**New Phytologist Supporting Information**

Article title: Nucleocytoplasmic shuttling of ETHYLENE RESPONSE FACTOR 5 mediated by nitric oxide suppresses ethylene biosynthesis in apple fruit
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The following Supporting Information is available for this article:

**Fig. S1** Ethylene production, fruit firmness and nitric oxide (NO) content in apple fruit treated with S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP).

**Fig. S2** Ethylene production, fruit firmness, Nitric oxide (NO) content and gene expression in apple fruit treated with S-nitrosoglutathione (GSNO).

**Fig. S3** *MdERF5* gene expression in apple fruit.

**Fig. S4** * MdACO1* gene expression in *MdERF5*-silenced calli.

**Fig. S5** Subcellular localization of *MdERF5* with SNP treatment.

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**Fig. S8** *MdPP2C57* interacts with *MdERF5N/D* in yeast cells.

**Fig. S9** Subcellular co-localization of *MdPP2C57* and *MdERF5*.

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Table S4 Nitric oxide (NO)-regulated PP2C identified from RNA-seq data derived from untreated apple fruit and fruit treated with S-nitrosoglutathione (GSNO).
Fig. S1 Fruit collected on the day of commercial harvest (145 DAFB, days after full bloom) in 2017 were treated with GSNO (50, 100, or 200 µM) and stored at room temperature for 25 days. (a) to (c), After treatment, ethylene production (a), fruit firmness (b), and endogenous NO content (c) were measured. (d) to (f), Fruit were treated with SNP (50, 100, or 200 µM). Numbers under the x-axes indicate the days of storage at room temperature after harvest; 0 indicates the day of commercial harvest. For RT-qPCR analysis, three biological replicates were analyzed as described in the Methods section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test.
Fig. S2 Apple fruit were collected at the commercial harvest stage (145 DAFB, days after full bloom) in 2018, treated with GSNO, and stored at room temperature for 20 days (a). Ethylene production (b), NO content (c) and fruit firmness were measured (d), and the expression levels of MdACS1 (e) and MdACO1 (f) were investigated by RT-qPCR. Scale bars, 1 cm. Untreated, fruit not receiving any treatment; GSNO, fruit treated with GSNO. Numbers under the x-axis indicate the days of storage at room temperature after harvest; 0 indicates the day of commercial harvest. Three biological replicates were analyzed as described in the Methods section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test (**P<0.01).
Fig. S3 Apple fruit were collected at the commercial harvest stage in 2017 or 2018, treated with S-nitrosoglutathione (GSNO), and stored at room temperature. (a) and (b) The expression levels of *MdERF5* were investigated by RT-qPCR. Untreated, fruit not receiving any treatment; GSNO, fruit treated with GSNO. Numbers under the x-axis indicate the days of storage at room temperature after harvest; 0 indicates fruit harvested on the day of commercial harvest. (c), RT-qPCR was used to examine *MdERF5* expression during apple fruit development in 2019. Three biological replicates were analyzed as described in the Methods section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test (**P<0.01, *P<0.05).
Fig. S4 *MdERF5* expression was silenced by RNAi in apple fruit calli (*MdERF5*-AN) via *A. tumefaciens*-mediated transformation. *MdACO1* expression was investigated by RT-qPCR in *MdERF5*-suppressed calli. Calli infected with empty vector (pRI101) were used as controls. Three biological replicates were analyzed as described in the Methods section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test. n.s., no significant difference.
Fig. S5 Confocal and brightfield images of *N. benthamiana* cells expressing *MdERF5*. *MdERF5* was driven by the native promoter and GFP was fused to its C terminus (*ProMdERF5::MdERF5-GFP*). An mCherry-labelled nuclear marker (*NF-YA4-mCherry*) was expressed together with *ProMdERF5::MdERF5-GFP*. 0, 2, 12 h indicate the time after SNP-treatment as described in Methods section. Untreated, tobacco leaves not receiving any treatment; SNP, tobacco leaves treated with sodium nitroprusside (SNP). The experiment was performed three times independently, and representative results are shown. Scale bars, 50 μM.
Fig. S6 Original figure of phosphorylated MdERF5 protein levels analyzed by phos-tag gel analysis. P-MdERF5 bands indicate phosphorylated MdERF5 protein. Untreated, fruit not receiving any treatment; GSNO, fruit treated with GSNO. Numbers under the panel indicate the days of storage at room temperature after harvest; 0 indicates the day of commercial harvest.
Fig. S7 Confocal and brightfield images of *N. benthamiana* cells expressing *MdERF5* treated with Type-1 and Type-2A serine/threonine protein phosphatase (PP2A)–specific inhibitor, okadaic acid (OA) and S-nitrosoglutathione (GSNO). *MdERF5* was driven by its native promoter and GFP was fused to its C terminus (*ProMdERF5::MdERF5-GFP*). An mCherry-labelled nuclear marker (*NF-YA4-mCherry*) was expressed together with *ProMdERF5::MdERF5-GFP*. Untreated, tobacco leaves not receiving any treatment; GSNO, tobacco leaves treated with GSNO alone; OA, tobacco leaves treated with OA alone; OA + GSNO, tobacco leaves treated with OA followed by GSNO. The experiment was performed three times independently, and representative results are shown. Scale bars, 50 μM.
**Fig. S8** The MdERF5 protein sequence was divided into three fragments (MdERF5N/D/C) and the interaction of MdPP2C57 with MdERF5N/D/C was investigated using a yeast two-hybrid assay. DDO, SD medium lacking Trp and Leu; TDO, SD medium lacking Trp, Leu and His; TDO/X/A, TDO medium containing X-α-gal and aureobasidin A. SV40 and P53 were used as positive controls, and AD vectors as negative controls. Blue color indicates protein interaction.
Fig. S9 Confocal images of *N. benthamiana* leaves expressing *MdERF5* and *MdPP2C57*. *MdERF5* was driven by its native promoter and *GFP* was fused to its C terminus (*ProMdERF5::MdERF5-GFP*). *MdPP2C57* was driven by its native promoter and *mCherry* was fused to its C terminus (*ProMdPP2C57::MdPP2C57-mCherry*). Tobacco leaves were co-infiltrated with *ProMdERF5::MdERF5-GFP* and *ProMdPP2C57::MdPP2C57-mCherry*. Untreated, tobacco leaves not receiving any treatment; GSNO, tobacco leaves treated with *S*-nitrosoglutathione (GSNO). The experiment was performed three times independently, and representative results are shown. Scale bars, 50 μM.
**Fig. S10** MdPP2C57 mediates the nucleocytoplasmic shuttling of MdERF5. *MdERF5* was driven by its native promoter and *GFP* was fused to its C terminus (*ProMdERF5::MdERF5-GFP*). An overexpression *MdPP2C57* vector (*MdPP2C57-pRI101*) was expressed together with *ProMdERF5::MdERF5-GFP*. Empty vector *pRI101* was used as a control. *NF-YA4-mCherry* was used as a nuclear marker. Scale bars, 50 μM.
Fig. S11 Prediction of NLS. The NLS sequence was predicted with the cNLS mapper program (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and is shown in red. The phosphorylation site S260 is indicated by asterisk.
**Fig. S12** (a) and (b) Confocal and brightfield images of *N. benthamiana* cells expressing *MdERF5* treated with S-nitrosoglutathione (GSNO). *MdACO1* was driven by its native promoter and GFP was fused to its C terminus (*ProMdACO1::MdACO1-GFP*). An mCherry-labelled nuclear marker (*NF-YA4-mCherry*) was expressed together with *ProMdACO1::MdACO1-GFP* (a). Co-infiltration of *ProMdACO1::GFP* with *NF-YA4-mCherry* represents the control. An mCherry-labelled plasma membrane marker (*PM-mcherry*, CD3–1007) was expressed together with *ProMdACO1::MdACO1-GFP* (b). Co-infiltration of *ProMdACO1::GFP* with *PM-mcherry* represents the control. Untreated, tobacco leaves not receiving any treatment; GSNO, tobacco leaves treated with GSNO. The experiment was performed three times independently, and representative results are shown. Scale bars, 50 μM.
Fig. S13 Confocal images of *N. benthamiana* leaves expressing *MdERF5* and *MdACO1*. *MdERF5* was driven by its native promoter and *GFP* was fused to its C terminus (*ProMdERF5::MdERF5-GFP*). *MdACO1* was driven by its native promoter and *mCherry* was fused to its C terminus (*ProMdMdACO1::MdMdACO1-mCherry*). Tobacco leaves were co-infiltrated with *ProMdERF5::MdERF5-GFP* and *ProMdMdACO1::MdMdACO1-mCherry*. Untreated, tobacco leaves not receiving any treatment; GSNO, tobacco leaves treated with S-nitrosoglutathione (GSNO). The experiment was performed three times independently, and representative results are shown. Scale bars, 50 μM.
**Fig. S14** A coimmunoprecipitation (co-IP) assay showing that S-nitrosoglutathione (GSNO) treatment enhanced the interaction between MdERF5 and MdACO1. MdERF5 fused to a Myc tag (MdERF5-Myc) and MdACO1 fused to a GFP tag (MdACO1-GFP) were overexpressed in tobacco leaves and a GFP antibody was used for immunoprecipitation analysis. Myc and GFP antibodies were used in an immunoblot analysis. The band detected by the Myc antibody in the precipitated protein sample indicates the interaction between MdERF5 and MdACO1 (lane 5) and GSNO treatment enhances this interaction (lane 7).
Fig. S15 Amino acid sequence shown by the black rectangle (162-254 aa) represents the 2-oxoglutarate (2OG) and Fe²⁺-dependent oxygenase domain of the enzyme family. The Fe²⁺ binding site (H177, D179 and H234) is indicated by solid line.
Fig. S16 ACC oxidase activity in apple fruit treated with S-nitrosoglutathione (GSNO). Apple fruit were collected at the commercial harvest stage in 2017 (a) and 2018 (b), treated with GSNO, and stored at room temperature. Untreated, fruit not receiving any treatment; GSNO, fruit treated with GSNO. Numbers under the x-axes indicate the days of storage at room temperature after harvest; 0 indicates the day of commercial harvest. Three biological replicates were analyzed as described in the Methods S6 section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test (**P<0.01, *P<0.05).
Fig. S17 (a) Three fragments of the MdERF5 coding sequence were used in a Yeast one-hybrid (Y1H) analysis showing that the middle region of MdERF5 (MdERF5D) binds to the *MdACS1* promoter (*ProMdACS1*). AbA (aureobasidin A), a yeast cell growth inhibitor, was used as a screening marker. The basal concentration of AbA was 200 ng ml\(^{-1}\). Rec-P53 and the *P53* promoter were used as positive controls. The empty vector and *ProMdACS1* were used as negative controls. (b) The *MdACS1* promoter was divided into five fragments (P1–P5) and used with MdERF5 in a Y1H assay. The basal concentration of AbA was 250 ng ml\(^{-1}\) for P5 and 200 ng ml\(^{-1}\) for the other fragments. DRE, dehydration-responsive element.
Fig. S18 MdERF5 expression was overexpressed in apple fruit calli (MdERF5-GFP) via A. tumefaciens-mediated transformation.MdERF5 expression was investigated by RT-qPCR. Calli infected with empty vector (pRI101-GFP) were used as controls. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test (***P<0.01).
**Fig. S19** Electrophoretic mobility shift assay (EMSA) showing that MdERF5 binds to the DRE motif in the *MdACS1* promoter (lane 2). Increasing amounts of MdACO1 protein did not interfere with MdERF5 binding to the *MdACS1* promoter (lanes 4-7). The hot probe was a biotin-labeled fragment containing the DRE motif, and the cold probe was a non-labeled competitive probe. GST-tagged MdERF5 and His-tagged MdACO1 were purified.
Fig. S20 Apple fruit collected on the day of commercial harvest (145 DAFB, days after full bloom) had *MdERF5* silenced (*MdERF5*-AN) by *Agrobacterium tumefaciens*-mediated transient transformation. Fruit infiltrated with an empty pRI101 vector were used as controls (pRI101). *MdERF5*-AN and control fruits were immediately treated with S-nitrosoglutathione (GSNO) and then stored at room temperature for 20 d (a). Scale bars, 1 cm. *MdERF5* expression was examined by RT-qPCR (b). Ethylene production (c), *MdACS1* expression (d), and ACO enzyme activity (e) were investigated. Untreated, fruit not receiving any treatment; GSNO, fruit treated with GSNO. For RT-qPCR, three biological replicates were analyzed as described in the Methods section. Values represent means ± SE. Each dot represents a biologically independent sample. Statistical significance was determined using a Student’s t-test (**P<0.01, *P<0.05).
Methods S1 Quantification of endogenous NO content

NO quantification was performed based on Hu et al. (2003) with modifications (Hu et al., 2003). NO is metabolized to nitrite and nitrate under acidic conditions, and quantitation of these stable anions is used to indirectly determine the amount of NO originally present. Six g of apple pulp were collected and homogenized with 12 mL of 50 mM acetic acid buffer (pH 3.6) containing 4% Zn(Ac)$_2$. The resulting suspension was centrifuged at 4°C and 10,000 g for 10 min and the NO content of the supernatant was measured by adding 1 mL with 1 mL of Greiss reagent containing 1% sulfanilamide (Sigma-Aldrich), 0.1 % N-(l-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in 5% phosphoric acid for 30 min at room temperature. This step converted nitrite into a purple azo dye, which was quantified spectrophotometrically at 550 nm. Different concentrations of NaNO$_2$ were used to prepare a standard curve. Five biological replicates were analyzed. Statistical significance was determined using a Student’s t-test (**P<0.01).

Methods S2 Y2H assay

A cDNA library was constructed with mRNA from GD fruit harvested at commercial maturity using the Make Your Own Mate & Plate Library System (Cat. no. 630489; Clontech, Mountain View, CA, USA). The $MdERF5$ CDS was introduced into the pGBKT7 vector enclosed in the kit using NdeI and EcoRI sites. The recombinant plasmid was used as bait to screen the cDNA library.

$MdERF5$ (332 aa), $MdERF5N$ (aa 1–180), $MdERF5D$ (aa 181–260) and $MdERF5C$ (aa 261–332) sequences were introduced into the activation domain (AD) vector (pGADT7) using the NdeI and EcoRI restriction sites. The $MdACO1$ (314 aa), $MdACO1N$ (aa 1–99), $MdACO1M$ (aa 100–161), $MdACO1D$ (aa 162–254) and $MdACO1C$ (aa 255–314) sequences were ligated to the binding domain (BD) in the pGBKT7 vector using the NdeI and EcoRI restriction sites. The full length $MdPP2C57$ sequence was ligated to the binding domain (BD) in the pGBKT7 vector using the NdeI and EcoRI restriction sites. The BD and AD vectors were co-transformed into the Y2H Gold yeast (Saccharomyces cerevisiae) strain. The detection of interactions between two proteins was conducted using the Matchmaker™ Gold Y2H Library Screening System kit.

Methods S3 Co-IP assay

For the co-IP assay, the $MdERF5$ CDS was ligated into the pCAMBIA1307 vector (BioVector) using the XbaI and BamHI sites to allow expression of the $MdERF5$ protein with a Myc tag driven by the CaMV 35S promoter. The $MdACO1$ CDS was cloned into the KpnI and EcoRI sites downstream from the GFP sequence and the CaMV 35S promoter in the pRI101 vector. The recombinant Pro35S::$MdERF5$-Myc and Pro35S::$MdACO1$-GFP constructs were infiltrated into N. benthamiana leaves using A. tumefaciens infiltration as previously described (Li et al., 2017). SNP (1 mM) was injected into infiltrated N. benthamiana leaves 3 h before sampling. Protein extracted from the infiltrated N. benthamiana leaves was used for co-IP analysis. A Pierce co-IP kit (catalog no. 26149; Thermo Scientific, Waltham, MA, USA) was used to immuno-precipitate
MdACO1-GFP using 10 μL of anti-GFP antibody (1mg mL⁻¹; Transgen Biotech). The precipitate was analyzed by immunoblot analysis with the anti-Myc antibody (1mg mL⁻¹; Transgen Biotech) diluted 1:3,000.

In addition, the recombinant Pro35S::MdACO1-GFP construct was introduced into apple calli as previously described (Xie et al., 2012), and the transgenic calli were used for co-IP analysis. The immuno-precipitation assay was performed as described above. The precipitate was analyzed by immunoblot analysis with the anti-MdERF5 antibody. The calli infiltrated with Pro35S::GFP were used as the negative control.

Methods S4 Firefly Luc complementation imaging assay

The MdPP2C57 and MdACO1 CDS were inserted into the pcAMBIA1300-nLuc vector using the KpnI and SalI restriction sites. The MdERF5 CDS was inserted into the pcAMBIA1300-cLuc vector using the KpnI and PstI restriction sites. A. tumefaciens strain EHA105 carrying the indicated constructs was cultured to OD₆₀₀ 0.5, combined with different volumes of a similarly adjusted culture to make the combinations shown in Fig. 5c or 7e and incubated at room temperature for 3 h before infiltration into N. benthamiana leaves. Luciferase activity was detected 3 d after infiltration using the Night SHADELB 985 imaging system (BertholdTechnologies). Thirty minutes before detection, 0.2 mM luciferin (Promega) was infiltrated into the same positions as where A. tumefaciens had been infiltrated. GSNO (100 μM) was injected into infiltrated N. benthamiana leaves 3 h before imaging. The infiltration in each assay was repeated 3 times as three biological replicates.

Methods S5 Measurements of ACO activity

MdACO1 enzyme activity was measured as previously described (Ji et al., 2021). Purified MdACO1-His protein (0.2 μg) was added to 2 mL of incubation buffer (pH 7.2) containing 10% (v/v) glycerol (Solarbio), 0.1 mM ACC (Sigma-Aldrich, St Louis, MO, USA), 5 mM Na-ascorbate (Sangon Biotech), 80 μM FeSO₄ (Sangon Biotech), 15 mM 500 μg catalase (Worthington, http://www.worthington-biochem.com), 15 mM NaHCO₃ (Sangon Biotech) and 2 mM dithiothreitol (DTT; Solarbio), and the mixture was incubated at 30°C for 2 h in a 15-mL gas-tight glass tube with a septum, shaking at 120 rpm. Next, 1 mL of gas was extracted from the headspace of the tube with a 1-mL syringe for measurement of ethylene production as previously described (Li et al., 2014). To investigate the effect of MdERF5 on MdACO1 activity, different amounts of purified MdERF5-GST (0, 0.2, 0.4 and 0.6 μg) were mixed with 0.2 μg of MdACO1-His and incubated at 4°C for 1 h, shaking at 100 rpm. Purified 0.2 μg MdACO1-His with different amounts of purified GST protein (0, 0.2, 0.4 and 0.6 μg) was used as a control. The mixture was then added to incubation buffer and incubated to measure ethylene production as described above. The ACO activity of apple fruit extracts was measured as previously described (Ji et al., 2021).

Methods S6 GUS analysis
The *MdERF5* or *MdPP2C57* CDS regions were cloned into the pRI101 vector (Xiao *et al.*, 2013) using the *Kpn*I and *EcoRI* restriction sites to generate the effector constructs. The reporter constructs were generated using the *MdACS1* (1,191 bp) promoter sequence cloned upstream from the GUS reporter gene in the pBI101 vector. The reporter and effector vectors were transformed into *A. tumefaciens* strain EHA105, and *N. benthamiana* leaves were used for co-infiltration. The co-infiltration and examination of GUS activity were performed according to Ji *et al.* (2021) (Ji *et al.*, 2021). The infiltration in each assay was repeated 3 times as three biological replicates, and a Student’s *t* test was employed to determine the statistical significance.

**Methods S7** *A. tumefaciens* infiltration

To silence *MdERF5* expression *in planta*, the full length *MdERF5* CDS was ligated into the pRI101 vector in the reverse direction to generate the antisense *MdERF5*-AN construct. To silence *MdERF5* in apple fruit calli, *MdERF5*-AN was transformed into *A. tumefaciens* strain EHA105 by electroporation. A single transformed *A. tumefaciens* colony was picked and inoculated in 5 mL YEP medium (1% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.0) containing kanamycin (50 μg ml⁻¹) and rifampicin (50 μg ml⁻¹), and grown at 28°C for at least 12 h with shaking at 180 rpm. One milliliter of the culture was then transferred to 50 mL of YEP medium containing kanamycin (50 μg ml⁻¹) and rifampicin (50 μg ml⁻¹) and was grown at 28°C for at least 12 h with shaking at 180 rpm. The cell culture was centrifuged at 10,000 g for 1 min at 24°C to collect the cells, the supernatant was removed and the pellet was suspended in 10 ml of liquid MS medium containing 100 μM acetosyringone, and adjusted to an OD₆₀₀ of 0.7. The suspension was then kept at 24°C for 3 h and immediately used for infiltration. The apple calli were dipped into the suspension, left to shake at 28°C for 5 min at 180 rpm and then left to stand for 20 min at room temperature for transfection. The calli were grown in the dark at 24°C on solid MS medium containing 100 μM acetosyringone for 2 days and then grown on solid MS medium containing 2,4-D, 6-BA, kanamycin (50 μg ml⁻¹) and cephalosporin (400 μg ml⁻¹) for one month. The new calli used for ethylene measurements and RNA or protein extraction were prepared as described above.

To silence *MdERF5* in apple fruit, the *A. tumefaciens* carrying pRI101-MdERF5-AN was prepared as above and suspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6 and 1 mM acetosyringone), and OD₆₀₀ was adjusted to 1.0. The suspension was then kept at 24°C for 4 h and immediately infiltrated. The infiltration assay was performed on apple fruit still attached to trees ~14 d before commercial harvest. To silence *MdERF5* expression 500 μL of the suspension was collected using a 1-mL sterile syringe and injected into the fruit to a depth of 0.5 cm, with 8 injections performed into each fruit. Infiltrated fruit were harvested 14 DAI, treated with 100 μM of GSNO as above, stored at room temperature for 15 d and sampled every 5 d. One fruit was used as a biological replicate and at least three fruit were used to measure ethylene production or gene expression at each sampling point. A Student’s *t* test was employed to determine the statistical significance.
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