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ORIGINAL ARTICLE

Activating glutamate decarboxylase activity by removing the autoinhibitory domain leads to hyper γ-aminobutyric acid (GABA) accumulation in tomato fruit

Mariko Takayama[1] · Chiaki Matsukura[1] · Tohru Ariizumi[1] · Hiroshi Ezura[1]

Abstract

Key message **The C-terminal extension region of *SlGAD3* is likely involved in autoinhibition, and removing this domain increases GABA levels in tomato fruits.**

Abstract γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid with several health-promoting benefits. In many plants including tomato, GABA is synthesized via decarboxylation of glutamate in a reaction catalyzed by glutamate decarboxylase (GAD), which generally contains a C-terminal autoinhibitory domain. We previously generated transgenic tomato plants in which tomato *GAD3* (*SlGAD3*) was expressed using the *35S* promoter/NOS terminator expression cassette (*35S-SlGAD3-NOS*), yielding a four- to fivefold increase in GABA levels in red-ripe fruits compared to the control. In this study, to further increase GABA accumulation in tomato fruits, we expressed *SlGAD3* with (*SlGAD3^{OX}*) or without (*SlGAD3ΔC^{OX}*) a putative autoinhibitory domain in tomato using the fruit ripening-specific E8 promoter and the *Arabidopsis* heat shock protein 18.2 (HSP) terminator. Although the GABA levels in *SlGAD3^{OX}* fruits were equivalent to those in *35S-SlGAD3-NOS* fruits, GABA levels in *SlGAD3ΔC^{OX}* fruits increased by 11- to 18-fold compared to control plants, indicating that removing the autoinhibitory domain increases GABA biosynthesis activity. Furthermore, the increased GABA levels were accompanied by a drastic reduction in glutamate and aspartate levels, indicating that enhanced GABA biosynthesis affects amino acid metabolism in ripe-fruits. Moreover, *SlGAD3ΔC^{OX}* fruits exhibited an orange-ripe phenotype, which was associated with reduced levels of both carotenoid and mRNA transcripts of ethylene-responsive carotenogenic genes, suggesting that over activation of GAD influences ethylene sensitivity. Our strategy utilizing the E8 promoter and HSP terminator expression cassette, together with *SlGAD3* C-terminal deletion, would facilitate the production of tomato fruits with increased GABA levels.

Keywords E8 promoter · Fruit ripening · GABA · HSP terminator · Tomato

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Introduction

γ-Aminobutyric acid (GABA) is a four-carbon, non-protein amino acid that widely occurs in animals, plants, and bacteria. In humans, GABA functions as an inhibitory neurotransmitter (Owens and Kriegstein 2002) as well as a functional compound that confers several health benefits, such as lowering blood pressure, inducing relaxation, and reducing sleeplessness, depression, and autonomic disorders (Okada et al. 2000; Inoue et al. 2003; Abdou et al. 2006); therefore, a variety of GABA-enriched foods have been developed (Tsushida et al. 1987; Okada et al. 2000; Inoue et al. 2003; Park and Oh 2007).

Among natural food resources, tomatoes accumulate relatively high levels of GABA in the fruits (Matsumoto

et al. 1997), perhaps due to their high capacity to biosynthesize and/or store GABA. We previously investigated the mechanism underlying GABA accumulation in tomato fruits and attempted to further increase GABA accumulation in tomatoes (Akihiro et al. 2008; Yin et al. 2010; Koike et al. 2013; Takayama et al. 2015). In various higher plants, GABA is primarily biosynthesized from glutamate by the enzyme glutamate decarboxylase (GAD) and is catabolized to succinic semialdehyde (SSA) by GABA transaminase (GABA-T) (Bouché and Fromm 2004). At least three homologs of *GAD* and *GABA-T* genes are present in the tomato genome, i.e., *SlGAD1-3* and *SlGABA-T1-3*, respectively (Akihiro et al. 2008). Genetic suppression analyses of these genes revealed that *SlGAD2/3* and *SlGABA-T1* are likely the key regulatory genes for GABA biosynthesis and catabolism, respectively, in the fruit (Koike et al. 2013; Takayama et al. 2015). Although suppressing *SlGABA-T1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter was effective for increasing fruit GABA levels (up to 9.2-fold wild-type levels at the red stage), this process also induced severe dwarfism and infertility (Koike et al. 2013). To avoid the negative effects of systemic suppression of *SlGABA-T1*, we also generated transgenic tomato plants in which *SlGABA-T1* was suppressed under the control of the E8 promoter, a strong, inducible promoter specific to ripening tomato fruits (Deikman et al. 1998). The resulting transgenic plants exhibited normal development, but the GABA levels in red-ripe fruits were lower than those in transgenic fruits in which *SlGABA-T1* was suppressed driven by the 35S promoter, even though the levels were still higher than those in the wild-type (WT) (Koike et al. 2013).

Increasing GABA biosynthesis appears to represent an effective alternative approach for improving GABA levels in tomato fruits. Indeed, we previously reported that overexpressing *SlGAD3* in tomato fruits using the CaMV 35S promoter/nopaline synthase (NOS) terminator expression cassette led to an increase in GABA levels (up to 5.2-fold wild-type levels) at the red-ripe stage, demonstrating that *SlGAD3* is a good candidate for genetic engineering to increase GABA accumulation in tomatoes (Takayama et al. 2015).

Plant *GAD* genes generally possess an extension region (consisting of 30–50 amino acid residues) at the C-terminus known as the Ca^{2+}/calmodulin (CaM) binding domain, which autoinhibits the activity of this enzyme at physiological pH, although its autoinhibitory function is de-repressed under low cellular pH or by binding of Ca^{2+}/CaM to this domain (Snedden et al. 1995; Snedden et al. 1996; Yap et al. 2003). Therefore, when plants are exposed to stresses that induce the accumulation of high concentrations of H^+ (low pH) and/or Ca^{2+} in plant cells, GABA biosynthesis becomes more active due to the suppression of

GAD autoinhibition (Shelp et al. 1999). Transgenic studies revealed that removing this autoinhibitory domain results in extremely high levels of GABA production in plant cells, most likely resulting from increased GAD enzyme activity (Baum et al. 1996; Akama and Takaiwa 2007). However, the role of the autoinhibitory domain in tomato *GAD* genes has not been investigated to date. In the present study, we generated transgenic tomato plants in which the full-length coding sequence of *SlGAD3* with or without the C-terminal extension region (*SlGAD3ΔC*) was overexpressed under the control of the fruit-specific E8 promoter and the heat shock protein 18.2 (HSP) terminator from *Arabidopsis thaliana*, which probably improves mRNA stability via a post-transcriptional process and was shown to increase gene expression in tomato (Nagaya et al. 2010; Hirai et al. 2011b; Kurokawa et al. 2013). We found that deleting the C-terminal autoinhibitory domain from *SlGAD3* greatly increased GABA levels in fruits, accompanied by both delayed ethylene production and reduced ethylene sensitivity. We discuss the methodology used to increase GABA levels by genetic engineering and propose that there is a molecular connection between GABA and ethylene.

Materials and methods

Plant material and growth condition

The tomato (*Solanum lycopersicum* L.) cv. 'Micro-Tom' seeds used in this study were provided by the National Bioresource Project (Saito et al. 2011). Seeds of WT and T_1 transgenic plants were germinated on moistened filter paper and transferred to Rockwool cubes (5 × 5 × 5 cm). The plants were grown under fluorescent light with a 16 h light (60 μmol/m²/s)/8 h dark photoperiod at 25 °C and were supplied with standard nutrient solution (Otsuka A; Otsuka Chemical Co., Ltd., Osaka, Japan). Fruits at the mature green (MG; 24 days after flowering), breaker (Br), Br + 3 days (Br + 3), Br + 7 days (Br + 7), Br + 10 days (Br + 10), Br + 15 days (Br + 15), and Br + 30 days (Br + 30) stage were used in this study.

Vector construction and tomato transformation

Two binary vectors derived from the previously reported binary vector E8-MIR-HSP containing the E8 promoter and HSP terminator (Kurokawa et al. 2013) were constructed in this study. The full-length coding sequence of *SlGAD3* (Solyc01g005000) and this sequence lacking the C-terminal 87 nucleotides (designated *SlGAD3ΔC*) was amplified by PCR using gene-specific primers containing some additional sequences at their 5′ ends (details are

shown in Fig. S1 and Table S1). The E8-MIR-HSP plasmid was digested with *Bam*HI and *Sac*I, and the amplified fragments of *SlGAD3* or *SlGAD3ΔC* were fused by the In-Fusion cloning reaction (Clontech, CA, USA) according to the manufacturer's instructions. The resulting constructs were designated *SlGAD3^{OX}* and *SlGAD3ΔC^{OX}*, respectively (Fig. 2a). These constructs were transformed into *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al. 1985) and used for tomato transformation (Sun et al. 2006). Regenerated plants that survived on Murashige and Skoog (MS) medium containing kanamycin (100 mg l^{-1}) were transferred to Rockwool cubes (5 × 5 × 5 cm) and grown under the conditions described above.

qRT-PCR analysis

Fruits at the MG, Br + 3, and Br + 10 stages were harvested and frozen in liquid nitrogen after removing seeds and jelly tissues. Frozen fruits were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using an RNeasy Plant Mini kit (Qiagen, Tokyo, Japan) with RNase-free DNase (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). The resulting first-strand cDNA was diluted tenfold in RNase-free water and used as a template for qRT-PCR. The qRT-PCR was performed using a Takara Thermal Cycler Dice Real Time System TP800 with SYBR Premix Ex Taq II (Takara-Bio Inc., Otsu, Japan). The reaction conditions were as follows: 95 °C for 30 s for initial denaturation, followed by 40 cycles of 5 s of denaturation at 95 °C and 30 s of annealing/extension at 60 °C (54 °C for *SlGAD3*). The tomato ubiquitin gene (*SlUBI3*; GenBank accession no. X58253) was used as an internal control. The gene-specific primers used in this experiment are shown in Table S1.

Determination of free amino acid contents

Free amino acid contents in MG and Br + 10 fruits were determined using an amino acid analyzer (JLC-500/V2, Japan Electron Optics Laboratory) as described previously (Koike et al. 2013). As a quick method for screening GABA contents in fruits, the GABase enzymatic assay was also carried out as described by Jakoby (1962) and Saito et al. (2008).

Determination of GAD activity

Br + 10 fruits were harvested from T$_0$ plants and immediately frozen in liquid nitrogen after removing seeds and jelly tissues. Frozen fruits were then ground to a fine powder in liquid nitrogen using a mortar and pestle. To extract crude protein, powdered fruits were homogenized in fivefold volumes of ice-cold extraction buffer containing 50 mM Tris–HCl buffer (pH 8.2), 3 mM dithiothreitol (DTT), 1.25 mM EDTA, 2.5 mM MgCl$_2$, 10 % (v/v) glycerol, 6 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, 2 % (w/v) polyvinylpyrrolidone, 2 μg ml^{-1} pridoxal-5-phosphate, 1 mM phenylmethylsulphonyl fluoride and 2.5 μg ml^{-1} leupeptin and pepstatin as described in Clark et al. (2009). The homogenates were then centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant was concentrated using Amicon ultra-4 (10 kDa, Millipore) and desalted with PD-10 columns (GE Healthcare). GAD enzymatic activity was assayed based on GABA production during the glutamate-dependent GAD reaction as described by Akama and Takaiwa (2007) and Akihiro et al. (2008) with slight modifications. The reaction was initiated by adding 60 μl of the crude protein (adjusted to 0.4–0.6 mg ml^{-1}) in 240 μl of GAD reaction buffer containing 100 mM of an adequate buffer (Bis–Tris for pH 7.0; MES for pH 5.8), 1 mM DTT, 5 mM glutamate, 0.5 mM pyridoxal-5-phosphate, 0.5 mM CaCl$_2$, 0.1 M bovine calmodulin (Sigma-Aldrich, Missouri) and 10 % (v/v) glycerol. The mixture was incubated at 30 °C for 180 min and boiled for 10 min to stop the reaction. GABA production was measured by the 'GABase' method as described by Jakoby (1962) with slight modifications.

Determination of lycopene and β-carotene contents

Lycopene and β-carotene contents in Br + 10 fruits were measured as described previously (Kitagawa et al. 2005; Mubarok et al. 2015). Pigments were extracted from powdered fruits with acetone-hexane (4:6) solution, and the optical density of the supernatant was simultaneously measured in a spectrophotometer at 663 nm (A_{663}), 645 nm (A_{645}), 505 nm (A_{505}), and 453 nm (A_{453}). Lycopene (C_{LYC}) and β-carotene (C_{CAR}) were calculated according to the following formula:

$$C_{LYC} \text{ (mg/100 gFW)} = (-0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}) \times \text{dilution factor.}$$

$$C_{CAR} \text{ (mg/100 gFW)} = (0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}) \times \text{dilution factor.}$$

Determination of ethylene production

Fruits were harvested at different developmental stages (MG, Br, Br + 3, Br + 10, and Br + 15). Each fruit was placed in a 50 ml glass vial and pre-incubated for more than 3 h to reduce ethylene evolution triggered by harvest

shock. Subsequently, each vial was sealed with a cap containing a septum and incubated for 1 h. Finally, 1 ml of headspace gas was sampled from the vial, and the ethylene production rate was measured by injecting it into a gas chromatograph (GC-14B, Shimadzu).

Results

Comparison of the C-terminal region of GAD proteins from various plant species

Tomato genome contains at least three GAD genes (SlGAD1-3; Akihiro et al. 2008). Like other higher plants, the encoded proteins possess an extension region at the C-terminus, which generally functions as a CaM-binding domain as well as an autoinhibitory domain (Baum et al. 1993; Arazi et al. 1995; Gut et al. 2009). When the amino acid sequences of the C-terminal region of SlGADs are compared with those from previously characterized plants, SlGADs represent several typical features involving CaM-binding (Fig. 1). For example, SlGAD1 and SlGAD2 conserve a tryptophan (W) residue and lysine (K) clusters that are likely involved in hydrophobic and electrostatic interactions, respectively, in CaM/GAD complex formation (Arazi et al. 1995; Gut et al. 2009). These residues are highly conserved in PhGAD, AtGAD1, OsGAD1 and MdGAD1 that are the typical isoforms with CaM-binding ability, but not (or less) in MdGAD3 and OsGAD2, the isoforms without CaM-binding ability (Akama and Takaiwa 2007; Trobacher et al. 2013). Although SlGAD3 contains only one lysine residue, it conserves a tryptophan residue (Fig. 1). In addition, all SlGADs possess one of the two putative pseudo-substrate glutamate (E) residues

characterized in PhGAD (Fig. 1; Yap et al. 2003), suggesting that they might have function as an autoinhibitory domain. In this study, we first focused on SlGAD3, which is the main isoform involving GABA biosynthesis in tomato fruits, and examined the effects of its C-terminal truncation on GABA accumulation in tomato fruits.

Generation of GABA hyper-accumulating transgenic tomato lines

To investigate the role of the C-terminal extension region in SlGAD3, we constructed two types of plant expression vectors, in which the full-length coding sequence of SlGAD3 with or without the C-terminal 87 nucleotides (corresponding to the putative CaM-binding/autoinhibitory domain) was inserted into the E8 promoter/HSP terminator cassette (designated $SlGAD3^{OX}$ and $SlGAD3\Delta C^{OX}$, respectively; Fig. 2a). Using Agrobacterium-mediated method, ten and fifteen transgenic plants were obtained for $SlGAD3^{OX}$ and $SlGAD3\Delta C^{OX}$, respectively (Fig. S2). Among them, three independent lines with significantly increased levels of SlGAD3 (ΔC) expression in Br + 10 fruits were selected (Fig. 2b). To examine the effects of increased SlGAD3 or SlGAD3ΔC expression on fruit GABA accumulation, we determined the GABA levels using the GABase enzymatic assay (Jakoby 1962; Saito et al. 2008). The GABA levels in Br + 10 fruits of $SlGAD3^{OX}$ (lines 8b, 9, and A) were significantly higher than those of the WT, ranging from 12–15 μmol gFW^{-1}, which corresponded to six to sevenfold higher levels than those of the WT (Fig. 2c). Notably, $SlGAD3\Delta C^{OX}$ lines (lines 68, 140a, and 153) accumulated much higher levels of GABA, i.e., 23–26 μmol gFW^{-1}, corresponding to 11- to 12-fold higher levels than those of the WT and 1.9-fold


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                          •      •       *              ━━━━━
PhGAD   469  VHKKTDSEVQLEMITAWKKFVEEKKKK-TNRVC-  500
AtGAD1  472  TVKKSDIDKQRDIITGWKKFVADRKK--TSGIC-  502
OsGAD1  473  VTKKSVLETEREIFAYWRDQV---KKKQT-GIC-  501
OsGAD2  469  AGEEASIRVVKSEAVPVRKSVPLVAGKTK-GVC-  500
MdGAD1  477  TVHKSETEAEQEIVSRWKGIV---KKR---GAC-  503
MdGAD2  470  ESKKSDLEKTREITIVWRKFVMARKQK-MNVVC-  501
MdGAD3  478  ENCHHKVSANGAAYGNGGTDTENGQMTDGAK-CY  510
SlGAD1  474  HFHMDTVETQKDIIKHWRKIAG--KK--TSGVC-  502
SlGAD2  471  AHHKTDREWELQVTEAWKKFVADKKKNKTMGVC-  503
SlGAD3  456  NEKKTEIEVQRAIAEFWKKYVLARK----ASIC-  484
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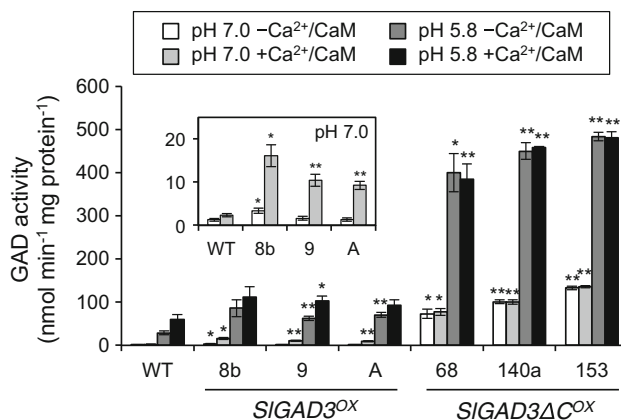
Fig. 1 Multiple alignment of the C-terminal extension regions of plant GADs. The C-terminal regions of tomato GADs (SlGAD1-3) were compared with those from previously characterized plant, such as petunia (PhGAD), Arabidopsis (AtGAD1), rice (OsGAD1 and 2) and apple (MdGAD1-3). The conserved tryptophan (W) residue and lysine (K) clusters involved in CaM-binding (Arazi et al. 1995; Gut et al. 2009) are indicated by an *asterisk* and a *black line*, respectively. The two pseudo-substrate glutamate (E) residues that are likely involved in autoinhibition in PhGAD (Yap et al. 2003) are indicated as *filled circles*
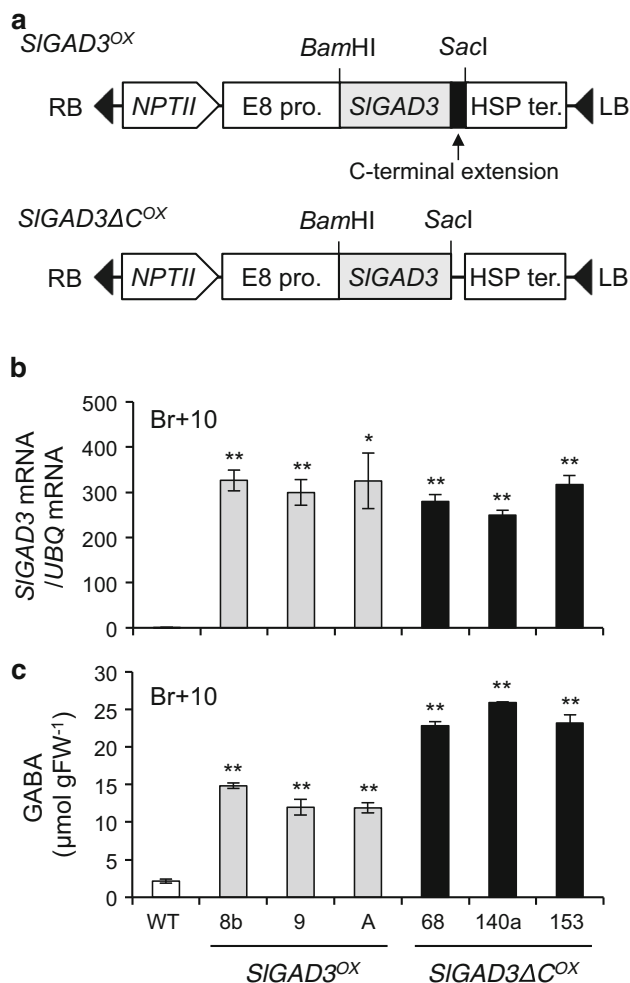
Fig. 2 Generation of *SlGAD3* and *SlGAD3ΔC* overexpressing lines. **a** Maps of T-DNA used for tomato transformation. RB and LB, right and left borders of T-DNA; *NPTII*, *neomycin phosphotransferase II* gene; E8 pro., tomato E8 gene promoter; HSP ter., *Arabidopsis* heat shock protein gene terminator. **b** qRT-PCR analysis of *SlGAD3* in T$_0$ transgenic fruits at the Br + 10 stage. The expression levels of WT fruits were set to a baseline value of 1. *Slubiquitin3* (UBI) was used for normalization. **c** GABA contents determined by the GABase enzymatic assay using the fruit samples used in **b**. The mean ± SE of three biological replicates are shown. *Asterisks* indicate a significant difference between WT and transgenic lines according to Student's *t* test (*$P < 0.05$ and **$P < 0.01$)

Fig. 3 GAD activity in T$_0$ *SlGAD3^{OX}* and T$_0$ *SlGAD3ΔC^{OX}* fruits. Crude proteins extracted from Br + 10 fruits were used for GAD enzymatic assay. The mean ± SE of three biological replicates are shown. *Asterisks* indicate a significant difference between WT and transgenic lines according to Student's *t* test (*$P < 0.05$ and **$P < 0.01$)

higher levels than those of the *SlGAD3^{OX}* lines (Fig. 2c). To confirm whether the increased GABA levels in transgenic tomato fruits are correlated with the GAD enzymatic activity, we extracted crude proteins from Br + 10 fruits and used them for GAD enzymatic assay (Fig. 3). As expected, higher GAD activity was found in the fruit of *SlGAD3^{OX}* (lines 8b, 9, and A) and *SlGAD3ΔC^{OX}* (lines 68, 140a, and 153). Notably, *SlGAD3ΔC^{OX}* fruits exhibited the highest activity in all conditions we tested (pH 7.0 or pH 5.8, with or without Ca^{2+}/CaM); the average activity was 80.5- and 48.9-fold higher at pH 7.0 without Ca^{2+}/CaM,

45.2- and 8.8-fold higher at pH 7.0 with Ca^{2+}/CaM, 15.5- and 6.1-fold higher at pH 5.8 without Ca^{2+}/CaM, and 7.3- and 4.3-fold higher at pH 5.8 with Ca^{2+}/CaM, when compared with that of the WT and *SlGAD3^{OX}* lines, respectively (Fig. 3).

Although the fruit appearance of *SlGAD3^{OX}* (lines 8b, 9, and A) was indistinguishable from that of the WT (Fig. 4a), all three *SlGAD3ΔC^{OX}* (lines 68, 140a, and 153) did not form red-ripe fruits, but formed fruits exhibiting orange-ripe phenotype at the Br + 10 stage (Fig. 4b). The lycopene levels in Br + 10 fruits of *SlGAD3ΔC^{OX}* were 37–63 % those of the WT, whereas no significant decrease was observed in those of *SlGAD3^{OX}* (Fig. 4c).

SlGAD3 (ΔC) expression and free amino acid contents in T$_1$ SlGAD3ΔC^{OX} fruits
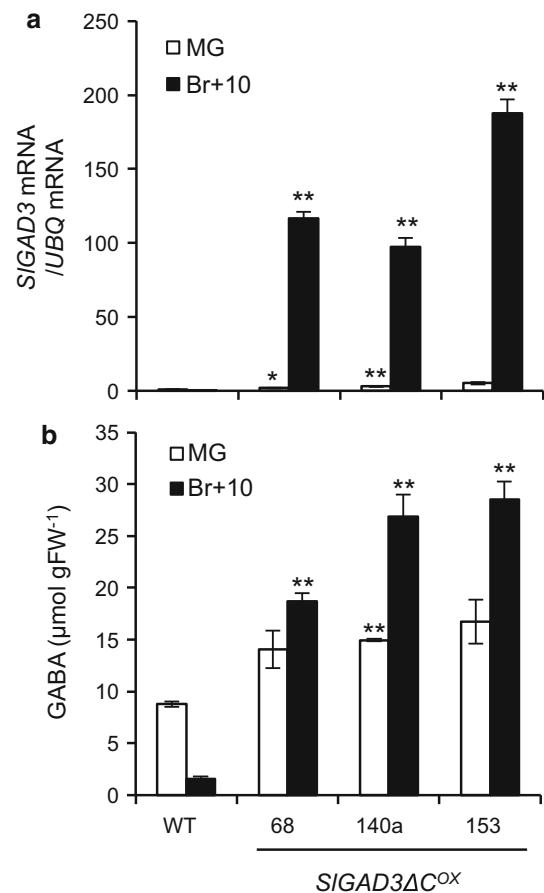
To analyze the stability of GABA accumulation, we compared the T$_1$ progenies of three *SlGAD3ΔC^{OX}* lines (68, 140a, and 153) with those of the WT control. Using fruits at the MG and Br + 10 stages, we analyzed *SlGAD3* (ΔC) mRNA transcript levels to confirm that mRNA induction occurred in T$_1$ *SlGAD3ΔC^{OX}* fruits. The *SlGAD3* (ΔC) mRNA levels in transgenic lines 68 and 140a were significantly higher than those of the WT at the MG stage, and all three transgenic lines had much higher levels at the Br + 10 stage, ranging from 800- to 1500-fold WT levels (Fig. 5a). Next, we determined the GABA levels in MG and Br + 10 fruits using an amino acid analyzer. In general, GABA levels in WT 'Micro-Tom' fruits are high at the MG stage and decrease rapidly as the fruit reaches maturation during the ripening stage (Akihiro et al. 2008). Consistent with this finding, the GABA level in WT fruits
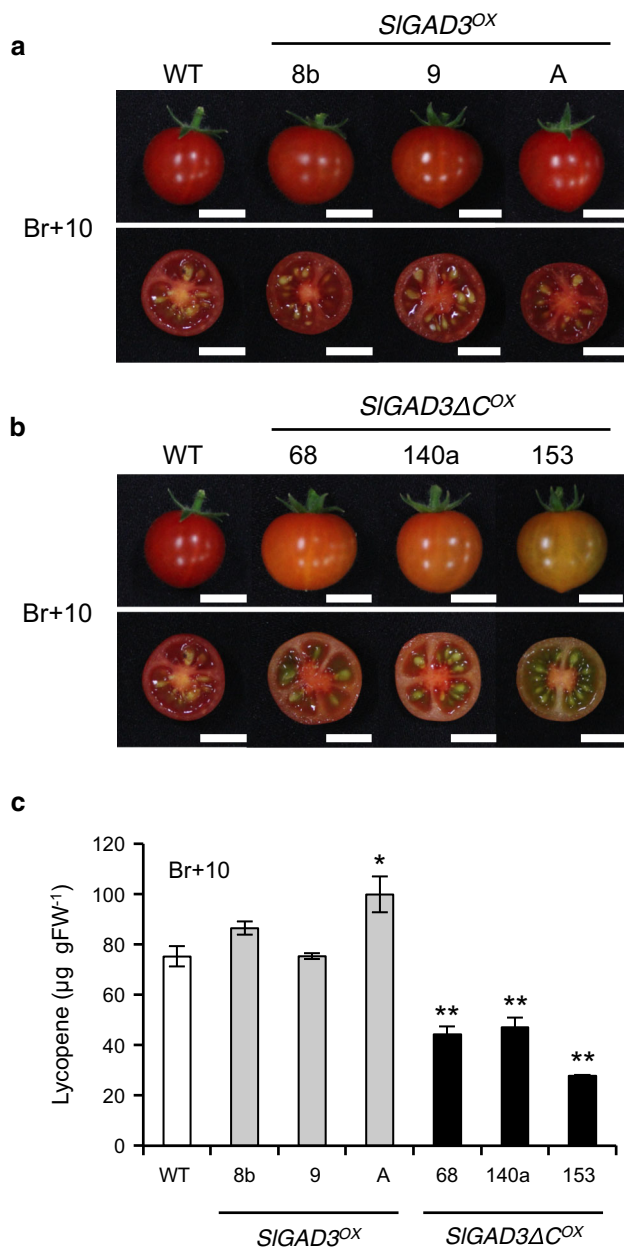
Fig. 4 Fruit appearance of T_0 *SlGAD3OX* and T_0 *SlGAD3ΔCOX* lines. Br + 10 fruits of *SlGAD3OX* (**a**) and *SlGAD3ΔCOX* (**b**) lines are shown. *Bar* 1 cm. **c** Lycopene contents in the fruits of WT and T_0 transgenic lines at the Br + 10 stage. The mean ± SE of three biological replicates are shown. *Asterisks* indicate a significant difference between WT and transgenic lines according to Student's *t* test (*$P < 0.05$ and **$P < 0.01$)

Fig. 5 *SlGAD3* mRNA levels and GABA contents in T_1 *SlGAD3ΔCOX* fruits. Fruits at the MG and Br + 10 stage were analyzed. **a** qRT-PCR analysis of *SlGAD3*. The expression levels in WT MG fruits were set to a baseline value of 1. *Slubiquitin3* (UBI) was used for normalization. **b** GABA contents determined using an amino acid analyzer. The same fruit samples used in **a** were used. The mean ± SE of three biological replicates are shown. *Asterisks* indicate a significant difference between WT and transgenic lines according to Student's *t* test (*$P < 0.05$ and **$P < 0.01$). *MG* mature green, *Br + 10* 10 days after breaker

decreased by 82 % from MG to the Br + 10 stage (Fig. 5b). By contrast, all transgenic lines accumulated much higher levels of GABA at the Br + 10 stage, ranging from 19 to 29 μmol gFW^{-1}, which corresponded to 12- to 18-fold higher levels than WT (1.6 μmol gFW^{-1}) at the Br + 10 stage (Fig. 5b).

We also analyzed the levels of other free amino acids in Br + 10 fruits to determine whether enhanced GABA

biosynthesis affected amino acid metabolism (Table 1). Notably, the levels of aspartate and glutamate were significantly lower in all transgenic lines compared to WT, i.e., 5–12 and 5–9 % of WT levels, respectively (Table 1). By contrast, the levels of alanine, cysteine, and phenylalanine were significantly higher than those of the WT, even though these levels accounted for less than 6 % of total free amino acids in the transgenic lines. In the transgenic lines, GABA comprised up to 81 % of total free amino acids at the Br + 10 stage. This percentage was much higher than that of the WT, not only at the Br + 10 stage (6.2 %; Table 1), but also at the MG stage (33 %; data not shown), when GABA reached maximum levels in WT fruits.

We also determined pH and total soluble solid (TSS) contents in Br + 10 fruits of *SlGAD3ΔCOX* lines. pH and TSS are commonly used as the indicators to estimate

Table 1 Free amino acid levels (μmol gFW^{-1}) in the Br + 10 fruits of WT and $SlGAD3\Delta C^{OX}$ lines

Amino acid	WT	$SlGAD3\Delta C^{OX}$		
		68	140a	153
Alanine	0.41 ± 0.00 (1.6)	1.10 ± 0.06** (4.3)	1.02 ± 0.06* (2.7)	0.79 ± 0.08* (2.2)
Asparagine	1.24 ± 0.07 (4.9)	0.70 ± 0.05** (2.7)	1.07 ± 0.07 (2.9)	0.63 ± 0.03** (1.8)
Aspartate	14.39 ± 0.43 (56.6)	1.78 ± 0.12** (6.9)	2.36 ± 0.07** (6.3)	1.05 ± 0.27** (3.0)
Cysteine	0.01 ± 0.01 (0.0)	0.06 ± 0.00** (0.2)	0.08 ± 0.01** (0.2)	0.06 ± 0.00** (0.2)
GABA	1.58 ± 0.25 (6.2)	18.70 ± 0.78** (72.5)	26.87 ± 2.12** (72.1)	28.56 ± 1.69** (80.7)
Glutamate	2.00 ± 0.07 (7.9)	0.17 ± 0.01** (0.7)	0.25 ± 0.00** (0.7)	0.15 ± 0.02** (0.4)
Glutamine	4.24 ± 0.13 (16.7)	1.85 ± 0.15** (7.2)	3.78 ± 0.40 (10.1)	1.97 ± 0.22** (5.6)
Glycine	0.04 ± 0.00 (0.1)	0.10 ± 0.01 (0.4)	0.10 ± 0.01** (0.3)	0.09 ± 0.01* (0.2)
Histidine	0.17 ± 0.00 (0.7)	0.12 ± 0.01* (0.4)	0.18 ± 0.00 (0.5)	0.15 ± 0.00* (0.4)
Isoleucine	0.05 ± 0.01 (0.2)	0.06 ± 0.01 (0.2)	0.11 ± 0.02* (0.3)	0.12 ± 0.02* (0.3)
Leucine	0.09 ± 0.00 (0.4)	0.06 ± 0.01** (0.2)	0.06 ± 0.01 (0.2)	0.08 ± 0.01 (0.2)
Lysine	0.16 ± 0.01 (0.6)	0.06 ± 0.01** (0.2)	0.10 ± 0.01** (0.3)	0.10 ± 0.01** (0.3)
Methionine	0.02 ± 0.00 (0.1)	0.03 ± 0.01 (0.1)	0.05 ± 0.00** (0.1)	0.05 ± 0.01** (0.2)
Phenylalanine	0.16 ± 0.02 (0.6)	0.27 ± 0.01* (1.1)	0.36 ± 0.01** (1.0)	0.53 ± 0.01** (1.5)
Serine	0.29 ± 0.02 (1.1)	0.32 ± 0.02 (1.2)	0.31 ± 0.01 (0.8)	0.29 ± 0.02 (0.8)
Threonine	0.19 ± 0.01 (0.8)	0.18 ± 0.02 (0.7)	0.27 ± 0.02* (0.7)	0.34 ± 0.04* (1.0)
Tyrosine	0.02 ± 0.00 (0.1)	0.01 ± 0.00 (0.1)	0.03 ± 0.00 (0.1)	0.04 ± 0.00** (0.1)
Valine	0.27 ± 0.01 (1.1)	0.20 ± 0.00** (0.8)	0.25 ± 0.02 (0.7)	0.31 ± 0.01* (0.9)
Total	25.44 ± 0.74 (100)	25.79 ± 1.23 (100)	37.28 ± 1.92 (100)	35.41 ± 1.07 (100)

Values are the mean ± SE of three independent fruits

Asterisks indicate a significant difference between WT and transgenic lines according to Student's t test (* $P < 0.05$, ** $P < 0.01$)

% of total free amino acids are shown in brackets

organic acid and sugar levels, respectively. Compared with WT, all three transgenic lines showed significantly higher pH, suggesting that over production of GABA also affected organic acid levels (Fig. S3a). By contrast, no significant difference was observed in TSS contents (Fig. S3b).

### Fruit pigmentation of T_1 $SlGAD3\Delta C^{OX}$ lines

Although the overall plant growth (plant height and days to flowering) and fruit development (days from flowering to the Br stage, and fruit weight) of the T_1 $SlGAD3\Delta C^{OX}$ lines were indistinguishable from those of the WT (Fig. S4), transgenic mature fruits failed to progress to the red-ripe stage, instead exhibiting an orange-ripe phenotype similar to that of the T_0 generation (Fig. 6a). At the Br + 10 stage, three $SlGAD3\Delta C^{OX}$ lines showed significantly low levels of lycopene, i.e., 28–57 % of WT levels, while β-carotene levels were equivalent to the WT level except for line 153 (Fig. 6c). Even when the fruits remained on the vine until 30 days after the Br stage, the exocarp and mesocarp tissue of the three $SlGAD3\Delta C^{OX}$ lines did not turn red completely (Fig. 6b). However, some transgenic seeds had germinated in the Br + 30 fruit like in the WT, most likely due to over-ripening, suggesting that fruit coloration was defective, whereas seed maturation and development were

functional in $SlGAD3\Delta C^{OX}$ lines (Fig. 6b). Also, when the off vine harvested fruits were stored at 25 °C, both $SlGAD3\Delta C^{OX}$ and WT fruits began to shrink in two weeks (Fig. S5), indicating that the fruit shelf life of $SlGAD3\Delta C^{OX}$ lines is unchanged.

Delayed ethylene production in $SlGAD3\Delta C^{OX}$ fruits

The plant hormone ethylene plays an essential role in tomato fruit ripening, and inhibiting its biosynthesis represses many aspects of ripening-related physiological processes, such as softening, aroma development, and red-ripe coloration (Oeller et al. 1991; Klee et al. 1991). To determine whether the reduced lycopene levels in $SlGAD3\Delta C^{OX}$ fruits could be attributed to suppressed ethylene production, we analyzed the ethylene production rates at different stages of fruit development. In WT fruits, a typical climacteric rise in ethylene production was observed, peaking at the Br + 3 stage and then declining (Fig. 7a). By contrast, in $SlGAD3\Delta C^{OX}$ fruits, a climacteric rise in ethylene production was detected from the Br stage (like WT), but ethylene levels increased slowly and peaked at the Br + 10 stage (Fig. 7a). However, ethylene production in transgenic fruits at the Br + 10 stage was almost equivalent to that of the WT at the Br + 3 stage (Fig. 7a).

Fig. 6 Appearance of WT and T$_1$ *SlGAD3ΔC*^{OX} fruits. Fruits were ripened on the vine and harvested at the Br + 10 (**a**) and Br + 30 (**b**) stages. *Bar* 1 cm. **c** Lycopene and β-carotene contents in the fruits of WT and T$_1$ *SlGAD3ΔC*^{OX} transgenic lines at the Br + 10 stage. The mean ± SE of three biological replicates are shown. *Asterisks* indicate a significant difference between WT and transgenic lines according to Student's *t* test (*$P < 0.05$ and **$P < 0.01$)
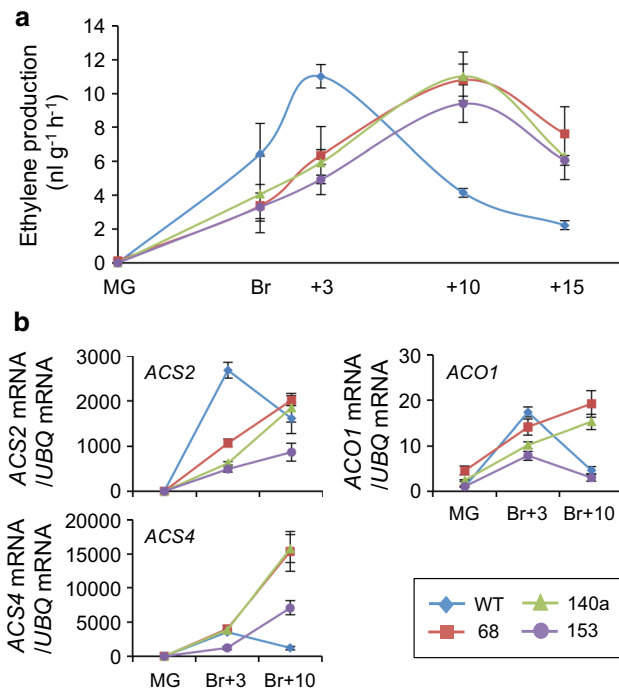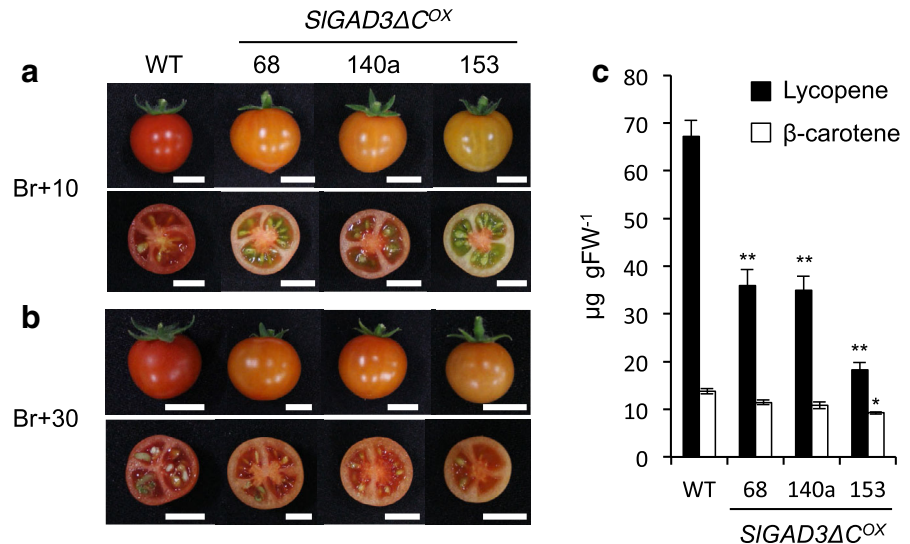
Fig. 7 Ethylene production in the fruits of WT and T$_1$ *SlGAD3ΔC*^{OX} transgenic lines. **a** Ethylene production rates measured at different developmental stages. The mean ± SE of five biological replicates are shown. **b** Relative expression levels of ethylene biosynthesis genes (*ACS2*, *ACS4*, and *ACO1*) determined by qRT-PCR. The expression levels in the WT MG fruits were set to a baseline value of 1. *Slubiquitin3* (UBI) was used for normalization. The mean ± SE of three biological replicates are shown

To examine whether delayed ethylene production resulted from reduced production of mRNA transcripts of ethylene biosynthetic genes, we analyzed the mRNA levels of two 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) genes, *ACS2* and *ACS4*, and one ACC oxidase

(ACO) gene, *ACO1*, in fruits at three developmental stages, MG, Br + 3, and Br + 10 (Van de Poel et al. 2012). In accordance with ethylene production, in WT fruits, the mRNA levels of *ACS2*, *ACS4*, and *ACO1* peaked at the Br + 3 stage and then declined (Fig. 7a, b). By contrast, in *SlGAD3ΔC*^{OX} fruits, the mRNA levels of these genes were highest at the Br + 10 stage, except for *ACO1* in line 153, which peaked at the Br + 3 stage (Fig. 7a, b).

Expression of carotenoid biosynthesis genes in T$_1$ *SlGAD3ΔC*^{OX} fruits

Although the timing of the climacteric rise in ethylene production was delayed in *SlGAD3ΔC*^{OX} fruits, ethylene levels in these fruits were equivalent to those of the WT until the Br + 10 stage (Fig. 7a). However, *SlGAD3ΔC*^{OX} fruits never reached the red-ripe stage, instead remaining orange, even at the Br + 30 stage (Fig. 6b). To examine whether this phenotype is associated with reduced carotenoid accumulation, we analyzed the mRNA levels of the phytoene synthase 1 (*PSY1*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), carotene isomerase (*CRTISO*), chloroplast-specific lycopene β-cyclase (*LCY-B*), and chromoplast-specific lycopene β-cyclase (*CYC-B*) genes, all of which are involved in carotenoid biosynthesis (Pecker et al. 1996; Ronen et al. 2000; Liu et al. 2015). In WT fruits, the mRNA levels of all six genes peaked at the Br + 3 stage and then declined (Fig. 8a). By contrast, in transgenic fruits, the expression of all genes except *LCY-B* remained at low levels until the Br + 3 stage and then increased slightly at the Br + 10 stage (Fig. 8a). These results suggest that *SlGAD3ΔC* overexpression widely affects the expressions of carotenoid biosynthesis genes in ripening fruits.

Fig. 8 Relative expression levels of carotenoid biosynthesis genes in the fruit of WT and T_1 $SlGAD3\Delta C^{OX}$ transgenic lines. The mRNA levels of carotenoid biosynthesis genes (*PSY1*, *PDS*, *ZDS*, *CRTISO*, *LCY-B* and *CYC-B*) (**a**) and ripening-related transcription factors (*RIN*, *TAGL1*, *FUL1*, *FUL2*, and *SlERF6*) (**b**) were determined by qRT-PCR. The expression levels in WT MG fruits were set to a baseline value of 1. *Slubiquitin3* (UBI) was used for normalization. The mean ± SE of three biological replicates are shown
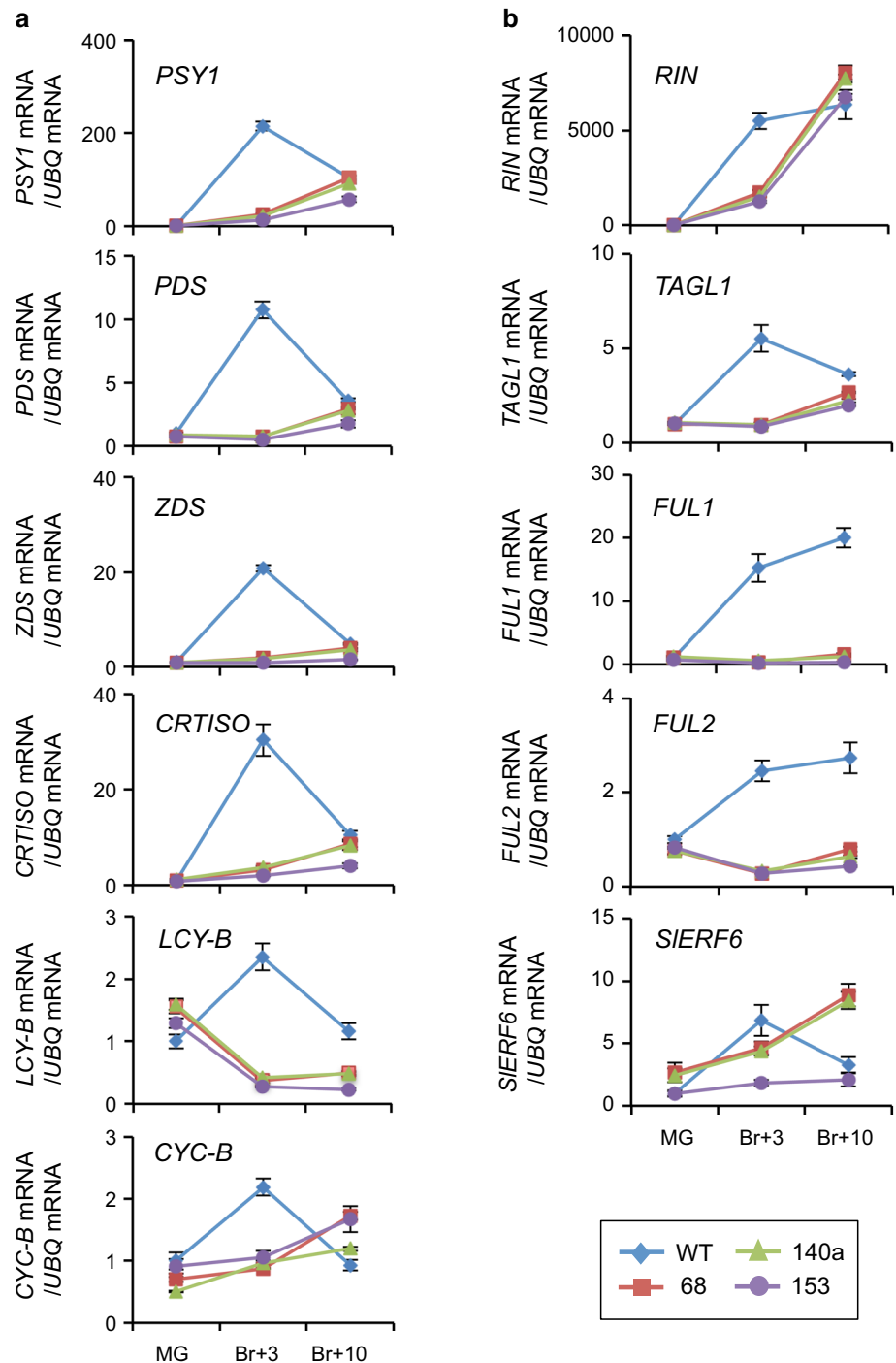
We also examined the mRNA levels of *RIN*, *TAGL1*, *FUL1* (*TDR4*), *FUL2* (*MBP7*), and *SlERF6* that encode transcription factors having a broad effect on fruit ripening including carotenogenesis. In WT fruits, these genes were all upregulated from MG to Br + 3 stage. By contrast, in transgenic fruits, the mRNA levels of these genes were consistently low throughout ripening, except that the expressions of *RIN* and *ERF6* were upregulated from Br + 3 to Br + 10 stage (Fig. 8b).

Discussion

The E8 promoter/HSP terminator expression cassette strongly induces the expression of *SlGAD3* in ripening tomato fruit

GAD mRNA expression and GAD enzyme activity are generally high in green tomato fruits, and they decline after Br stage. In addition, GABA accumulation is well

correlated with these mRNA and enzyme profiles; the final GABA content in mature red-ripe fruits of cv. 'Micro-Tom' is 1–3 μmol/gFW (Akihiro et al. 2008; Sorrequieta et al. 2013; Koike et al. 2013). Therefore, producing tomato plants with increased *GAD* mRNA levels or GAD enzyme activity at the ripening stage via genetic engineering represents a feasible strategy for improving GABA levels in red-ripe tomato fruits. Previously, we generated transgenic tomato plants in which *SlGAD3* was expressed using the CaMV 35S promoter/NOS terminator expression cassette (*35S-SlGAD3-NOS*; Takayama et al. 2015). The GABA levels in these transgenic fruits were 5.2-fold higher than those of WT at the red-ripe stage (Takayama et al. 2015), indicating that overexpressing *SlGAD3* is an effective approach for increasing GABA accumulation in tomato fruits. In the current study, we further explored the possibility of increasing GABA levels in red-ripe tomato fruits using alternative approaches.

First, we modified the expression cassette. To induce the *SlGAD3* expression more strongly and specifically in ripening fruits, we replaced CaMV35S promoter into fruit-specific E8 promoter, and NOS terminator into *Arabidopsis* HSP terminator. As a result, $SlGAD3^{OX}$ plants exhibited significantly higher levels (300-fold of WT levels) of *SlGAD3* expression in Br + 10 fruits (Fig. 2b). Although the E8 promoter generally induces lower levels of mRNA accumulation compared to the CaMV 35S promoter in red stage fruits (Nambeesan et al. 2010; Hirai et al. 2011a), the level of *SlGAD3* upregulation in $SlGAD3^{OX}$ fruits was substantially higher than that of the previously generated *35S-SlGAD3-NOS* lines, which exhibited an approximately 200-fold increase in *SlGAD3* mRNA levels compared to the WT (Takayama et al. 2015). As studies in tobacco, rice, and tomato have shown that the *Arabidopsis* HSP terminator is more effective than the NOS terminator in increasing mRNA accumulation of transgene (Nagaya et al. 2010; Hirai et al. 2011b; Kurokawa et al. 2013), it is suggested that the strong induction of *SlGAD3* in $SlGAD3^{OX}$ fruits was achieved by utilizing HSP terminator. However, although $SlGAD3^{OX}$ fruits had higher levels of *SlGAD3* mRNA, the GABA levels were comparable to those of previously generated *35S-SlGAD3-NOS* fruits at the red stage (Fig. 2c; Takayama et al. 2015), indicating that GABA levels are not solely determined by *SlGAD3* mRNA levels in red-ripe fruits.

C-terminal truncation of *SlGAD3* increases GABA production in tomato fruits

We next examined the effects of C-terminal truncation of *SlGAD3* on GABA accumulation. Although we found no prominent difference between *SlGAD3* (*ΔC*) mRNA levels of $SlGAD3^{OX}$ and $SlGAD3ΔC^{OX}$ lines (approximately

300-fold WT levels; Fig. 2b), the GABA levels in $SlGAD3ΔC^{OX}$ fruits were 1.9-fold higher than those of $SlGAD3^{OX}$ lines at the Br + 10 stage (Fig. 2c). In addition, Br + 10 fruits of $SlGAD3ΔC^{OX}$ lines exhibited higher GAD activities compared with those of $SlGAD3^{OX}$ lines in all conditions we tested (Fig. 3). These results suggest that the C-terminal extension region of SlGAD3 also functions as an autoinhibitory domain, like that of other plant GADs (Baum et al. 1996; Akama and Takaiwa 2007). The high productivity of GABA in $SlGAD3ΔC^{OX}$ fruits was maintained in T_1 progenies that accumulated 19–29 μmol gFW^{-1} at the Br + 10 stage (Fig. 5b; Table 1). These levels of GABA were much higher than those of the WT (1.6 μmol gFW^{-1}), as well as those of the previously generated *35S-SlGAD3-NOS* plants and plants in which *SlGABA-T1* was suppressed by RNAi under the control of the CaMV 35S promoter and NOS terminator, which exhibited levels up to 5.2- and 9.2-fold higher than that of the WT, respectively (Koike et al. 2013; Takayama et al. 2015). These results indicate that removal of the C-terminal extension region of SlGAD3 is a useful way to strongly increase GABA accumulation in tomato fruits.

Enhanced GABA biosynthesis in tomato fruits influences ethylene production and sensitivity

In the present study, we did not find any morphological abnormalities in both $SlGAD3^{OX}$ and $SlGAD3ΔC^{OX}$ lines, probably due to the use of fruit-specific E8 promoter. However, $SlGAD3ΔC^{OX}$ lines showed an abnormality in fruit ripening. Unlike the fruit of the WT which became red-ripe until Br + 10 stage, that of $SlGAD3ΔC^{OX}$ lines remained orange even at the Br + 30 stage when the fruits were ripen on the vine (Fig. 4). This orange-ripe phenotype was accompanied by a reduction in lycopene levels, which was correlated with the reduced mRNA levels of carotenogenic genes (Figs. 4, 6, 8a). Taking consideration of that $SlGAD3^{OX}$ fruits displayed a normal red-ripe phenotype, it is hypothesized that the orange-ripe phenotype in $SlGAD3ΔC^{OX}$ lines might be attributed to the metabolic disturbance caused by over activation of GAD via C-terminal truncation. In fruits of the WT, GABA comprised up to 6.2 % of total free amino acids at the Br + 10 stage, whereas GABA in T_1 $SlGAD3ΔC^{OX}$ fruits comprised up to 81 % of total free amino acids at the same stage (Table 1). By contrast, the levels of aspartate and glutamate, the two major amino acids in WT fruits, were considerably decreased in all transgenic lines (Table 1). The decline in glutamate level could be caused by enhanced GAD activity, because glutamate is the direct precursor of GABA. Although aspartate is not the direct precursor of GABA, it is mainly biosynthesized from glutamate by aspartate aminotransferase (Forde and Lea 2007). Thus, rapid

consumption of glutamate might have affected the aspartate biosynthesis in $SlGAD3\Delta C^{OX}$ fruits. It is reported that transgenic tobacco plants expressing a petunia GAD lacking the C-terminal extension region possessed extremely high GABA and low glutamate levels (Baum et al. 1996). These transgenic plants exhibited severe developmental abnormalities correlated with attenuated cell elongation in stems. As the possible causes of this phenotype, the authors pointed to the lower levels of glutamate, because glutamate is directly or indirectly involved in a number of pathways, including biosynthesis of gibberellins as well as post-translational modifications of cell wall proteins (Baum et al. 1996). Similarly, transgenic rice plants expressing a C-terminal truncated $OsGAD2$ had aberrant phenotypes such as dwarfism, etiolated leaves and sterility (Akama and Takaiwa 2007). As high levels of GABA were detected in those transgenic plants, the authors suggested that an imbalance of amino acids in cells would be involved in aberrant phenotypes. Interestingly, transgenic tomato fruits in which transcription factors FUL1 and FUL2 were simultaneously suppressed exhibited the orange-ripe phenotype, with highly reduced lycopene levels (Bemer et al. 2012). These fruits also exhibited increased GABA levels (twofold higher than WT levels) and strongly reduced glutamate levels (eightfold lower than WT levels), possibly due to the upregulated expression of three $SlGAD$ genes ($SlGAD1$, $SlGAD2$, and $SlGAD3$) (Bemer et al. 2012). As the GABA levels in FUL1/FUL2 suppressed fruits were less elevated compared with those of the red-ripen $GAD3^{OX}$ fruits, it is possible that reduction in glutamate levels is also attributed to the orange-ripe phenotype. However, the relationships between glutamate and carotenoid accumulation are still unclear. In tomato fruits, ethylene plays a crucial role in accelerating fruit ripening and carotenoid accumulation (Oeller et al. 1991; Klee et al. 1991; Wilkinson et al. 1997). We found that although the climacteric ethylene production in $SlGAD3\Delta C^{OX}$ fruits was delayed, this phytohormone eventually reached the maximum level at the Br + 10 stage, which was equivalent to the results from the WT at the Br + 3 stage (Fig. 7a). The ethylene production rates were, at least in part, correlated with the expression of two ethylene biosynthesis genes, $ACS2$ and $ACS4$ (Fig. 7b), suggesting that over activation of GAD via C-terminal truncation affects ethylene production through transcriptional control of these biosynthesis genes.

We also found that $SlGAD3\Delta C^{OX}$ fruits did not turn red-ripe even at the Br + 30 stage when the fruits had already been exposed to high levels of ethylene (at Br + 10 stage for $SlGAD3\Delta C^{OX}$ fruits) that were almost equivalent to the WT maximum levels (at Br + 3 stage for WT fruits) (Figs. 6b, 7a). Indeed, mature fruits of the ethylene insensitive mutants $Sletr1$, $Never$ $ripe$ (Nr), and $ripening$

$inhibitor$ (rin), as well as transgenic tomato in which an ethylene biosynthesis gene is suppressed, also fail to produce fruit with red-ripe coloration (Oeller et al. 1991; Alba et al. 2005; Okabe et al. 2011). Therefore, it is possible that the failure of $SlGAD3\Delta C^{OX}$ to form red-ripe fruit is not only due to delayed ethylene production, but also to reduced ethylene sensitivity.

Enhanced GABA biosynthesis in tomato fruits affects the expression of ripening-associated genes

In addition to ethylene, MADS-box transcription factors are also key regulators of tomato fruit ripening. For example, RIN is one of the earliest acting factors that regulate fruit ripening in both ethylene-dependent and -independent pathways (Fujisawa et al. 2013). The recently identified transcription factors TAGL1, FUL1, and FUL2 also play crucial roles in regulating ripening processes such as ethylene biosynthesis and lycopene accumulation (Itkin et al. 2009; Bemer et al. 2012; Shima et al. 2013; Fujisawa et al. 2014). SlERF6 is known as a negative regulator of both carotenoid accumulations and ethylene synthesis (Lee et al. 2012). Thus, in the present study, we examined the mRNA levels of these transcription factors in $SlGAD3\Delta C^{OX}$ fruits (Fig. 8b). Compared to the WT, $SlGAD3\Delta C^{OX}$ fruits exhibited strong reductions in $TAGL1$, $FUL1$, and $FUL2$ expression at the Br + 3 and Br + 10 stages, while RIN was only suppressed at Br + 3, and $ERF6$ were upregulated from Br + 3 to Br + 10 stage (Fig. 8b). RIN, TAGL1, FUL1, and FUL2 can bind to the promoter region of $ACS2$ (Itkin et al. 2009; Fujisawa et al. 2011; Shima et al. 2013), suggesting that the reduced levels of $ACS2$ expression observed in $SlGAD3\Delta C^{OX}$ fruits were caused by the reduced expression of these transcription factor genes (Figs. 7b, 8b). Similarly, carotenogenic genes $PSY1$ and $CRTISO$ are targeted by RIN and FUL1 and by RIN, FUL1, and FUL2, respectively (Fujisawa et al. 2014). Thus, the reduced expression of these transcription factors might affect the expression of carotenogenic genes in $SlGAD3\Delta C^{OX}$ fruits (Fig. 8). Although the mechanism by which RIN, $TAGL1$, FUL, and $FUL2$ are suppressed in $SlGAD3\Delta C^{OX}$ fruits remains unknown, we found that over activation of GAD via C-terminal truncation alters the process of fruit ripening by modulating the expression of multiple genes involved in fruit ripening.

Conclusion

This study demonstrates that hyperaccumulation of GABA in tomato fruits could be achieved using the E8 promoter and HSP terminator expression cassette coupled with C-terminal truncation of $SlGAD3$. Using this strategy, we

succeeded in increasing GABA accumulation in tomato fruits to a level of 29 μmol gFW^{-1} (295 mg 100 gFW^{-1}). This level is much higher than those of previously generated transgenic tomato fruits with increased levels of GABA (Koike et al. 2013; Takayama et al. 2015) and is higher than that of *S. pennellii*, a wild species of tomato that accumulates high levels of GABA (approximately 200 mg 100 gFW^{-1} when determined by our protocol; data not shown) (Schauer et al. 2005). Daily intake of 10–20 mg of GABA is effective in reducing blood pressure in adults with mild hypertension (Fukuwatari et al. 2001; Kazami et al. 2002; Inoue et al. 2003). This amount of GABA is equivalent to the level present in 3.4–6.8 gFW of transgenic fruit (corresponding to 1–3 fruits), suggesting that the transgenic tomato fruit produced in the current study contains sufficient levels of GABA to contribute to human health.

Author contribution statement   HE, CM, and TA conceived and designed research. MT conducted experiments and analyzed data. MT, CM, TA, and HE wrote manuscript. All authors read and approved the manuscript.

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