renders PTs more sensitive to ethylene signaling. PT growth stimulation in LOF mutants was modulated with a low dose of 1-MCP, and a significant decrease in PT length was observed in *etr3-ko*, whereas no significant decrease in PT length was observed in WT and WTB. Here, we confirm results regarding the effects of 1-MCP on PT growth in *P. inflata* (Holden et al., 2003), but unlike *Arabidopsis* (Jia et al., 2018), tomato PT growth does not appear to be governed by *ETR1* alone. Altogether, it seems that ethylene signaling partially governs PT elongation. Indeed, blocking ethylene action by 1-MCP led to minimal PT growth, whatever the *ETR* alteration.

Before these experiments with LOF and GOF mutants, we ran preliminary trials with WT at various doses of ethylene and 1-MCP to determine optimal concentrations (Figure S7). The best stimulation of PT growth by ethylene was observed at 100 ppm, but adding ethylene up to 1000 ppm led to a decreasing effect on PT growth, may be due to slight toxicity. For 1-MCP, the dose chosen for all trials was 2 ppm, as the PT growth decrease seems to saturate beyond 4 ppm. It can be postulated that the remaining PT growth is independent of ethylene signaling.

### **Transcriptome profiling of *NR* and *etr3-ko* PTs revealed that the GOF mutation has a stronger impact than the LOF mutation**

Then we detected gene expression changes in *ETR3* mutants using a RNA-seq survey. We showed that the number of DEGs between *NR* and WTB was higher than between *etr3-ko* and WT. These observations are logical considering that in *NR*, the ethylene-insensitive *ETR3*, dominates the signaling of the surrounding *ETRs* and blocks ethylene signaling (Chen et al., 2019), while the *etr3-ko* mutation impairs only one out of the three mostly expressed *ETRs* in PTs.

In addition, the expression of *ETR3* was repressed in *etr3-ko*, without any consequence as a functional *ETR3* protein is not produced. This negative feedback on other *ETRs* is not similar to recent observations in *etr7-ko* fruit tissues in which the expression of *ETR3*, *ETR4*, *ETR5* and *ETR6* was increased at the inception of ripening (Chen et al., 2020a). Thus, it is possible that the regulation of *ETR* expression is tissue-dependent. It is also possible that *ETR3* and *ETR7* do not exert similar feedback on *ETR* expression. In *NR*, the *ETR4* and *ETR5* transcripts are up-regulated. The related proteins would increase the potential blockage of PT growth.

## Cell wall remodeling is a main output of ethylene signaling in pollen tubes: A focus on pectins

The transcriptomics study revealed the importance of the changes observed in the expression levels of cell wall-related genes in both *NR* and *etr3-ko* PTs. The expression of genes encoding proteins involved in HG remodeling was the most impacted, as expected since HGs are abundant pectin components of PT cell walls (Dehors et al., 2019). Consequently, two kinds of enzymes could be modulated: HG-modifying enzymes demethylating or deacetylating HGs, such as *PMEs* and *PAEs*; or enzymes degrading HGs, thus producing oligogalacturonides, such as *PGs* or *PLs* (Hocq et al., 2017). In *NR* PTs, seven *PME*-, four *PG*-, and four *PL*-encoding genes were down-regulated. This observation is consistent with PT length reduction. Indeed, PT elongation requires cell wall flexibility brought in part by the active remodeling of HGs at the PT tip, such as demethylation and reduction of HG chain length. When these modifications do not happen, the cell wall remains stiff, thus inhibiting PT elongation. Conversely, only a few genes encoding pectin-related genes were DEGs in *etr3-ko* PTs. Four *PMEs* were only slightly up-regulated, suggesting a little effect on HG methylesterification, but sufficient to explain the fastest growth compared to the other lines. Only one of these four *PMEs* was clearly down-regulated in *NR*, *Solyc05g054360*. Its level of expression went down from a log2 of 0.74 in *etr3-ko* to 0.1 in *NR*. Thus, the massive decrease in *PME* expression levels in *NR* was related to other genes.

Besides, transcript levels of *TBL*-encoding genes were strongly modified in *NR* and in *etr3-ko*. The precise role of the *Arabidopsis* 46 *TBLs* is unknown. However, they were grouped in five clusters, and clusters IV and V are linked to *O*-acetylation of hemicelluloses, like xyloglucan, mannan and xylan, whereas the other clusters gather *TBLs* possibly involved in *O*-acetylation of pectins (Stranne et al., 2018). The two *TBL* genes up-regulated in *NR* are homologous to *Arabidopsis* *TBLs* of cluster IV and V (*Solyc07g053330*, 2-fold; *Solyc10g009590*, 52-fold), suggesting a re-organization of the cell wall to cope with changes occurring at the pectin level. The third *TBL* gene with modified level of transcripts is down-regulated (about 2-fold) and is homologous to *Arabidopsis* *TBL* genes of cluster II among which the *TBL44/PMR5* gene was shown to play a role in pectin esterification (Vogel et al., 2004). The expression of the *Solyc07g053330* gene, coding for a putative cell wall polysaccharide *O*-acetyltransferase, was lower in *etr3-ko* than in *NR*, also suggesting modifications of hemicelluloses.

Since pectins are critical components for cell wall changes at the PT apex (Mollet et al, 2013), mainly by affecting cell wall rheological properties (Parre and Geitmann, 2005), and since regulation of genes involved in HG remodeling was affected in *NR* and *etr3-ko*, our study focused

on HG distribution in PT cell walls. In WT, the weakly methylesterified HGs are located in the PT shank where they can be cross-linked by  $\text{Ca}^{2+}$  ions, promoting cell wall stiffness and providing mechanical support for the tip-polarized elongation (Dardelle et al., 2010; Chebli et al., 2012; Mollet et al., 2013; Dehors et al., 2019). In contrast, the highly methylesterified HGs are mainly located at the PT apical region providing sufficient flexibility required for the turgor-driven PT elongation (Parre & Geitmann, 2005; Vogler et al., 2013; Mollet et al., 2013; Hepler et al., 2013)

The surprising LM20 labeling has revealed rings of highly methylesterified HGs all along *NR* PTs. These observations could be related to the down-regulation of eight *PMEs* and associated to the pulsating-growth of PTs. As such, in *Arabidopsis pme48* pollen grains, in which *PME* activity was reduced and the degree of methylesterification of HGs was higher than in WT, germination was delayed and PT length was reduced (Leroux et al., 2015). On the other hand, in *Solanum chacoense*, exogenous application of *PME* to germinating pollen grains induced a reduction of PT growth, an increase of cell wall stiffness and a reduction of its visco-elasticity (Parre and Geitmann, 2005), suggesting that tight regulations of *PME* activity are required for proper cell wall remodeling and PT growth. The fact that no change in global *PME* activity was observed among the various lines is not surprising, as temporal and spatial changes revealed by the immunolocalization of methylesterified HG are more refined than the measurement of global activity after crushing whole PTs.

The rate of PT elongation is determined by the equilibrium between turgor-pressure and cell wall ability to extend under this pressure (Kroeger et al, 2011). We observed that PTs exhibited larger diameters in *NR* than in WT in liquid medium only. In contrast, in *etr3-ko*, the diameter of PTs in liquid medium was smaller than in WT. The down-regulation of *PMEs* in *NR* presumably did not allow the proper demethylesterification of HGs in the sub-apical dome of the tip and consequently the normal  $\text{Ca}^{2+}$  bridging of deesterified HGs, thus decreasing the stiffness of the cell wall in the shank of PTs. This led to an increase of PT diameter under the internal turgor-pressure. This increase was also observed in liquid medium in *pme48* (Leroux et al., 2015). These data demonstrate that solid medium can provide a mechanical support to PTs and that the stiffness of the medium can also differently modulate PT growth (Gossot & Geitmann, 2007; Reimann et al., 2020).

Taken together, our results suggest that ethylene signaling can modify the cell wall structure of PTs by regulating the transcription of genes encoding enzymes involved in pectin and hemicellulose modifications. These changes, especially those observed in the degree of

methylesterification of HGs, induce a reduction in the length of PTs and an increase in their diameters when ethylene perception is constitutive as in *NR*, and the reverse phenotype when one of the major ETRs present in PTs is inactive like in *etr3-ko*.

### **Calcium signaling is a main target of ethylene signaling in pollen tubes**

Tip-focused  $\text{Ca}^{2+}$  gradients generated by interplay between  $\text{Ca}^{2+}$  pumps and channels are observed during PT elongation and correlated with pulses in growth rates (Pierson et al., 1996; and for a recent review see Zheng et al., 2019). In our study, more than 30 genes coding for proteins involved in  $\text{Ca}^{2+}$  homeostasis and decoding were de-regulated in the two *ETR* mutants. Such a high DEG number indicates the tight link that exists between ethylene and  $\text{Ca}^{2+}$  signaling, and highlights the importance of the latter in modulating tomato PT elongation. More than 25 of these DEGs were repressed in *NR*.

Interestingly, a few DEGs were inversely de-regulated in *NR* and *etr3-ko*, in agreement with the observed opposite growth and tip-focused  $\text{Ca}^{2+}$  gradient phenotypes. This is the case for the calreticulin gene (*Solyc01g100380*), which expression is down-regulated in *etr3-ko* and up-regulated in *NR*. This protein was already shown to be important for  $\text{Ca}^{2+}$  homeostasis during PT growth in *P. hybrida* (Suwinska et al., 2017). Conversely, *CPK17* (*Solyc12g099790*) is up-regulated in *etr3-ko* and down-regulated in *NR*. *CPK17* was shown to be preferentially expressed in *Arabidopsis* PTs and to have a redundant function with *CPK34* during PT growth and tropism (Myers et al., 2009). The PTs of the double mutant *cpk17/cpk34* display a slower growth and impaired tropism. The *CPK17* ortholog being up-regulated in *etr3-ko* and down-regulated in *NR* could fit with longer PTs observed in *etr3-ko* and shorter PTs observed in *NR*. Genes encoding two other  $\text{Ca}^{2+}$  effectors, *CPK20* (*Solyc10g076900*) and *calcineurin B-like protein3* (*CBL3*) (*Solyc07g065820*) are also up-regulated in *etr3-ko* PTs. These two  $\text{Ca}^{2+}$  effectors have been reported to be associated with PT growth by activating the slow anion channel SLAH3 at the PT tip (Gutermuth et al., 2013), whereas CBL3 activates CIPK12 to promote a fast PT growth (Steinhorst et al., 2015). The gene (*Solyc10g086060*) that showed the strongest amplitude between inhibition (in *NR*) and stimulation (in *etr3-ko*) is homologous to the *Arabidopsis* gene *At5g03040*. This gene encodes the  $\text{Ca}^{2+}$ -calmodulin binding protein IQD2, of the large IQD family. These family members participate in microtubule organization during plant development and are modulated by auxin (Bürstenbinder et al., 2017). Here, it appears that removing the ETR3, in the LOF, leads to the up-regulation of *IQD2* that could promote PT expansion by favoring the cytoskeleton organization required for a proper elongation of the cell.

Regarding *NR*, there were only a few up-regulated genes: two encoding Ca<sup>2+</sup>-ATPases, *calcium-transporting ATPase 4 (ECA4) (Solyc11g072880)*, *ACA9 (Solyc03g123890)* and one encoding CML7 (*Solyc06g083000*). Considering that PTs were shorter in *NR* than in *etr3-ko*, we can speculate that these ATPases and CML7 could contribute to dissipate the Ca<sup>2+</sup> gradient by extruding the Ca<sup>2+</sup> ions from the cytosol. Indeed, in *NR*, the Fluo-4 fluorescence is systematically lower than in *WTB*, indicating a lower Ca<sup>2+</sup> concentration in PT tips. However, we cannot exclude a membrane permeability disturbance due to the mutation, which would affect the loading of the probe in PTs.

In *NR*, there were down-regulated genes encoding the CMLs 3, 13, 16, 27, 28 and 46 (*Solyc05g050750*, *Solyc11g072270*, *Solyc01g010020*, *Solyc06g068960*, *Solyc02g063340* and *Solyc02g067220*, respectively) and two genes encoding cyclic nucleotide-gated ion channels, CNGC8 and 16 (*Solyc03g116850* and *Solyc03g114110*, respectively). CNGCs together with iGLUR channels are the main players in generating the Ca<sup>2+</sup> influx in PTs (Michard et al., 2017; Gao et al., 2016; Tunc-Ozdemir et al, 2013). CNGC8 was recently shown to be involved in PT growth by antagonizing CNGC18 (Pan et al., 2019). With respect to shorter PTs observed in *NR*, a reduced activity of these CNGC channels in conjunction with a supposed increased activity of Ca<sup>2+</sup>-ATPases could result in a disruption/mitigation of the tip-focused Ca<sup>2+</sup> gradient as confirmed by the lower Fluo-4 fluorescence observed in PT tips. Concerning CMLs, little is known about their targets in plants but most of the genes cited above have been already shown to be up-regulated during pollen germination and/or PT growth (Wang et al 2008). Moreover, it is not excluded that one of these down-regulated genes encode CMLs that could participate in either a positive or a negative regulation of one of the two identified Ca<sup>2+</sup>-ATPases as already shown (Astegno et al., 2017). In addition, several other genes belonging to the Ca<sup>2+</sup> toolkit are also down-regulated including several genes encoding CIPKs or cation exchangers. These are known to be directly involved in the regulation of ions channels governing ion homeostasis during PT growth. The *Arabidopsis* gene homologous to the down-regulated *annexin 5* gene (*Solyc04g008270*) was shown to be involved in pollen development and PT growth by promoting Ca<sup>2+</sup>-dependent membrane trafficking necessary for PT elongation (Zhu et al., 2014). The additional experiment, using La<sup>3+</sup> to block calcium fluxes, proved that calcium is downstream of the ethylene signal, as no PT elongation was observed in any of the tomato lines in the presence of lanthanum, whatever the signals delivered by ETRs.

Overall, the calcium signaling-related DEGs in the two *ETR* mutants similarly point out the crucial role of ethylene in controlling the tip-focused Ca<sup>2+</sup> gradient required for PT growth. The fact that

the application of lanthanum inhibits calcium transport in all lines, wild types and loss- or gain-of-function *ETR* mutants, is a proof that calcium transport is downstream of the ethylene signaling.

In summary (Figure 6F), our work suggests that *ETR* signaling blocks PT growth, in absence of a sufficient amount of ethylene. This blockade is notably mediated by cell wall modifying enzymes acting on the methylesterification status of HGs, and by an alteration of  $\text{Ca}^{2+}$  loading in PTs. Changes in ethylene signaling is also altering expression of other gene families, particularly related to hormone metabolism, that were not detailed in this study. Contributions of the ethylene precursor, 1-aminocyclopropane carboxylic acid, also has been suggested to play crucial roles in ovular PT attraction (Mou et al., 2020). Ethylene has long been known to be involved in many plant development processes, and its contribution to fruit set has been under scrutiny over the last decades (An et al., 2020). Here, we unravel part of its crucial role in PT growth.

By altering ethylene perception, we show that ethylene signal acts on both cell wall- and calcium-related genes. Specific effects on HG methylesterification and calcium signaling were demonstrated.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

Tomato lines (*Solanum lycopersicum* cv MicroTom): the *etr3-ko* and *etr4-ko* mutant lines were generated in our laboratory using the CRISPR-Cas9 technology, as described (Chen et al., 2020a), these *ko* lines are Loss-Of-Function (LOF). Briefly the sgRNAs “GAATCCTGTGATTGCATTGAGG” and “GCGATGTAAGTGTGATGATGAGG” were designed to knock out *ETR3* and *ETR4*, respectively. The *etr3-ko* used in this study has an adenine missing at the 4<sup>th</sup> position before the PAM (protospacer adjacent motif) resulting in a frameshift mutation. For *etr4-ko*, a guanine was deleted at the 4<sup>th</sup> position before the PAM. The comparisons of the amino acid sequences of the mutated *ETR* proteins in *etr3-ko* and *etr4-ko* to their wild types are shown in Figure S8. Unfortunately, we did not succeed in the mutagenesis of *ETR5*. WTB and *NR* mutant lines were obtained from the L.E. Pereira Peres laboratory (Carvalho et al., 2011), the *NR* line is a Gain-Of-Function line (GOF), due to a point-mutation in *ETR3*. All lines are MicroTom cultivar, but WTB and *NR* might have evolved slightly differently over the last 20 years since they were distributed by the Avi Levy’s laboratory (Weizmann Institute, Israel). Thus, we used two wild-type lines, WT and WTB. WT is the wild type we used in the laboratory over the last 20 years, with which we performed the CRISPR-Cas9 constructs for *etr-ko*. In WTB, the “B” stands for Brazil. Plant sowing and growing conditions are detailed in Chen et al. (2020a).

### ***In vitro* pollen grain germination assay**

For germination assays, pollen was sampled from flowers at one and two days post-anthesis (DPA) (Figure S9), at second and third positions of flower clusters. Pollen grains from 10 anthers were collected in 1.5 mL centrifuge tubes using an electric toothbrush body (Oral-B, France). The germination medium (GM) as in Firon et al. (2012), containing 0.5 % (w/v) agarose or not. Pollen grains were spread over solid GM or suspended in 0.5 mL of liquid GM, and incubated at 25°C for various times in the dark. Images were acquired with an inverted microscope (Leitz DMIRB, Leica Microsystems, Germany) equipped with a 10M pixel CMOS camera (MC 190HD, Leica), with a 10x/0.22NA NPLAN objective (Leica). PT length and diameter were measured with ImageJ (Abràmoff et al., 2004). The lanthanum was applied at 50 µM LaCl<sub>3</sub> in liquid GM as described in Qu et al. (2016).

### **Ethylene and 1-MCP**

Pollen grains were spread on solid GM as described above, the Petri dishes were sealed with an electric tape, and ethylene or 1-MCP were injected in the headspace at the desired concentration, and incubated for 4 h before germination or PT length measurements. Preliminary experiments were performed to validate optimal concentrations, as in Hen et al. (2020). Endogenous ethylene measurements by PTs were performed by sampling the headspace in a 2 mL brown glass vials containing around 10 mg of pollen grains in 0.5 mL of liquid GM, 4 h after imbibition, and analysing the headspace gas sample as previously detailed (Chen et al., 2020b). A vial with GM only was used as control. Numbers of pollen grains per µL were assessed by microscopy.

### **RNA extraction and RT-qPCR**

Pollen grains (20 mg) were suspended 0.5 mL of liquid GM. After 4 h of germination at 25°C in the dark, PTs were centrifuged at 16,000 g for 5 min. The supernatant was removed and the pellet was frozen into liquid nitrogen and stored at -80°C. Samples were ground with a ball grinder using Tissue Lyser II (Qiagen, France) for 1 min. The pollen powder (20 mg) was used to extract total RNA using the ReliaPrep™ RNA Tissue Miniprep System kit (Promega, France) and treated with DNase I (Invitrogen, France). RT-qPCR analyses were performed as described (Chen et al., 2020a). Primers are listed in Table S1. *ETR6* transcripts were always below detection threshold. Thus, *ETR6* was not used in correlation analysis between RNA-seq reads and RT-qPCR results.

## RNA-seq analyses

For each line (WT, WTB, *etr3-ko* and *NR*), four biological replicates were prepared, and RNA quality was checked on Agilent 2100 Bioanalyzer System, using the RNA 6000 Nano kit protocol (Agilent Technologies, Germany). RNA samples with RNA Integrity Number (RIN) values greater than 7.5 were sent to the GENEWIZ company (Germany) for sequencing (standard service, 2x150bp, HiSeq Illumina Platform). Raw sequenced reads were treated using the pipeline described in Figure S10, implemented inside a data-driven computational pipeline called NextFlow (Di Tommaso et al., 2017): raw sequenced reads in Fastq format were cleaned after quality check using fastQC version 0.11.05 (Andrews, 2010). To use only high-quality sequences, reads were cleaned by removing all left sequencing adaptors and by trimming low quality bases. This step was made using Trim Galore 0.6 ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)), a wrapping tool based on Cutadapt (Martin, 2011). Cleaned sequences were mapped to an improved reference tomato genome produced and assembled by the GBF lab with the annotation Slmic1.1 (<http://tomatogenome.gbfwebtools.fr/>) using STAR 2.5.1b, a spliced-aware mapper (Dobin et al., 2013). The mapping parameters took into account each library size when paired-end sequencing was performed. Finally, featureCounts 1.6.0 (Liao et al., 2014) was used to calculate read counts for each gene from the mapping file. The homologies between Slmic 1.1 (Sly nomenclature) and ITAG 2.4 (Solyc) annotation ([https://solgenomics.net/organism/Solanum\\_lycopersicum/](https://solgenomics.net/organism/Solanum_lycopersicum/)) is available at <http://tomatogenome.gbfwebtools.fr/correspondence>, and in Table S3A. The homologies between Slmic 1.1 and ITAG 4.1 are available in Table S3B, and all RNA sequences are available from the European Nucleotide Archive, accession number E-MTAB-9660.

## Differential expression and downstream analyses

Without *a priori* knowledge of gene expression variances, we optimized our experimental design with four biological replicates for each condition of interest (Lamarre et al., 2018). Differential expression (DE) analysis was performed with R software using DESeq2 package with default Relative Log Expression (RLE) normalization method, encompassing biases caused by library size and relative size of transcriptomes (Love et al., 2014; Maza et al., 2013, Maza, 2016). False discovery rate was limited by using a threshold for adjusted p-values equal to 0.05 (Benjamini and Hochberg, 1995). To visualize distances between samples, a PCA of normalized expressions was performed with plotPCA function (DESeq2 package), to check the homogeneity of the biological replicates and relative distances between conditions. The RLE normalization method used in DESeq2 for the DE analysis, considering that transcripts have the same length in all

conditions, do not normalize counts by transcript length. Thus, normalized counts generated by DESeq2 need to be normalized by transcript length to allow comparison between transcript levels within a condition. Then, DESeq2 normalized samples were multiplied by 100 (rough magnitude of read lengths, in bp) and divided by the transcript length (in bp). Finally, the mean of the biological replicates for a given condition was calculated. This mean relates to the number of transcripts produced in this condition (Zouine et al., 2017). Venn diagrams were generated with JVENN software (<http://jvenn.toulouse.inra.fr/app/example.html>). The RNA-seq results are available in Table S3.

The MAPMAN annotation (<https://mapman.gabipd.org/mapman>) and the *ProtAnnDB* (<http://www.polebio.lrsv.ups-tlse.fr/ProtAnnDB/>) tools were used to identify functional categories of genes and to determine genes that could be related to calcium signaling and cell wall modification. TAIR 11 annotation was selected to search for homologous genes in *Arabidopsis* by using BLAST+ tool, version 2.9.0. The calcium signaling-related genes are listed in Table S5. The proteins predicted to be secreted and having no more than one transmembrane domain and no intracellular retention signal have been selected as cell-wall related proteins (Table S4). They have been named following the nomenclature of *WallProtDB*, a database dedicated to cell wall proteins identified in cell wall proteomes (San Clemente and Jamet, 2015) and the CAZY database (<http://www.cazy.org/>).

### **Pectin immunolabeling and PME activity measurement**

The immunolabeling was performed as described (Hocq et al., 2020). Briefly, after 4 h of growth in liquid GM, PTs were treated with a fixation medium: 100 mM PIPES, 4 mM MgSO<sub>4</sub>, 7 H<sub>2</sub>O, 4 mM EGTA, 10% (w/v) sucrose, and 5% of paraformaldehyde pH 7.5 and stored at 4°C until use. The fixed PTs were centrifuged at 4,000 *g* for 7 min and washed 3 times with 250 µL PBS, pH 7.4, incubated in a blocking solution (PBS containing 3% fat-free milk) for 30 min at room temperature. After three washes, PTs were incubated overnight with the primary antibody, LM19 or LM20, for weakly or highly methylesterified HGs, respectively (PlantProbes, <http://www.plantprobes.net/>) (Verhertbruggen et al., 2009) at 1:5 dilution in PBS at 4°C in the dark. After washing the pellet 3 times, it was incubated for 3 h at 30°C in the dark with the secondary Alexa488 anti-rat antibody at 1:50 dilution in PBS, followed by 3 washes with PBS. Controls were incubated with the secondary antibody only. Images were acquired with a Leica DMI6000 B inverted microscope equipped with DFC 450C camera and LAS V3.8 software. Alexa488 was detected with a filter cube L5, λ excitation at 460-500 nm and λ emission at 510–540 nm. All the samples were observed at the same exposure time.

For PME assays, the protein extraction from PT was carried out with 2M NaCl buffer according to Baldwin et al. (2014). After desalting, protein concentration was measured with the Bradford assay (Bio-Rad, France). The total PME activity was assayed according to Baldwin et al. (2014) by quantifying the amount of methanol released from citrus pectin with a degree of methylesterification > 85% (Sigma-Aldrich, France). The mean  $\pm$  SD of PME activity is from 3 biological replicates.

### **Intracellular calcium detection and imaging**

Intracellular  $\text{Ca}^{2+}$  was detected using Fluo-4 Acetoxymethyl ester (Fluo-4/AM, Invitrogen, USA) as described (Qu et al., 2015) with the following adjustments. Stock aliquots of fluo-4/AM were made of 50  $\mu\text{g}$  fluo-4/AM dissolved in 91.2  $\mu\text{L}$  anhydrous dimethyl sulfoxide solution, stored at  $-20^{\circ}\text{C}$ . PTs were imbibed for 1 h in liquid GM at a concentration of  $8 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$ , treated with a final concentration of 1  $\mu\text{M}$  fluo-4/AM, and incubated for 30 min at  $22^{\circ}\text{C}$  in the dark. Images were acquired with CLSM confocal microscope Leica SP8, driven by LAS X software, with water immersion objective 25x/0.95. Fluo-4/AM was excited at 488 nm and fluorescence was collected on photomultiplier between 500 and 690 nm. Images were acquired on one plan, every 2 s for a 30 min total duration. The maximum fluorescence intensity observed over the time course, for one PT apex, was selected for quantification with ImageJ (Abràmoff et al., 2004), using the mean grey value, over a constant area, shown in figures with a yellow dashed line. The mean of maximum fluorescence of 10 individual PTs for each genotype was measured.

### **DATA AVAILABILITY**

All RNA-seq data were deposited in the European Nucleotide Archive under the accession number E-MTAB-9660.

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## **AUTHOR CONTRIBUTIONS**

RAA and CC conceived the study, YC and RAA performed the CRISPR mutations, and RAA, PF, EM and AD performed the RNA-seq. RAA, CM, EJ and JCM managed experiments and analyses related to calcium, pollen culture and cell wall. All co-authors wrote and approved the manuscript.

## **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## **SUPPORTING INFORMATION**

Figure S1. Flower morphology of *Solanum lycopersicum* (cv. MicroTom).

Figure S2. Tomato pollen tube (PT) growth as a function of time after imbibition.

Figure S3. Quality indicators of RNA-seq experiment.

Figure S4. Expression levels of PME genes, and calcium related genes in germinating tomato pollen grains.

Figure S5. Immunolabeling of weakly methylesterified HG epitopes probed with LM19, and PME activity.

Figure S6.  $\text{Ca}^{2+}$  gradients in apical regions of pollen tubes and lanthanum, ethylene and 1-MCP trials.

Figure S7. Effects of ethylene and 1-MCP concentrations on the rate of pollen germination and the pollen tube growth of WT

Figure S8. *etr3*-ko and *etr4*-ko tomato protein sequences.

Figure S9. Pollen viability at different stages of flower development.

Figure S10. Overview of the RNA-Seq analysis pipeline.

Table S1. Genes and primer sequences used for RT-qPCR analyses.

Table S2. Correlation between RNAseq and RT-qPCR data, and feedback of ETR mutations on ETR expressions

Table S3. Global list of sequenced RNAs (tab A), correspondence between the Sly and Solyc of ITAG 4.1 (tab B).

Table S4. Comparison between the levels of expression of cell wall-related genes in the four tomato lines.

Table S5. Comparison between the levels of expression of calcium signaling-related genes in the four tomato lines.

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## FIGURE CAPTIONS

### Figure 1. Regulation of germinating pollen grains by ethylene

**(A)** Expression levels of *ETR* genes in germinating tomato pollen grains. Total RNA was extracted from WT PTs 4 h after pollen harvest and imbibition, n = 4 biological replicates, LOD stands for lower limit of detection, Relative expression was calculated with regard to *ETR1*. The sequences of the gene-specific primers used for PCR are listed in Table S1. **(B)** PT length in WT, WTB, NR and *etr3-ko*, 4 h after pollen grain imbibition; and effects of 100 ppm ethylene and 2 ppm 1-MCP treatments on PT length. n = 90 PTs. **(C)** Ethylene production by germinating pollen grains, 4 h after pollen grain imbibition, n = 6 biological replicates. For all panels, error bars show SE, different small letters indicate a significant difference between means at  $P = 0.05$  (Tukey's HSD for panels A and B, Dunn's test for panel C).

### Figure 2. Transcriptome profiling of *etr3-ko* and NR PTs.

**(A)** PCA of normalized samples. Axes 1 and 2 represent 68% of data information. Colored dots underline the four conditions of interest and the four biological replicates analysed by RNAseq. See Figure S3 for additional RNAseq quality indicators. **(B)** Venn diagrams showing the numbers of differentially expressed genes (DEGs) in NR vs WTB and *etr3-ko* vs WT, up-regulated DEGs, and down-regulated DEGs, base means of normalized counts > 5, and adjusted P-value <0.05. **(C)** ETRs are differentially expressed in *etr3-ko* and NR. Bar plot representing log<sub>2</sub>(fold change) shows differences in expression level between (i) NR vs WTB, and (ii) *etr3-ko* vs WT, \* shows significant changes at  $P < 0.05$  using Tukey HSD. **(D)** Functional classification of DEGs. The down-regulated genes are in blue (log<sub>2</sub> fold change < 0,  $P < 0.05$ ), and the up-regulated genes in red (log<sub>2</sub> fold change > 0,  $P < 0.05$ ). Four biological replicates were analyzed.

**Figure 3. Changes in the level of transcript accumulation of genes possibly related to pectin modifications, in NR (upper part) and *etr3-ko* (lower part) compared to WTB and WT,**

**respectively.** These bar plots represent log<sub>2</sub> (fold change) for the pectin-related DEGs ( $P < 0.05$ ). They are grouped in different families: PG (polygalacturonases), PME (pectin methylesterases), PAE (pectin acylesterases), PL (pectate lyases), PMEI (PME inhibitors), TBL (trichome birefringence-like) and PAC (Proline-rich Arabinogalactan proteins conserved Cysteines). The pectin-related genes are listed in Table S4C.

**Figure 4. Changes in the level of transcripts related to calcium signaling, in *NR* (upper part) and *etr3-ko* (lower part) compared to *WTB* and *WT*, respectively** These bar plots represent log<sub>2</sub> (fold change) for the calcium-related DEGs ( $P < 0.05$ ). The genes are grouped in families: CIPKs (CBL-interacting protein kinases), CPKs (calcium-dependent protein kinases), CaMBPs (calmodulin binding proteins), CaBPs (calcium-binding proteins), CMLs (calmodulin-like proteins). The calcium signaling-related genes are listed in Table S5.

**Figure 5. Immunolocalization of highly methylesterified homogalacturonan (HG) epitopes. (A)** Immunolabeling of PTs of *WT*, *etr3-ko*, *WTB* and *NR* using LM20. **(B)** Fluorescence intensity was measured at PT apex.  $n = 80$  PTs for each line, error bars show SE, different letters show significant differences at  $P = 0.05$  (Dunn's test). The area delimited by a yellow dashed line (as shown on *WTB*) indicates the chosen zone for measuring the fluorescence mean intensity. **(C)** A representative picture of PTs cultured in the liquid growth medium for each line. **(D)** Measurement of PT length and diameter, in solid and liquid growth medium, 4 h after pollen grain imbibition, in the four tomato lines,  $n = 50$  PTs for each line. Error bars show SE and different letters show significant differences at  $P = 0.05$  (Tukey's HSD). Scale bars = 50  $\mu\text{m}$  in panels A and C.

**Figure 6. Effects of *ETR* mutations on the  $\text{Ca}^{2+}$  gradient in the apical regions of pollen tubes. (A, B, C, D)** Representative images of  $\text{Ca}^{2+}$  gradient at maximal fluorescence over a 30 min growth period in *WT*, *etr3-ko*, *WTB* and *NR* respectively, scale bars = 10  $\mu\text{m}$ . **(E)** Means of maximum fluorescence intensity measured in PT apex loaded with Fluo-4/AM over a 30 min growth period,  $n = 10$  PTs, error bars are SE, different small letters show significant differences at  $P = 0.05$  (Tukey's HSD). The zone delimited by a yellow dashed line indicates the region where the maximum fluorescence intensity has been measured. **(F)** Model showing that ethylene signaling regulates PT elongation through cell wall reshuffling by affecting activities of pectin methylesterases (PME) and through modifications in calcium gradient by affecting calcium channels (CNGC), calcium pumps (ACA) and  $\text{Ca}^{2+}$  effectors (CPK). Only enzyme families showing strong regulation in *ETR* LOF or GOF mutants are represented.











